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CHANGES IN THE VOLATILE FRACTION COMPOSITION OF PORT WINES DURING AGING: A MECHANISTIC APPROACH

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Food Engineering

by

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Place: Escola Superior de Biotecnologia – Universidade Católica Portuguesa

Supervision: Doutor António César Silva Ferreira

June, 2013

Mãe espero que isto te faça sorrir
Espero que estejas feliz com a minha vida
Com todas as escolhas que fiz
Como mudei ao longo deste caminho
Porque sei que sempre acreditaste em todos os meus sonhos
E devo-te tudo a ti

Resumo

Os produtos alimentares durante o seu tempo de vida útil sofrem modificações químicas provocadas por conjuntos de reações reguladas por princípios termodinâmicos, sendo estas tradicionalmente agrupadas em diversos mecanismos. No processamento alimentar destaca-se a reação de Maillard que é dos mais descritos nos alimentos. O conhecimento da "mecânica" das reações e da sua possível interligação é fulcral para a compreensão e consequentemente para a monitorização da qualidade alimentar.

Neste contexto, surge a motivação deste trabalho: elucidar sobre a possível interligação entre a reação de Maillard e a oxidação durante o envelhecimento do Vinho do Porto. De que forma estes mecanismos são afectados por parâmetros tecnológicos, como o oxigénio e a temperatura. E por fim saber de que forma podem ser controlados e assim providenciar formas de gerir o processo de envelhecimento e consequentemente a qualidade do produto.

Foram usados vinhos envelhecidos durante 63 dias sob diferentes condições de oxigénio e temperatura e vinhos de diferentes idades que foram analisados por cromatografia gasosa acoplada a um detetor de ionização por chama e por espectrometria de massa. Compostos marcadores da reação de Maillard, oxidação e de um possível mecanismo que resulta da junção dos 2 que dá origem ao sotolon foram quantificados e calculados os respetivos parâmetros cinéticos, posteriormente usados para através de simulações de Monte Carlo prever a composição de vinhos armazenados em diferentes condições. Os perfis cromatográficos i.e. vetores de intensidade vs. tempos de retenção foram usados como impressões digitais químicos para encontrar mais marcadores relacionados com o processo. Foi criada uma nova técnica de visualização de cromatogramas que consiste na construção de uma rede com conexões (correlações de Pearson) entre tempos de retenção (compostos).

Os parâmetros cinéticos demonstraram que a formação de sotolon é dependente do oxigénio e da temperatura, observando-se um efeito sinérgico entre ambos o que sustenta a hipótese da sua formação estar relacionada com o mecanismos de Maillard e a oxidação. As simulações demonstram que o efeito da temperatura tem maior impacto em vinhos armazenados em recipientes com elevada permeabilidade ao oxigénio (barris) do que naqueles com baixa permeabilidade (garrafas com rolha de cortiça).

O uso dos perfis cromatográficos permite classificar as amostras de diferentes idades e encontrar mais marcadores relacionados com o processo. A reconstrução de redes é útil na priorização da identificação dos biomarcadores assim como permite uma visualização das cinéticas destes, compostos próximos têm a mesma expressão temporal e consequentemente obedecem á mesma ordem cinética e verificou-se que compostos agrupados são sensíveis aos mesmos parâmetros tecnológicos (oxigénio e temperatura).

O controlo de parâmetros tecnológicos permite modular o perfil sensorial do vinho do Porto e consequentemente a sua qualidade. Este conhecimento tem elevado valor para a indústria porque permite gerir a qualidade e consequentemente aumentar o valor do produto.

Abstract

A food product during shelf life suffers chemical changes, caused by sets of reactions regulated by thermodynamics principles forming several mechanisms. In food processing, Maillard reaction is probably the most described. The knowledge of the different mechanism and their possible interconnections is central to understand and consequently monitor food quality.

It is within this context, that the motivation of this thesis arose: to provide insights about the inter-connections between the Maillard reaction and oxidation during Port wine aging and how these mechanisms will be affected by technological parameters such as temperature and oxygen. Conclusively know how they can be controlled and thus provide ways to manage the aging process and consequently the quality of the product.

In this context wines used were aged for 63 days under different conditions of temperature and oxygen and wines of different ages were analyzed by gas chromatography coupled to a flame ionization detector and to mass spectrometry. Marker compounds of Maillard reaction, oxidation and from a possible mechanism that outcomes from the previous ones which leads to sotolon were quantified and was calculated the respective kinetic parameters, these were used for through Monte Carlo simulations predict the composition of wine stored in different conditions of oxygen and temperature.

The chromatographic profiles (vectors of intensity vs. retention times) were used as chemical fingerprints to find out more markers related with the process. A new approach for chromatographic data visualization was created consisting in a network of connections (Pearson correlations) between retention times (compounds).

The kinetic parameters have shown that the formation of sotolon is highly dependent upon oxygen and temperature. Kinetics models with Monte Carlo simulations where applied and on the basis of the modeling predictions, it would seem that the temperature of a cellar would have a more significant impact on the Port wines stored in containers where the oxygen intake is higher (barrels) when compared to containers with low oxygen permeability (bottles with cork stoppers).

The use of chromatographic profiles as samples fingerprints allows classifying samples of different ages and finding more markers related to the process. The network reconstruction provides considerably more information in an effort to understand the probable kinetic contexts of the molecules represented by peaks in each chromatogram.

The technology developed in the study allows the modulation of the Port sensory profiles and consequently contributes to quality improvement. This knowledge has high value for the industry because it allows the management of the quality and therefore the increment of the product value.

Acknowledgements

It is difficult to overstate my gratitude to my MSc. Supervisor, Dr. António César Ferreira. With his enthusiasm, his inspiration, and his great efforts to explain things clearly and simply, he helped to make this work fun for me. Throughout this time, he provided encouragement, sound advice, good teaching, good company, and lots of good ideas. I would have been lost without him. He was the driver for my development in research.

To Symington Family Estates for the availability of supply all the Port wine samples used in the work and to Corksupply, SA, Portugal for oxygen measurement support.

Life as a post graduate student is not always easy. Nevertheless, I was lucky enough to meet some colleagues who inspired and helped me to overcome these difficulties. These friends are Joana Tomé, Carla Oliveira, Carla Teixeira and Rosa Martins.

Last but not the least, I owe a debt of gratitude to my family members for all their encouragement.

To Pedro for sharing my dreams and for always being there.

To my father for the support in the last years.

To my grandfather, who had a great influence in the person I am today.

To a very special person, Rosa. Without her this work never have been possible. Thank you for the patience and love expressed during this time and for being around when I needed it most.

And finally to the most important person in the entire world, the centre of my life, without whom nothing would have been possible, my mother. Always present in my heart and with me all the time.

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MVA	Multivariate Analysis
PCA	Principal Component Analysis
PLS	Partial Least Squares
PLS-R	Partial Least Squares Regression
PC1	First Principal Component
PC2	Second Principal Component
HCA	Hierarquical Cluster Analysis
GC	Gas Chromatography
FID	Flame ionization detection
MS	Mass spectrometry
SPME	Solid Phase Microextraction
LLE	Liquid-Liquid Extraction
t	Time
T	Temperature
k	Rate constant
Ea	Activation energy
C_0	Compound initial concentration
MC	Monte Carlo
COW	Correlation Optimized Warping
HMF	5-hydroxymethylfurfural
5MF	5-methylfurfural

1.Introduction

1.1.Wine Flavour

The olfactory area in humans is about 2,5 cm^2 wide and contains a number of about 50 million receptor cells with 8-20 cilia down in a layer of mucus of about 60 microns thick. Only volatile substances, soluble in mucus, can reach the receptors and interact with them and finally produce sensation (Sarafoleanu *et al.*, 2009).

Humans have only 350 functional genes for olfactory receptors comparing with other mammals, which have 1100 active genes. These genes are structured in clusters of 10 and are located in different chromosomes. Nevertheless, this reduced number of genes for olfactory receptors is balanced by the amazing capacity of human brain processing. The olfactory nerve transmits olfactory impulses from the olfactory epithelium of the nose to the brain (Monkhouse, 2006). Language and speech plays an important role in the perception and discrimination of the odours. The human being able to learn to like certain things because of how they smell. In food industry, flavours have a great importance. *"The nose smells what the eyes see"* because sometimes a simple chemical ingredient added can make a type of food to look like another.

A huge example is the wine tasters, they analyse orthonasal and retronasal perception, associates them with other flavours from his memory, and are capable to identify constituents separately. The flavour of wine is a sensory perception that diverges with the individual, the context of the consumer experience and the chemical composition of the product. The final reaction is the consequence of complex chemosensory relations that are difficult to predict because of the influences of many variables (Fleet, 2003).

Aroma is an important factor in quality control of all foods but in wines this factor is probably the most important. The chemical compounds responsible for the aroma are more than 800 and are present in different ranges of concentrations, and with different volatilities and polarities (Arrehnius *et al.*, 1996).

This large number of compounds contributes to sensorial wine complexity. During wine production, from grape to ageing, complex reactions occur; this allows differentiating proper characteristics of wine.

1.2. Port Wine

Port wine is a fortified wine, produced in a specific area in North Portugal called Douro Region.

According to *Decreto-Lei nº 104/85 de 10 de Abril* there are 48 grape varieties permitted in the production of Port wine divided into two categories: recommended and authorized. This simple fact goes a long away to explaining the great variation in quality and character of Ports within the same basic style. However based on the research work of ADVID ("Associação para o Desenvolvimento da

Viticultura Duriense"), five types of varieties were selected ("top five"), which are now recommended for future plantings: Touriga Nacional, Tinto Cão, Tinta Roriz, Tinta Barroca and Touriga Francesa.

The harvest of the grapes in Douro starts at the end of September and for the most part is still carried out manually. Grapes usually arrive at the winery in baskets holding or in special steel containers. After the vinification process the grapes are weighted, tested for potential alcohol (according to sugar content) and visually inspected. Once accepted grapes start to be process. The production of a good quality port depends on the complete and fast extraction of both the color and the flavor from the tannins of the berry skins. These must be extracted before adding fortifying spirit after two or three days stopping must fermentation.

The most traditional way to produce must is the use of the lagar, a low granite trough, in which grapes are trodden and fermented. Lagares are progressively filled during the day and in the evening pickers arrive to tread them. About 24-36 hours later, yeasts activate the fermentation of the sugars contained in the grapes. Alcohol and gas have the effect of pumping the skins and the solid material to the surface, encouraging the extraction of phenolics and creating a cap on the must.

Port wines derive its sweetness from unfermented sugars, when the respective concentration of the fermenting juice has dropped to about 90 grams per litter of sugar, the alcoholic strength will normally be between 6 and 8%, depending on the richness of the juice, which in turn is related upon the grape variety and vintage. The vinification is stopped by adding grape-distilled spirit of 77% alcohol adding alcoholic strength ending with a final alcohol content of about 19-21% (v/v).

According to sugar content Port wines can be classified in extra dry (<40 g/L), dry (40-65 g/L), semi dry (65-90 g/L), sweet (90-130 g/L) and very sweet (>130 g/L). After the vinification process wines are blended and maturated in oak casks.

The diversity of different styles offered by Port wines makes up for is uniqueness among other fortified wines, contributing for is brand recognition around the world. Its different styles derive essentially from the various ways in which it can be aged. Its remarkable ageing potential and the fact that it is fortified mean that Port will continue to improve in cask or bottle for much longer than most wines. The choice of ageing period and ageing vessel will determine what the Port tastes like. A Port wine aged in wood in contact with air will evolve more quickly than which ages in bottle. Ports can be broken down into two groups: wood aged which aged in cask, normally made of oak, and bottle aged Ports which, as the name indicates, spend most of their lives maturating in bottle. Within the wood aged Port family there are Tawny Reserve Port, Tawny 10, 20, 30 or 40 years old Port and Colheita Port. The bottle-aged family of Ports is made up mainly of Ruby Reserve Port, Late Bottled Vintage Port (LBV), Vintage Port and Port Single Quinta Vintage (Figure 1.2.1.).

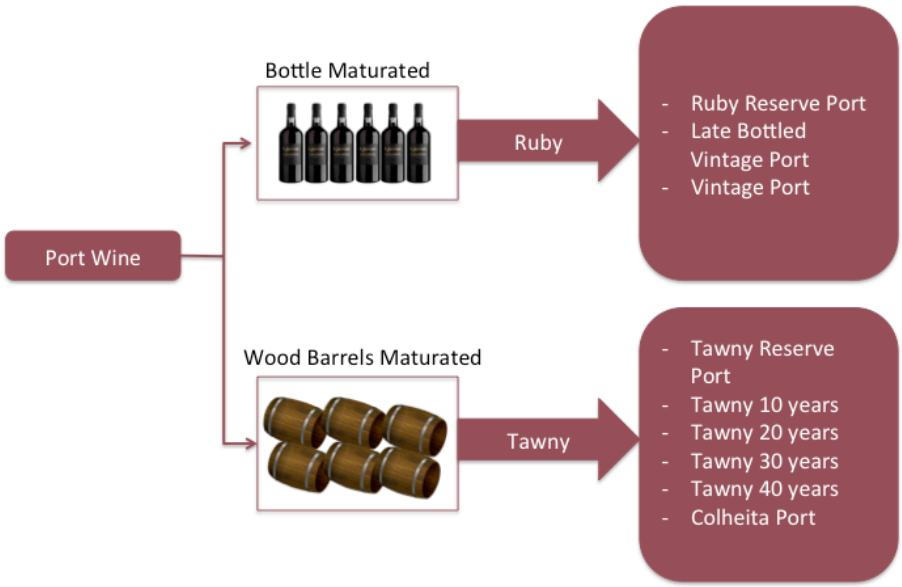

Figure 1.2.1. Port wine categories

Aging potential is the ability of a wine to improve with age. People have the perception that older wines are better (Verdú Jover *et al.*, 2004) leading to a common portuguese popular expression "the older the better". Age is therefore a quality signal for consumers, although most wines are not made to age nowadays.

The aging of fortified wines is based on the development of color, the extraction of small amounts of oak components, and the evaporative loss of volatile spirit components.

1.3. Wine Flavour Generation Mechanism

Wine aroma complexity, is due to the diversity of the mechanisms involved in their development i.e., grape metabolism, fermentation process, and wine ageing. The grape metabolism depends not only of the grape variety but also on the soil type, on the climate conditions and on the vineyard management techniques. A part of the varietal aroma impression is related to the amino acid profile of the variety (Hernández-Orte *et al.*, 2002) and the most significant part is related to specific odourless precursors (Williams Patrick *et al.*, 1989). These precursors can be glycosides, polyhydroxylated molecules (Williams *et al.*, 1980) or cysteine-derivatives (Tominaga *et al.*, 1998).

Biochemical or enzymatic phenomena occurs mainly prior to fermentation during the extraction of the juice and maceration while, chemical reactions occur mainly after fermentation, and during ageing of the wine (barrel and bottle).

Wines are consumed after a period of aging that may take place in wooden casks, in the bottle, or in both successively. For many wines, this period of aging is a necessary stage in the production process. The effect of aging is to modify the various organoleptic properties of the wine, making some more and others less intense (Singleton Vernon and Cilliers Johannes, 1995). This process implies a significant financial cost that must be recovered in the final price of the wine. For this reason, it is of

commercial interest to characterize the chemical reactions that take place during the processes of aging.

Wine aging is easily seen to be not an entity but a set of changes, several reactions act as a whole complex and connected system implicating several mechanisms. The systems thermodynamics of these reactions will regulate the shelf life of the product traditionally slow and for flavour to be modified relatively few molecules of key compounds need to be changed. Formation/degradation of varietal compounds, oxidation, reduction, esterification/hydrolysis are mechanism that occur during wine maturation.

Rather extensive oxidative and associated changes, including browning, are desirable in Ports, moreover in white wines any evidence of these characteristics made the wines unattractive (Silva Ferreira *et al.*, 2003).

Some of the reactions/events are largely acknowledged like Maillard reaction and oxidation. Some of these mechanisms are more significant during age, and some molecules are more related with aging then others, in this study we focused on them. Some furanic compounds and Strecker aldehydes from Maillard reaction, from oxidation some aldehydes and acetals, and an important molecule responsible for the aged character of Ports the sotolon. However we will also refer other compounds that undergo an evolution with age but not with so much detail, like esters, alcohols and lactones.

Generally, aging of wines leads to a loss of the characteristic aromas linked to the grape varietal and fermentation, and to the formation of new aromas characteristic of older wines or atypical aromas associated with wine deterioration (Lambropoulos and Roussis, 2007). During aging most monoterpenes decrease in concentrations over time due to acid-catalyzed reactions, however, some increase in concentration may contribute to the aging bouquet of wine. The concentration of norisoprenoides have also been show to increase in aged wines, including β-damascenone and 1,1,6-trimethyl-1,2-dihydronaphtalene (TDN). TDN is formed during wine aging and causes distinctive “kerosene” and “petrol-like” aromas in aged wines (Humpf *et al.*, 1991).

The major fermentation aroma constituents are ethanol, higher alcohols, and esters. Alcohols are produced by yeast and bacteria during fermentation. Ethanol is the main product of grape sugar conversion by yeasts and is capable of suppressing “fruitiness” in wines, by masking the perceptions of esters (Escudero *et al.*, 2007) and probably not through a change in volatility (Guth and Sies, 2001). Glycerol is also produced by yeasts and, although odourless, also has a slightly “sweet” taste (Noble and Bursick, 1984). Higher alcohols or fusel alcohols are produced by yeast, and probably contribute with “fruit” characteristics at optimal levels. Excessive concentrations of higher alcohols results in a strong “pungent smell and taste (Nyjânen, 1986). During aging higher alcohols are relatively stable in wine (Marais, 1978) (Blake *et al.*, 2009).

Free or saturated volatile fatty acids generally contribute with negative characteristics to wine, but are rarely above their aroma thresholds. Acetic acid makes up about 90% of the volatile fatty acids produced by yeasts and bacteria. During aging volatile fatty acid stability is not uniform, with some compounds reportedly increasing, while others remain stable or decrease in wines over time (Blake*, et*

al., 2009). This may be in part due to the chemical hydrolysis of some fatty acid ethyl esters, which can result in the formation of acid compounds.

Esters represent the largest and most important compounds produced during fermentation. They are present in all wines and are considered to significantly influence wine aroma and quality by contributing with "fruity" characteristics of wine (Étievant, 1991). The two types of esters produced are acetate esters and fatty acid ethyl esters. There are also a number of organic acid ethyl esters in wine that increase during aging. Ester formation can occur either by chemical reactions, which are slow and contribute little to wines, or, much more importantly, via microbial intracellular enzymatic reactions during fermentation (Mason and Dufour, 2000).

Acetate esters decrease in concentrations during wine aging through chemical hydrolysis. This leads to a loss of "fruity" flavours in aged wines (Marais, 1978). The stability of fatty acid ethyl esters differs depending on the structure of the fatty acid carbon chain. Straight-chain fatty acid ethyl esters decrease in concentration over time, whereas, branched-chain fatty acid ethyl esters are stable and their concentration can increase during wine aging (Díaz-Maroto *et al.*, 2005).

Storage of wine in oak barrels also results in modified aroma profiles, mostly due to the extraction of aroma compounds from the wood into the wine. Cis- and trans-oak lactones are the most important oak-derived aroma compounds. They impart aromas of "vanilla" and "coconut-like" aromas to wine (Jarauta *et al.*, 2005). Other aldehydes extracted from oak include furfural and 4-methylfurfural, which have aromas of "sweet", "butterscotch" and "woody" (Campo *et al.*, 2008). 2-Furanmethanethiol has an aroma reminiscent of "roasted coffe" (Blanchard *et al.*, 2001). Guaiacol and 4-methylguaiacol impart "smoky" aromas to wine, and are indicative of the level of toasting or charring of oak barrels (Jarauta*, et al.*, 2005).

Aroma changes at room temperature caused by non-enzymatic reactions are observed only after prolonged storage. The Maillard reaction and the related Strecker degradation of aminoacids all play a part. The large number of volatile compounds formed by the degradation of only one or two constituents is characteristic of a non-enzymatic reaction. This reaction provides volatile carbonyl compounds.

The Maillard reaction is a reaction between a reducing sugar and an amino acid, has been named after the French chemist Louis Maillard (1912) who first described it, but only in 1953 Hodge does the first scheme (Hodge, 1953) (Figure 1.3.1.).

All reaction can be divided into the Amadori / Heyns and Strecker degradation. Nucleophilic compounds like aminoacids or amines easily add to the carbonyl function of reducing carbohydrates with the formation of imines (Schiff bases), this can rearrange via the 1,2-eneaminols corresponding to the 1,2-enediol. This rearrangement leads to an aminoketose called an Amadori compound. Amadori products are only intermediates formed in the course of the Maillard reaction.

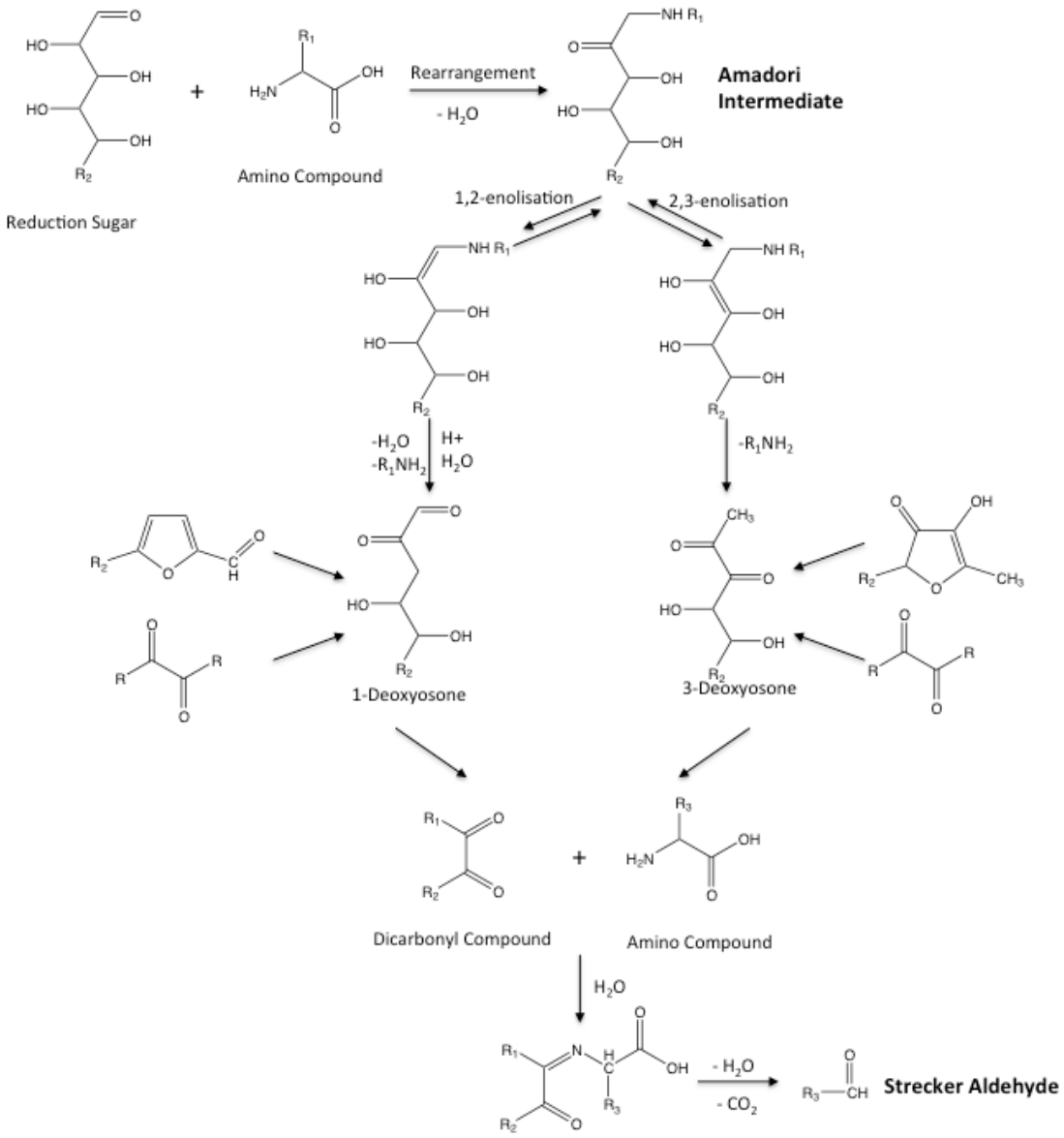

Figure 1.3.1. Maillard reaction squeme, adapted from Hodge (1953).

Unlike the acidic and alkaline sugar degradation reactions, the Amadori compounds are degraded via 1,2enolisation via 3-deoxy-1,2-deulose or 2,2enolisation via 1-deoxy-2,3-dicarbonyls. The best-known compounds of 3-deoxyosone degradation are HMF and furfural. Besides the Maillard reaction they can be originated from the dehydration of sugars in acidic medium and caramelization (Antonelli *et al.*, 2004) and can be present in higher concentration in wines aged in oak barrels, because they are formed during toasting of oak and can be released during wine aging. These compounds are also reported in different types of wines namely in fortified wines (Cutzach *et al.*, 1999) (Ho *et al.*, 1999). The HMF can react with acetic acid and originate 5-(acetoxymethyl) furfural and with ethanol to form 5-(ethoxymethyl) furfural.

The second step of reaction, Strecker degradation is the most important in relation to flavour formation; in this step the α-dicarbonyls compounds, like the deoxyosones react with the aminoacids. This reaction leads to the formation of aldehydes (Strecker aldehydes), CO_2 and α-aminoketones on

oxidative decarboxylation of the α-aminoacids. The aldehydes, which have one C-atom less than the amino acids, possess a considerable aroma potential, depending on the amino acid degraded.

Most of the odour products from the Maillard reaction are sulphur-, oxygen-, and nitrogen-containing heterocycles. In wine are many studies related to maillard aroma, Pipris-Nicolau, *et al.* (2000) studied the reaction between four α-dicarbonyls compounds (diacetyl, pentan-2,3-dione, glyoxal and methylglyoxal) and aminoacids present in wines the principal odours detected dependent of the aminoacid are present in Table 1.3.1..

Table 1.3.1. Identified reaction products and principal odours detected in synthetic amino acid solutions in the presence of α-dicarbonyl compounds, (Pipris-Nicolau*, et al*., 2000).

Substrates	Reaction Products
Cysteine	Pyrazines, methylpyrazines, methylthiazoles, acetylthiazoles, acetylthiazolines, acetylthiazolidines, trimethyloxazole, and dimethylethyloxazoles
Methionine	Methanethiol, dimethyl disulfide, methional
Valine	2-methylpropanal
Leucine	3-methylbutanal
Isoleucine	2-methylbutanal
Phenylalanine	Benzaldehyde, phenylacetaldeyde

Methional and phenylacetaldeyde are related to the typical aroma of oxidative, spoiled white wine (Silva Ferreira *et al.*, 2002). Besides the Maillard reaction these two compounds can be formed from the direct oxidation of the respective alcohol (Marchand *et al.*, 2000) or by the reaction of an o-quinone with the aminoacid (Rizzi, 2006).

This leads to another mechanism the chemical oxidation. Oxygen contained in the air can be dissolved into the wine during different manipulations; in general, any rapid oxygenation (during wine-making) will generate a deviation, while slow oxygenation (aging) will allow the wine to develop in complexity (Kilmartin, 2009).

The principal compounds responsible for oxygen consumption are polyphenols, therefore explaining the different oxidation capacities between white and red wine. These compounds are usually divided into flavonoids and non-flavonoid compounds. The flavonoids have a more-or-less intense yellow pigments and it structure are characterized by two benzene cycles bonded by an oxygenated heterocycle derived either from the 2-phenyl chromone nucleous (flavones and flavonols) or the 2-phenyl chromanone nucleus (flavanones and flavanols). The most common wine flavonoid compounds are flavonols, flavan-3-ols and anthocyanins. The non-flavonoid compounds are mainly derivatives of benzoic acid and of cinnamic acid. Polyphenols containing a 1,2-diphenol (an o-catechol moiety) or a 1,2,3-triphenol (a galloyl group) are the most easily oxidized (Kilmartin, 2009).

Chemical oxidation is similar to enzymatic, except that a metal ion is required in place of the enzyme (Danilewicz, 2003). The direct interaction between molecular oxygen and organic molecules is "spin forbidden" due to the arrangement of electrons in the oxygen molecule. Conversion of molecular oxygen from its lowest energy state to a higher energy state is required before a reaction can occur. As an *o-dihydroxyphenol* reacts with O_2 to produce its quinone, only one atom of oxygen is needed and the second appears as hydrogen peroxide (Singleton Vernon and Cilliers Johannes, 1995). Under acidic conditions, this hydrogen peroxide oxidizes additional substances, including ethanol, which would otherwise not readily autooxidise.

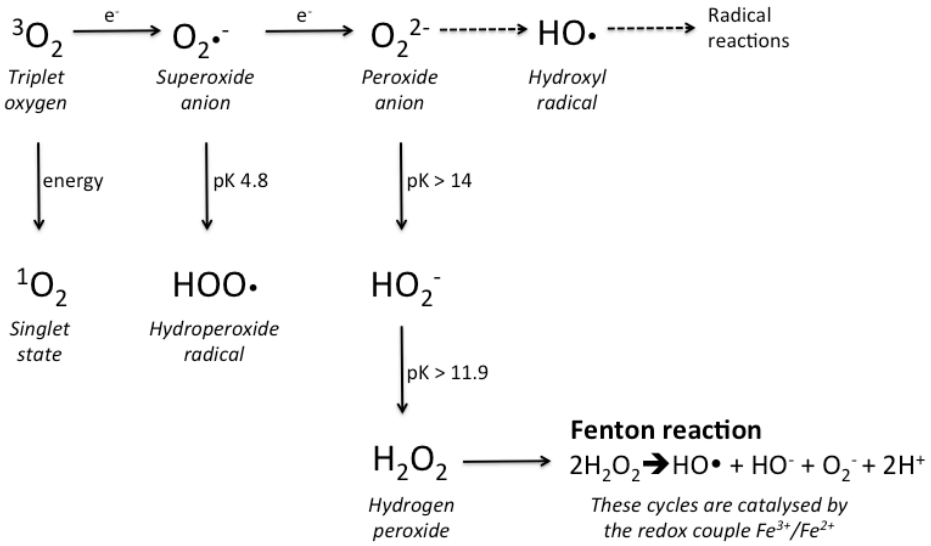

Figure 1.3.2. Oxygen reactive species.

Due to the poor direct reactivity of oxygen with organic molecules, the oxidising potential of molecular oxygen is harnessed by the generation of reactive oxygen species (ROS) that constitute a reductive ladder of oxidation (Figure 1.3.2.). The initial transfer of an electron leads to the formation of superoxide ion, $O_2^{\bullet -}$, which at wine pH exists as the hydroperoxide radical (OOH•). This step requires a catalyst, presumably a transition state metal such as iron (Waterhouse and Laurie, 2006). The transfer of a second electron would then produce a peroxide (H_2O_2) being the specific form generated in wine. The next reduction creates an oxidative agent even more reactive than the previous one, namely the hydroxyl radical (OH•), via the Fenton reaction between hydrogen peroxide and ferrous iron salts. The last reaction produces water, the final product of oxygen reduction (Danilewicz, 2003).
The aldehydes produced by coupled polyphenol oxidation are very important in wine aging. They provide links between various flavonoid polyphenols to produce polymeric pigments that explain the change in red wine hue with age (Alcalde-Eon *et al.*, 2006).

Many compounds are formed during this process (du Toit *et al.*, 2006) (Escudero *et al.*, 2002) (Silva Ferreira*, et al.*, 2003). Aldehydes and mostly acetaldehyde, resulting essentially from ethanol oxidation, are important intermediates in the chemical transformations occurring in red wine, leading to colour and flavour changes. When an aldehyde reacts with an alcohol an acetal is formed. Two groups of acetals can be formed: by the acetalization between an aldehyde and a monoalcohol or by an

aldehyde and a polyol. This second reaction leads to the formation of cyclic acetals like 1,3-dioxane and 1,3-dioxolane. The most important acetal found in wine is diethoxyethane, this compound results from a reaction between ethanal and ethyl alcohol.

Acetaldehyde can also react with glycerol under acid conditions leads to the formation of four isomers: *cis*- and *trans*-5-hydroxy-2-methyl-1,3-dioxane and *cis*- and *trans*-4-hydroxymethyl-2-methyl-1,3-dioxolane (Silva Ferreira *et al.*, 2002). These four compounds increase during aging.

Other molecules were found related to oxidation: 3-(methylthio) propionaldehyde and 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN). However the wine oxidation can lead to the removal of existing aroma compounds, particularly those containing sulphur, they can react with quinones by a Michael-type addition reaction, resulting in the loss of wine varietal character (Nikolantonaki *et al.*, 2012).

Sotolon (3-hydroxy-4,5-dimethyl-2(5H)-furanone) is a volatile compound with an intense odour of curry. It was identified for the first time in 1967 by Sulser *et al.* (1967) in vegetable protein hydrolisates with an aroma reminiscent of walnuts.

Until today this compound were identified in many types of wines. Jura wines (Dubois *et al.*, 1976), Botrytized wines (Masuda *et al.*, 1984), Port wines (Silva Ferreira *et al.*, 2003), Madeira wines (Câmara *et al.*, 2004) and in dry white wines (Lavigne *et al.*, 2008).

In Port wines sotolon was recognized as the key molecule in the "perceived age" of barrel storage Port wine and consequently in the aroma quality of the product, it concentration can rise from a few dozen µg/L in a young wine to 1 mg/L in wines older than 50 years. The odour threshold value was estimated at 19 µg/L (Ferreira *et al.*, 2005).

The formation mechanism of this compound is not totally fully understood. Maillard reaction was testes to be a potential "via" of formation, were tested binary mixtures of cysteine, and three sugars, ribose (Hofmann and Schieberle, 1995), glucose and rhamnose (Hofmann and Schieberle, 1997). According to Hoffman and Schieberle (1995) heating an aqueous solution (145ºC, 20 min pH, 5.0) containing hydroxyacetaldehyde and butane-2,3-dione (diacetyl) generated a significant amount of sotolon. Dubois *et al.* (1976) detected sotolon after heating (100ºC, 24h) a dilute solution (HCL, 6N) containing pyruvic acid and 2-ketobutyric acid. Silva Ferreira *et al.* (2003) observed too a higher correlation between age and sotolon concentration with 5-methylfurfural. Câmara *et al.* (2004) related in Madeira wine a higher correlation of other furanic derivatives, HMF and furfural with sotolon concentration. This suggests a connection between sotolon and Maillard reaction.

On other hand vary authors connect the sotolon formation with oxidation (Silva Ferreira*, et al.*, 2003), (Escudero *et al.*, 2011) (Cutzach*, et al.*, 1999), (Silva Ferreira*, et al.*, 2003), (Pham *et al.*, 1995).

Cutzach suggest that sotolon can be produced by an aldol condensation between glutamic and pyruvic acid in fortified wines (Cutzach*, et al.*, 1999). Pisarnitzky, *et al.* (1987) relates that higher levels of sotolon on sherry and Madeira wines are from a strict oxidative mechanism, based on the peroxidation of acetaldehyde. Konig, *et al.* (1999) reported that sotolon are formed by the oxidative degradation of ascorbic acid in an acidic medium containing ethanol.

Takahashi, *et al.* (1976) and Cutzach, *et al.* (1998) studied the role of the aldol condensation reaction between 2-ketobutyric acid and acetaldehyde in the sotolon formation in a specific model dilute

solution (high ethanol and reducing sugar levels) during oxidative ageing. However Silva Ferreira*, et al.* (2003) observed that sotolon and ketobutyric acid don't have any correlation contrary to what is observed to acetaldehyde in Port wine. In a forced ageing protocol in white wine when samples were supplemented with oxygen and high temperature to simulate typical oxidation spoiled aroma, sotolon is one of the compounds with highest rate (Silva Ferreira *et al.*, 2003).

Both parameters, oxygen and temperature seems to influence the sotolon concentration, which suggest that this molecule is an hybrid compound, this means that can have origin in a connection between oxidation and maillard reaction.

An example of a similar compound is methional; it can be originate by oxidation of methionol or by Strecker degradation of methionine. It was observed that the main pathway for the methional formation was via Strecker degradation (Silva Ferreira, Guedes de Pinho, Rodrigues, & Hogg, 2002).

1.4. Chemical Kinetic

Quality is a very elusive concept, which depends on many factors. The production management view is to maintain quality during production. In a food technologist perspective, quality is the result of the ability to control chemical, physical and microbiological changes during processing and storage. For this kinetic modeling is gaining increasing interest in different fields of research (Martins *et al.*, 2000).

Multiple reactions taking place during food storage. Some of them are able to produce the compounds for flavor, texture, and nutritional value, others result in spoilage or undesirable, harmful substances. Chemical reactions occur when sufficient energy is brought to the molecules of one or several compounds in proximity of each other, producing collisions that result in breakage or formation of bonds among the atoms in those molecules.

Complex reaction networks are commonly encountered in the chemical process industry. These complex reaction networks are efficiently analysed using kinetic modelling for better understanding of the reaction mechanism.

Kinetic parameters estimation is integral to the analysis of complex reaction networks. It is thus not surprising that kinetic parameter estimation is important in the design, optimization and control of chemical processes. Chemical kinetic studies implies that changes occur in foods can be captured in mathematical models containing characteristic kinetic parameters, such as activation energy and rate constants.

Chemical reaction kinetics deals with the rates of chemical processes. The huge variety of chemical species, types of reactions, and the accompanying potential energy involved means that the timescale over which chemical reactions occur covers many orders of magnitude, from very slow reaction to extremely fast reactions.

A large portion of the field of chemical kinetics can be described by the Arrhenius equation.

$$k = Ae^{\frac{-Ea}{RT}} \quad Eq.\,1.4.1.$$

The Arrhenius equation relates the rate constant k, a measure of the extension of the reaction at that temperature of an elementary reaction to the absolute temperature T; R is the gas constant. The parameter E_a is the activation energy, which constitutes a measure of sensitivity towards temperature, with dimensions of energy per mole, and A is the pre exponential factor, which has the units of k. If k is a first-order rate constant, A has the unit $second^{-1}$, so it is called the frequency factor.

The description implies that A and E_a are temperature independent, an implication that is difficult to test because of small temperature range usually employed in such studies (Connors, 1990).

The process of estimating ambient stability involves estimating the reaction rate at different temperatures, and then extrapolating to the desired temperature (Waterman and Adami, 2005).

However chemical mechanisms usually are cascades of consecutive and parallel reaction steps, which involve many reaction products and intermediates. Multi-response has proved to be a powerful tool in unravelling complex chemical reactions. The multi-response modelling techniques allow us to study a entire mechanism as a whole which allow a more detailed and more informative about the reaction mechanism analysis, since the reactants degradation is analysed simultaneously with the intermediates and end products formation.

1.5. Metabolomics

Presently in science the world of “omics” are very important and refers to a discipline of science and engineering for analysing the interactions of biological information in various fields. Thus the different “omic” technologies are inter-related in that: transcriptomics (gene expression) assesses changes in the transcriptome (the entire complement of RNA produced by DNA transcription of a cell, tissue or organism at a particular time point), proteomics studies the total protein complement (the proteome) and metabolomics studies the complement of small molecules.

Metabolomics is defined as the study of “as-many-small-metabolites-as-possible” in a system (Cevallos-Cevallos *et al.*, 2009). The main objective of this study field is collect as many information as possible in objects, and find interactions between them, after this engineering the networks and the objects to understand and manipulate the regulatory mechanism. Metabolomic studies can be divided into two groups: target and no target analysis. Target analysis refers to analytical projects wherein the goal is to quantify a relatively small number of specific analytes of interest, so the user can ignore the remaining components of complex samples. Non-target techniques aim to comprehensively analyse entire complex chromatograms to discover important analytes or chemical fingerprints while requiring few user inputs and minimizing the need for prior information about the samples.

In agricultural and food products, typical quality parameters are sensory properties, shelf life, safety, health, nutritional value, and crop yield per area and disease resistance. It is known that these parameters are importantly determined by the metabolites in the crops and food products.

Metabolomics in a food quality/authenticity perspective allows an identification of several food constituents to asses both food adulteration and food quality. Can be useful in the detection of adulterated on contaminated food products. Similar chemical composition characteristics can also be

exploited to distinguish between food products with desirable characteristics that cannot otherwise be detected by flavour, aroma or colour. Food quality also impacts food quality control. In fact, metabolomic techniques may find their greatest use in the food industry in monitoring quality control.

In the case of wines, meticulous controls are required to assess factors (e.g. geographical origins, grape varieties, vintages and oenological practices) as a way of evaluating quality and detecting fraudulent adulterations (Arvanitoyannis *et al.*, 1999). The parameters influencing quality cannot be described in a simple manner from given individual compounds of the sample but they result from complex combinations of hundreds of compounds. Metabolomics are used as an important tool to study complex systems and a huge number of data. Table 1.5.1. shows some metabolomic studies in wine published in the last three years.

Separation and detection of the metabolites have been considered the key steps in metabolite profiling for characterization studies. Separation techniques such as liquid chromatography (LC), in its high performance (HPLC) or ultra performance (UPLC) forms, gas chromatography (GC), capillary electrophoresis (CE) are coupled to detection techniques such as mass spectrometry (MS), nuclear magnetic resonance (NMR) and others (Table 1.5.1.) are used. An emerging trend in wine analysis relies on MS for describing complex aroma properties associated with volatile components, which comprise hundreds of substances.

However important analyte information and chemical variations in chromatographic data are often obscured by irrelevant variations from, for example, noise and background interferences. Preprocessing of the raw data reduces chemically irrelevant variations with the goal of improving accuracy and precision of qualitative and quantitative analyses. Data should be aligned before comparison to correct instrumental deviations on retention/migration times. Pre-processing tecniques such as alignment has been show to drastically improve the performance of multivariate analysis techniques (MVA) (Son *et al.*, 2008).

Chemometrics provides a useful tool for the characterisation of wine, evaluating several parameters. In contrast to the use of single-element concentrations, multivariate statistical methods allow verifying the contribution of each variable to the model, and its capacity to discriminate one category from another.

Data has a huge number of variables and PCA and PLS are MVA visualization techniques that allow for the interpretation of multidimensional data sets. When multivariate analysis involves large datasets, variable selection processes play an important role because they eliminate the less significant or non-informative variables. The overall aim of any variable selection technique is to capture variables from the original dataset that are most specifically related to the problem of interest and to exclude those variables that are affected by other sources of variation.

PCA is a non-supervised technique that decomposes the original variables of a data set into two matrices: the score and the loading matrices. The scores matrix contains information about the samples, which are described in terms of their projection onto the principal components. The loading matrix contains information about the variables which are also described in terms of their projection

onto the principal components. The loadings can also be interpreted as the contribution of the variables for the observed scores distribution.

PLS is a supervised technique that allows sample discrimination by reduction of dimensionality while maximizing correlation between variables (Wold *et al.*, 2001).

Characterization of wines based on analytical methods combined with chemometric treatment of data provides excellent robustness and efficiency. Physico-chemical parameters, concentrations of wine components and instrumental signals can be used as multivariate data. Because of the multi parametric nature of wines, chemometrics makes interpretation of data more feasible in samples classification and biomarkers selection.

Table 1.5.1. Recent methods for the characterization of wines.

Samples	Characterization studies	Instrumental techniques	Data analysed	Chemometric methods	Ref.
***V.labrusca* wines**	Compounds important on wine flavour	ESI-MS	Acid and phenolic compounds	PCA	(Biasoto *et al.*, 2010)
Albariño wines	Geographical origin	GC-FID, sensory descriptive analysis	36 volatile compounds	PCA, PLSR	(Vilanova *et al.*, 2010)
Aglianico wines	"Terroir" influence	^{1}H-NMR		PCA, PLS-DA, HCA	(Mazzei *et al.*, 2010)
Madeira wine	Ageing	GC-MS	37 volatile compounds	PCA	(Pereira *et al.*, 2011)
Wines	Sensory attributes	^{1}H-NMR		PCA, PLS-DA	(Rochfort *et al.*, 2010)
Grenache red wines	Oxygen impact	HPLC-DAD-MS	Phenolic compounds	PCA	(Wirth *et al.*, 2010)

Samples	Characterization studies	Instrumental techniques	Data analysed	Chemometric methods	Ref
Italian wines (Barbera d'Alba and Dolcetto d'Alba)	Wine authentication	NIR, UV-vis spectrometry, HS-MS-e-nose		PCA, LDA	(Casale *et al.*, 2010)
Italian wines (Barbera d'Alba and Dolcetto d'Alba)	Wine authentication	NIR, UV-vis spectrometry, HS-MS-e-nose		PCA, LDA	(Casale*, et al.*, 2010)
Amarone wine	Correlation between metabolic content and ageing.	^{1}H-NMR		PCA, PLS-DA	(Consonni *et al.*, 2011)
Wines	Classification of smoke tainted wines.	MIR spectroscopy, GC-MS		PCA, LDA	(Fudge *et al.*, 2012)
Valpolicella, Amarone and Recioto wines	Differentiate between brands.	HS-SPME-GC-MS	54 volatile compounds	PCA, HCA, CTA	(Dall'Asta *et al.*, 2011)

Samples	Characterization studies	Instrumental techniques	Data analysed	Chemometric methods	Ref
Godello wines	Correlation between instrumental and sensorial analyses.	GC-MS	37 volatile compounds	PCA, PLS	(González Álvarez *et al.*, 2011)
Shiraz wines	Geographic origin	UV-vis, NIR and MIR spectroscopy		PCA, LDA, SIMCA	(Riovanto *et al.*, 2011)
Chardonnay wines	Effects of temperature and packaging type on the chemical properties	Colour analysis, HS-SPME-GC-MS	Colour, 30 volatile compounds	PCA, PLS	(Hopfer *et al.*, 2012)
Lambrusco wines	Authenticity	^{1}H-NMR		PCA, PLS-DA	(Papotti *et al.*, 2012)
Spanish region wines	Wine characterization	CZE (capillary zone electrophorese)	20 polyphenols	PCA	(Franquet-Griell *et al.*, 2012)

Samples	Characterization studies	Instrumental techniques	Data analysed	Chemometric methods	Ref
Spanish white wines	Differentiate wines from different brands.	Coupled plasma optical emission spectrometry	Metals	PCA, support vector machine classification.	(Jurado *et al.*, 2012)
Sauvignon blanc	Effects of different vinification techniques	^{1}H-NMR		PCA	(Baiano *et al.*, 2012)
Sauvignon blanc	Evaluation of key odorants	GC-O GC-MS	Volatile compounds	PCA	(Benkwitz *et al.*, 2012)
Rioja wines	Geographical origin	^{1}H-NMR	31 compounds	PCA, ECVA (extended canonical variate analysis)	(Lopez-Rituerto *et al.*, 2012)
Chilean wines	Varietal discrimination	ESI-FT-MS		PCA, LDA	(Villagra *et al.*, 2012)

Samples	Characterization studies	Instrumental techniques	Data analysed	Chemometric methods	Ref
Garnacha Tintorera wines	Correlation between sensorial and chemicals profiles.	GC-MS Aromatic profile	70 volatile compounds	PLS-2	(Noguerol-Pato *et al.*, 2012)
Spanish appellations wines	Discrimination of wines based on oenological practices.	UV-vis	Phenolic compounds	PCA, PLS1-DA, PLS2-DA	(Serrano-Lourido *et al.*, 2012)

PCA – principal component analysis; PLS – partial least squares; DA –discriminant analysis; LDA – linear discriminant analysis; HCA – Hierarquical Cluster Analysis; CRT – Classification Tree Analysis; SIMCA – Soft Independent Modelling of Class Analogy; GC- gas chromatography; FID –flame ionization detector; MS –mass spectrometry; H-NMR – proton nuclear magnetic resonance; HPLC- high pressure liquid chromatography; DAD-diode array detector; NIR – Near-infrared; UV-vis – ultra violet- visible; MIR – mid infrared; HS – headspace; SPME - solid phase micro extraction; O –olfactometry; ESI – electrospray ionization; FT-Fourier transform.

2. Materials and Methods

2.1. Samples

Sample Group 1 (GC-MS)

Thirty-four Port wines between the ages of 1 and 129 years were used for the construction of the database. Wines were made according to standard traditional Port winemaking procedures and all the wines used for the database creation were aged in "pipas" (550 L spent-oak barrels). For the kinetic study (isothermal protocol), 16 litters of Port wine with pH=3.4, 2.5 mg/L dissolved oxygen, a free SO_2 level of 17 mg/L, 105 g/L of reducing sugars and 20.5% alcohol and produced in the year of the experiment (without any oak contact) were used. Samples were provided by Symington.

Sample Group 2 (GC-FID)

For GC-FID analyses 37 samples were used in this study with ages between 2 and 60 years. All wines were matured in oak barrels. The wines were made following standard traditional winemaking procedures for Port wine and have been provided by IVDP (Instituto dos Vinho do Douro e Porto).

Experimental Set-up

The determination of the kinetics of aging Port wine was done under differing constant storage temperatures and oxygen concentrations (Figure 2.1.1.). Wines were stored at 20, 30, 35 and 40ºC in temperature-controlled incubators. The oxygen treatments included 0 (F1), 3 (F2), 5 (F3) and 10 (F4) saturations. For each combination of oxygen/temperature, used glass vessels filled with 500 mL of wine were used. Oxygen saturation was obtained by stirring the sample vigorously for about 1 hour until an oxygen concentration of about 8-9 mg/L was reached. This was performed in a laminar flow chamber under UV light to prevent microbial contamination. The F1 group was never supplemented with O_2. F2 were saturated at sample day 14, 35 and 56. F3 were saturated in the beginning of the experiment and in sample day 14, 28, 42 and 56. F4 were saturated with O_2 at all sampling day points (0,14, 21, 28, 35, 42, 49, 56 and 63). This forced aging experimental protocol was performed in duplicate for practical reason. Not all samples were analyzed by GC-MS on the replicate trial and were used as a crosscheck procedure.

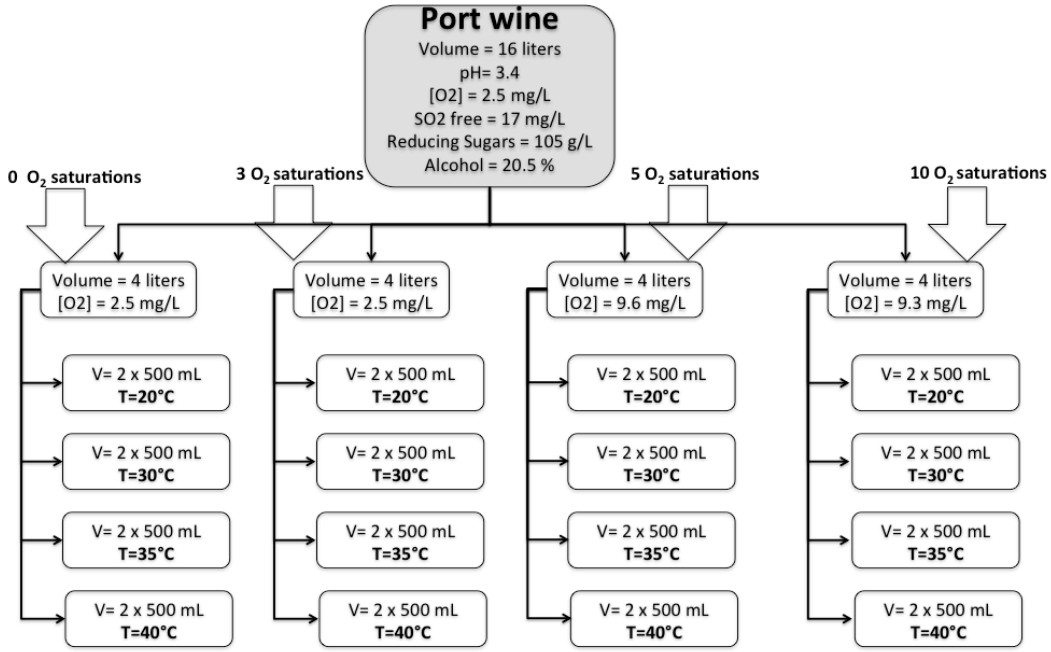

Figure 2.1.1. Experimental Design

2.2. Analytical procedure.

2.2.1. Dissolved Oxygen Measures

The oxygen concentration was measured using a Fibox 3 LCD fiber optic oxygen transmitter, a polymer optical fiber and planar oxygen sensitive spots (5 mm sensor spots PSt3), from PreSens Precision Sensing GmbH (Germany). The sensor was positioned in the center of the 500 mL glass vessel and remained in contact with the wine at all times. Oxygen levels were measured at 9 intervals (weekly) during the 63 day isothermal storage.

2.2.2. Volatiles extraction – GC-MS Analysis

The extraction procedure was based on the method described previously by Silva Ferreira*, et al.,* (2003). To describe it briefly, 50 mL sample of Port is spiked with 50 µL of 3-octanol in a hydro alcoholic solution (427 mg/L) as the internal standard. Anhydrous sodium sulphate (5 g) is added to increase ionic strength after which the wine is extracted twice with 5 mL dichloromethane. The two organic phases obtained are combined and dried over anhydrous sodium sulphate. Two milliliters of this organic extract are concentrated to 0.4 mL under a constant nitrogen stream.

Extracts were analysed using a Varian 450 gas chromatograph, equipped with a mass spectral detector, Varian 240-MS and the Saturn GC-MS workstation software version 5.51.The column used was Stabilwax-DA (60m x 0.25mm x 0.25µm) fused silica (Restek, USA). The injector port was heated to 220 °C. The injection volume was 1 µL in splitless mode and the split vent was opened after 30

seconds. The carrier gas was helium C-60 (Gasin, Portugal), at a constant flow of 1 mL/min. The oven temperature was 40 °C (for 1 min), then increased at 2 °C/min intervals to 220 °C, and held there for 20 min. All mass spectra were acquired in the electron impact (EI) mode (ionization energy, 70 eV; source temperature, 180°C). The ion trap temperatures were 230, 45 and 170 °C respectively. The mass range was m/z 33-350, with a scan rate of 6 scans/s in full-scan mode. The emission current was 50 µA and the electron multiplier was set in relative mode to autotune procedure. The maximum ionization time was 25000 µs, with an ionization storage level of m/z 35.

Compound identification was achieved by comparing retention times and mass spectra obtained from a sample containing pure, authentic standards. Kovats indices were calculated (van Den Dool & Dec. Kratz, 1963) and also used as validation when compared to mass spectra as reported in the NIST 05 MS Library Database. Two injections of dichloromethane extracts was carried out. Compound quantification was performed based on standard calibration curves.

2.2.3. Volatiles Extraction – GC-FID Analysis

A liquid-liquid extraction was performed to extract the volatile fraction from each sample. The procedure used was as follows: 5 g of anhydrous sodium sulphate and 50 µL of internal standard (3-octanol) were added to 50 mL of sample and were extracted twice with 5 mL of diclorometh ane using a magnetic stir bar for 5 minutes per extraction and 2 mL of the resulting organic phase were concentrated under a nitrogen stream 4 times. The extract was then analysed by GC (Agilent 5980, USA) with FID detection. Two microliters of the extract were injected. Chromatographic conditions were the following; column BP-21 (50 m x 0,25 mm x 0,25 μm) fused silica (SGE, Portugal); hydrogen (5.0, Air-liquide, Portugal); 1.2 mL/min flow rate; injector temperature, 220ºC; oven temperature, 40 ºC for 1 min programmed at a rate of 2ºC/min to 220 ºC, maintained during 30 min; splitless time, 0.5 min; split flow, 30 mL/min.

In order to facilitate identification, the Kovat's index for each peak was calculated as described by Van den Dool and Kratz (1963). This determination was performed on polar phase columns, BP21 (50 m x 0.25 mm x 0.25 μm).

2.2.4. Amino Acids Analysis.

Twenty one amino acids were analysed in the Port wine samples: aspartic acid, glutamic acid, cysteine, asparagine, histidine, serine, glycine, arginine, threonine, alanine, g-aminobutyric acid, tyrosine, ethanolamine, valine, methionine, tryptophan, phenylalanine, isoleucine, leucine, ornithine and lysine. The methodology used was that described by Pipris-Nicolau *et al.* (2001).

2.2.5. Ethanal Analysis

The samples were analysed by solid phase micro extraction (SPME). To a 20ml headspace vial was added a 5ml sample and 50 μL of 4-methyl-2-pentanol (1 g/L) as internal standard. To this was added 0.5g of sodium sulphate. The program consists of inserting the fiber into the headspace for 20min at 40ºC with agitation, then the fiber was transfer to the injector for desorption at 220ºC for 5min in a Varian 3900 GC gas chromatograph equipped with a CP-1177 Split/Splitless injector and a Flame Ionization Detector (FID) at 220ºC. The column was a CP-WAX 57CB (25*0.25mm*0.2μm) from Varian. The injector port and the detector were heated to 220°C. The split vent was opened after 30s. The oven temperature was 40°C (for 5 minutes), then increased at 3°C/min to 67°C, and then increased at 15°C/min to 200°C (holding time of 1.0 minutes). The carrier gas was Helium C-60 (Gasin, Portugal), at 2 mL/min, constant flow.

2.3. Kinetic Modelling

2.3.1. Regression Analysis

Kinetics studies were done using R 2.13.0 for Macintosh.

Non-linear regression for model fitting was performed on all data, using the one-step methodology (Martins *et al.*, 2008) by maximizing the likelihood function for all temperatures and using bootstrap sampling to estimate the Prediction Residuals Sum of Squares (PRESS) criterion (Hastie and Tibshirami, 1996), (Bates and Watts, 1998). The semi-studentised residuals were also inspected for outliers and randomness, and also being tested for normality. The lack of fit test was performed to determine the adequacy of the regression model, and the studentised effect (t ratio) ($\beta i / s\beta i$) was studied at a 5% confidence level (Neter *et al.*, 1996), (Martins and Silva, 2004).

The general rate law for compound formation is:

$$r = \frac{d\,[compound]}{dt} = kobs\,[compound] \quad \text{(Eq. 2.3.1.1.)}$$

The most common kinetic models reported in literature to describe the kinetics of compound formation are zero, first or second order reaction models.

$C = C_0 + kt$ (zero-order) (Eq. 2.3.1.2)

$C=C_0 . \exp(kt)$ (first-order) (Eq. 2.3.1.3.)

$1/C = 1/C_0 + kt$ (second-order) (Eq. 2.3.1.4.)

where C_0 is the initial compound concentration.

Oxygen related degradation occurs via multiple pathways and/or reactions, but it can be considered that O_2 is depleted by the first-order reaction kinetics, where its apparent kinetic rate is an average value of all oxidation reactions that occurs in Port wine during storage at any given temperature.

$[O_2]$ (t) = $[O_2]_0$. exp (-kapp.t) (Eq. 2.3.1.5.)

where $[O_2]_0$ is the initial oxygen concentration (mg/L) and k_{app} (day^{-1}) is the apparent kinetic rate at the given time, t in days.
Therefore k_{app} may follow the Arrhenius behavior with temperature.

$$k_{app} = k_{ref} \cdot \exp\left[-\frac{Ea}{R}\left(\frac{1}{T}-\frac{1}{T_{ref}}\right).t\right] \quad \text{(Eq. 2.3.1.6.)}$$

where k_{ref} (day^{-1}) is the kinetic rate at the reference temperature T_{ref} (K) and Ea represents the Arrhenius activation energy ($J.mol^{-1}$), and R is the universal gas constant ($J.mol^{-1}.K^{-1}$).
At any temperature, it is possible to assume that sotolon formation is proportional to the oxygen consumption.

$$\frac{d[Sot]}{dt} \alpha \frac{d[O_2]}{dt} \quad (Eq.\ 2.3.1.7.)$$

where [Sot] (µg/L) is the sotolon concentration, $[O_2]$ (mg/L) the oxygen concentration and t the storage time (days).
Taking this into consideration it is possible to derive the following mass balance:

$[Sot]_0 + b\,[O_2]_0 \rightarrow [Sot]_t + b\,[O_2]_t$ (Eq. 2.3.1.8.)

where b is the proportional coefficient for O_2 consumption vs. sotolon formation.
This balance makes it possible to derive the following sotolon formation kinetics:

$[Sot]_t = [Sot]_0 + b\,[O_2]_0\,[1-\exp(-k_{app}.t)]$ (Eq. 2.3.1.9.)

However, for practical reasons in Port wine production, it is easier to rather compute the consumed oxygen. By using the same mass balance presented in equation 2.3.1.8, it is possible to find a direct theoretical relationship between the consumed oxygen and the compound formation, which can express its concentration as a function of the consumed O_2.

$[Sot]_t = [Sot]_0 + f(\text{ Consumed } O_2)$ (Eq. 2.3.1.10.)

Such behavior is possible to be traduced empirically into first-order kinetics, such as:

$$[Sot]_t = [Sot]_0 + [O_2]_{consumed} \cdot k_{ref} \cdot \exp\left[-\frac{Ea}{R}\left(\frac{1}{T}-\frac{1}{T_{ref}}\right).t\right] \quad \text{(Eq. 2.3.1.11.)}$$

where the time-temperature integral must be computed during regression analysis (Arabshahi and Lund, 1985), (Hastie and Tibshirani, 1996), (Ricardo and James, 2006).

Therefore, by monitoring the oxygen consumption and temperature during storage (aging), it is possible to estimate the compound concentration and predict it under new storage conditions.
The remainder of the studied compounds (*cis* and *trans* dioxanes, furfural, 5MF) and HMF also tend to follow first order reversible kinetics (Baig and Rehman, 2006).

$$C(t) = C_{eq} - (C_{eq} - C_0) \exp\left[-\frac{Ea}{R}.\left(\frac{1}{T} - \frac{1}{Tref}\right).t\right] \quad \text{(Eq. 2.3.1.12.)}$$

where C_{eq} is the final concentration of these compound found in Port wines older than 25 years.

2.3.2. Feature Space Analysis

For the construction of the Port wine database, 37 Port wines (ages ranging from 0 - 129 years) were analyzed according to the method as explained above. Ninety volatile compounds (alcohols, esters, acids, sulfur compounds, norisoprenoides and acetals) were quantified and used for the construction of the database.

The discriminant feature space was determined by performing singular value decomposition on scaled compositional data. Relevant compounds, capable of discriminating the wines, were obtained through the singular values above the first singular value of the randomized by sample compositional table (Nomikos and MacGregor, 1994). Furthermore, Q-statistics (Qα at 5% significance) (Westerhuis *et al.*, 2000), (Conlin *et al.*, 2000) and T-hotling statistics (TH,α at 5% significance) (Kindratenko *et al.*, 1997), (Joe Qin, 2003) were used to measure the distance to the average composition. The accelerated aging of Port wines in this study were then projected into the feature space and analyzed for feature proximity by the k-nn (k's nearest neighbor) algorithm and the classification probability of each accelerated wine was assessed by bootstrapping (Hahn and Shapiro, 1997).

2.3.3. Monte Carlo Simulation

The Monte Carlo (MC) method is widely used to determine the extent of a deterministic effect, given a number of stochastic inputs (Gardiner, 1997). We used the MC simulation to estimate the uncertainty effect on the amount of oxygen that permeates through a cork stopper or barrel at a given period of time in order to estimate its effect on sotolon concentrations during simulated storage. The following combinations were considered for the simulated wines: i) Temperatures of 5, 10, 15 and 20°C (typical wine cellars temperatures including two extreme values of a low (5ºC) and a high (20ºC) temperature); ii) a barrel permeability of around 10 ± 2 mg/L per year (AWRI, 2004); iii) a cork O_2 permeability of approximately 1.5 ± 0.75 mg/L per year (Lopes *et al.*, 2006), (Lopes *et al.*, 2009).Therefore the distribution of the chemical composition computed assumes that all permeated O_2 are consumed through chemical reactions in the wine. The MC results were afterwards compared to the Port wine database.

The Monte Carlo (MC) algorithm uses the derived kinetics to estimate the chemical composition in terms of: i) oxygen; ii) sotolon; iii) furfural; iv) 5MF; v) HMF; and vi) *cis* and *trans* dioxanes. As sotolon formation is dependent on the O_2 permeability and consumption, the MC method was implemented as follows:

Step 1: generate MC samples based on the statistical distribution of O_2 permeability and kinetic parameters; Step 2: for each MC case study: a) calculate $[O_2]$; b) calculate the sotolon formation based on temperature and content of O_2 - in this case we consider that inside barrels or bottles the daily O_2 intake is well below the saturation point of 9 mg/L and therefore all O_2 taken in (IO_2) is either dissolved or consumed and computed by: $dO_2\ dt + k[O_2] - IO_2 = 0$ (pseudo function determine O_2 in Algorithm 1); c) estimate the remainder of the studied chemical parameters (furfural, HMF, 5MF, *cis* and *trans* dioxane); Step 3: Study the MC results by projecting the results into the Port wine database feature space and classify them by Hierarchical Cluster Analysis (HCA).

2.4. Data pre-processing.

The ASCII file of GC-FID chromatographic data obtained from each sample was extracted and a matrix created containing all of the chromatograms. The intensities were normalized by dividing each value by the intensity of the internal standard (3-octanol). The raw dataset (GC-FID) was then imported into The Unscrambler®X 10.1 (Camo, Sweden), where the first stage of the alignment of chromatograms was performed using Correlation Optimized Warping (GC-FID-COW). This algorithm aligns chromatograms by means of sectional linear stretching and compression, which shifts the peaks of one chromatogram to correlate with those of the other chromatograms in the dataset (Gong *et al.*, 2004). The saturated peaks were then removed and the baseline corrected (GC-FID-COW-saturated removed and baseline correction). The resulting matrix (GC-FID-X) was then used for multivariate data analysis as described in the statistical analysis section. The same data pre-processing were used for GC-MS data.

2.5. Statistical Analysis – Multivariate Analysis

The GC-FID data was analysed with PCA and PLS-R using either Qlucore (Lund, Sweden) or SIMCA-P+ 12.0.1 (Umetrics, Norway). PCA shows similarities between samples projected on a plane and makes it possible to determine which variables determine these similarities and in what way. PLS is used to extract factors related to one or more response values. PLS validation was performed by cross-validation method.

The data for the wines, which were stored for 63 days at four different temperatures and 4 oxygen regimens, were submitted for two-way analysis of variance (ANOVA), using 'temperature' and 'oxygen' conditions as fixed factors. The statistical significance was set at the conventional 5% level. Differences between two variables were tested using student's t-test ($p<0.05$).

2.6. Kinetic Network Reconstruction.

In order to attempt to reconstruct the underlying kinetic network, the fingerprint needed to be further compressed to a single value for each putative molecule detected by GC-FID. Thus, each chromatographic peak needed to be replaced with a single value for the intensity and retention-time, at the apex of each peak and therefore a more refined alignment procedure was required. This was achieved as follows: An "average chromatogram" was created by taking the mean of the values at each elution point in the GC-FID-X matrix. The average chromatogram and the sample chromatograms were smoothed with the Savitzky-Golay method (settings: left=15, right=15, polynomial degree=0) (Savitzky and Golay, 1964) as implemented in The Unscrambler ®X 10.1 (Camo, Sweden). The wavelet method of Du et al. (Du *et al.*, 2006), which was originally developed for peak calling in peptide mass-spec data, was adapted for finding peak centres in chromatographic data and a Mexican hat wavelet used to determine the location of all of the peaks in the average and sample chromatograms. A custom built Perl program was integrated with the R-based wavelet method to achieve this. The distances between the locations of all of the peaks in each sample chromatogram and the locations of the peaks in the average chromatogram were calculated. It was observed that there was a correlation between peak height and the amount of peak centre shift that occurred across chromatograms and we thus devised a two-step process for aligning sample peaks to those of the average chromatogram. If the (internal-standard-normalized) height of the average peak was greater than 2 and the distance to the nearest sample peak was less than 0.3 minutes then the intensity value of the sample peak was assigned as the sample value at the average peak location. However, if the (internal-standard-normalized) height of the average peak was less than 2 and the distance to the nearest sample peak was less than 0.15 minutes then the intensity value of the sample peak was assigned as the sample value at the average peak retention time. This algorithm was implemented in Perl.

As a result of this process a new matrix was created that contained the retention time of all peaks in the average chromatogram and the intensity values of all of the peak centres from each sample aligned to these average retention times. Thus a vector was created for each peak (presumably representing a compound) across all samples. An all-against-all comparison was done calculating the Pearson correlation between each and every peak vector. As such, one is able to track the increase or decrease of compounds (peaks) during the aging process and determine the correlative relationships amongst them. We applied a Pearson correlation threshold of 0.8 and represented the remaining relationships as a mathematical graph in order to form a correlation network with the nodes representing peaks and the edges weighted with the Pearson correlations between the peak vectors. In order to reconstruct the most likely kinetic network underlying the set of chemical reactions involved in the aging process, a maximum spanning tree was created by transforming the edge weights into inverse correlations (by taking the difference between the number 1 and the absolute correlation values) and the subsequent use of a minimum spanning tree (mst) algorithm (Dijkstra, 1959) on the this inverse correlation network. A minimum spanning tree represents the shortest possible path

through a graph and, as such, selects for the smallest inverse correlation (i.e highest correlation) pairs between all nodes in the network. The resulting networks were visualized in Cytoscape.

3. Results and Discussion

Kinetic studies are important to establish temporal relationships between Port constituents and infer the chemical network of reactions to better understand the connection between several mechanisms. Beyond other compounds sotolon is considered the “driver” of Port quality and is thought to its formation is related to temperature and to the presence of oxygen, therefore, these two parameters are critical concerning Port quality. The fact that both parameters influence this compound to a large extent would suggest that this molecule is an “hybrid compound”, meaning it can have origin in a connection between oxidation and maillard reactions.

This problem has an important consequence for the management of Port storage especially in the case of “Vintage” (bottles) and “Tawny” (barrel) Port styles, where the correct temperature and O_2 permeability are needed in order to attain the desired sensory quality of the aged wine. Although sotolon is the key odorant of Port wine, the quality of the end product is also dependent on other relevant chemicals.

3.1. Oxygen consumption

The oxygen consumption in the Port wines over time follows first-order rate law as shown in Figure 3.1.1.

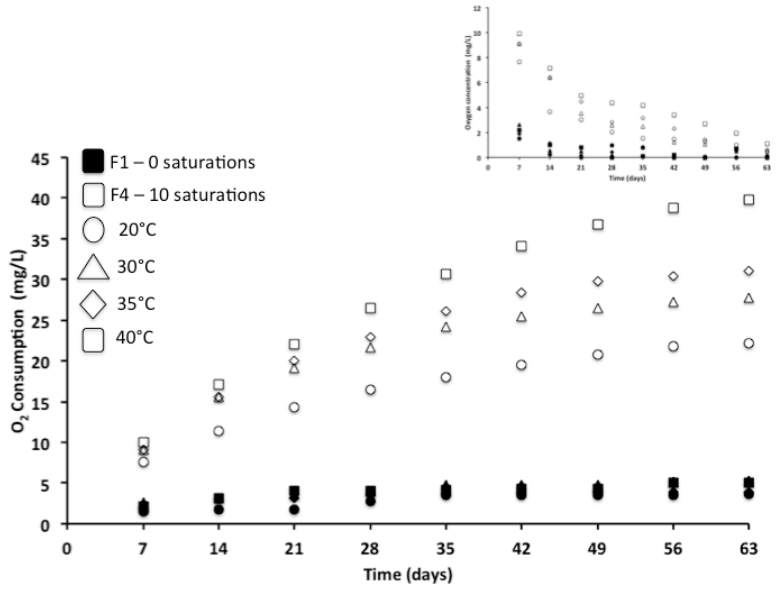

Figure 3.1.1. Oxygen consumption as a function of time at different temperatures and O_2 saturations and oxygen uptake in each week.

In fact, in this study, and based on previous works (Ferreira*, et al.*, 2005) as well as the experimental observations, we assumed that the O_2 consumption and the formation of sotolon and other molecules, the reactions obey a first-order rate law. Only treatments with 0 and 10 saturations are shown in figure 2 to prevent clutter. The fact that oxygen consumption is of first order reaction kinetics has important

implications regarding the effects on the oxygen consumption rate (Figure 3.1.1.). At 10 saturations, significant O_2 consumption rates are observed whereas 0 saturation leads to extremely low rates of O_2 consumption. Three and 5 saturations (not shown) follow the same behavior and are located in order between 0 and 10 saturations. Oxygen consumption was more rapid at the beginning of the trial up to 21 days after which the consumption slowed down. The dissolved oxygen reacts with compounds which are present at high concentration at the onset of oxygenation which will cause the faster consumption rate. The consumption rate for the F4 samples (supplemented every week) slowed down as there was a lack of substrate (compounds capable of reacting with oxygen) in the later period of the trial. The oxygen consumption rates were high at all the studied temperatures with kinetic rates of 0.010, 0.012, 0.012 and 0.018 per day at 20, 30, 35 and 40 ºC respectively.

We are aware that by changing the composition of the matrix, deviations in the kinetic values will occur. The behavior of the compound will however be similar and these deviations will not affect the main purpose of the study which is to know how certain parameters (oxygen and temperature) will influence the formation of sotolon.

The activation energy (Ea), which constitutes a measure of sensitivity towards temperature, is in addition very low, Ea = 3,5 kJ/mol, but is close to values that has been previously reported for free radicals (3 kJ/mol) (Formosinho, 1983).

As O_2 consumption tends to lower values, the time needed to consume all the oxygen increases at low concentrations, therefore having a significant effect on the Port wine aging process and consequently on the formation of molecules dependent on the presence of oxygen.

3.2. Sotolon Formation

The formation of sotolon in the Port wines is presented in Figure 3.2.1.

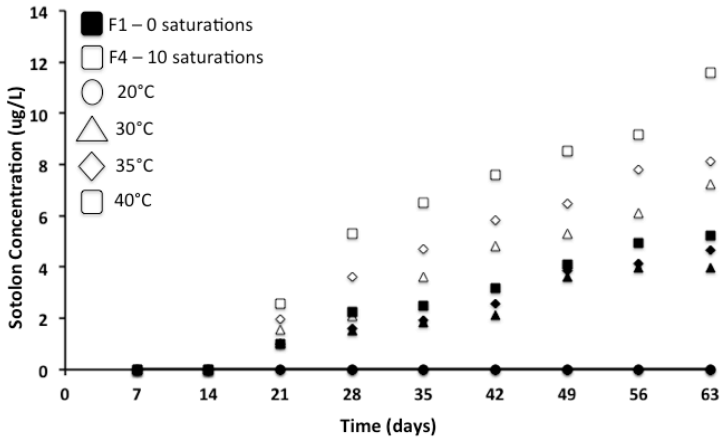

Figure 3.2.1. Port wine kinetics; Sotolon concentration as a function of time at different temperatures and O_2 saturations.

A first order kinetics that is dependent on the consumed oxygen, model equations 2.3.1.8. and 2.3.1.9. for the isothermal experiment were fitted to the experimental data. The low PRESS, correlation

coefficient, t-values and the lack of fit test results ($p < 0.01$) confirm the model's validity for predicting and simulation.

There was an increase in sotolon concentration over time. It is clear that consumed oxygen had a significant impact on the formation of sotolon. Samples that have been submitted to oxygenation had a higher rate of sotolon increase when compared to the samples that did not undergo forced oxidation. These samples also had a higher sotolon concentration at the end of the experiment.

Temperature also played a critical role in the formation of this compound as more sotolon was formed at higher temperatures. As example, at 10 saturations sotolon formation was higher at 40ºC when compared to 30ºC. This effect was also observed at 0 saturations.

In terms of kinetics, the sotolon formation rate at 40ºC was high when compared with the other temperatures ($k = 0.0157$ µgSot.mg/O_2 per day), this rate however changed as a function of oxygen consumption (Figure 3.2.1.). The rate of formation values obtained are in agreement with the values previously obtained by Silva Ferreira *et al.* (2005).

Following an increased number of O_2 saturations, the rate of formation increased linearly according to the consumed O_2. Temperature, as stated before, had a significant impact on the sotolon formation, which is reflected in the Ea value (92 ± 5 kJ/mol). In fact, this value is higher than in most oxidative processes, where the Ea value is in the range of 20 kJ/mol (Formosinho, 1983). An increase in storage temperature and the consumed oxygen concentration would thus lead to an increase in sotolon formation. For example, at 20 ºC the ratio between sotolon formation rate and O_2 consumption is 0.08, whereas it is 0.2 at 40 ºC (a 247% increase).

This effect is visually portrayed in a surface plot (Figure 3.2.2.) where the sotolon formation rate (RSot) is plotted against oxygen and temperature. RSot is given by: $RSot = CO_2.kapp$, which states that RSot is directly proportional to O_2 consumption rate and dependent on storage temperature.

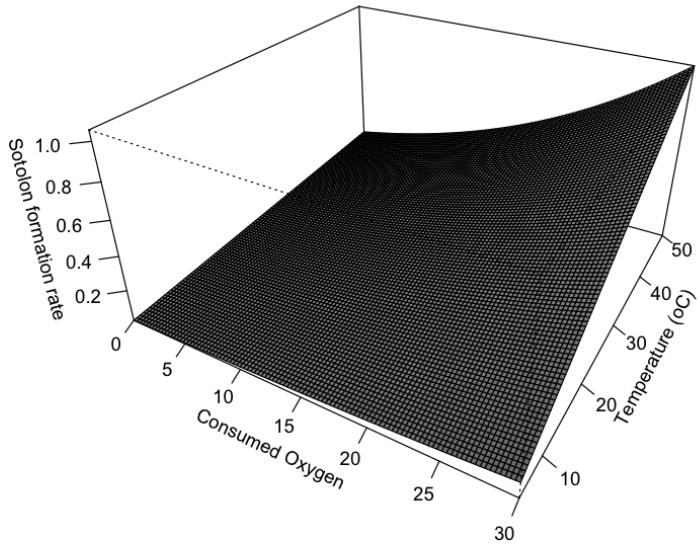

Figure 3.2.2. Sotolon formation rate as function of the consumed O_2 and storage temperature.

3.3. Maillard and oxidation related compounds formation

Additional compounds known to be connected to oxygen (i.e. cis dioxane and trans dioxane) and temperature (HMF, furfural and 5MF) were also investigated.

The influence of oxygen, temperature and time on *cis* dioxane and furfural are respectively on Figure 3.3.1.. Kinetic rates and model parameters are presented in Table 3.3.1. and 3.3.2..

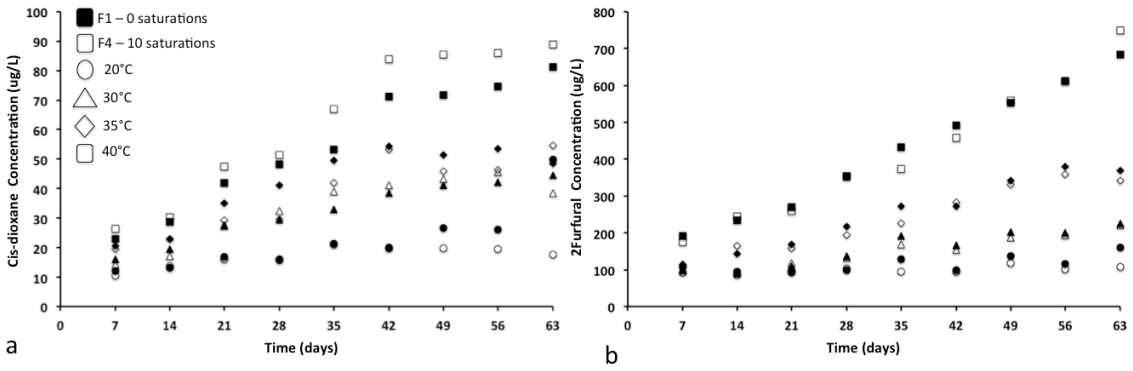

Figure 3.3.1. *Cis* dioxane and furfural concentration as a function of time at different temperatures and O_2 saturations.

These compounds were fitted to a first order reversible kinetic as discussed in the materials and methods. Statistical significance of this empirical model is also assured by the same parameters as for sotolon and O_2 consumption (see Table 3.3.1. and 3.3.2.). These compounds present considerably lower kinetic rates when compared to sotolon, such as furfural at k20 ºC = 6.7e-6 per day, HMF at k20 ºC = 2.1e-5 per day and *cis* dioxane at k20 ºC = 7.2e-4 per day (Table 1). Two groups can therefore be considered in terms of temperature sensitivity: (i) high Ea: furfural (169 kJ/mol), 5MF (169 kJ/mol) and HMF (230 kJ/mol); and (ii) low Ea: *cis* dioxane (76 kJ/mol) and *trans* dioxane (86 kJ/mol). Furfural, 5MF and HMF are well known compounds with their formation being linked to high temperatures, such as thermal processing or the Maillard reaction. Their high Ea and low kinetic rates in Port wines indicate that these compounds are not formed in a significant concentration during aging. The Ea of *cis* and *trans* dioxanes are within the range of most oxidation reactions (Formosinho, 1983). Dioxanes are formed by the reaction of acetaldehyde and glycerol. Acetaldehyde is known to be an oxidation marker and an important marker of the age of Port wine (Silva Ferreira*, et al.*, 2002). Thus it would make sense that the activation Ea for dioxanes are lower, as the precursor is present in high concentrations in oxidative conditions and not therefore dependent on temperature.

Table 3.3.1. Isothermal kinetic rates for sotolon, furfural, 5MF, HMF, Cis Dioxane and Trans Dioxane.

	Kinetic Rate (day^{-1})			
Temperature (°C)	**20**	**30**	**35**	**40**
Sotolon	0.0047 ± 0.00041	0.0048 ± 0.00042	0.0083 ± 0.00061	0.016 ± 0.00065
Furfural	(6.7 ± 0.54)e-6	(5.6 ± 1.0)e-5	(8.7 ± 2.0)e-5	(1.9 ± 0.32)e-4
5MF	(9.4± 0.60)e-5	0.0014 ± 0.00051	0.0022 ± 0.00053	0.0043 ± 0.00081
HMF	(2.1 ± 0.19)e-5	(3.4 ± 0.20)e-4	(3.9 ± 2.4)e-4	(1.7 ± 0.44)e-3
***Cis* Dioxane**	(7.2 ± 0.75)e-4	0.0016 ± 0.00012	0.0021 ± 0.00025	0.0042 ± 0.00023
***Trans* Dioxane**	(2.2 ± 0.43)e-5	(6.5 ± 0.71)e-4	0.0010 ± 0.00011	0.0020 ± 0.00011

Table 3.3.2. Global optimization models for isothermal for: furfural, 5MF, HMF, Cis Dioxane and Trans Dioxane.

	Kinetic parameters		
	Furfural	**5-MethylFurfural**	**HMF**
C_0 (µg/L)	164 ± 9.70	7.25 ± 0.269	(15.6 ± 1.23)e2
k_{ref} ($days^{-1}$)	(6.71 ± 0.543)e-6	(9.41 ± 0.601)e-5	(2.12 ± 0.193)e-5
Ea (kJ/mol)	169 ± 26.6	169 ± 31.8	230 ± 57.9
	***Cis* Dioxane**	***Trans* Dioxane**	
C_0 (µg/L)	3.71 ± 0.191	1.57 ± 0.0998	
K_{ref} ($days^{-1}$)	(7.18± 0.748)e-4	(2.20 ± 0.428)e-5	
Ea (kJ/mol)	75.8 ± 13.1	85.5 ± 13.2	

3.4. Monte Carlo storage simulations

For the simulations of wine composition, the container was chosen based on O_2 permeability of 1.5 ± 0.75 mg/L per year for cork stoppers, and 10 ± 2 mg/L per year for barrels. Storage temperatures were chosen as 5, 10, 15 or 20 ºC. The time of storage was also selected as 2, 5, 10, 15 or 20 years. The Monte Carlo (MC) algorithm uses the above derived kinetics to estimate the chemical composition in terms of: i) oxygen; ii) sotolon; iii) furfural; iv) 5MF; v) HMF; and vi) *cis* and *trans* dioxane. We assume that inside barrels or bottles, the daily O_2 intake is well below the saturation point (8-9 mg/L) and therefore all of the O_2 intake is either dissolved or consumed.

Table 3.4.1. and 3.4.2. are simulated Port wine composition according to the temperature, time aged and the containers used (barrel or bottle) obtained with Monte Carlo simulations. Figure 3.4.1. shows an example of a simulated Port wine stored at 15 ºC for 10 years (Table 3.4.1.).

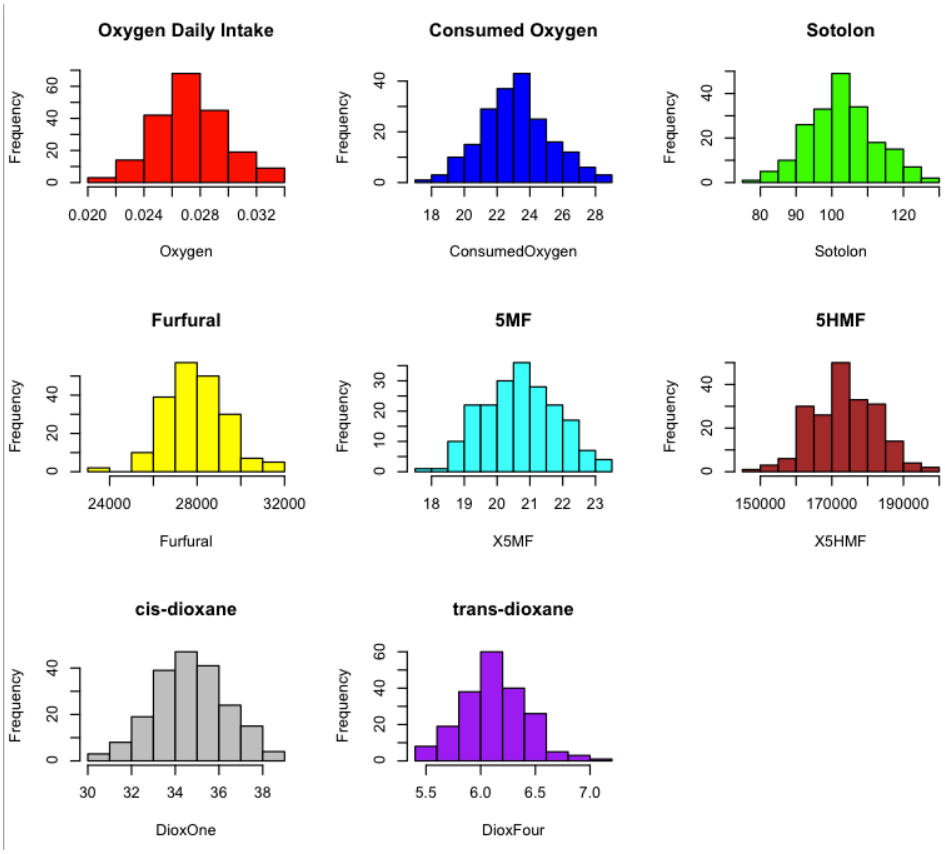

Figure 3.4.1. Port wine kinetic rate chart and expected scenario for T=15ºC, t=10 years and O_2 permeability=10±2 mg/L year (barrel).

Figure 3.4.2. is a dendogram of the Port database which included Port samples of various ages (indicated in black and the age reported in the sample name). Monte Carlo predictions for wines stored for 10 years at 10 and 20 ºC were then included in the dendogram. Samples indicated in color are the simulated aged Port wines. These wines have been included in the dendogram according to all the parameters measured. This would allow the positioning of the wines within the database and thus make predictions as to what age the samples would be associated with.

Wines in the database are mostly clustered by age (Figure 3.4.2.). This classification is mainly influenced by the presence of sotolon and *cis* and *trans* dioxanes. It is possible to observe three very distinct groups of wines according to age: i) extremely old (around 129 years); ii) 10 to 76 years old; and iii) young (0 to 5 years).

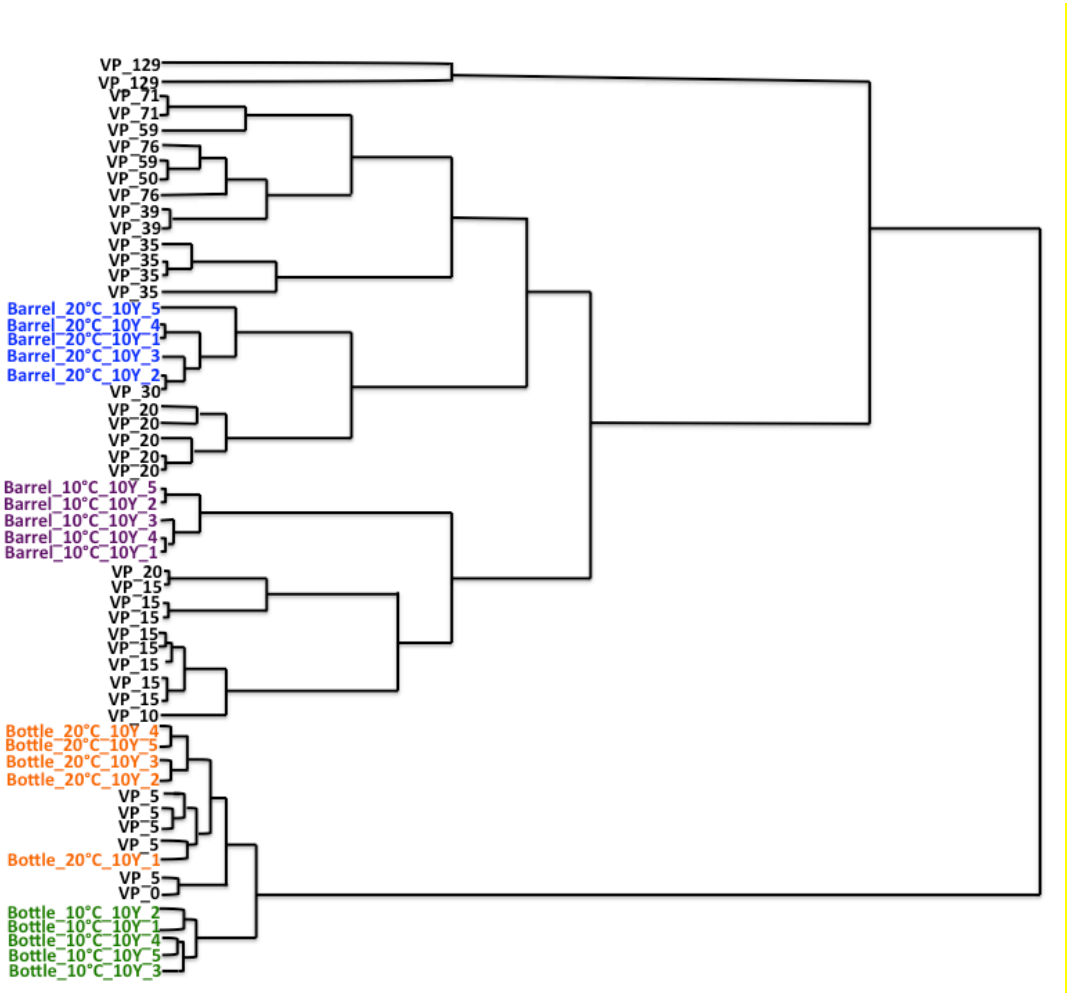

Figure 3.4.2. Dendogram of Monte Carlo predictions for 10 years ols wines storage in bottles and barrels at 10 and 20ºC with the normal aged wines.

When comparing the simulated wines aged in bottles and barrel, it is evident that the barrel-aged wines are associated with the older Port wines (between 20 and 35 years). On the other hand, the bottle aged wines are associated with much younger Port wines (between 0 and 10 years). This separation is most likely due to the oxygen availability in the different vessels. When considering the barrel aged wines, there is a clear separation between the two temperatures. A higher temperature (20 ºC) was associated with wines of 30 to 35 years of age, while a lower storage temperatures led to wines associated with wines of about 10 to 20 years of age. For the bottle aged wines, the wines stored at 10 ºC were associated with young Port wines (not aged at all). The wines stored at 20 ºC were associated with Port wines in the 5 year old cluster.

Results further show that in bottles (O_2 ingress through cork) with an O_2 intake of 1.5 ± 0.75 mg/L per year led to a bigger range (larger error) of sotolon concentration at the end of the 10 year storage period when compared to wines aged in barrels. In Table 3.4.1. and 3.4.2. it is possible to observe that the wines stored in bottles has a sotolon concentration of 0.1 to 118 μg/L as usually found in Port wines of about 10 years of age, whereas, during storage in the barrel, an increase in sotolon concentration were observed to the amount of 1 to 800 μg/L.

It would seem as if the presence of oxygen was the more important parameter in the aging character of Port wines. The separation between temperatures (within a specific oxygen management e.g. barrel or bottle) were higher for the oxygen exposed treatment (barrel) when compared to the low oxygen exposure (bottle). It could be concluded that these two parameters work in a synergistic mechanism and amplify the aging effect.

This study showed the kinetics of oxygen consumption. In a wine with a high O_2 concentration, the rate of consumption was higher than when compared to a wine with a low O_2 concentration. At lower concentrations, the consumption rate was much slower, resulting in a longer consumption time. Higher initial O_2 concentrations also resulted in wines with a more pronounced aged bouquet.

Sotolon is mainly responsible for this aged bouquet and this study shows the importance of oxygen consumption in the formation of this compound, as also reported in other studies (Ferreira*, et al.*, 2005), (Lavigne*, et al.*, 2008). It would seem as if temperature, combined with the presence of O_2, could have a synergistic effect, causing a significant increase in the formation of this compound. This study also confirmed the dependency of the formation of other molecules on temperature and/or oxygen.

Table 3.4.1. Monte Carlo simulation results for isothermal storage of Port wine inside barrels.

				Barrel			
Temperature (ºC)	**Age (year)**	**Sotolon (µg/L)**	***Cis* Dioxane (mg/L)**	***Trans* Dioxane (mg/L)**	**5MF (µg/L)**	**furfural (mg/L)**	**HMF (mg/L)**
5	2	0.980±0.108	6.14±0.293	0.99±0.0484	8.02±0.417	11.8±0.606	0.999±0.0474
5	5	6.11±0.710	12.2±0.600	1.59±0.0763	9.34±0.494	29.4±1.34	24.2±1.21
5	10	24.4±2.37	20.6±1.01	2.64±0.134	11.3±0.562	58.0±2.76	48.6±2.51
5	15	55.0±5.84	27.4±1.25	3.56±0.182	13.3±0.675	87.7±4.32	72.4±3.62
5	20	98.1±10.1	32.4±1.61	4.51±0.213	15.2±0.805	117±6.14	96.7±4.75
10	2	2.04±0.198	9.40±0.478	1.34±0.0663	10.2±0.551	42.3±2.07	5.55 ±2.66
10	5	12.6±1.22	19.1±0.983	2.52±0.130	14.5±0.745	105±5.23	139±6.38
10	10	50.2±5.13	30.3±1.51	4.33±0.214	21.8±1.08	211±11.3	278±13.9
10	15	115±5.11	37.8±1.83	5.89±0.305	28.8±1.46	317±14.9	415±20.5
10	20	203±20.7	42.7±2.12	7.37±0.368	35.7±1.92	424±19.6	556±26.8
15	2	3.20±0.271	14.6±0.728	2.05±0.103	35.7±1.92	147±6.41	302±14.9
15	5	25.8±2.71	27.9±1.29	4.10±0.182	32.2±1.64	367±18.9	759±37.2
15	10	102±10.3	40.2±2.05	7.02±0.329	55.9±2.67	728±36.5	(1.51±0.818)e2
15	15	233±23.0	46.1±2.19	9.38±0.513	78.2.±4.11	1096±54.9	(22.3±1.14)e2
15	20	412±45.7	48.6±2.27	11.2±0.615	98.7±4.83	1459±75.9	(29.8±1.57)e2
20	2	8.22±0.797	21.6±1.04	3.24±0.154	39.9±1.96	489±25.8	(15.4±0.683)e2
20	5	50.8±4.99	37.6±1.89	6.59±0.336	84.8±4.18	(12.2±0.587)e2	(38.3±1.91)e2
20	10	202±19.7	47.4±2.34	10.7±0.562	150±6.99	(24.1±1.24)e2	(74.6±3.87)e2
20	15	462±46.7	50.3±2.55	13.6±0.656	206±9.80	(36.1±1.85)e2	(110±5.66)e2
20	20	806±85.2	50.8±2.44	15.3±0.877	253±13.0	(47.4±2.15)e2	(143±7.84)e2

Table 3.4.2. Monte Carlo simulation results for isothermal storage of Port wine inside bottles.

		Bottle					
Temperature (°C)	**Age (year)**	**Sotolon (µg/L)**	***Cis* Dioxane (mg/L)**	***Trans* Dioxane (mg/L)**	**5MF (µg/L)**	**furfural (mg/L)**	**HMF (mg/L)**
5	2	0.140±0.0781	6.14±0.299	0.940±0.0469	8.07±0.401	11.1±0.0605	9.83±0.503
5	5	0.935±0.463	12.2±0.586	1.56±0.0805	9.24±0.468	29.2±0.155	24.3±1.113
5	10	3.60±1.82	20.6±1.06	2.60±0.132	11.3±0.645	58.4±0.280	48.6±2.34
5	15	8.00±3.88	27.1±1.33	3.58±0.178	13.3±0.658	88.0±4.38	72.2±3.45
5	20	15.8±7.155	32.4±1.58	4.47±0.231	15.3±0.828	117±5.56	96.8±4.67
10	2	0.319±0.149	9.46±0.476	1.35±0.0669	10.1±0.546	42.5±2.42	55.7±3.02
10	5	1.84±0.897	19.1±1.97	2.53±0.125	14.5±0.696	106±5.37	139±6.89
10	10	7.53±3.91	30.1±1.54	4.35±0.215	21.9±1.09	212±11.4	279±14.0
10	15	17.7±9.14	37.6±1.96	5.93±0.296	28.9±1.50	316±16.9	417±22.2
10	20	29.5±15.5	42.6±2.04	7.34±0.325	35.9±1.65	424±22.1	556±29.6
15	2	0.578±0.307	14.6±0.657	2.06±0.102	17.3±0.984	147±6.86	303±15.1
15	5	4.01±2.03	27.9±1.45	4.13±0.208	32.2±1.51	369±17.0	751±36.1
15	10	15.2±6.74	40.2±1.71	6.98±0.343	55.4±2.63	734±38.0	(15.1±0.774)e2
15	15	32.2±17.4	46.0±2.65	9.38±0.465	78.1±4.09	(11.0±5.34)e2	(22.3±1.11)e2
15	20	60.5±33.4	48.6±2.38	11.3±0.544	99.6±4.81	(14.6±8.44)e2	(30.0±1.58)e2
20	2	1.19±0.576	21.7±1.16	3.24±0.165	39.9±2.06	469±24	(15.4±0.764)e2
20	5	7.38±3.92	37.7±1.87	6.58±0.330	84.6±4.52	(12.2±0.599)e2	(38.1±1.93)e2
20	10	30.6±14.5	47.6±2.45	10.7±0.562	150±7.23	(24.1±1.10)e2	(74.6±3.59)e2
20	15	67.2±36.9	50.2±2.43	13.4±0.721	206±10.2	(36.0±1.84)e2	(109±5.08)e2
20	20	119±63.4	50.8±2.42	15.3±0.826	250±12.5	(47.8±2.52)e2	(144±6.52)e2

3.6. Multivariate approach

Wine aging is a complex system, which requires more information to be analysed in order to better understand the mechanisms at play. Given this, a technology that are able to capture information about a broader range of compounds participating in the aging process are necessary in order to achieve a better understanding thereof.

The aim of this section of the study is to validate the feasibility of using the entire chromatographic feature as a screening procedure to classify complex chemical mixtures, such as wine samples, to identify which compounds are responsible for differences and to perform network reconstructions that may indicate underlying kinetic relationships and mechanisms.

3.6.1. Unsupervised Approach - Principal Component Analysis.

The initial goal for the use of PCA was to examine the intrinsic variation in the data set prior to alignment in order to determine if the volatile fraction of the samples followed a trend related to age. However, when using the GC-FID matrix described above some samples did not follow the latent age variable described by PC1, namely the 4 and 60 year old samples (score plot not shown). The analyses global workflow is described in Figure 3.6.1.1.

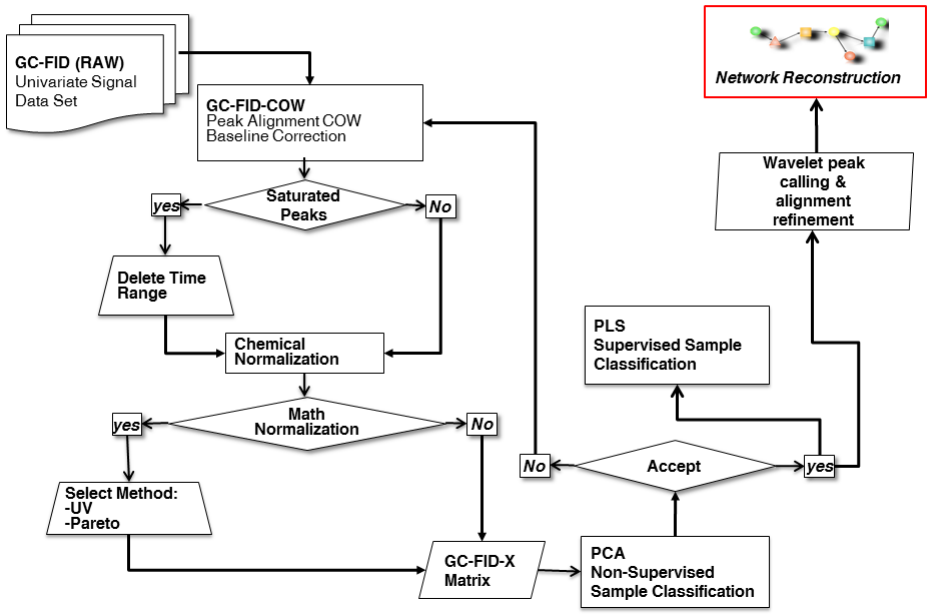

Figure3.6.1.1. Proposed workflow for univariate (chromatographic) signal processing.

The loading plot in Figure 3.6.1.2. shows higher levels of acetic acid, 2,3-butanediol, diethyl succinate, diethyl malate, phenylethanol and succinic mono ethyl ester present in the older samples. The esterification process appears to be the most prevalent reaction amongst the compounds apparent in the loading plot. The organic acids naturally present in grape must, such as malic acid and

those present as the result of fermentation, such as lactic, succinic and acetic acids all react with ethanol to yield the esters seen in the loading plot (Ribéreau-Gayon *et al.*, 2006). However, these molecules are out of the detector's linear response range, so they needed to be eliminated. Furthermore, the chromatograms must be aligned because an unavoidable characteristic of all chromatographic data is that the retention times for the peaks in the chromatograms shift slightly from one analysis to another. To address this problem correlation optimized warping (COW) was used to align all of the chromatograms.

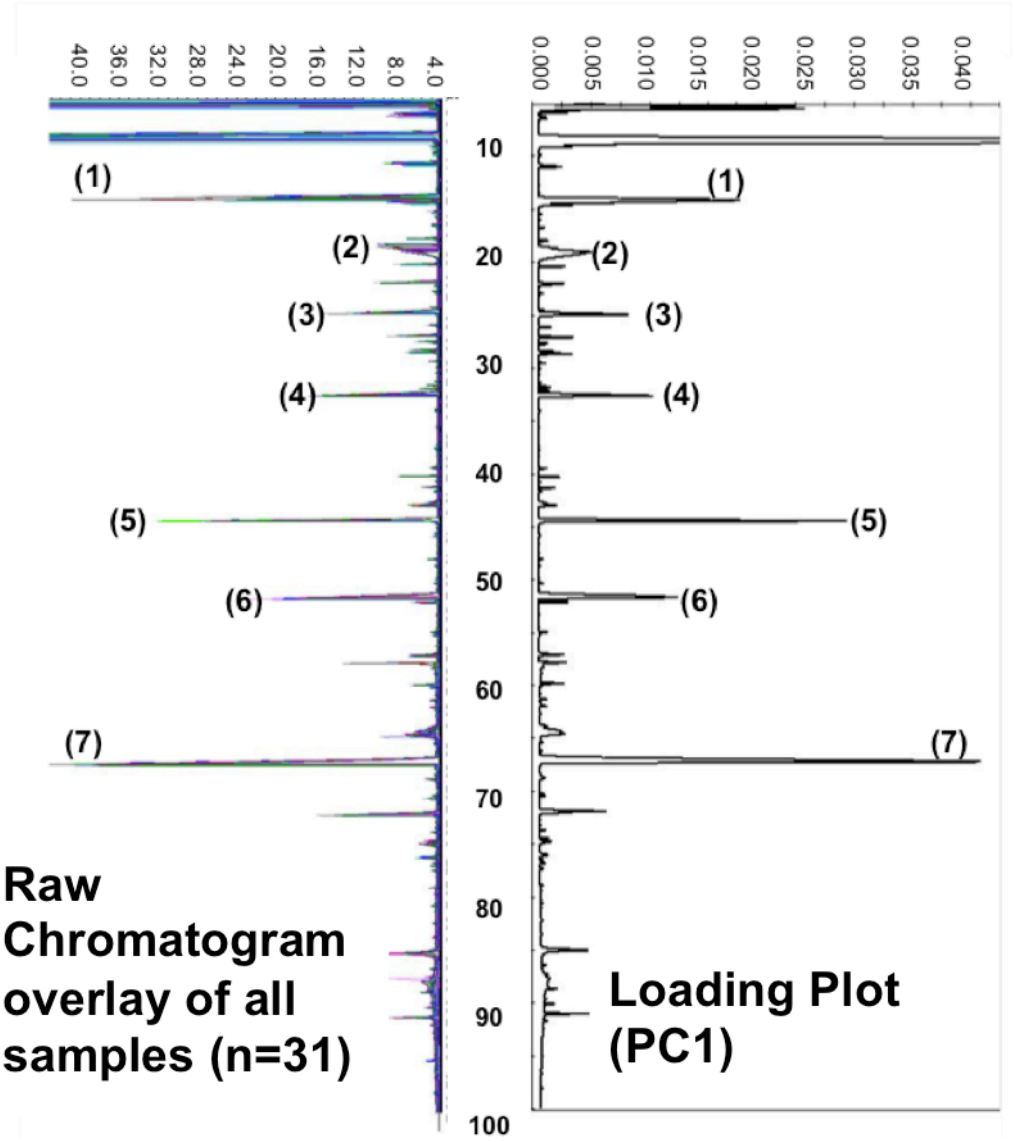

Figure 3.6.1.2. Raw chromatogram overlay of all samples (n=31) and Loading plot (PC1) representing the average chromatogram GC-FID chromatogram. (1) ethyl lactate, (2) acetic acid, (3) 2,3-butanediol, (4) diethyl succinate, (5) 2-phenylethanol, (6) diethyl malate and (7) succinic monoethyl ester.

A new fingerprint was created (GC-FID-COW), by the removal of saturated peaks and baseline correction to yield the GC-FID-X matrix, which was subsequently analysed by PCA. The new score plot shows the same latent age variable described by PC1, but the explained variance of the first two components is 74% (Figure 3.6.1.3.A) and the samples that did not previously follow the age vector now do so after alignment.

The score-plots in Figure 3.6.1.3.A and B show a clear trend related to wine age, suggesting that the chemical mechanisms are correlated with time, across the first principal component with the first two components explaining 74% of the variance. It appears that the latent age vector remains intact whether the data is mathematically normalised or not.

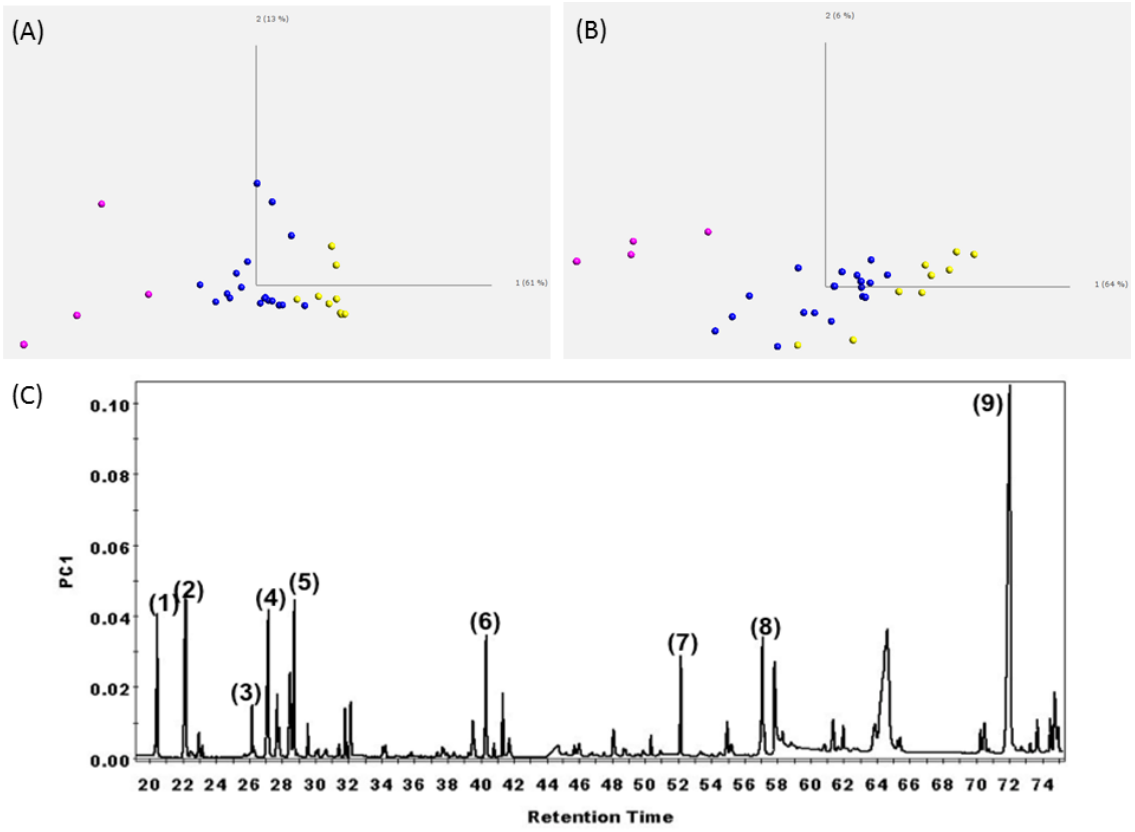

Figure 3.6.1.3. PCA score plots of cleaned and COW-aligned chromatograms: (A) un-normalized (B) normalized. Colours denote wines of age 2 to 7 years (yellow), 10 to 42 years (blue) and 48 to 60 years (pink). (C) Loading plot of PC1 with 9 of the peaks identified as (1) furfural, (2) *cis* dioxane, (3) benzaldehyde, (4) 5MF, (5) *cis* dioxolane, (6) *trans* dioxolane, (7) octanoic acid, (8) unknown and (9) HMF.

It is important to note that the data for both the PCA and PLS analyses was not mathematically centered or normalized as is commonly done to give all variables equal impact on the model. Centering is usually used as a matter of convenience for display and mathematically has no impact on the multivariate model. Looking at the loadings (Figure 3.6.1.3.C) the data was not center in order to not have negative scores along PC1 and thus to have no negative peaks on the loading plot in order to keep the loading plot looking as much like a normal chromatogram as possible. Attention was paid that the choice of not to normalize means that peaks with higher intensities will have a larger impact on the model and be more apparent in the loading plots shown in Figure 3.6.1.2. and 3.6.1.3. However, this allows the patterns visible in the loading plots to be recognizable to an analytical chemist and therefore is easily read and interpreted as a normal chromatogram. Mathematical normalization unfortunately makes the standard chromatographic patterns unrecognizable as it rescales every peak to the same amount of variance. In addition, as can be seen from Figure

3.6.1.3.A and B, normalization does not change the fact that PC1 comprises the age vector with the biggest affect of normalization changing the sample distribution along PC2, which is likely to represent vintage and vinification technology effects. This suggests that the aging of wine largely overwhelms the differences between wines that are present due to the season they were made in or variations amongst the approaches used to make them (different yeast strains, temperatures, crushing mechanisms, fermentation tanks, barrels used for maturation, etc.). The primary purpose of PCA and PLS in the pipeline is as a graphical user interface for analytical chemists to use as a screening step for univariate data sets. As such, we strove to present the analytical chemist with a multivariate interpretative environment with which they would be as familiar as possible, namely chromatographic fingerprints with which they have large experience. For this part of the analysis, the visual representation of the chromatographic loading plots outweighs the assignment of equal weights to every variable in the model. Furthermore, we address this variable normalization issue with the use of network reconstruction via Pearson correlation networks and maximum spanning trees. In the network analysis, every peak is analyzed and has an equal opportunity to form a part of the network and Pearson correlation includes vector normalization.

During aging there are likely to be several different mechanisms involved, including oxidation and Maillard reactions. In PC1 the samples correlate with the age of the wine, which points out that the overall kinetic system overrides any one specific mechanism. As such, the connections between the mechanisms at play are more relevant to sample classification than the contributions of any individual mechanism.

After alignment, compounds which appear to correlate with port wine aging as found in the loading plots were: *cis* dioxane, *cis* dioxolane, *trans* dioxolane, *trans* dioxane, octanoic acid and HMF as shown in Figure 3.6.1.3.C.

The *cis* and *trans* dioxane and dioxolane are formed by the condensation reaction between glycerol and acetaldehyde. These molecules were identified in Port wine by Silva Ferreira*, et al.* (2002) who noted that they increased with age, and, as such, could be used as age markers for port wine kept under oxidative conditions. Furanic compounds, HMF, 5MF and furfural are thought to be products from the Maillard reaction, formed by the fragmentation or cyclization of 3-deoxyosone, a highly reactive intermediary of the reaction (van Boekel, 2006). Barrel oak can also be a source of HMF and furfural (Moutounet *et al.*, 1989).

3.6.2. Supervised Approach - Partial Least Squares Analysis.

Partial Least Squares (PLS) analysis was used on the GC-FID-X matrix in an effort to associate specific peaks/fingerprint regions with mechanisms known to be involved in aging. It is worth noting that PLS was not used in its traditional role as a method with which to build calibrated, predictive models (that would therefore be built with training sets and validated with independent test sets). Rather, the goal of our use of PLS-1 was simply as a method with which to perform principal-component-based regressions in an effort to identify sets of peaks that were associated with known

mechanistic markers or potential precursors for volatile compounds. Molecules that are thought to be associated with different mechanisms were selected and quantified from each sample and used as markers to try and find other compounds in GC-FID-X that may be related with the same mechanism. Acetaldehyde and HMF were used as markers for oxidation and the Maillard reaction respectively. Sotolon was also used in an effort to gather more information about its origin. The concentration of each of the marker molecules was determined for each sample and the resulting vectors used as a second data block in PLS.

The resulting PLS coefficient plots show the variables which correlate with each mechanism-marker. Some variables have a positive value, which means that these have kinetic vectors which correlate with that of the mechanism-marker, and some have negative values, which indicate that they have an inverse correlation with the kinetic vector of the mechanism-marker.

For sotolon the correlation is 0.89 (over 7 components) for molecules such as furfural, 5MF, cis dioxane, cis-dioxolane, trans-dioxane, trans-dioxolane and HMF. We also found some organic acids with negative correlations, which indicate that they were being consumed as sotolon was being formed (Figure 3.6.2.1.). The model had a correlation of 0.91 for HMF (a Maillard reaction marker) and 0.92 to acetaldehyde (an oxidation marker) for 7 latent variables. The PLS loading plot for sotolon was very similar to those seen for HMF and acetaldehyde which means that the mechanisms are correlated, and during aging contribute in the same way to the dynamics of the overall process.

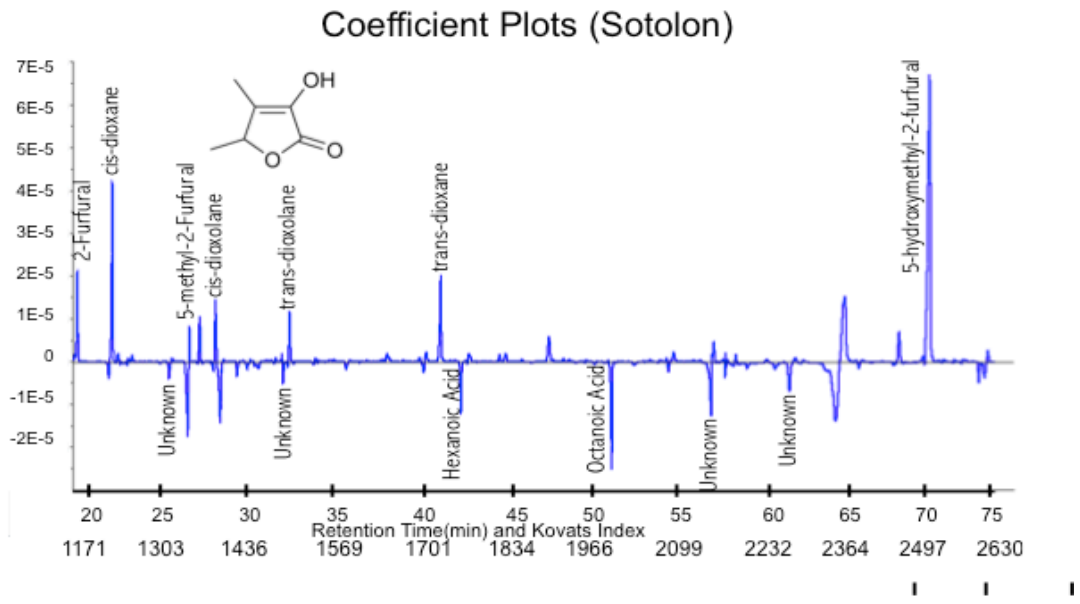

Figure3.6.2.1. PLS b-coefficients for Sotolon as the Y vector with 7 latent variables.

Some amino acids, namely, valine, alanine, arginine, glutamine and aspartate, had relatively high (0.69-0.83) inverse correlations with a number of peaks in the volatile profile which were themselves correlated to Maillard reaction markers such as such as HMF and furfural. Thus it seems likely that these amino acids are major Maillard aroma precursors.

3.6.3. Network Reconstruction.

Figure 3.6.3.1. shows the maximum spanning tree derived from the correlation network between all peaks. Each node represents the center of a peak (Kovats index) and each edge represents the best correlation between the peaks. Fold changes between 2 year old and 60 year old wines were calculated for each peak and the nodes colored accordingly with shades of red representing increasing concentration and blue representing decreasing concentration. The thickness of each edge has been scaled to represent the level of correlation (thicker lines mean higher correlation values). Furthermore, the size of each node has been scaled to represent the number of correlations it had with other peaks above a threshold of 0.8.

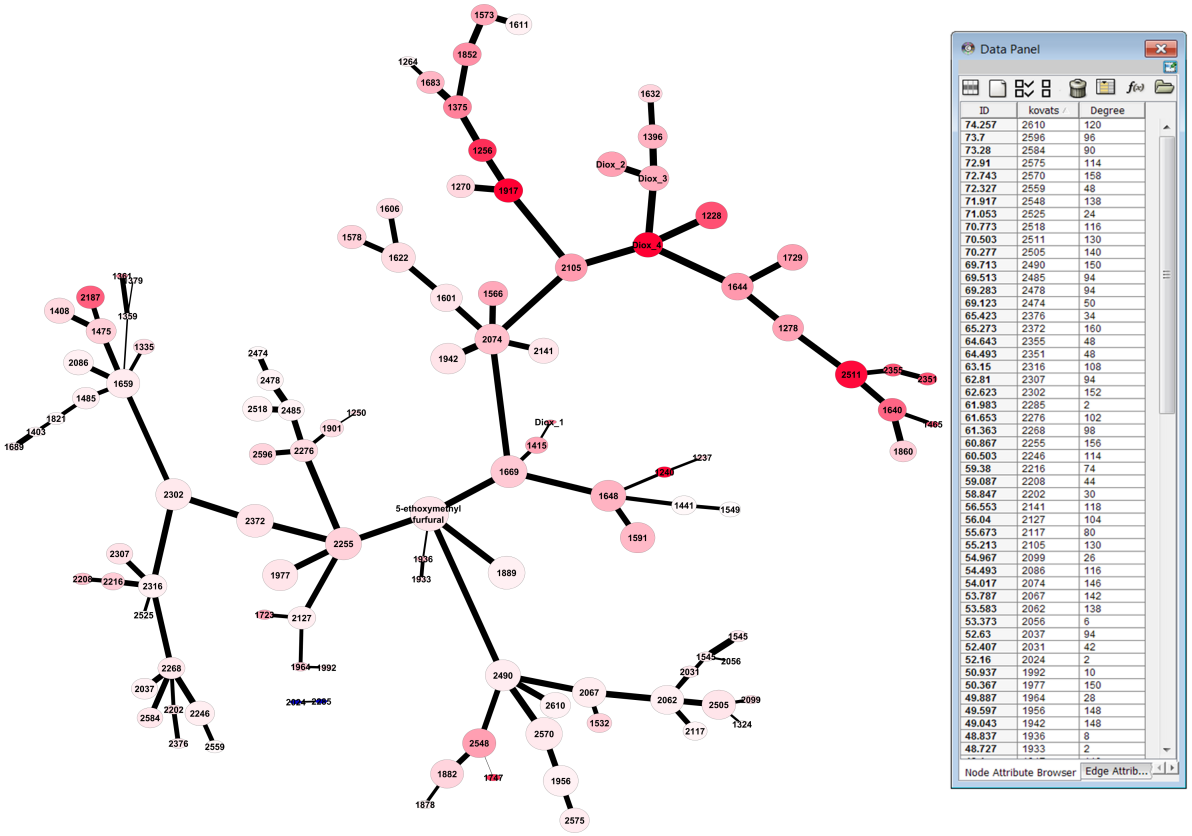

Figure 3.6.3.1. Putative Kinetic Network. Nodes are coloured in shades of red based on the fold change from 2 to 60 years. Node sizes are scaled by the number of other nodes (peaks) that are correlated to them above a Pearson threshold of 0.8. Edge thickness is scale by the degree of correlation between its two nodes. (Dioxanes in the network are labelled as follows: cis dioxane: Diox 1; cisdioxolane: Diox 2; trans dioxolane: Diox 3 and, trans dioxane: Diox 4).

Using pure standards as markers for Maillard and oxidation together with the Kovats index it is possible to explore and extract more information from the proposed network. In fact the cyclic acetals of glycerol and ethanal (oxidation products) cluster together on the upper part of Figure 3.6.3.1. In addition, 5-ethoxymethylfurfural, an ester of a major Amadori product (HMF), links two major branches of the network. As such, the network illustrates the aging process with the continuous formation of substances absent in young wines, which explains the aging character of wine.

There are no doubt intermediate compounds for some reactions that were not detected by FID. The network is robust to this missing data, as the intervening steps will simply be represented by a

lower correlation value of an edge between compounds that were detected. This correlative approach of course is not proof of causation but rather serves as a useful tool for hypothesis generation in order to prioritize the identification of unknown compounds represented by the peaks.

By using correlation values to targeted compounds (or other variables such as age) it was found that we could highlight the regions of the network that are closely associated with them and therefore likely involved in their formation or consumption. In order to explore regions of the network that may be related to age and particular mechanisms, Pearson correlation values between the peaks and each of the target vectors (sample age, sotolon, acetaldehyde, HMF, glutamate and alanine) were loaded into cytoscape as node annotations. Alanine and glutamate were selected as target vectors because they were the amino acids best correlated with the GC-FID-X matrix. By sorting the nodes by correlation values and selecting the nodes corresponding to a correlation value with a target above some threshold, portions of the network which correlated with each target vector could be visualized in aqua as shown in Figure 3.6.3.2.

Figure 3.6.3.2.A shows the nodes with a 0.86 Pearson correlation to the age of the wines. There is a clearly defined subnetwork that corresponds to age and represents compounds involved in the aging process. It was clear in the PCA diagrams that there are a group of compounds that correspond to aging which are responsible for the first principal component. It is likely that the compounds responsible for the second principle component are due to differences between the vintages of the starting wines. Can be seen the same pattern in this network where there are a number of compounds that do not correlate well with age and are likely reflecting vintage differences amongst the wines.

Figures 3.6.3.2. B, C and D show the regions of the network (colored as aqua) that correlate (Pearson threshold 0.86) with sotolon, HMF and acetaldehyde, respectively. It is clear that there is considerable overlap between the subnetworks correlated with these three compounds and as such it is possible that there is a mixture of oxidation and maillard reactions at play in forming these compounds. The subnetwork that correlates with age at a Pearson threshold of 0.86 in Figure 3.6.3.2.A clearly overlaps to a very large degree with the subnetworks defined by the correlations to acetaldehyde, HMF and sotolon. The arrow in Figure 3.6.3.2.A shows the node that negatively correlates to both alanine and glutamate (-0.8 Pearson threshold) and as such likely represents the entry point to the volatile network. The fact that the anti-correlation is relatively low (-0.8 to -0.83) probably indicates that there are one to several intermediates between the amino acids and their products' entry into the volatile network.

The network representation captures some of the dynamics of the aging process based on the underlying kinetics. In fact those compounds that are highly correlated to one another (>0.8) are likely to have the same kinetic order and the network thus enables one to screen molecules according to their kinetic parameters.

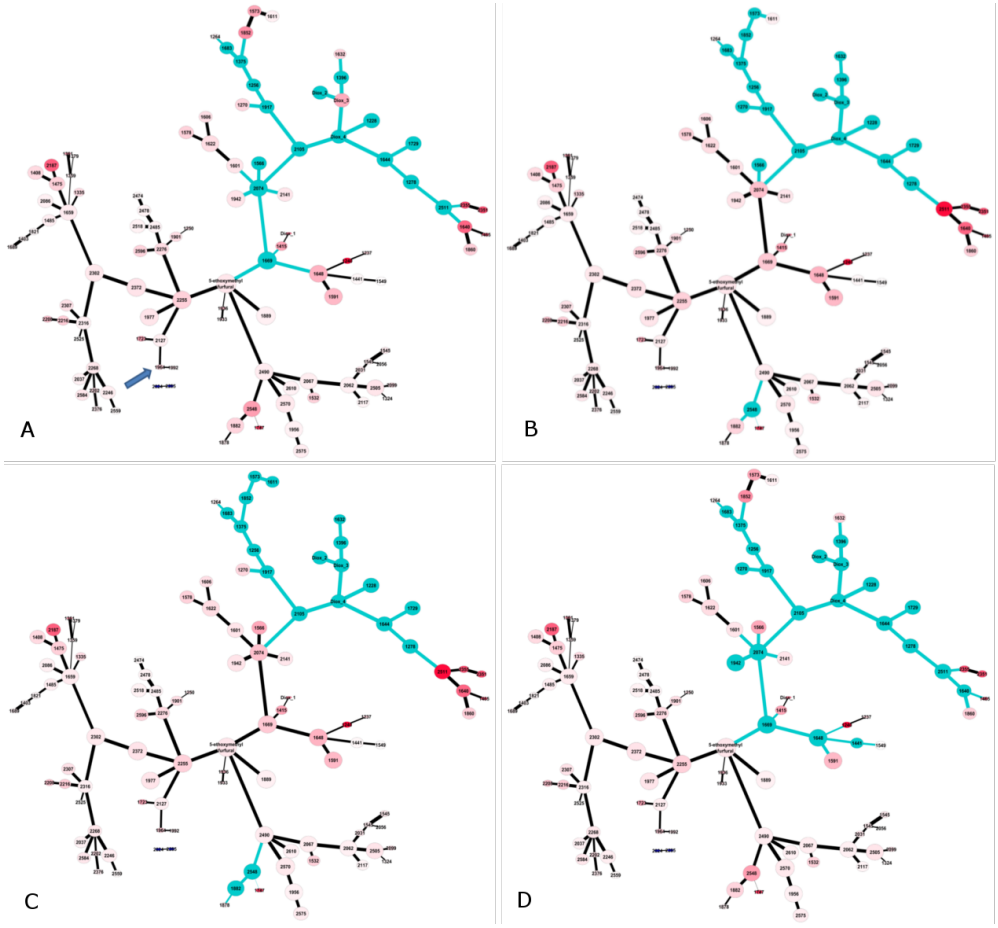

Figure 3.6.3.2. Subnetworks correlating to A) Age, B) Sotolon, C) HMF and D) Acetaldehyde. Nodes (compounds) with strong Pearson correlations to these target vectors are colored with aqua.

The network is an approximate representation of the underlying chemical reaction network during ageing. The higher the level of correlation (and therefore the nearer any two peaks are to each other in the network) the higher the probability is that they participate in the same or neighboring reactions. The correlation between compounds drops as you move further away in a chemical reaction network, as the intervening kinetics of each reaction will cause differences at each step.

Based on this assumption and to validate the pipeline a network with GC-MS data was constructed using the Total Ion Current (TIC) as the feature, i.e. m/z dimension was collapsed. Several compounds related with the representative branches of each possible mechanism were identified using pure standards together with the Kovats index, to be able to explore and extract more information from the proposed network. Quantification was then performed on the perturbed wines in order to determine their dependence on oxygen and temperature and to calculate the kinetic parameters.

Figure 3.6.3.2. shows the network derived from the correlation between all peaks, it is possible to observe in the figure three main branches a, b and c.

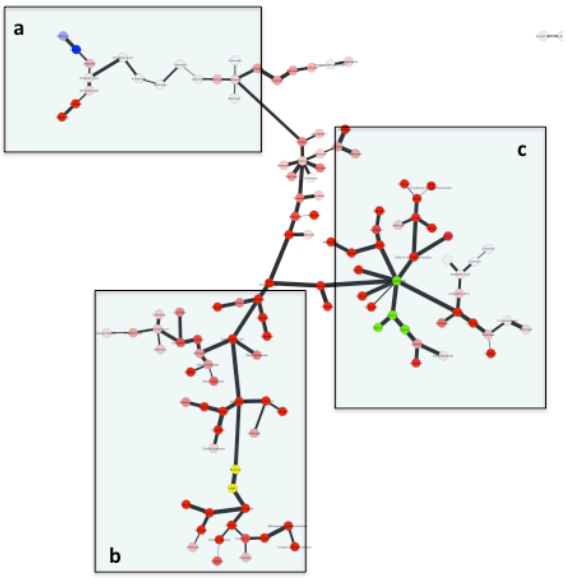

Figure 3.6.3.3. GC-MS Putative Kinetic Network.

It was observed that branch A (figure 3.6.3.3.) included compounds such as hexanoic, octanoic and decanoic acids, which cluster together. The nodes are colored in shades of red representing increasing concentration and blue representing decreasing concentration, which show the compounds identified did not undergo significant changes during ageing.

The ANOVA analysis on perturbed wines shows that acids are not dependent on temperature or oxygen.

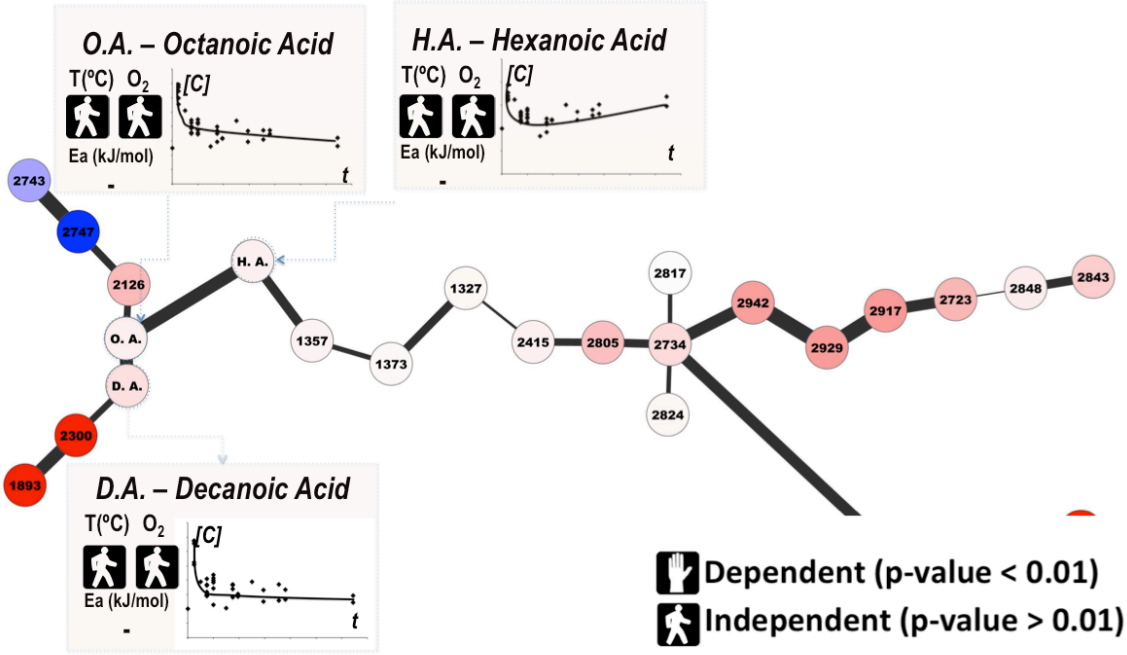

Figure 3.6.3.4. Putative Kinetic Network. Branch a.

In fact its concentration during wine ageing is regulated by the esterification/hydrolysis between the acid and the correspondent ester, acids may be formed due to hydrolysis, be lost through chemical esterification, or remain at a near constant equilibrium concentration depending on their initial, post-fermentation levels (Ramney and Ough, 1980). When the system is in balance, there is a constant correlation between the concentrations of the substances present, governed by the mass action law.

In branch B (figure 3.6.3.3.) are located two compounds that are demonstrated to be highly sensitive to temperature due to their high activation energy (HMF and furfural), which suggests that the other compounds in the branch may also be sensitive to temperature.

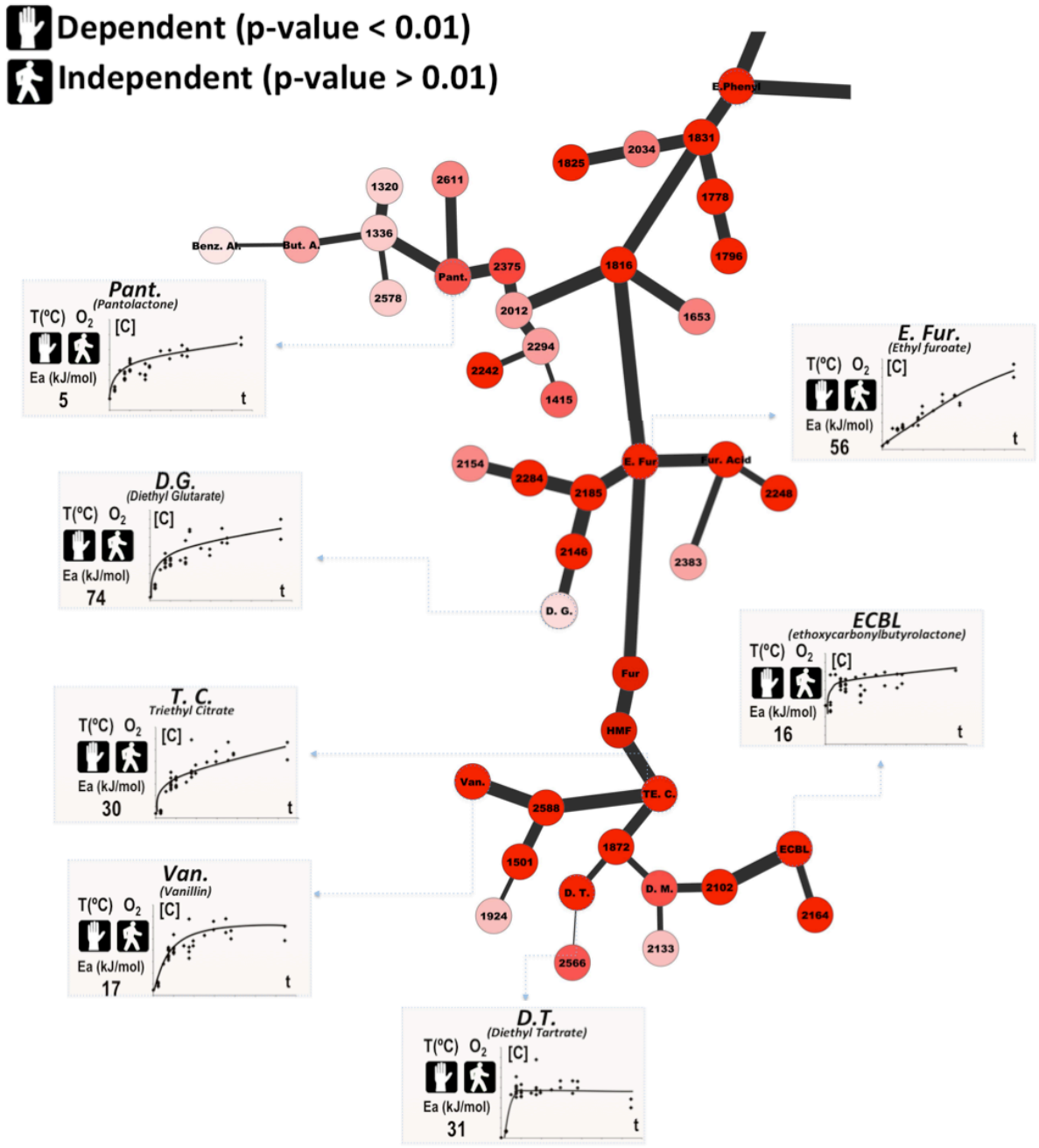

Figure 3.6.3.5. Putative Kinetic Network. Branch b.

A summary of the ANOVA analysis results is present on the figure as well as the time expression of each compound identified. All the compounds identified tend to increase during ageing time and ANOVA shows that significant differences were established across the temperature conditions, meaning that the compounds identified were all dependent on temperature.

It is possible to observe three sub-branches apparently related to activation energy value. The branch of vanillin, γ-ethoxycarbonyl-γ-butyrolactone , diethyl tartrate and triethyl citrate have an Ea between 16 and 36 kJ/mol.

The organic acids esters, diethyl tartrate and triethyl citrate, due to the high concentration of the respective acids (tartaric and citric acids) tend to increase, by the reaction between the acid and the ethanol (esterification reaction). According to Ribéreau-Gayon and Peynoud (1936) polyprotic acids such as tartaric, citric and malic esterified more rapidly than the monoprotic acids acetic, propanoic and butanoic and the temperature accelerates the reaction (Ramney and Ough, 1980).

The γ-ethoxycarbonyl-γ-butyrolactone is a lactone that probably results from glutamic acid or related compounds, and is dependent on wine ageing and contributes with a cherry aroma to wine (Wurz *et al.*, 1988). The formation of the lactone is a esterification reaction. Vanillin (4-hydroxy-3-methoxybenzaldeyde) is a phenolic aldehyde related with barrel ageing, its sensitivity on temperature in wines that were not subject to wood contact is still unknown.

Diethyl glutarate and ethyl-2-furoate are located in a second sub branch. These two compounds have activation energies of 74 and 56 kJ/mol and therefore higher than the posterior sub-branch. Ethyl-2-furoate is also dependent on temperature its formation is related with Maillard reaction.

In this branch are located compounds related with temperature and apparently there is a cluster based on the activation energy values.

In branch C are clustered compounds related with oxygen such as benzaldehyde formed by the oxidation of benzyl alcohol and dioxanes and dioxolanes formed by the reaction of ethanal and glycerol.

A region of the branch can be observed that correlates sotolon and 3-hydroxy-2-methyl-4H-pyran-4-one (maltol). These two compounds have a certain similarity in their chemical structures, i.e. a furanone and a pyranone respectively and the two have a very sweet aroma, normally classified in the same group of aromas with flavors of “burnt sugar, caramel and maple” although there is different orders of magnitude on the sensorial impact of both compounds, ppb for sotolon and ppm for maltol.

Sotolon is dependent on temperature and oxygen which suggests that maltol is dependent on the same parameters and in fact by the ANOVA analysis it is observed that the compound is sensitive to both parameters and has a very high activation energy when compared with sotolon, which means that maltol is much more sensitive to temperature than sotolon.

Yaylayan et al observed that maltol formation is related with 1-deoxyosone, an important intermediary of Maillard reaction (Yaylayan and Mandeville, 1994). However there are no studies on the impact of oxidation on its formation.

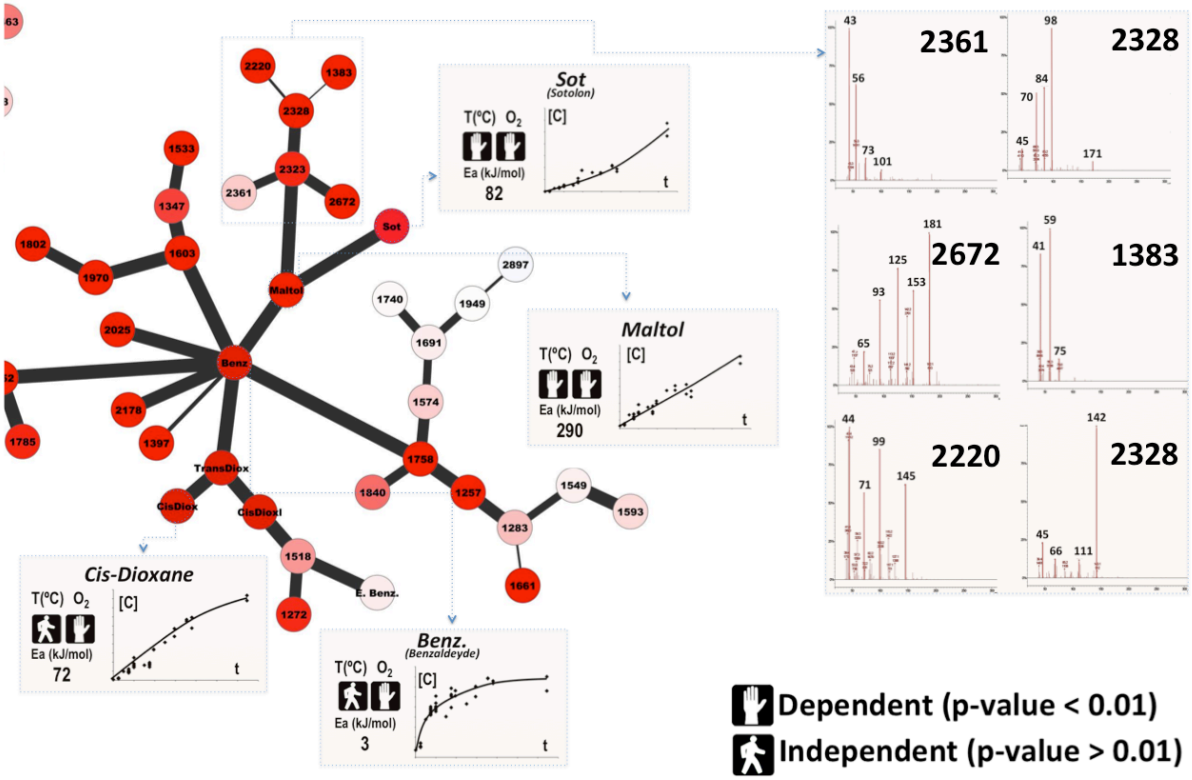

Figure 3.6.3.6. Putative Kinetic Network. Branched c.

Other compounds are presented on this sub-branch of the network connected with sotolon and maltol. The mass spectra of the compounds are presented in figure 3.6.3.4. These compounds are probably dependent on oxygen and temperature and their identification can be useful in the unraveling of the connection between Maillard mechanism and oxidation.

These observations validate the assumptions made that compounds grouped on the same branch of the network have the same time expression and consequently the same kinetic order. It was also observed that compounds grouped together are sensitive to the same technological parameter.

Conclusions

The goal of this thesis was the study of the chemical mechanisms occurring during Port wine ageing and the results presented clearly demonstrate the usefulness of the information to monitor the process.

This study has shown the importance of oxygen consumption and temperature on the formation of sotolon. Temperature combined with the presence of oxygen could have a synergistic effect, causing a significant increase in the formation of this compound. This confirms that there is a connection between Maillard reaction and oxidation on the molecule formation during Port ageing. This study also confirms the dependency of the formation of other molecules upon the temperature (HMF, furfural and 5MF) and oxygen (cis and trans dioxanes).

Once the amount of oxygen introduced in wine strictly depends upon the winemaking practices, the right oxygen management allows for the modulation of the chemical and sensorial profiles of Port wines. In fact, it can be known in which manner the oxygen and temperature affect the quality of Port wines. It is indeed much more important to control these factors for barrel aged wines (especially for temperature) than for bottle-aged wines because of the high levels of dissolved oxygen in the former.

The untargeted approach allows the performance of sample classification and contextualization and monitoring of the ageing process. The approach described can be used to predict potential molecules involved in the Port wine ageing by the deconvolution of time and the kinetics of different ageing mechanism.

The network reconstruction is very useful in visualizing the relationships between all of the compounds detected via GC-FID and GC-MS and their changes in concentration over time. And further, since untargeted approaches provide a larger number of compounds related to the process, the network reconstruction allows the identification of the most relevant candidates to be prioritized.

This view of the data provides considerably more information in an effort to understand the probable kinetic contexts of the molecules represented by peaks in each chromatogram. The approach described should indeed be a very powerful tool for the further study of mechanism and kinetic networks in complex systems.

All the results will allow the producer to manage the ageing process, taking into account the desired Port style, providing oxygen and temperature conditions and subsequently predict the age of Ports based on a chemical profile. In an industry perspective this information is very useful because it allows the modulation of the chemical and sensorial profile of Ports and consequently influences the product value.

Future Work

This thesis exemplifies and explains how potentially powerful the combination between analytical techniques, kinetics and multivariate statistical methodologies, namely networks reconstruction, can be in terms of wine characterization and mechanism contextualization.
Additional work should to be carried out, including the following issues:

i) Increase the number of compounds in the Port wine database used in the Monte Carlo simulations.

ii) Calculate kinetic parameters for some phenolics compounds, relevant substrates for wine oxidation.

iii) Focus on the identification of more compounds presented in the network.

iv) Select some network branches and develop small reaction mechanism, and try to validate regions of the network with multiresponse kinetic modelling in model solutions to decrease the complexity presented in the wines.

Annexes

Data is not disclosed due to confidentiality issues.

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