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PORTO

CHEMIOMICS: A SYSTEMATIC CHEMISTRY APPROACH TO UNRAVEL THE INTERFACE PATHWAYS BETWEEN OXIDATION AND MAILLARD MECHANISM RESPONSIBLE FOR FLAVOUR MODULATION DURING WINE STORAGE

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology, with specialization in Food Science and Engineering

Ana Rita Araújo da Silva Monteiro Monforte

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RESUMO

O vinho é uma bebida consumida mundialmente com alto valor comercial, cujo preço pode aumentar ou diminuir com o envelhecimento dependendo do tipo de vinho. No entanto, o conhecimento dos mecanismos químicos que ocorrem durante o processo que resultam em um perfil químico/sensorial específico permanece limitado. Essa falta de conhecimento limita significativamente a capacidade de melhorar a qualidade e consistência do produto. Por esse motivo, desvendar as alterações químicas que ocorrem durante o tempo de armazenamento, responsáveis pelas características do vinho, constitui uma tarefa crítica quando se tenta abordar questões relacionadas à autenticidade e qualidade sensorial. Estas consideráveis modificações químicas estão relacionadas, em particular, com a formação de substâncias ativas do aroma, como os aldeídos de Strecker

Dependendo das condições de armazenamento, nomeadamente relacionadas com a concentração de oxigénio dissolvido, a disponibilidade, qualidade e quantidade de antioxidantes, e as concentrações de moléculas precursores de compostos odorantes, o perfil químico ao longo do armazenamento irá ser diferente.

Nesse sentido, nesta tese vários estudos foram realizados para avaliar o impacto dos referidos fatores na formação dos aldeídos de Strecker, em particular o fenilacetaldeído (capítulos 3, 4, 5 e 6) e outras moléculas (capítulos 7 e 8). Os estudos realizados basearam-se numa abordagem holística de forma a avaliar vários fatores ao mesmo tempo assim como a interação entre eles.

Nos capítulos 3, 4, 5 e 6, foram realizados estudos em soluções modelo de vinho e os resultados demonstraram que a presença de metais é relevante na promoção da degradação de Strecker por vias de oxidação de fenólicos no pH do vinho. Além disso, esta rota é a principal via para a formação de fenilacetaldeído. Pela primeira vez, foi demonstrado que a presença de glicose inibe a formação de fenilacetaldeído nos sistemas modelo de vinho e no vinho branco. Pela quantificação da quinona, foi demonstrado que a glicose afeta diretamente sua concentração, o que sugere que no vinho branco a glicose tem um efeito antioxidante ao inibir a formação de o-quinonas.

Para estudar o fenômeno em sistemas de alta complexidade, nos capítulos 7 e 8, foram estudados vinhos brancos sujeitos a envelhecimento por diversos períodos de tempo e submetidos a tratamento por aplicação de resinas catiónicas.

Palavras chave: oxidação, aldeídos, Strecker, chemiomics, vinho.

ABSTRACT

Wine is a widely consumed beverage with a high commercial value, which price can increase with aging for a certain type. However, the knowledge of the chemical processes occurring during aging that result in a specific chemical/sensory profile remains limited. This lack of knowledge and understanding significantly limits the ability to improve product quality and consistency. For that reason, unravelling the chemical changes occurring during aging that are responsible for wine flavour, constitutes a critical task when one attempts to address issues related with authenticity and sensory quality. These considerable chemical modifications are related in particular with the formation of aroma active substances such as Strecker aldehydes.

Depending on the storage conditions, namely related with dissolved oxygen, and availability, quality and quantity of antioxidants as well as precursors concentrations of key odorants the chemical profile of wines changes. In that regards these work presents several studies in order to evaluate the impact of such factors in the formation of Strecker aldehydes, in particular phenylacetaldehyde (Chapters 3, 4, 5 and 6) and other molecules (Chapter 7 and 8).

In chapters 3, 4, 5 and 6, studies were performed in wine model solutions and the results demonstrated that the presence of metals, are relevant in promoting the Strecker degradation through phenolics oxidation pathways at wine pH. Also, this route is the major pathway for phenylacetaldehyde formation. For the first time it was demonstrated that the presence of glucose inhibits the formation of phenylacetaldehyde in both wine model systems and in white wine. By quinone quantitation it was shown that glucose affects directly their concentration, which suggest that in white wine glucose has an antioxidant effect by inhibiting o-quinones formation.

In order to study the phenomenon in high complex systems, in chapters 7 and 8, aged white wines were studied.

Keywords: oxidation, aldehydes, Strecker, chemiomics, wine

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Embora não estejam presentes fisicamente, não posso deixar de referir o meu avô e a minha mãe.

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Esta tese é para ti!

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LIST OF ABBREVIATIONS

3,4-DHB	3,4-dihydroxybenzoic acid
ANOVA	Analysis of variance
ARP	Amadori rearrangement product
AUC	Area under the curve
C0	Initial reactant concentration
Cat	Catechin
CE	Capillary-electrophoresis
CP	Center point
DI	Direct Injection
DoE	Design of experiments
DVB/CAR/PDMS	Divinylbenzene/carboxen/polydimethylsiloxane
EasyOx	Easily oxidized compounds
EDTA	Ethylenediaminetetraacetic acid
FC	Fold changes
FCCD	Face-centered composite design
FID	Flame Ionization Detector
FTICR	Fourier transformed ion cyclotron resonance
Gal	Gallic acid
GAQ	Gallic acid o-quinone
GC	Gas Chromatography
Glu	Glucose
HMF	5-(hydroxymethyl)furfural
HPLC	High Performance Liquid Chromatography
HRMS	High resolution mass spectrometry
HS-SPME	Headspace solid phase microextraction
LC	Liquid Chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
MDA	Mean decrease accur
MG	Methyl glyoxal
MR	Maillard reaction

MRPs	Maillard Reaction Products
MS	Mass Spectrometry
MVA	Multivariate Analysis
NMR	Nuclear magnetic resonance
OPD	o-phenylenediamine
OPLS-DA	Orthogonal Partial Least Squares-Discriminant Analysis
PA	Phenylacetaldehyde
PCA	Principal Component Analysis
PFBHA	O-(2,3,4,5,6- Pentafluorobenzyl)hydroxylamine
Phe	Phenylalanine
PhenOx	Total oxidizable compounds
PLS-DA	Partial Least Squares-Discriminant Analysis
PLS-R	Partial Least Squares-Regression
PO	Phenolic oxidation
PTR	Proton transfer reaction
R^2	Coefficient of determination
RF	Random forests
RI	Refractive Index
RMSECV	Root-mean-square error of cross validation
ROC	Receiver operating characteristic curves
ROS	Reactive oxygen specie
RSD	Relative standard deviation
RSM	Response surface methodology
SA	Strecker aldehydes
SD	Strecker Degradation
SO_2	Sulphur Dioxide
SPE	Solid phase extraction
SVM	Support vector machines
TDN	1, 1, 6, -trimethyl-1,2-dihydronapthalene
TOF	Time-of-flight
UPLC	Ultra Performance Liquid Chromatography
VIP	Variable importance on projection

CHAPTER 1 GENERAL INTRODUCTION

CONTEXT AND MOTIVATION

The organoleptic stability of beverages during storage has been, and still is, a major concern in the Fast-Moving Consumer Goods (FMCG) industry. In fact, consumer acceptability of a specific type of wine depends on a complex network of chemical reactions occurring during storage, which may result in significant flavour modifications. Several different chemical mechanisms are known to contribute to the generation of powerful sensory active compounds in beverages. Interestingly, the same mechanism may impart simultaneously positive and negative aroma notes. For example, phenylacetaldehyde when present in white wine can give a negative contribution to the aroma quality whereas in red port wines are well related to desirable flavour characteristics associated to honey like notes (Silva Ferreira *et al.*, 2002a; António César Silva Ferreira *et al.*, 2003).

Since the Maillard review made by Hodge in 1953 (Hodge, 1953), the Strecker degradation (SD) has mainly been viewed as a corollary of Maillard Reaction where the primary reactants have been considered to be amino containing compounds and reducing sugars. However, in addition to carbohydrate reactants, it is also known that other major food ingredients like polyphenolic compounds can participate in SD to produce novel flavour compounds. Polyphenolic compounds are well related with oxidation mechanism that causes profound modifications on the chemical profile of wine, however the interaction of these compounds with other, formed from other chemical reactions such as Maillard are not fully understood. Structurally, many flavonoids are catechol derivatives that upon oxidation to *o*-quinones may function as precursors of volatile flavour compounds via the SD by reacting with amino acids (Rizzi, 2006a; Delgado, Zamora and Hidalgo, 2015). However, quinones are able to react with other nucleophiles and per se have not received much attention as participants in the SD especially with regard to food systems (Nikolantonaki and Waterhouse, 2012).

A major drawback of the mechanistic studies is that they are focus on target compounds and those only represent a fraction of its chemical complexity and many of them calculate the kinetic parameters just by fitting simple kinetic models, which gives no insight in the reaction mechanism (van Boekel, 2001). Hence kinetic modelling coupled with design of experiments could be a useful technique that helps in building mechanistic models, which reaction route prevails and what kinds of products are formed.

Moreover this "targeted" type of analysis is often inadequate to understand the underlying chemical networks responsible for generating the compounds present in complex matrices

such as wine. In contrast to the use of single-element concentrations, new methods emerge with the aim of generate system-wide data sets for all levels of the chemical information transfer chain, commonly referred as "untarget analysis". These studies have demonstrated that the restricted target analysis of specified metabolites misses a part of the molecular information regarding the entire system and that untargeted approach can be a powerful tool for the molecular fingerprinting of complex beverages such as wine (Castro *et al.*, 2014).

On a chemical perspective, wine can be described as being multi-scale, i.e. several orders of magnitude and multivariate, i.e. large diversity of chemical substances, and because of that the first requirement to achieve the entire systems complexity is to have available techniques that are as comprehensive as possible for chemical analyses. As the chemistry of different metabolites is very heterogeneous, isolating and measuring them all together is very hard, and most chemical studies are really 'chemical profiling' of subsets of chemical classes. Therefore, data fusion of data acquired from an array of detectors will be a critical Task. Taken together, such data can allow the reconstruction of in silico networks (Jacobson, Monforte and Ferreira, 2013). With the recent development of new technologies in the chemical sciences, as well as improvements in statistical and interpretation tools, this type of studies have become a feasible option, and a unique opportunity exists to approach the analysis of chemical systems in a holistic manner – Chemiomics.

LITERATURE REVIEW

1.1. Chemiomics and Wine Quality

Chemiomics studies applied to food aim to build understanding on product quality mainly as a function of process and storage conditions (Monforte, Jacobson and Silva Ferreira, 2015). Wine quality is a complex, multidimensional and relative concept that can change over time. Changes in quality impact product shelf life, defined as the length of time a wine can be stored whilst still maintaining an acceptable quality or specific functionality (Young and O'Sullivan, 2011). Several factors can affect changes in wine quality due to chemical changes. Factors can be categorized in intrinsic and extrinsic factors (Kong and Singh, 2011). Intrinsic factors are related with the product characteristics such as: product formulation and characteristics, pH, water activity, moisture, additives and preservatives. Extrinsic factors are characteristics of the environment and include temperature, relative humidity, light exposure, composition of gaseous atmosphere within packaging. A scheme was proposed to explain the dimensions of wine quality (Figure 1.1) (Charters and Pettigrew, 2007).

Therefore, in order to understand and consequently be capable to monitor wine quality is of paramount importance to study the occurrence and concentrations of chemical molecules in relation to their extrinsic factors and their respective mechanisms of formation.
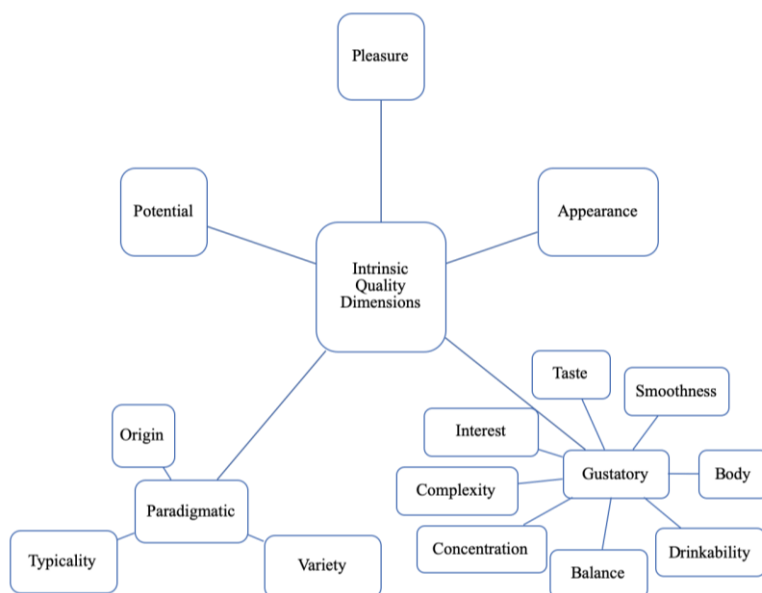
Figure 1.1 Intrinsic dimensions of wine quality adapted from (Charters and Pettigrew, 2007).

1.1.1. Chemiomics Studies in Wine

Several reviews are available in the literature covering the impact in quality of several factors such as viticulture practices and processing and fermentation (Lloyd, Johnson and Herderich, 2015).

Regarding storage conditions: humidity, light exposure, oxygen and packaging are critical factors in wine modifications during storage. Several studies were performed in the last years in order to have a holistic view of the process occurring during storage of wine. Mainly related with oxygen consumption and wine stability.

The effect of oxygen and iron to Sangiovese wines were evaluated using UPLC-Q-TOF-MS. Correlations between oxygen doses and metal contents were observed and changes in the concentration of primary metabolites in particular: arginine, proline, tryptophan and raffinose and secondary metabolites, allowed to formulate new hypotheses regarding the formation and reactivity of wine pigments during micro-oxygenation (Arapitsas *et al.*, 2012a).

Several studies were performed by using GC-MS and GC-FID data in Port wines to better understand the ageing process in particular to extract volatile compounds markers of the oxidative process: sotolon, dioxanes and dioxolanes, furfural, 5-methylfurfural were found as markers of storage for long periods of time (>100 years old) (Jacobson, Monforte and Ferreira, 2013; Monforte, Jacobson and Silva Ferreira, 2015). Furthermore the same compounds were found to be related with oxygen and temperature in a metabolomics pipeline developed with MetAlign as a preprocessing step in order to extract metabolites related with Port degradation in a forced aged protocol (Castro *et al.*, 2014).

HILIC-LC-MS have been used to evaluate differences in wines stored under different conditions (cellar and domestic storage) for 24 months. XCMS and OPLS-DA were used for data pre-processing and features extraction. It was found that polar metabolites and nitrogen containing compounds were the most impacted. 4-Amino-heptenedoic acid and its ethyl ester were detected for the first time in wines stored in cellar conditions (Arapitsas *et al.*, 2016).

Oxygen ingress was evaluated in Champagne wines during ageing on lees, samples were characterized by FTICR-MS and compositional networks were used to aid in the identification of hypothetical structures related with wine oxygenation. Results shown a discrimination of wines according to the vintage, and regardless of their oxygenation level, demonstrating that sensibility to oxygen was wine dependent (Roullier-Gall *et al.*, 2016).

The impact of glutathione one wines oxidative stability was studied using sensory combined with FTICR-MS, where Spearman rank was used to combine both types of information. Van Krevelen diagrams were used to detect groups of metabolites more affected by the perturbations and amino acids, aromatic compounds with S- and peptides were the most affected. According with the authors know the chemical compositions and origin of wines antioxidant metabolome is crucial to estimate wines ageing potential (Nikolantonaki *et al.*, 2018).

The oxidation of oaked chardonnay classified in three groups according with browning (absorbance at 420nm) were evaluated using NMR and GC-MS. From 155 compounds detected, acetaldehyde, 3-methylbutanal, phenylacetaldehyde, methional, 3-penten-2-one, β-damascenone were confirmed as markers of oxidation. Moreover new compounds were identified as markers of the process: pentanal, 3-methyl-2-butanone, 3-penten-2-one, 2-methyltetrahydrofuran-3-one, , ethyl 2-methylbutanoate and vinyl decanoate (Pinto *et al.*, 2018).

In five different wines from several vintages phenolic compounds markers of oxidation were found to be discriminant in the detection of the oxidative status of white wines preliminary classified in low and high oxidation classes. In particular 3-methylcatechol, cyanidin 3-O-6″-p-coumaroyl-glucoside, delphinidin 3-O-glucoside, quercetin 3-O-glucosyl-xy-loside, dihydroquercetin, and quercetin 3-O-glucuronide were the molecules found (Romanini *et al.*, 2019).

Chardonnay wines were produced with two different amounts of SO_2, oxidation was promoted by electrochemical simulation and on-line and off-line FTICR-MS acquisitions were performed. Results confirmed that several sulfur-containing and nitrogen-containing compounds were the most sensitive to oxidation (Roullier-Gall *et al.*, 2019).

The combination of sensory evaluation, chemical and metabolomics analyses in combination with the study of oxygen transfer through the bottleneck/stopper, demonstrate the importance of the glass/cork interface. Metabolomics analysis show that non-oxidative markers were sulfonated compounds, and oxidation markers were characterized by a significantly reduced contributions of compounds containing sulphur and nitrogen atoms (Karbowiak *et al.*, 2019).

1.2. The importance of aroma compounds in quality perception

From the intrinsic dimensions of quality, five were selected has the most relevant ones, being the gustatory factor, related with aroma and taste one of them (Charters and Pettigrew, 2007).

Olfactory perceptions lead us to the importance of aromas in foods. Aroma compounds are volatile substances which are perceived by the odor receptor sites. Nevertheless, from all the volatile compounds, molecules considered as aroma substances are primarily those which are present in food in concentrations higher than the odor thresholds.

Molecules with impact on food quality, might contribute to the typical odor of one specific product, while in another can cause a faulty odor, resulting in an off-flavour (Belitz, Grosch and Schieberle, 2004). An off-flavour can arise through foreign aroma substances, that are normally not present in a food, loss of key odorants, or changes in the concentration of individual aroma substances (Reineccius, 1994). Compounds that provide the characteristic aroma of the food are called key odorants (character impact aroma compounds). Some compounds such as methional and phenylacetaldehyde are considered key compounds in potatoes and honey, respectively but an off-flavour when present in white wines.

Non-enzymatic browning is very important to the production of desirable foods, it is also a primary source of undesirable aromas. In Table 1.1, an overview of non-enzymatic reactions shows the main reactions with impact in food sensorial properties.

Rarely food products improve in sensory quality with storage with some exceptions, like for example cheese and wine. In the case of the wine a new layer of complexity in the definition of the "sensory characteristics" is added, due to the difficulties related with changes in the sensory profile across raw material (grapes), process of vinification and storage.

According to literature from a technological point of view related with wine organoleptic characteristics, factors affecting wine quality could be divided in i) factors of quality regarding the grape (raw material) and ii) factors of quality regarding the process. Briefly, flavour in wine can be divided in three, dependent in its origin, varietal, fermentative and ageing.

Table 1.1 Overview of chemical reactions in foods with impact in quality adapted from (Van Boekel, 2008).

Example	Type	Consequences
Nonenzymatic browning	Chemical reaction Maillard reaction	Colour, taste and aroma, nutritive value
Fat oxidation	Chemical reaction	Loss of essential fatty acids, rancid flavour.
Phenolics oxidation	Chemical reaction	Off-flavours, mainly due to the formation of aldehydes and ketones
Hydrolysis	Chemical reaction	Changes in flavour, vitamin content

1.3. Wine – Storage and Ageing Impact on Flavour Quality

The shelf-life in a wine is the primary concern of the wine industry, and in the case of a young wine it is directly linked to its resistance to oxidation, aside from the problems caused by different sources of yeast and bacteria.

Depending on the type of wine, ageing can be seen as a way to produce high-quality wines (red wines) or in the case of white wines a way to decrease the quality of the product.

Regarding red wines good agreement between wine professionals on the assessment of aging potential of red Burgundy wines were associated with high astringency, high concentration of anthocyanins and polyphenols and a red saturated colour (Jaffré *et al.*, 2009).

The first published studies regarding white wine deterioration were focused on the browning, i.e. in the chromatic changes (Simpson, 1983; Singleton, 1987; Cheynier *et al.*, 1990; Gómez, Martínez and Laencina, 1995). Later it was demonstrated that oxidative browning affected not only chromatic characteristics but the aromatic profile. Earlier, it was thought that acetaldehyde and its acetals were the main aromas generated during wine oxidation (Wildenradt and Singleton, 1974). But approximately 2 decades later it was demonstrated by several authors, that acetaldehyde wasn't the main contributor for the aromatic oxidation faults. Oxidative storage affected mainly unsaturated aldehydes (trans-2-octenal, trans-2-nonenal, trans-2-decenal) and wine browning was mainly correlated with furfural formation (Ferreira *et al.*, 1997). The same research group conducted, three years later, a sensorial study and detected 5 compounds with maximum dilution factors in oxidized wines namely: 2,4,5-trimethyl-1,3-dioxolane, methional, eugenol and sotolon (Escudero, Cacho and Ferreira, 2000). The role of methional was further studied and it was demonstrated its sensorial impact by adding it to the oxidized wine and leading to a "cooked vegetables" off flavour. The impact of the compound in oxidized wines related with non-oxidized wines, was more than 200 odor units and their perception limit were found to be 0.5 ppb in a synthetic wine. It was proposed that methional could be formed via Strecker degradation of methionine or by the direct peroxidation of methionol (Escudero *et al.*, 2000).

More recently, it was found that when submitting a white wine to a forced aged protocol a synergistic effect of oxygen and temperature in the deterioration of wine, in particular in the decrease of terpene alcohols and norisoprenoids (floral aromas) and in the development of off flavours such as "honey-like", "boiled potato" and "farm feed" associated with the presence of phenylacetaldehyde, methional and TDN (Silva Ferreira *et al.*, 2002a). Sensorial tests proved the role of the above molecules more sotolon in the typical oxidation-spoiled aroma (A C Silva Ferreira *et al.*, 2003).

Ageing in white wines leads to the formation of aromas that are transversal to all types of wines, contrary to varietal aromas, meaning that common mechanisms are responsible for their formation. Maillard reaction and phenolics oxidation are the main mechanisms responsible for changes in the aroma profile occurring during ageing.

1.4. Mechanisms of Flavour Formation: Maillard Reaction

Maillard Reaction was first reported by Louis-Camille Maillard in 1912 (Maillard, 1912). The reaction includes a cascade of several steps initiated by a condensation of amino group with a reducing sugar, resulting in the formation of intermediaries, including aroma components and high molecular weight brown polymers. The reaction can be divided in three main steps:

1.   Initial Stage: sugar-amine condensation and Amadori rearrangement.

2.   Intermediate Stage: Sugar dehydration and fragmentation and amino acid degradation

3.   Final Stage: Polymerization of intermediates to produce fluorescent and coloured polymers known as melanoidins.

Nevertheless this classification was considered too general and simplistic to accommodate recent information obtained (Yaylayan, 1997). The mechanism for intermediates formation through the Maillard reaction, is dependent on three 'principal precursors': sugars, amino acids and Amadori or Heyn's products. The nature and the relative ratios of these precursors constitutes the 'parent pool' and will determine the pathways of the MR under specific conditions. It was demonstrated that besides the reactants (type & concentration), temperature, pH and water activity are key factors regarding the capacity for the MR to occur (Ames, 1990). In relation to aroma, the type of reactants determines the type of flavour formed while the extrinsic factors influence the kinetics of the reaction (Figure 1.2) (van Boekel, 2006).

So far, only a few products of Maillard reaction have been quantified in foods, being the Amadori products the most identified (> 90% of the MRPs). Amadori compounds derived from the condensation of amino acids with reducing sugars in the Maillard reaction and are important flavour and colour precursors (D. Mills *et al.*, 1969). Amadori compounds were for the first time identified in white wine and confirmed as important compounds in oxidative browning (Hashiba, 1978).

The Maillard reaction is initiated by a condensation reaction between a reduction sugar and a compound with a free amino group (amino acids, peptides, proteins) given a N-substituted glycosamide which can rearrange to form the Amadori rearrangement product (ARP) (Hodge, 1955). At pH below 7 ARP suffers 1,2-enolisation leading to the formation of

furfural or hidroxymethylfurfural depending on the type of sugar, pentoses or hexoses respectively. Further along with enolization reactions, ARP and its dicarbonyl derivatives can undergo retro-aldol reactions producing C2, C3, C4 and C5 sugar fragments. Capable to act as Strecker degradation substrates. Nevertheless, it was proposed a new mechanism for the formation of Strecker aldehydes via a direct degradation of the ARP without the presence of α-dicarbonyls, catalyzed by oxygen and metal ions (Hofmann and Schieberle, 2000).
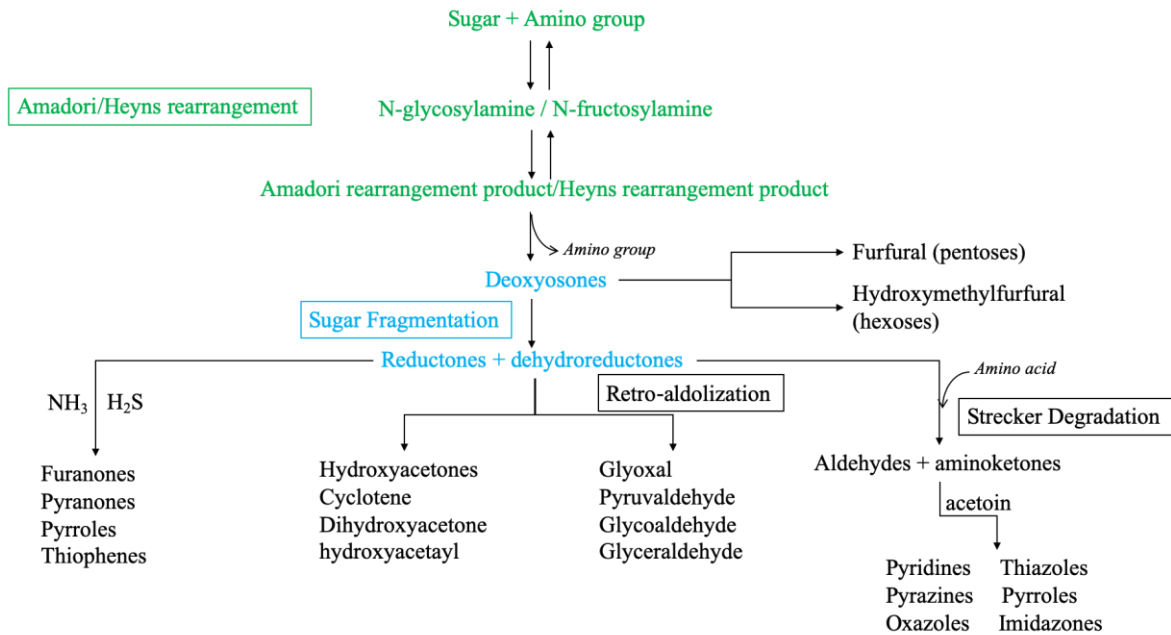
Figure 1.2 Overview of Maillard reaction and flavour compounds as end products adapted from (van Boekel, 2006).

The first evidences of the role of Maillard reaction in wines, were observed in the aroma of sweet fortified wines following heat treatment (Deibner and Benard, 1956). Later MR molecules formation in that type of wines were attributed to the average temperatures and the high amount of sugars (Cutzach, Chatonnet and Dubourdieu, 1999).

The formation of heterocycles related with MR in wine were related to the generation of notes described as 'popcorn', 'hazelnut', 'toasted' and 'roasted' (Marchand, de Revel and Bertrand, 2000; de Revel, Marchand and Bertrand, 2009). In sparkling wines, HMF, a marker of Maillard reaction in wines stored without wood contact, were considered a suitable

marker to reveal conditions of storage due to its linear behavior with storage time at all studied temperatures (4, 16 and 20 ºC) (Serra-Cayuela *et al.*, 2014).

In Port wines, and dry white wines submitted to a forced aged protocol, 3-deoxysonone an important intermediate of the reaction were identified and quantified. Results demonstrated a negative correlation of the compound with the age of Ports (Oliveira *et al.*, 2016).

Elucidation of the progress of Maillard reactions in foods is complicated; the presence of several reactants as well as the dynamic conditions found in wines, processing and storage conditions all contribute to a complex chemical landscape (Lund and Ray, 2017).

1.5. Mechanisms of Flavour Formation: Oxidation

Wine is capable of reacting with a considerable amount of oxygen; red rather more so than the white, as the principal initial reactants are phenolic compounds. It plays a central role during several steps of production, in particular, during storage imparting modifications occurring at the level of flavour, colour and taste (Ribéreau-Gayon, 1933). In red wines a degree of oxidation could be beneficial to red wines contributing to colour stabilization, softening astringency and bitterness and decrease vegetative and green character. But in white wines oxygen is responsible for undesirable effects, mainly due to the increased occurrence of reduced or oxidized aroma off-flavours as well as low colour stability.

It was recognized several years ago that the reactions involved in the modifications of wine by O_2 were complex and difficult to understood (Rossi and Singleton, 1966) lacking a comprehensive understanding.

"Although the fundamental chemistry of oxidation reactions has been well characterized in many food systems, the complexity of wine and its many interacting components have prevented a comprehensive understanding (Waterhouse and Laurie, 2006)*"*

The first postulated mechanisms were proposed involving the nonenzymatic autoxidation of vicinal dihydroxy phenols, resulting in the production of hydrogen peroxide capable to convert ethanol in acetaldehyde (Figure 1.3) (Wildenradt and Singleton, 1974).
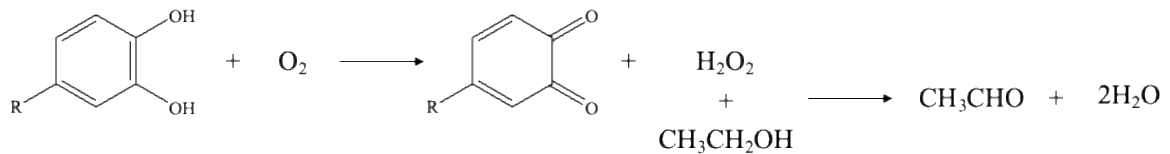
Figure 1.3 Autoxidation of catechol derivatives in wine produces acetaldehyde by coupled oxidation of ethanol adapted from (Wildenradt and Singleton, 1974).

From the first oxidation studies (before 1987) several observations were made:

- Polyphenols are the main substrates for oxygen in wine (Ribéreau-Gayon, 1933).

- Multivalence metals in wine (iron, copper and manganese) were considered key catalysts in wine oxidation (Ribéreau-Gayon, 1947).

- The golden and brown colour in white wines come exclusively from phenolics (Rossi and Singleton, 1966);

- Polyphenols are able to polymerize as a result of oxidation (McDonald and Hamilton, 1973);

- Dry wines protected against oxidation do not suffer browning (Singleton and Kramling, 1976);

- Browning in white wine was more closely related with flavonoid content (Simpson, 1983);

- The rate of oxygen dissolved in wines is slower in the beginning and faster after (Singleton and Kramling, 1976);

- In wines with excess of oxygen the colour changes to amber, oxidized flavours are produced, and precipitations are caused, mainly in red wines (Singleton, 1987);

- Oxidation rarely improves white wine, but improvement occurs in red wines (Singleton, 1987).

The first studies regarding oxidation in white wine as a major organoleptic fault started in the 1990s beginning with studies regarding changes in wine colour.

A particular study focuses on the role of metals (iron copper and manganese) in several phenolic fractions, demonstrate that O_2 and metals concentration have an impact in

decreasing total phenolic content and tannin fraction but not on the anthocyanin content. At that time acetaldehyde were considered the "volatile proxy" for oxidation in wines, as shown in Figure X. In the same study it was observed that acetaldehyde increases in the first days of oxidation and then decreases under extreme conditions. The authors added to the conclusion that manganese favors acetaldehyde formation while iron induces their polymerization with phenolic compounds (Cacho *et al.*, 1995).

Moreover the addition of iron to a wine model solution containing catechin resulted in the formation of yellow pigments with maxima absorbances in the 440-460 nm region, demonstrating the role of flavanols in the browning of wines (Oszmianski, Cheynier and Moutounet, 1996). Other hypotheses were established to explain colour changes during storage time, in particular the formation of xanthylium-type polymers by the condensation between flavanols-flavanols, flavanols-anthocyanins (M. Francia-Aricha *et al.*, 1997) and phenolics-acetaldehyde (Saucier, Little and Glories, 1997).

After 2000s two approaches started to appear, one aiming to understand the key factors with impact on the sensory perception of wines and the second a molecular approach aiming to clarify the mechanisms involved in the oxidation of wine.

1.5.1. Molecular Oxygen and Reactive Oxygen Species

The solubility of oxygen from air into wine saturated at room temperature and atmospheric is 8 mg/L, increasing about 10% at temperatures 5°C lower (Singleton, 1987).

Oxygen (O_2) in nature is in its minimum energy triplet form ($^3O_2$), the ground state of molecular oxygen a stable and unreactive form. In this configuration the molecule cannot react directly with most of all organic molecules, neither change electrons in order to form bonds. But excitation of this oxygen form is possible when induced by some factors capable to give the energy needed to transform the triplet form in its singlet state ($^1O_2$).

In wine the reduction of the triplet to singlet form is due to the acceptance of an electron from a metal catalyst such as Fe^{2+}/Fe^{3+} (Felipe Laurie and L. Waterhouse, 2006). A ladder of reactive oxygen species (ROS) is formed as observed in Figure 1.4.

These radical oxygen molecular species all become more reactive as their reduction level increases *triplet oxygen < superoxide anion < hydroperoxide radical < peroxide anion < hydroxyl radical*.
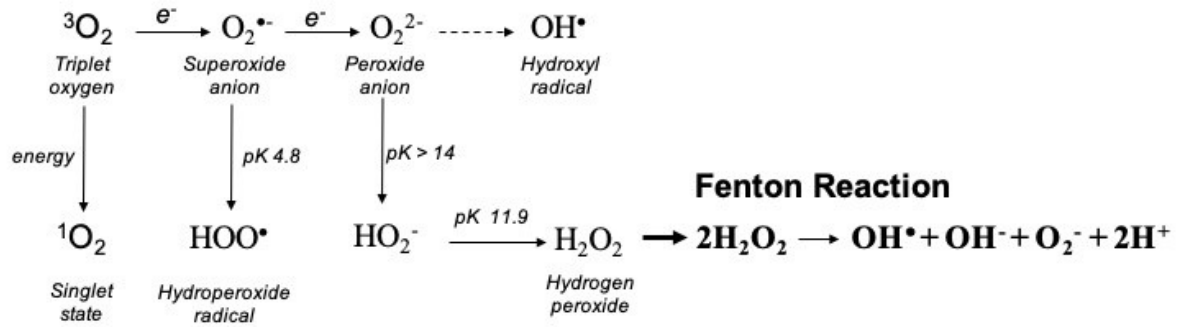
Figure 1.4 Ladder of oxygen reduction adapted from (Karbowiak et al., 2009).

Hydrogen peroxide is capable to react with a ferrous or cuprous salt to give a hydroxyl radical, a very potent specie capable of nonspecifically oxidizing all organic constituents in proportion to their concentration in wine. The fate of H_2O_2 can be changed by sulfur dioxide (SO_2) capable to diverting peroxide from the Fenton route (J. Elias and L. Waterhouse, 2010).

Preferred substrates are the oxidation of ethanol in acetaldehyde and the organic acids into keto acids. For example glucose and glyceraldehyde yield carbon dioxide, lactic acid gives pyruvic acid and tartaric acid can yield diketosuccinic acid, hydroxy malonic acid and glyoxylic acid (Danilewicz, 2003).

Electron paramagnetic resonance spin trapping confirm that wine oxidation is mediated by radical processes and that several key steps are catalyzed by transition metals and the Fenton reaction has been confirmed by the appearance of hydroxyethyl radical (J. Elias *et al.*, 2009).

In wines undergoing micro-oxygenation, oxygen consumption increases when SO_2 was depleted, fact attributed to Fenton reaction due to the continuous formation of radicals (Gambuti *et al.*, 2015).

Comparison of oxygen consumptions were performed in several types of wines. Results show that for red wines oxygen consumption rates were higher in the beginning of the

saturation cycles (range between 0.5 and 8.2 in the beginning and 0.4 and 0.8 mg O_2/L/day). Meaning reaction orders higher than firs-order, suggesting several mechanisms involved in the oxidation mechanism. Moreover, initial rates were negatively correlated with to SO_2 consumption, suggesting that in the beginning the O_2 consumption rate could be controlled by an unknown antioxidant (Ferreira *et al.*, 2015).

Nonetheless, in rosé and white wines the oxygen consumption rates are below the values found in red wines and not so dramatic changes were observed in the beginning and after 30 days (0.3 and 0.8 for 5 days and 0.2 and 0.6 mg O_2/L/day after 30 days) (Carrascón *et al.*, 2017). The conditions of storage have a very important role on the ability of wine to age.

Wines capacity to interact with oxygen is largely dependent on its composition, which makes it very difficult to predict the impact of a given dose in wine characteristics. For example high pH and the presence of ellagitannins can increase oxygen consumption (Vivas and Glories, 1996). Consequently, oak wood could be used to influence oxidation-reduction of wines due to their antioxidant capacity and therefore modulate its oxidative stability. Independently of the vintage and the wine matrix a relationship was established between oxidative stability and oak barrel tannin potential (Nikolantonaki *et al.*, 2019).

That fact was studied recently, and the complexity of oxygen consumption is increased when oak wood is added to a wine (oak ships, days) due to the fact that they begin to soak, releasing to the wine the oxygen adhering to their surface. Kinetics if oxygen release from oak chips were evaluated and it was demonstrated the contribution of it to the ellagitannin disappearance (García-Estévez *et al.*, 2017).

In particular, the type of container and the closure will have a strong impact on the amount of oxygen capable to permeate to the wine after bottling. The oxygen exposure depends on the closure sealing capacity, for example in general in synthetic stoppers the oxygen entrance rate is higher compared with screw caps and technical corks. In natural corks the entrance of oxygen is dependent on the cell structure of the cork. It was demonstrated by storing a Sauvignon Blanc during 24 months that higher browning were conserved in synthetic corks while wines sealed under more airtight conditions (screw cap) and natural corks have the slowest rate of browning and less reduced and oxidized character (Lopes *et al.*, 2009).

Comparison of storage of red and white wines in PET bottles, Bag in Box and glass bottles, demonstrated that white wine conservation contrary to red wine was strongly affected in

PET bottles, while in PET bottles containing a O_2 scavenger as in Bag in Box no significant changes were observed when compared with glass bottles (Ghidossi *et al.*, 2012).

1.5.2. The Catechol-Quinone System and the Oxidation Process

The principal initial reactants for wine oxidation are polyphenols and ethanol. Polyphenol are oxidized to quinones and ethanol to acetaldehyde as shown in Figure 1.5.

Iron in its reduced ferrous state (Fe^{2+}), is first oxidized to the ferric state (Fe^{3+}) by oxygen and then reduced to the oxidation of the phenolic compound to quinone. Oxygen would be reduced to hydroperoxyl radical ($HO_2^{\bullet}$) that could again oxidize polyphenols to quinones, possible by Fe^{3+}. Nevertheless Fe^{2+} can reduce an intermediate Fe-oxygen complex capable to produce H_2O_2, that can be rapidly reduced by Fe^{2+} in hydroxyl radicals ($HO^{\bullet}$). As explained above this highly reactive radical will react immediately with ethanol and the elimination of the hydrogen peroxide would then produce acetaldehyde (Danilewicz, 2003, 2013; Danilewicz and Wallbridge, 2010).

With a typical total concentration of about 0.01% (total weight) for white wine and 0.2% for red wines, phenols play a key role in the overall antioxidant capacity of wines.

Briefly polyphenols include the cinnamic acid derivatives, flavanols and derivatives, which include oligomers, the proanthocyanins, and polymers (Waterhouse, 2002). Substances with pyrogallol and catechol groups, are the most abundant initially oxidizable wine constituents.

Several authors devoted their research in better understand the phenolic compounds more affected by oxidation reactions, one of the techniques used to rank phenolic compounds according with their reducing strength was cyclic voltammetry (A. Kilmartin, Zou and L. Waterhouse, 2001). Nowadays the method has considered an official AOAC international method for the determination of wine oxidation-reduction potentials. Nevertheless it was demonstrated that the electrochemical properties of platinum electrodes can change the reaction order by allowing ethanol oxidation to couple first with oxygen reduction, in the absence of other oxygen electroactive species (Danilewicz, Tunbridge and Kilmartin, 2019).
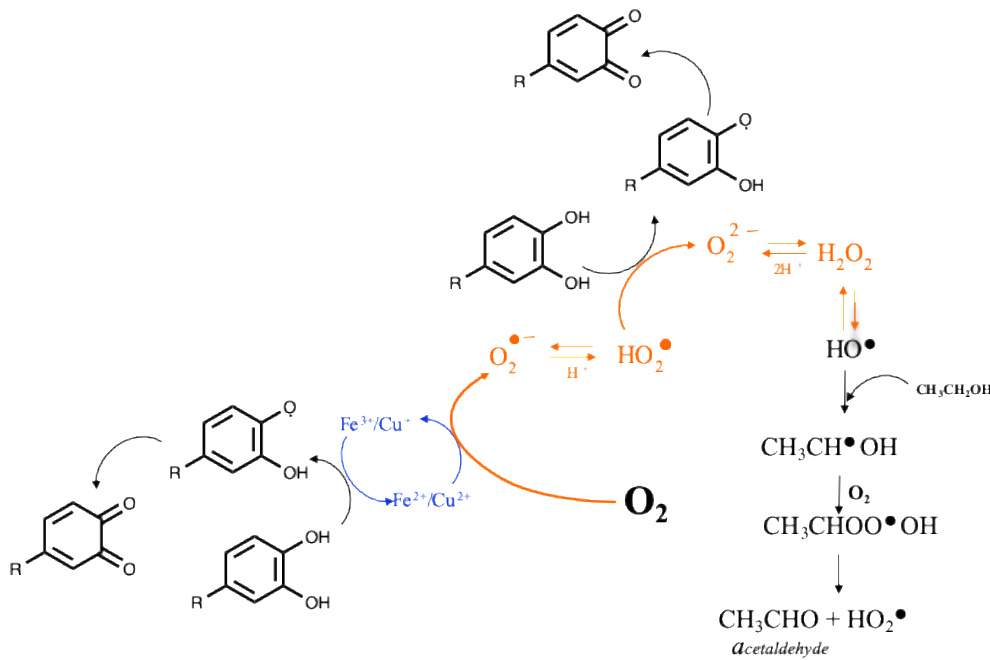
Figure 1.5 Proposed mechanism illustrating the reactions occurring due to oxygen consumption.

The product resulting from polyphenols oxidation are the quinones that are capable to react with several nucleophiles constituting a key mechanism to understand wine ageing. Antioxidants (sulfur dioxide, glutathione, ascorbic acid), desirable and undesirable aroma thiols, amino acids and polyphenols all represent nucleophilic species in wine capable to react with quinones (Nikolantonaki and Waterhouse, 2012). According with a nucleophilic scale with the quinone of 4-methyl catechol: methionine and phenylalanine were the weakest nucleophiles followed by phloroglucinol < 4-methyl-4-sulfanylpenta-2-one (4MSP) < 3 sulfanylhexan-1-ol (3SH) < 2-furanmethanethiol (2FMT) and the strongest reactants were sulfur dioxide, ascorbic acid, glutathione and hydrogen sulfide. Furthermore, nucleophiles, which reacts with quinones faster than the electron transfer of flavonoids, such as SO_2, can prevent the browning generated via the quinone reactions with flavonoids.

1.5.3. The Role of Metals

Transition metals such as iron and copper are important catalyzers of wine oxidation. Typical concentrations in wine are between 1 - 6 mg/l and 0.1 – 3.6 mg/L respectively. While Fe^{2+} is the reducing agent of oxygen to the production of hydrogen peroxide, the Fe^{3+} oxidizes polyphenols with the assistance of nucleophiles, such as sulfite, regenerating Fe^{2+} (Figure

1.6). A low $[Fe^{3+}/Fe^{2+}]$ ratio would indicate that polyphenols are capable of removing O_2 that is entering in the system while an increasingly ratio would indicate that a wine is oxidizing at faster rates. Moreover Fe^{3+} inhibits Fe^{2+} oxidation and as its concentration increases their production is slower down. Nevertheless, it was demonstrated that organic acid, in particular tartaric and malic acid, are capable to co-ordinate Fe^{3+}, forming dimeric complex facilitating Fe^{2+} oxidation. Due to the highest concentration of organic acids in comparison to polyphenols the complexes must form to some small extent to allow oxidation to occur (Danilewicz, 2014). The coordination of tartaric acid, lowers the reduction potential of the Fe^{3+}/Fe^{2+} turning the Fe^{3+} oxidation of the catechol disfavored (Danilewicz, 2011).
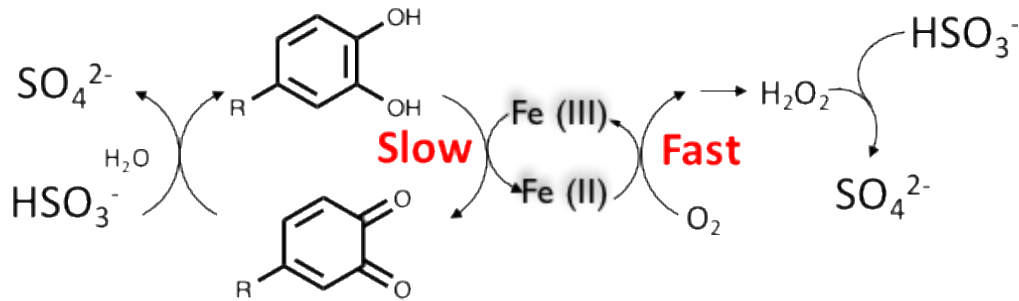
Figure 1.6 Proposed mechanism of catechol oxidation in wine: Fe(III)/Fe(II) redox cycling and involvement of SO2 adapted from (Danilewicz, 2016).
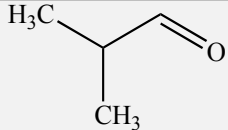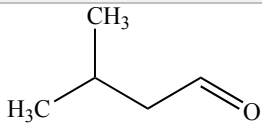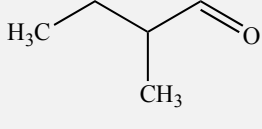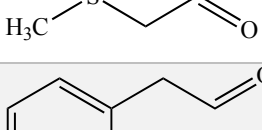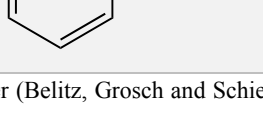
Recently a method was developed in order to evaluate if the ratio can be an indicator on the redox status of wines stored in stored using in box, or in bottles with screw cap or natural corks. The results shown that the Fe^{2+}/Fe^{3+} ratio was dependent on the type of storage (higher in bottles with screw caps than natural cork and boxes) and changed with oxygen exposure. As Fe^{2+} decreases with oxidation the ratio stabilizes which is imported the compositional changes between wines. Meaning that several factors can affect the capacity of wines to oxidize and metals by itself are not enough to establish the redox status of wines (Danilewicz, 2016).

1.6. The Impact of Strecker Aldehydes

Strecker aldehydes (SA) are important for flavour development in foods such as bread, coffee, cocoa, and roasted meat, however they are perceived as off-flavours in other food products such as wine, beer and processed milk.

Few Strecker degradation reactions have been of interest to researchers: 2-methylpropanal (derived from valine), 2-methylbutanal (derived from isoleucine), 3-methylbutanal (derived from leucine), methional (derived from methionine), and phenylacetaldehyde (derived from phenylalanine) (Table 1.2). Benzaldehyde is formed indirectly from phenylalanine with phenylacetaldehyde but is considered a SA (Guedes de Pinho and Silva Ferreira, 2006).

Table 1.2 Strecker aldehydes in wine, structures, precursor amino acid and odour thresholds found in water and in wine.

Strecker aldehyde	Structure	Precursor Amino acid	Odor threshold water/wine
2-methylpropanal		**Valine**	2 / 6 µg/L
3-methylbutanal		**Leucine**	3 / 4.6 µg/L
2-methylbutanal		**Isoleucine**	4 / 16 µg/L
Methional		**Methionine**	0.2 / 0.5 µg/L
Phenylacetaldehyde		**Phenylalanine**	4 / 1 µg/L

Odor thresholds in µg/L in water (Belitz, Grosch and Schieberle, 2004) and in wine model solution (Culleré, Cacho and Ferreira, 2007)

In 1999, Rizzi (Rizzi, 1999) presented a review regarding the contribution of SD to food flavour. The first studies were focus in milk (Patton and Josephson, 1953), cheese (Keeney and Day, 1957; Griffith and Hammond, 1989), cocoa beans (Purr, Helfenberger and Nadai, 1963; Darsley and Quesnel, 1972), chocolate (Rohan and Stewart, T, 1966), tea (Bokuchava and Popov, 1954) and tea leaves (Saijō and Takeo, 1970). Coincidently these first studies were performed in beverages and foods normally stored for long periods of time.

In beverages such as wine Strecker Aldehydes, in particular methional and phenylacetaldehyde, are among the oxidation-related aroma compounds that have drawn most attention, due to their aroma impact. The first studies recognizing the role of aldehydes in wine aroma were performed more than 30 years ago (Baró and Quiros, 1977).

Methional contributes with a "pungent" and "cooked vegetables" notes and their presence marks the end of wine shelf-life (Escudero, Cacho and Ferreira, 2000). It was observed that methional could reach more than 200 odor units when compared oxidized with non-oxidized wines. Moreover their formation was promoted when methionine and methional were added to wines (Escudero *et al.*, 2000).

Phenylacetaldehyde, contributes with a "honey-like" aroma, and its formation is related with the aroma deterioration of white wines (A C Silva Ferreira *et al.*, 2003). The formation of these compounds is greatly affected by oxygen concentration, temperature, pH and SO_2 (Silva Ferreira *et al.*, 2002b). Nevertheless, their mechanism of formation is not yet established since several pathways can contribute to their formation.

Phenylacetaldehyde and methional have been identified in several types of wines as indicated in Table 1.3.

Table 1.3 Phenylacetaldehyde and methional concentration and several types of wines.

Type of wine	Phenylacetaldehyde	Methional	Reference
Dry white wines	3 - 90	1 - 5	(Culleré, Cacho and Ferreira, 2007)
Red wines	5 - 40	0.8 - 9	(Ferreira *et al.*, 2014)
Pedro Ximenez	64 - 74	16 - 24	(Campo, Cacho and Ferreira, 2008)
Fino	36 - 42	8 - 20	(Campo, Cacho and Ferreira, 2008)
Sauternes	80 - 114	15 - 28	
Cava	16 - 18	16 - 18	
Sweet wines botrytis	23 - 136	4 - 50	(Sarrazin, Dubourdieu and Darriet, 2007)
Port Wines	60 - 100	n.d. - 17	(Culleré, Cacho and Ferreira, 2007)
Madeira Wines	45 - 126	n.d. - 24	(Campo *et al.*, 2006; Oliveira e Silva *et al.*, 2008)

1.6.1. Strecker Degradation Mechanism

Strecker degradation has been named in 1948 (Schönberg, Moubasher and Mostafa, 1948) approximately 50 years after the first observation that alloxan can react with an amino acid (alanine) to produce carbon dioxide and acetaldehyde (Strecker, 1862) as indicated in Figure 1.7.
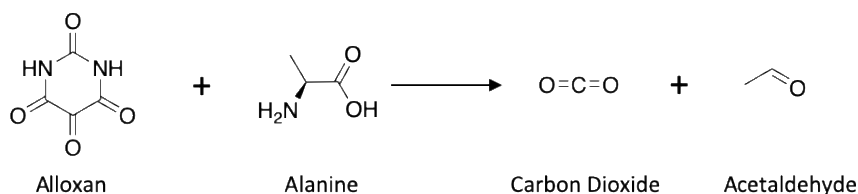
Figure 1.7 Mechanism of Strecker degradation between alloxan and alanine

According with the definition "Strecker degradation" refers to all degradations of α-amino acids to aldehydes and ketones containing one carbon atom less, whatever the degradant agent may be. But, it was demonstrated that aldehydes were easily formed with α - dicarbonyls or α -tricarbonyls and vinylogous compounds such as quinones and a mechanism was proposed (Figure 1.8) (Schönberg, Moubasher and Mostafa, 1948). The first studies reported Strecker degradation as a sub-section of the Maillard Reaction (MR) due to the fact that MR generates compounds easily converted to dicarbonyl structures (Hodge, 1953).
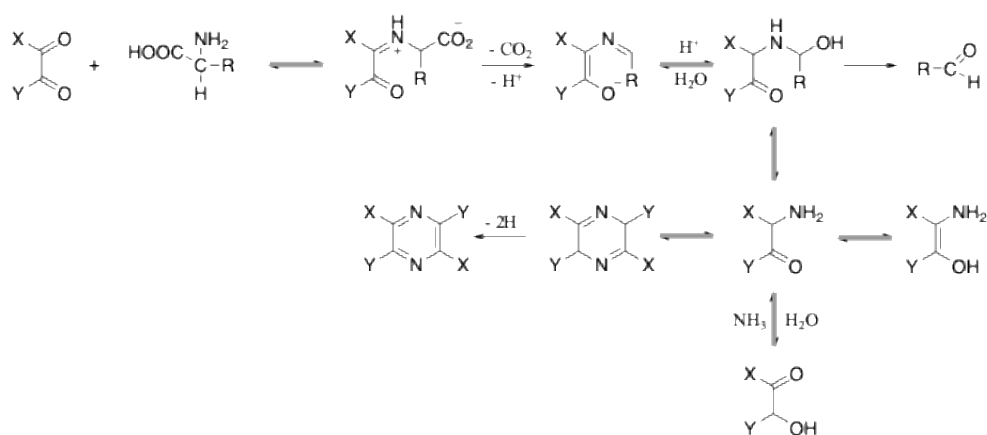
Figure 1.8 Mechanism of reaction between a dicarbonyl and an amino acid for the formation of a Strecker aldehyde adapted from (Schönberg, Moubasher and Mostafa, 1948).

1.6.2. Strecker degradation mediated by α-dicarbonyls

α-Dicarbonyl structures are of major interest in food chemistry and biochemistry. They play a central role in the course of MR as flavour and colour (melanoidins) are formed by the reaction of those key intermediates (Kroh, Fiedler and Wagner, 2008).

There are 11 dicarbonyl compounds formed from monosaccharides as indicated in Table 1.4 (Thornalley, 2005).

At low pH, quantitative measurements revealed that 3-deoxysosone and glyoxal were the first formed sugar degradation products while methyl glyoxal only appeared later in the reaction. Nevertheless the last proved to be the most effective in the generation of

phenylacetaldehyde at lower pH (3.0) and phenylacetic acid at higher pH (9.0) (Hofmann, Münch and Schieberle, 2000).

Table 1.4 α-dicarbonyls intermediates of Maillard Reaction

Glyoxal	Erythrosone	Glucosone
Methyl glyoxal	3-deoxyerythrosone	1-deoxyglucosone
Hydroxypyruvaldehyde	Ribosone	3-deoxyglucosone
	3-deoxyribosone	3,4-dideoxyglucosone-3-ene

The principal α-dicarbonyl compounds found in wine are: glyoxal, methylglyoxal, diacetyl and pentane-2,3-dione (De Revel and Bertrand, 1993). All these compounds could be synthetized by *Saccharomyces cerevisiae* during alcoholic fermentation and also by *Oenococcus oeni* during malolactic fermentation, except pentane-2,3-dione (Revel *et al.*, 2000).

The dicarbonyls values in red and white table wines ranged from 0.15 to 2 mg/L for glyoxal, from 0.1 to 1 mg/L for methyl glyoxal, from 0.5 to 10 mg/L for diacetyl and from 0.05 to 5 mg/L for pentane-2,3-dione (Revel *et al.*, 2000). While in sweet fortified wines (Madeira and Ports) the concentrations ranged from 4 and 30 mg/L for glyoxal, from 0.4 to 26 mg/L for methyl glyoxal, from 1 and 4 mg/L for diacetyl and 0.1 and 0.5 mg/L for pentane-2,3-dione (Silva Ferreira *et al.*, 2007).

It was demonstrated that in wine model conditions, by reacting 14 amino acids with 4 dicarbonyl compounds that Strecker degradation could happen in wine ageing conditions (low pH and low temperatures) (Pripis-Nicolau *et al.*, 2000). The most interesting molecules according with the authors were identified in the presence of cysteine with the production of heterocycles compounds (de Revel, Marchand and Bertrand, 2009). The referred compounds in particular methyl glyoxal and glyoxal could bind to SO_2 (Barbe *et al.*, 2000).

Moreover, it was observed that the blockage of α-dicarbonyls inhibited the accumulation of sensory-active aldehydes such as *trans*-2-nonenal and phenylacetaldehyde.

Trapping of dicarbonyls, in particular glyoxal and methyl glyoxal was a subject of study using several food models. In particular compounds such as: flavonoids (Totlani and Peterson, 2006; Sang *et al.*, 2007; Liu *et al.*, 2017; Wang *et al.*, 2017), hydroxytyrosol (Navarro *et al.*, 2015) and glutathione (Zheng, Chung and Kim, 2015).

1.6.3. Strecker degradation mediated by o-quinones

Besides α-dicarbonyls, it was found that *o*-quinones are reagents capable to suffer Strecker degradation. These compounds are oxidants and possess soft (α,β-unsaturated carbonyl) and hard (carbonyl) electrophilic centers. The molecule is composed by a conjugated cyclic dione in its aromatic structure, and depending on the position of the -C(=O)-group the molecule can be classified in *ortho-* or *para*-quinone (Kato and Suga, 2018).

In food systems involving fermentative processes such as tea, coffee, *o*-quinones can be formed by the action of *polyphenol oxidases* in phenolic compounds (Saijō and Takeo, 1970). Nevertheless it was demonstrated that *o*-quinones could be formed by the direct oxidation of the polyphenol in the presence of a strong oxidant in the absence of PPOs (Rizzi, 2006b) and react with several nucleophiles (Kutyrev, 1991). Since nucleophilic 1,4-Michael addition to a quinone represents a formal two electron reduction resulting in a catechol-nucleophile conjugate, their oxidant and electrophilic properties are closely related.

In wine, *o*-quinones are key reactive electrophilic oxidation intermediates. To understand the role of quinones in Strecker degradation substrates is crucial to understand the molecular reactions of O_2 as well as the factors involved in the underlying mechanism.

Reaction of Quinones with Amino acids

In 1979, Motoda investigated the SD of alanine, valine, leucine and isoleucine in the presence of catechin and a purified PPO extracted from a microorganism, it was observed the formation of the Strecker aldehyde 3-methyl-butanal from leucine (Motoda Tokyo (Japan)), no date).

The first mechanism suggested for the SD mediated by *o*-quinones is present in Figure 1.9. In the reaction the *ortho*-quinone reacts with the amino acid to form an amine/carbonyl

condensation product that can suffer decarboxylation followed by hydrolysis into the Strecker aldehyde and a transamination product.
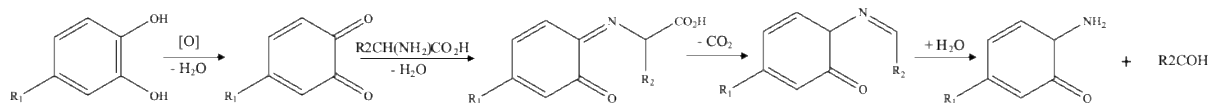
Figure 1.9 Proposed SD from quinones assisted decarboxylation/deamination of amino acids (Schonberg and Moubacher, 1952)

Rizzi later demonstrated that the formation of Strecker aldehydes could happen in model solutions with amino acid/polyphenol using ferrocyanide ion as oxidant in substitution of enzymes (Rizzi, 2006b). And proposed that the formation of a 4-amino catechol intermediate can act as an oxidation substrate and consume a second oxygen equivalent to form the SA.

However it was demonstrated that at wine pH the Michael addition of the amino acid to the *o*-quinone is not favored (Nikolantonaki and Waterhouse, 2012). And according with (Oliveira *et al.*, 2017) the formation of phenylacetaldehyde is higher from catechin > gallic acid and lower for caffeic acid. Furthermore, the structures from the mechanism proposed by Schonberg and Moubacher (1952) were identified by MS^n experiments (Figure 1.10).
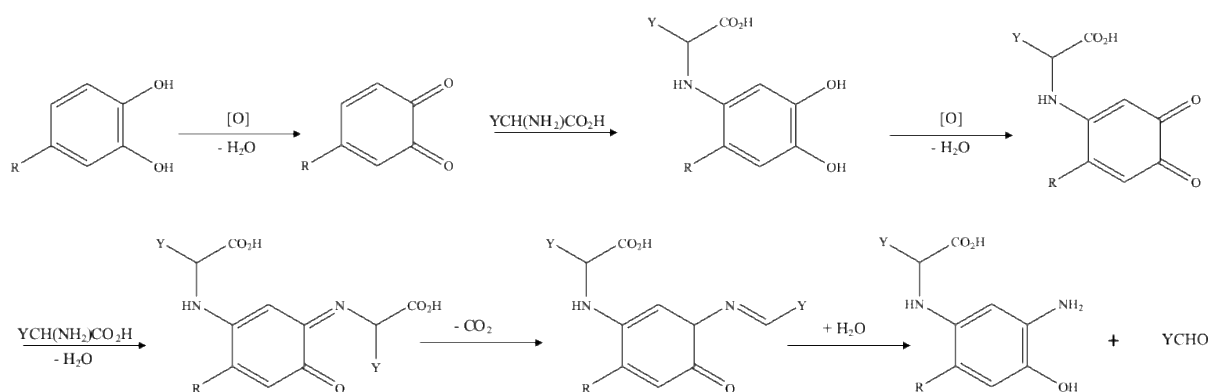
Figure 1.10 Proposed Michael addition reaction of o-quinones with amino acids to produce Strecker aldehydes(Rizzi, 2006b)

1.6.4. Bisulphite-Aldehydes Adducts

In order to protect wines from microbial spoilage as well as chemical oxidation, sulfur dioxide is used. Concentrations of added SO_2 to wine generally varied from 50 to 200 mg/L.

When SO_2 is added to wine, an equilibrium between the molecular forms of this compound is established. Part reacts with carbonyl compounds forming carbonyl/bisulfite (bound SO_2) and the other part forms the unbound or free SO_2. Most free part of SO_2 in wine is in its hydrogen sulfite form (HSO_3^-).

The main anti-oxidative activity of sulphur dioxide is due to the capacity to the bisulphite ion to react with hydrogen peroxide producing sulphuric acid, limiting in that way the oxidation of phenolic compounds and organic compounds (Danilewicz, 2003).

Sulfites are very potent antioxidants capable to accelerate oxygen consumption , due to their capacity in consuming the quinones produced during wine oxidation (Danilewicz, Seccombe and Whelan, 2008) and are capable to consume oxygen by facilitating the consumption of oxygen through the formation of the quinones and then returning the quinone to catechol.

Sulphur dioxide can bind with several wine components: acetaldehyde, anthocyanins, pyruvic acid, glutaric acid, glucose and certain phenolic compounds. In aqueous solutions bisulfite ion can bind to carbonyl compounds, when aldehydes are the targets lead to the formation of addition compounds known as hydroxyalkylsulphonic acids (HASA). The evaluation of 22 carbonyl compounds in sulfite synthetic wine and in real wines reveled that the molecular structure plays an important role in the formation of adducts, where aliphatic aldehydes were considered the most reactive (de Azevedo et al., 2007).

The formation of carbonyl compounds in red wines stored under different oxygen levels correlated with wine combined SO_2, suggesting that part of the increment was related with the release of the aldehyde from the bisulfite combination (Ferreira et al., 2014). The development of a method capable to determine free and bound forms of aldehydes allowed new developments on the subject (Bueno, Zapata and Ferreira, 2014). In order to gather more information related with bisulfite-aldehyde adducts the same group demonstrated that fermentation could be a major source of SA, and during the first steps of oxidation the bound forms could be cleaved resulting in the release of the aldehyde. Still it was concluded that this fact depends on the aldehyde and the wine (Bueno et al., 2016).

1.8. Chemiomics Pipeline

In the past 20 years a great development in different fields associated with high-throughput, *omics* technologies occurred. In which high throughput refers to technology wherein a large number of measurements can be taken in a fairly short time period.

The field of metabolomics has made remarkable progress and has implemented new tools that have offered mechanistic insights by allowing for the correlation of biochemical/chemical changes with phenotype (Patti, Yanes and Siuzdak, 2012).

The focus of chemiomics/metabolomics studies is shifting from categorisation chemical structures to finding chemical stories. Unlike genes, transcripts and proteins, chemical molecules are less tractable to catalogue and are chemically diverse (Baker, 2011).

Chemiomics/metabolomics can be separated in two categories: targeted (hypothesis-driven) and untargeted (data-driven) studies. Untargeted studies focused on the profiling of the total complement of molecules ('fingerprint') in a sample. While the targeted approach focuses on the quantification of selected groups of molecules.

However, it is assumed that is more productive to study a system as a whole, according to an integrated approach (untargeted), than to apply a reductionist approach by analysing the parts (targeted).

Both in targeted and untargeted approaches the main challenges are the chemical complexity and heterogeneity of molecules, the dynamic range of measuring technique, the throughput of the measurements, and the extraction protocols (Goodacre *et al.*, 2004).

In order to complement the faults a very well-established workflow is needed. The workflow of chemiomics could be divided in the i) data acquisition and ii) data processing and analysis. The first is related with the chemical platform chosen and the other is related with the best way of dealing with the data obtained, in order to get as much relevant information as possible (Figure 1.11).
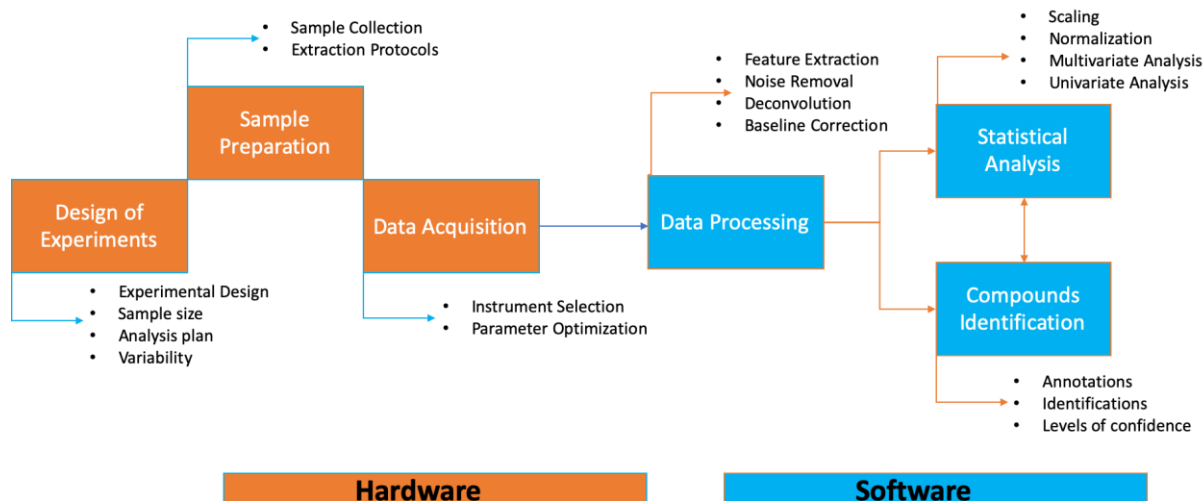
Figure 1.11 Workflow of chemiomics adapted from (Beisken, Eiden and Salek, 2015)

1.8.1. Design of Experiments

The goal of any research project is to obtain reliable results that could lead to proper conclusions. To achieve them, several guidelines need to be followed in order to prevent mistakes, one of these guidelines is to have a proper design of experiments (DoE) aiming to conduct experiments to evaluate the influence of certain factors (independent variables) on selected responses (dependent variables). In the DoE approach (Figure 1.12), the influence of each factor on the studied process is measured simultaneously at different levels, allowing to study single factors as well as interactions between them.

Depending on the amount of information needed several designs could be chosen factorial plans, central composite designs, Box-Behnken designs and response surface methodology are examples of designs.
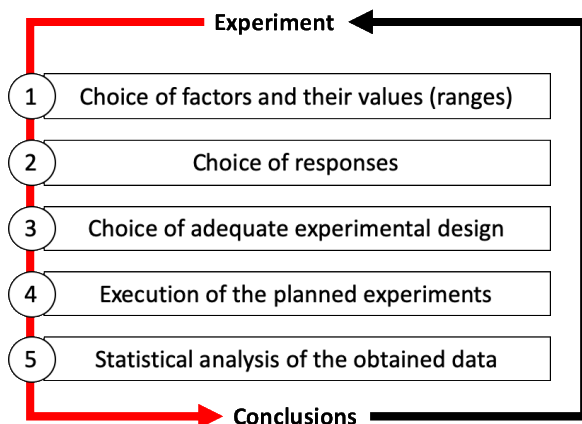
Figure 1.12 General scheme for conducting the experiment in accordance with the DoE methodology (Jacyna, Kordalewska and Markuszewski, 2019)

1.8.2. Sample Preparation

Sample preparation is common in many analyses and is developed to allow or to improve a specific analysis. This step could be the most time-consuming in an analysis and affects significantly the analytical information.

In metabolomic studies the choice of sample preparation method is extremely important because it affects both the observed metabolite content and the interpretation of the data. An ideal sample-preparation method for global metabolomics should be:

> i) Be as non-selective as possible to ensure adequate depth of molecules coverage;
>
> ii) Simple and fast to prevent metabolites loss and/or degradation during the preparation procedure and enable high throughput;
>
> iii) Be reproducible (Vuckovic, 2012).

A strong interaction exists between sample preparation and the analytical technique selected.

For certain techniques, such as NMR spectroscopy the sample preparation is minimal and analytical reproducibility has been demonstrated to be very good (C. Keun *et al.*, 2002). Still, in chromatography dependent on the type of analysis, sample preparation requires several steps and can be classified based on the number of criteria, as shown in Table 1.5.

The main goal of sample preparation in MS-based metabolomics approaches is the reduction of matrix effects, including ion suppression without causing chemical degradation of metabolites (Ibáñez *et al.*, 2013).

The initial extraction procedure is aimed at maximizing the amount and concentrations of the compounds of interest. Nevertheless, in untargeted metabolomics the nature of compounds of interest is mostly unknown. Hence several solvents and extraction methods should be tested and compared between the groups of samples. The most studied isolation/preconcentration steps includes liquid-liquid extraction (LLE), solid phase extraction (SPE), solid phase microextraction (SPME), simultaneous distillation extraction (SDE), supercritical fluid extraction (SFE), headspace solid phase microextraction (HS-SPME) and stir bar sorptive extraction (SBSE).

When GC-MS is used, derivatization is commonly applied in order to increase volatility of analytes. Derivatization is a two-step process and includes: oximation (conversion of aldehydes and ketones to oximes) followed by silation to increase volatility by reducing hydrophilicity of functional groups OH, SH or NH (Gullberg *et al.*, 2004).

Table 1.5 Criteria used for classification of sample preparation procedures.

Purpose of the operation	Physical nature of the sample	Type of sample	Use or absence of solvents	Type of chromatographic process	Property to be enhanced
Dissolution	Gas	Food/flavours	With solvents	GC	Solubility
Clean-up	Liquid	Oil	Solventless	HPLC	Volatility
Fractionation	Solid	Pharmaceutic	Using membrane	UPLC	UV-Vis Absorbance
Concentration	Heterogeneous	Biological Fluids		TLC	Fluorescence
Chemical Modification		Other		Other	Etc.

1.8.3. Data Acquisition

The application of advanced information rich, spectroscopic techniques to complex samples is essential for the generation of global metabolite profiles required for metabolomics. However there is currently no single technique that fulfils all the requirements of an ideal global metabolite profiling tool, yet the use of a suite of metabolite profiling techniques from the "metabolomics toolbox" is most likely to result in comprehensive metabolite profiles (Maria Lenz and D. Wilson, 2006).

The choice of the chemical platform is dependent on the type of sample and on the type of metabolites. Nevertheless, for untarget metabolomics the analysis should have a wider coverage in terms of the type and number of metabolites detected.

Holistic analysis require access to sensitive and powerful instruments; therefore, metabolomics nowadays typically includes GC, HPLC, UPLC, CE coupled to MS and NMR spectroscopy.

In the analysis of unknowns, MS and NMR provide complementary data and both are often required for full characterization. MS can provide the atomic formula of an analyte while NMR indicates the structural moieties those atoms are organized into. The analytical advantages and disadvantages, in terms of the structural information gained from each detector, and some of their contrasting features are presented (Table 1.6).

Table 1.6 Comparison between NMR and MS-based Metabolomics adapted from (Wishart, 2019)

NMR	MS
Non-destructive (sample)	Destructive (sample)
Robust instrumentation	Frail instrumentation
Simple sample preparation	Complex sample preparation
Poor to moderate sensitivity	Excellent sensitivity
Modest metabolite coverage	Extensive metabolite coverage
Small spectral databases	Large spectral databases

Nevertheless, to analyse different types of metabolites the choice of the most appropriates separation method (GC, LC or CE) is an important task (Figure 1.13).

A variety of mass spectrometers with different detectors (e.g., time -of-flight detectors and Fourier transform instruments) and configurations (e.g., tandem MS and hybrid instruments), which can be coupled with different separation strategies (e.g., gas chromatography and liquid chromatography) and ionization methods (e.g., positive or negative electrospray ionization and atmospheric pressure chemical ionization), can be used.
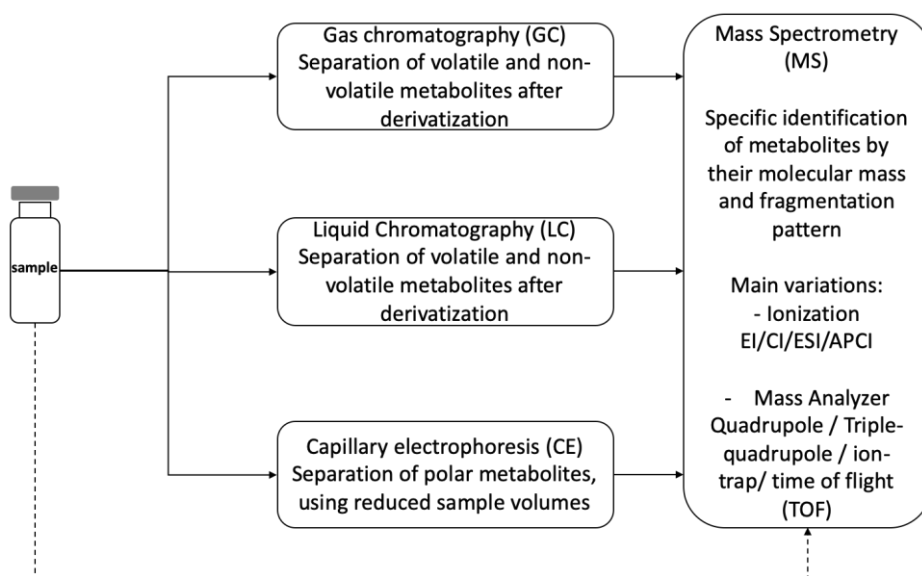
Figure 1.13 Most used MS analysis of metabolites. The sample be directly analysed by MS (dashed arrow), or it can be first resolved with different on-line separation techniques (Villas-Bôas et al., 2005).

Alternatively, no separation could be used by following a direct injection approach. For LC-MS, the advantage is that in principle no derivatization is required for the analysis of polar or high molecular weight metabolites, allowing a fast analysis of samples. While in GC-MS the analysis of medium polar and polar metabolites requires a derivatization step. The mass spectrometer acquires mass spectra from the column output at a specified scan rate. Due to the fact that each eluting compound gives rise to a number of mass signals (adducts, fragments and isotopic peaks), a metabolite induces several two-dimensional features. "Feature" is a term commonly used for a bounded, two dimensional (m/z and retention time) MS signal. While "peak" is used for one-dimensional signals. Regarding molecules

identification in LC-MS the molecular ion is most often detected. Additional MS/MS experiments are required to obtain more clues regarding the structures of metabolites.

Nowadays the use of high resolution detectors mass spectrometry (HRMS) as time-of-flight (TOF) and Fourier transformed ion cyclotron resonance (FTICR) allows the acquisition of metabolite profiles with high mass accuracy (Liu, Ser and W Locasale, 2014). Generally, these instruments measure the exact mass of analytes without fragmentation, however they can be combined with a quadrupole in which case fragmentation is possible and can add more selectivity to the method.

FTICR-MS is a powerful technique, mostly useful in direct infusion experiments, however a major disadvantage is that direct infusion do not allow to discriminate between isomeric and isobaric compounds (Roullier-Gall *et al.*, 2014).

More recently a new approach to increase the number of identified metabolites is to couple ion mobility (IM) analyser to LC and GC-MS to allow a higher number of features to be separated and detected (Chouinard *et al.*, 2017). The principle behind IMS is the separation of ionized analytes that migrate through an electric field at characteristic velocities according to mass, charge, size and shape.

1.8.4. Data Processing

The high throughput metabolomics analytical techniques available result in complex data sets, which are difficult to visualize and interpret. Meaning that the value of metabolomics analysis is determined not only on the performance of a given hardware, but on the subsequent treatment of data by the use of the appropriate software (Eick and Pohnert, 2015).

The technical improvements associated with the analytical platforms described in the hardware section lead to the generation of data structures of increasing size and complexity.

Due to inherently complex nature of metabolomics data and dependent on the data structures, several pipelines have been reported concatenating several tools (Arapitsas *et al.*, 2012b; Godelmann *et al.*, 2013; Castro *et al.*, 2014; Monforte, Jacobson and Silva Ferreira, 2015). Nevertheless, it is crucial a good understanding of the steps involved in each pipeline.

To benefit to the largest extent from the wealth of metabolomic data three challenges were recently proposed (Boccard and Rudaz, 2014):

1. Reducing data dimensionality for biomarker discovery;
2. Handling multiple data tables generated by several analytical platforms (data fusion);
3. Analyzing longitudinal metabolomic data with time-resolved models.

Data reduction is a critical task in metabolomics studies, variability, both instrumental and biological is a major factor that needs to be considered. Therefore, data handling and processing attempts to extract relevant information by removing noise and low-quality data points.

The majority of software tools for MS pre-processing requires data to be in open format e.g. mzXML and netCDF. After conversion the initial stages of data processing for LC-MS and GC-MS are similar. Typical data processing pipeline usually proceeds through multiple stages, including filtering, feature detection, alignment and normalization.

Filtering methods process the raw measurement signal with aim of removing effects like measurement noise or baseline. Typically, GC and LC-MS methods contains chemical and random noise. Chemical noise is caused by molecules in buffers and solvents and can be particularly strong at the beginning and end of the run, while the random noise is mainly related with the detector. Moreover, baseline variation across samples may happen due to instrumentation problems, external environmental sources or the separation process.

The purpose of feature detection stage of data processing is to identify all signals caused by true ions and avoid detection of false positives. This step also aims to provide as accurate quantitative information about ion concentrations as possible. Feature detection is an essential step in the metabolomic data processing pipeline, yet in practice rarely performed perfectly. This is therefore an important area for further method development (Katajamaa and Orešič, 2007).

Nowadays there several platforms available comprising all the steps needed for data processing including, baseline correction and feature detection, some of them are indicated in Table 1.7.

Two of the most used platforms are MZmine2 and XCMS, and recently a comparison between both were performed. Results shown that both packages were very good in performing peak picking, a high number of false positives in extracted ion chromatograms were detected (D. Myers *et al.*, 2017).

Table 1.7 Software tools commonly used for the pre-processing of metabolomics data.

Software	GC-MS	LC-MS	Reference
XCMS	✓	✓	(A. Smith *et al.*, 2006)
AMDIS	✓	✗	(Vey and Voigt, 2007)
MetAlign	✗	✓	(Lommen, 2009)
MzMine2	✓	✓	(Pluskal *et al.*, 2010)
mzMatch	✗	✓	(A. Scheltema *et al.*, 2011)
MSeasy	✓	✗	(Nicolè *et al.*, 2012)
MS-DIAL	✗	✓	(Tsugawa *et al.*, 2015)
ParaDise	✓	✗	(Johnsen *et al.*, 2017)

1.8.5. Data Analysis

Metabolomics experiments usually result in a large quantity of data (50-1000s of variables) for each sample ("fingerprint"). For multiple samples a two-dimensional data tables constructed, by stacking each sample on top of each other. Univariate and multivariate analysis techniques are routinely used to extract relevant information from the data with the aim of providing chemical/biochemical knowledge on the studied problem. Nevertheless univariate approaches, miss the systematic environment of metabolites and their inter-dependencies (Barupal, Fan and Fiehn, 2018). Yet univariate methods are sometimes used in combination with multivariate methods and be used as a filter to retain 'information-rich' features. The number of features considered could be significantly reduced down to those showing statistical significance in univariate methods (e.g. p-value < 0.05).

Different multi-dimensional and multivariate statistical analyses and pattern recognition programs have been developed to filter the large amounts of data in an effort to interpret

complex chemical information from the measurements (Issaq *et al.*, 2009). Multivariate analysis methods seek to capture not only changes of single metabolites between different groups, but also to utilize the dependency structures between the individual molecules.

Based on the specific objective of the analysis and data manipulation the studies can be classified as: discriminative, informative and/or predictive (Cevallos-Cevallos *et al.*, 2009) or can be classified in two strategies: unsupervised and supervised learning.

The aim of unsupervised analysis is to explore the structure of the experimental data in order to gather more information between relationships between samples and variables (metabolites) and to detect aberrant samples (outliers). This type of analyses does not require a priori knowledge about class membership and is normally the step that precede the application of supervised methods.

The majority of metabolomics studies make use of several multivariate models such as classic MVA tools include the principal component analysis, hierarchical cluster analysis, support vector machines, random forest, partial least square-discriminant analyses and other tools. Several reviews are available in the literature covering this subject (Barker and Rayens, 2003; Liland, 2011; Bro *et al.*, 2012, 2013; Boccard and Rudaz, 2014; Ghasemi-Varnamkhasti and Forina, 2014).

Commonly in the search for biomarkers, multivariate discriminate models between two classes of subjects/samples are used. One of the most used methods is Partial Least Squares-Discriminant Analysis (PLS-DA). If a statistically significant discrimination between two classes can be found, then the model parameters can be interpreted for their discriminating power and chemical biomarkers can be found (Szymańska *et al.*, 2012).

Contrary to PLS-DA support vector machines (SVM) is not influenced by the distribution of the different sample's classes, being a non-parametric machine learning technique. The method is based on the mapping of data into a high-dimensional space that allows for separation of two groups of samples into distinctive regions. In terms of feature selection variable importance on projection (VIP) for PLS-DA and recursive feature elimination from SVM were compared. Based on the reduced number of features the classification accuracies were measured, and it was shown that SVM outperformed PLS-DA for both parameters (Mahadevan *et al.*, 2008).

Another technique based on classification trees is random forests. These techniques can be used for classification or regression, and generates many decision trees, each of which is constructed using different sets of randomly selected input variables. The main advantages of RF is their ability to deal with large datasets without variable deletion, provide a feature importance measure of the predictor variables (mean decrease in accuracy) as well as the capacity to handle with missing data (Gromski *et al.*, 2015).

For MS data machine learning techniques were found to be more effective in variable selection, in particular, support vector machines-recursive feature elimination and mean decrease in accuracy provided by random forest when compared with variable importance for projection (VIP) coefficients employed in PLS-DA (Gromski *et al.*, 2014).

Nonetheless despite the conferred utility, powerfulness and versatility of MVA models, their performance might be fraught by the hi-dimensionality of the obtained data-sets i.e. datasets contain too much sparse data in terms of the number of input variable (Vinaixa *et al.*, 2012). This fact can result in the introduction of errors and a reduction in the predictive power of the models (i.e. overfitting). Therefore, using MVA models require intensive validation work.

Nowadays seems to be a trend toward the use of several techniques in combination, with strategies for combining data generated from more than one analytical platform.

Data Fusion

Data integration has been identified as being a bottleneck for future research in drug discovery, due to the complexity of multifactorial diseases demands (Searls, 2005).

Data fusion is the part of data analysis studying fusion of data sets of different origins (K. Smilde *et al.*, 2005). The data obtained from different techniques can be coupled together using three strategies: low-level, mid-level and high-level (Steinmetz, Sévila and Bellon-Maurel, 1999).

A review regarding data-fusion applied in food and beverage authentication and quality assessment provides a more detailed overview of data fusion strategies (Borràs *et al.*, 2015).

Low-level approach is based on the simple concatenation in the columns dimension of the data obtained in different techniques. The obtained matrix can be analysed with a single

model to obtain the final classification or prediction. Low-level data fusion also includes the "outer product of "of the signals. This approach consists in the multiplication of the data from the two techniques resulting in a three-dimension matrix where the first dimension corresponds to the number of samples, and the second and third dimension corresponds to the signals of the two techniques used. This approach normally generates a high number of variables compared with the number of samples which constitutes a drawback.

In order to reduce the number of variables mid-level fusion (feature level fusion) is based on the extraction of the relevant features for each technique separately. This extraction normally is performed by extracting the scores of PCA for each technique and concatenate them into a single matrix. The challenge of mid-level fusion is the selection of the number of relevant components from each technique.

High level data fusion is the less explored of the three techniques, is based on the classification outputs of all individual sensors. The classification output for each sensor is denominated "identity declaration", which are further concatenated. In high-level data fusion techniques based in probability estimation are used, such as Bayesian approach (Roussel *et al.*, 2003; Doeswijk *et al.*, 2011). However, this approach has the disadvantage of turning difficult the interpretation of the data.

Data Interpretation

Despite the technique used for acquisition or the MVA technique selected the detection of the unequivocal identification of biomarkers compounds represents perhaps the most important step, due to the fact that it is only after these compounds have been identified that their chemical/biological significance can be determined.

In metabolomics five different levels of molecules identification were established by the Metabolomics Society at the 2017 annual meeting of the Metabolomics Society (Brisbane, Australia) (Blaženović *et al.*, 2018) .

Level 0: Unambiguous 3D structure: Isolated, pure compound, including full stereochemistry.

Level 1: Confident 2D structure: uses reference standard match or full 2D structure elucidation.

Level 2: Probable structure: matched to literature data or databases by diagnostic evidence.

Level 3: Possible structure or class: Most likely structure, isomers possible, substance class or substructure match.

Level 4: Unknown feature of interest.

In a recent review a workflow for peaks identification was proposed (Tsugawa, 2018):

Step 1. Eliminate the possibility of false-positive peaks (isotopic ions, different adduct types, in-source fragments and background ions).

Step 2. Search spectral libraries

Step 3. Predict the molecular formula.

Step 4. Retrieve known/expected structures of a suggested formula followed by their ranking.

Step 5. Expand the chemical spaces for searching and predict the molecular scaffold.

Initial putative metabolite identification can be made on the basis of mass to charge ratio (m/z) of the mass spectral ion. Three approaches for molecules identification were recently reviewed: mass spectral database search, in silico fragmentation tools and orthogonal coupled techniques including retention time matching and ion mobility spectrometry (Blaženović *et al.*, 2018).

The chemical space of small molecules currently is covered in several databases (Table 1.8), the number of compounds reported exceeds 120 million compounds. Still, the highest percentage of metabolites found in untargeted studies remains unknown.

Table 1.8 Databases that help in metabolite identification for MS data.

Name	Web address
Human metabolome Database	www.hmdb.ca
MassBank	www.massbank.jp
Lipid Maps	www.lipidmaps.org
METLIN	metlin.scripps.edu
KEGG	https://www.genome.jp/kegg/
NIST Chemistry Web book	webbook.nist.gov/chemistry
PubChem	https://pubchem.ncbi.nlm.nih.gov
ChemSpider	http://www.chemspider.com

In silico fragmentation is used when no reference mass spectra is available, in that case matching the experimental spectra against a selection of in silico fragmentations calculated on candidates retrieved from known compounds is performed. For example MetFrag is a combinatorial fragmenter that uses candidate structures from PubChem, ChemSpider and KEGG (Ruttkies *et al.*, 2016).

OBJECTIVES

The aim of this thesis is to elucidate the interconnectivity between oxidation and Maillard mechanisms responsible for the formation of key compounds for wine quality during aging in particular Strecker aldehydes. As well as understand the impact of the reaction's extension on the overall chemical profile of white wines wine in a holistic approach.

The detailed objectives of the present proposal are outlined as follows:

- To identify and quantify the main factors with impact in the formation of Strecker Aldehydes.

- To reveal relevant kinetic data and determine the storage conditions dependence of the estimated parameters.

- To establish the reaction pathways and propose a mechanistic model from a quantitative point of view.

- To reveal relevant kinetic data and determine the storage conditions dependence of the estimated parameters.

- To develop an array of detectors and respective acquisition parameters capable to detect the systems changes over storage time and join together all the information (data fusion) – "Omic Sensor".

- To conduct computational analysis and use multivariate analysis techniques to extract biomarkers related with each mechanism.

- To validate each mechanism in real aged wines by the construction of correlation networks with all the compounds detected allowing the development of a risk-based strategy for designing, analyzing and controlling wine aging process.

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THESIS STRUCTURE

CHEMIOMICS: A SYSTEMS CHEMISTRY APPROACH TO UNRAVEL THE INTERFACE PATHWAYS BETWEEN OXIDATION AND MAILLARD MECHANISM

RESPONSIBLE FOR FLAVOUR MODULATION DURING WINE STORAGE

CHAPTER 1. GENERAL INTRODUCTION

CHAPTER 2. MATERIAL AND METHODS

Studies Performed in Wine Model Systems

CHAPTER 3. Strecker aldehydes formation in wine: new insights into the role of gallic acid, glucose and metals in phenylacetaldehyde formation

CHAPTER 4. Applicability of ptr-tof-ms to study the kinetics of strecker aldehydes formation in model solutions in dynamic conditions

CHAPTER 5. Response surface methodology: a tool to minimize aldehydes formation and oxygen consumption in wine model systems.

CHAPTER 6. Impact of phenolic compounds in strecker aldehydes formation in wine model systems: target and untargeted analysis

Studies Performed in White Wine

CHAPTER 3. Strecker aldehydes formation in wine: new insights into the role of gallic acid, glucose and metals in phenylacetaldehyde formation

CHAPTER 7. Discrimination of wine "oxidation-status" based on untarget peak picking approach with multi-class target coupled with machine learning algorithms

CHAPTER 8. Changes in white wine composition after treatment with cationic exchange resin: impact on wine oxidation after 8 years of bottle storage

CHAPTER 9. CONCLUSIONS AND FUTURE PRESPECTIVES

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CHAPTER 2 MATERIAL AND METHODS

2. MATERIAL AND METHODS

This manuscript is divided in two parts i) studies performed in wine model solutions (Chapter 3, 4, 5 and 6) and ii) studies performed in white wines (Chapter 7 and 8) as indicated in the scheme of Figure 2.1.

Material and Methods section is divided in two sections:

- Materials (Model solutions and wines)
- Methods (Analytical methods for characterization)

Detailed information regarding the preparation of model solutions as well as the methods used for data analyses are presented in each chapter.
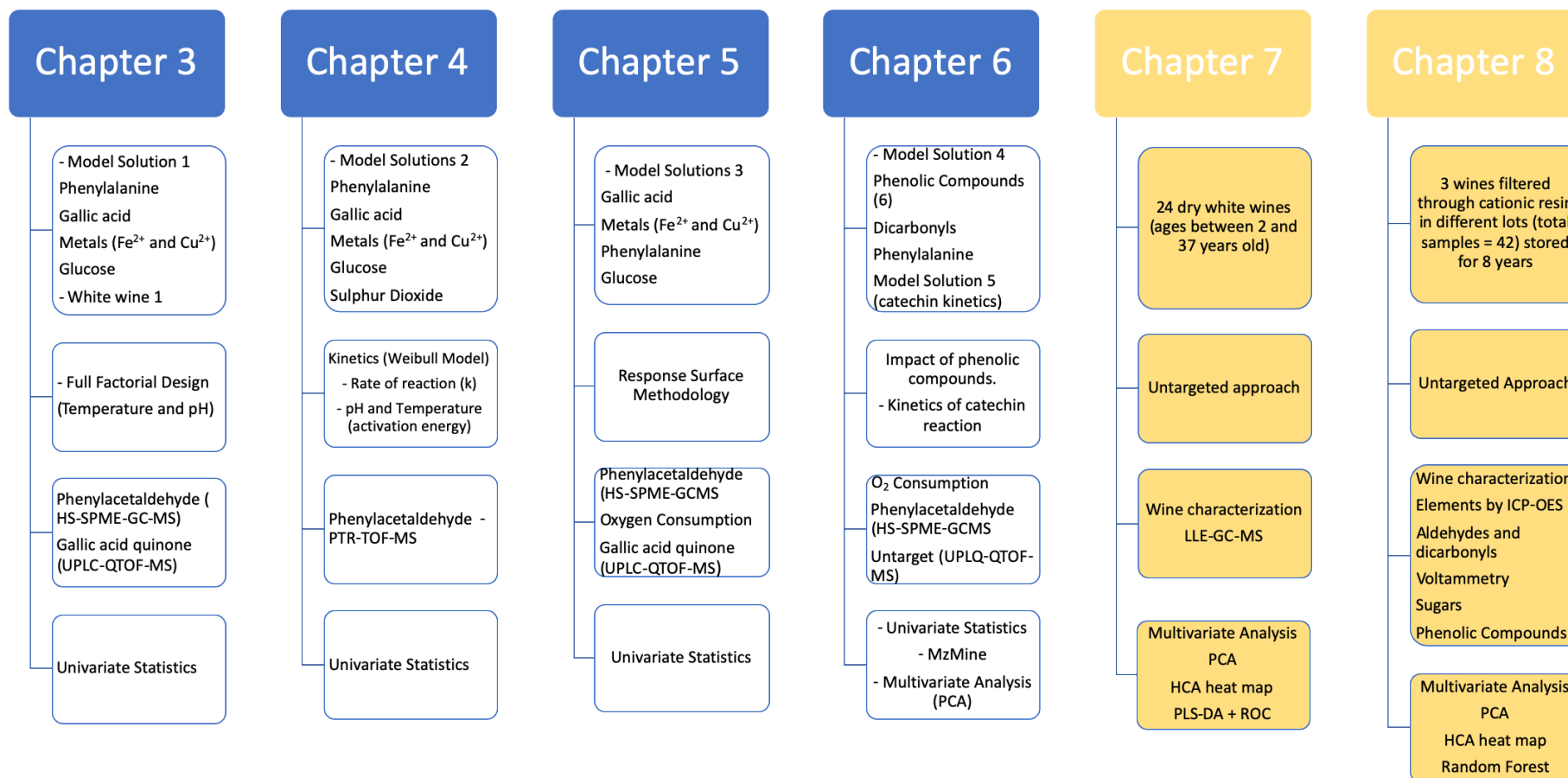
Figure 2.1 Material and methods used in each chapter.

2.1. Materials

2.1.1. Model Solutions

Model solutions were prepared according with Table 2.1.

Table 2.1 Model solutions composition for chapters 3, 4, 5 and 6.

	Chapter 3	**Chapter 4**	**Chapter 5**	**Chapter 6**
Ethanol	12% (v/v)		12% (v/v)	12% (v/v)
Tartaric acid	0.03 M	0.03 M	0.03 M	0.03 M
pH	3.4 and 7	3.4, 5 and 7	3.4	3.4
Phenylalanine	2.4 mM	2.4 mM	20 mM	2.4 mM
Gallic acid	2.4 mM	2.4 mM	2.4, 11.2 and 20mM	2.4 mM
Phenolics *	-	-	-	2.4 mM
Glucose	2.4 mM	2.4 mM	2.4, 11.2 and 20 mM	2.4 mM
Methyl glyoxal	-	-	-	2.4 mM
Fe^{2+}	6.3 µM	6.3 µM	6.3 and 12.6 µM	6.3 µM
Cu^{2+}	0.1 mM	0.1 mM	0.1 and 0.2 µM	0.1 mM
EDTA	50µM	-	50 µM	-
SO_2	-	50 mg/L	-	-
Temperature	80ºC or 40ºC	40, 50, 60, 70 and 80ºC	40ºC	80ºC
Trial Time	24 hours	1 hour	24 hours	24 hours
Volume	20 mL	50 mL	60 mL	20 mL

* catechin, epicatechin, caffeic acid, ferulic acid, gallic, acid and 3,4-dihydroxybenzoic acid (3,4-DHB)

All model systems comprising 12% (v/v) aqueous ethanol and tartaric acid (0.03M) with addition of phenylalanine (2.4 mM) buffered to pH 3.4 and 7 with NaOH (1M) were used for all experiments. The role of tartaric acid was not only to maintain acidity but in its role in coordinate preferentially to ferric ions to reduce the formal reduction potential of the Fe^{3+}/Fe^{2+} couple (Danilewicz, 2003). The metal ions, Cu^{2+} and Fe^{2+}, were added to the model system at the concentrations indicated in Table 2.1, in the form of Cu (II) sulfate·$5H_2O$ and Fe (II) sulfate·$7H_2O$. Chosen concentrations are normally found in white wines (Danilewicz, 2007). In the solutions with no metal addition, a complexing agent (50 µM EDTA solution) was also added to prove the catalytic effect of metals in the reaction.

Kinetic study of Catechin reactions

In parallel, three kinetic studies were also conducted. Equimolar solutions (2.4 mM) were prepared in wine model solutions in duplicate: i) phenylalanine + catechin + metals, ii) phenylalanine + catechin + glucose + metals, iii) phenylalanine + catechin + methyl glyoxal + metals. The metals Fe^{2+} and Cu^{2+} were added to all model systems at concentrations of 0.1 mM and 6.3 µM in the form of $FeSO_4.7H_2O$ and $CuSO_4.5H_2O$ respectively. The solutions were prepared in 20 mL vials, closed with an internal PTFE septum and an external screw cap, 20% of the vial volume were left with air. Samples were heated at 80ºC for 2, 4, 6, 8 and 24 hours. At each time sample was taken and cooled in ice water and then stored at -20ºC.

2.1.2. Wines

White Wine – Chapter 1

In order to evaluate the impact of the presence of sugar in the phenylacetaldehyde formation in real wine systems, glucose 10 g/L has been added to a young, dry white wine, produced in the Douro region (North Portugal) (pH=3.4; SO_2 free = 0.2 mM), following standard winemaking procedures, without any wood contact.

White Wines – Chapter 5

Twenty-four dry white wines were collected from seven producers from 4 regions of Portugal (Lisbon, Douro, Dão and Alentejo). Amongst them, 4 samples from C, 2 from DB, 4 from DQ, 2 from MS, 1 from TA, five from T and 6 from DV. The storage time includes wines with 2, 3, 4, 5, 6, 7, 8, 9,10, 14, 17, 23, 24 and 37 years old. Before bottling the wines had no contact with wood. Wines were stored in cellar conditions with natural corks.

Each sample was codified referring to its brand together with the storage time (wine DV stored for two years was codified as DV_2y).

White Wines – Chapter 6

A total of 42 samples of bottled white wine from three different regions in Portugal stored for 8 years were analysed. Wines were sourced from: Douro, Dão and Minho regions.

In order to study the impact of metals a cation exchange resin was used. The regeneration of the resin was performed with HCl (30% v/v) with a flow rate of 5L/hour and the clearing phase using distilled water (flow rate of 10 L/hour).

Wines were divided into 7 lots for each treatment (15 L each). One lot was representative of the control wine (untreated) and other represent the eluted wine (100%). The remaining five containing cation exchanged wine, were mixed in different proportions (5%, 10%, 15%, 20%, 25%) with the untreated wine. Each wine was sealed in three green glass bottles (0.750 L) with natural corks and stored in cellar conditions for 8 years. Two replicates for each wine was analysed.

2.2. Methods

Several methods were used for the characterization of both model solutions and wines. In Table 2.2, is represented the analysis performed in each chapter, represented by a check mark or a cross symbol.

Table 2.2 Measurements performed in each chapter

	Ch. 4	Ch. 5	Ch. 6	Ch. 7	Ch. 8	Ch. 9
Dissolved O$_2$	✓	✗	✓	✓	✓	✓
pH	✓	✓	✓	✓	✓	✓
Free and total SO$_2$	✗	✗	✗	✗	✗	✓
Colour	✗	✗	✗	✗	✓	✓
Voltammetry	✗	✗	✗	✗	✗	✓
ICP-OES	✗	✗	✗	✗	✗	✓
PTR-TOF-MS	✗	✓	✗	✗	✗	✗
HPLC-RI	✗	✗	✗	✗	✓	✓
DI-GC-FID	✗	✗	✗	✗	✓	✓
HS-SPME-GC-MS	✓	✗	✗	✓	✗	✗
DI-LLE-GC-MS	✗	✗	✓	✗	✓	✓
PFBHA-HS-GC-MS	✗	✗	✗	✗	✗	✓
UPLC-QTOF-MS	✓	✗	✓	✓	✓	✓

2.2.1. Dissolved Oxygen

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).

In model solutions experiments (Chap4, 6 and 7) a control sample (water) stored in the same conditions as the experimental samples were used to monitor if some oxygen ingress into the container, to be assured that oxygen entrance was not significant.

2.2.2. pH

Measurements of pH were performed using a pH meter Crison pH 2002, with a combined glass electrode with a precision of 0.1.

2.2.3. Free and Total Sulphur Dioxide

Free and total sulphur dioxide in wines were determined using the Ripper method (iodine titration) according with the Organisation Internationale de la Vigne et du Vin (OIV, 2007). The methodology consists on iodometric titrations of an acidified wine sample.

For free SO_2, 5 mL of 25% H_2SO_4 was added to 25 mL of wine and the titration was performed with iodine titrant solution (0.01 M).

To determine the total SO_2 content, 25 mL of 1 mol/L NaOH was added to 25 mL of wine 15 minutes prior to analysis, in order to perform alkaline hydrolysis and then 10 mL of 25% H_2SO_4 just before the titration.

Calculation of free and total SO_2 was performed using the equation:

$$SO_2(mg/L) = Vi \ x \ Ni \ x \ 25.6$$

Where Vi is the volume of iodine titrant used at the endpoint of the titration (mL) and Ni was the normality of the iodine titrant.

2.2.4. Colour Measurement – Visible Spectroscopy

For spectrophotometric measurements, a UV-9200 Ray Leigh was used. The measurements were performed with a standard 1 cm pathlength with reduced volume. Deionized water was used as a blank. Measurements were performed at 420, 520 and 620 nm and each sample were analysed in triplicate.

2.2.5. Voltammetry

Electrochemical analysis was performed with Nomasense Polyscan electrochemical analyser (Nomacorc, Belgium) using disposable screen-printed sensors in a three-electrode arrangement. Two indices were obtained: easily oxidized compounds (EasyOx) and total oxidizable compounds correlated to Folin Ciocalteau (PhenOx).

2.2.6. Elements – ICP-OES

Elements were determined using a Perkin Elmer Optima 7000 DV ICP-OES. The wine samples were diluted ten times in nitric acid (5%). Calibrations were performed in wine model solution (10% ethanol with 4 g/L of tartaric acid and pH=3.2). The dilution was validated with spike recovery studies.

2.2.7. Phenylacetaldehyde release - PTR-TOF-MS

A commercial PTR-TOF-MS 8000 (Ionicon Analytik GmbH, Innsbruck, Austria) instrument were used for measurements. The sampling time chosen was 0.1 ns for 60 minutes, resulting in 8000 cycles per sample. All measurements were carried out under inlet temperature of 125ºC, drift tube temperature of 90ºC, drift tube pressure of 3.8 mbar and drift voltage of 900 V with an electric field strength (E/N) of 131 Td (Townsend).

Mass axis calibration and calculation of peak areas were done using the software PTR-MS Viewer. In order to guarantee high mass accuracy throughout the analysis, the mass scale was calibrated following the peaks of known ions (H_3O^+, m/z=21.022; and NO^+). The raw data were subsequently corrected for transmission and peak areas converted into concentration (parts per billion by volume) using the primary ions H_3O^+ and $(H_2O)_2H^+$ measured by their respective ^{18}O isotopologues and using a common coefficient rate (k=2.10^{-9} cm^3/s).

Dead time correction, mass calibration, peak extraction and integration were performed using PTR-TOF DATA Analyzer software (v4.17). Three replicates were acquired for each condition and the spectra were averaged.

To assess for sensitivity, preliminary experiments were performed to control the linearity of the PTR-MS by injecting measured amounts of phenylacetaldehyde into the chamber. The compounds have been injected four times, and in the middle of the injection's blanks were performed to check for carry-over between measurements. Compound obtained by following m/z 121.065 corresponding to $C_8H_9O^+$.

2.2.8. Ethanol and Sugars – HPLC-RI

Free sugars (glucose and fructose) ethanol were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI). A Beckmann Model 126 with a autosampler and a RI detector was used. The chromatographic separation was achieved with an Aminex hpx-87H (300 x 7.8mm, Bio-Rad) operating at 35ºC (7971R Grace oven). The mobile phase used was deionized water with sulphuric acid (2.5mM) at a flow rate of 0.6 mL/min, and the injection volume was 20 µL. Sugars and ethanol identification was made by comparing the relative retention times of sample peaks with standards. Linearity and sensitivity of the HPLC analysis were determined and the method was validated by the instrumental precision, repeatability and accuracy.

2.2.9. Acetaldehyde – GC-FID

Acetaldehyde quantification was performed by gas chromatography coupled to a flame ionization detector (GC-FID). Sample preparation consist in add to 5 mL of wine 50 µL of 4-methyl-2-pentanol (10 g/L), as internal standard.

Sample was injected (5 µL) in a Varian 3900 GC equipped with a CP-1177 split/splitless injector and a FID detector at 220ºC. Separation was performed in a CP-WAX 57 (50m x 0.25 mm x 0.3 µm, Varian). The oven temperature was maintained at 40ºC for 5 minutes, then increased at 3ºC/min to 110ºC, and then increased at 20ºC/min to 220ºC (holding time of 1 minute). The carrier gas was Helium C-60 (Gasin, Portugal) at 1 mL/min (constant flow).

2.2.10. Phenylacetaldehyde – HS-SPME-GC-MS

Phenylacetaldehyde was analysed by headspace (HS) – solid phase micro extraction (SPME) using a SPME grey (DVB/CAR/PDMS) fiber (Supelco, Bellefonte, PA) and the volatiles were analyzed using a Varian 450 Gas Chromatograph, equipped with a mass spectral detector, Varian 240-MS and a CombiPAL auto sampler (CTC Analytics AG, Zwingen, Switzerland). The column used was a VF-WAXms (15m x 0.15mm, 0.15μm) (Varian). A 5 mL sample was spiked with 20 μL of 3-octanol (50 mg/L) as the internal standard. Anhydrous sodium sulphate (0.5 g) was added to increase ionic strength. Samples were pre-incubated in the CombiPAL oven at 40°C and 500 rpm for 2 min, then the fiber was exposed on the sample headspace for 10 minutes at 250 rpm and 40°C for extraction. The fiber was kept 10 mm from the bottom. Desorption of volatiles took place in the injector at 220°C for 15 minutes. The oven temperature was 40°C (1 min), and then increased 4 °C/min to 220 °C and held for 2.5 min (48.5 min). The carrier gas was Helium at 1 mL/min, with a constant flow. 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. All mass spectra were acquired in the electron impact (EI) mode (ionization energy, 70 eV; source temperature, 180°C). The analysis temperatures were trap 170°C, the manifold 50°C, transfer line 190°C and ion source 210°C. Compound identification was achieved by comparing retention times and mass spectra obtained from a sample containing pure, authentic standards. Compound quantitation was performed based on standard calibration curves (n=7) using m/z = 91 as quantifier and m/z=120 as qualifier. LOD and LOQ of the method for PA are: 30 and 100 μg/L respectively. The recovery is 99.8% and the RSD was 2.5%.

2.2.11. Volatiles – DI-LLE-GC-MS

Volatile compounds were extracted by a liquid-liquid. Briefly 5 g of anhydrous sodium sulphate and 50 μL of internal standard (3-octanol) (500 mg/L) was added to 50 mL of wine. Samples were extracted twice with dicloromethane (5 mL) using a magnetic stir bar for 5 minutes per extraction and 2 mL of the organic phase were concentrated under a nitrogen stream 4 times. The extract was injected (2 μL) into the GC-MS (Varian 450 gas chromatograph, coupled to a Varian 240-MS mass spectrometer). Chromatographic

conditions were the following, column BP-21 (50m x 0.25mm x 0.25μm) fused silica; oven temperature 40ºC for 1 minute at a rate of 2ºC/min to 220ºC.

Retention indices for each peak was calculated by the injection of a series of alkanes (C3-C30).

2.2.12. Aldehydes and α-dicarbonyls-PFBHA-HS-GC-MS

The analysis of Strecker aldehydes, alkanals and α-dicarbonyls was performed according with (Moreira *et al.*, 2019). Briefly wine (2mL) was placed in a 20 mL vial, 2 g/L of PFBHA was added. Sample was incubated at 40ºC for 10 minutes and molecules were extracted with PDMS/DVB fiber for 20min at the same temperature. Compounds were analyzed using a Varian 240-MS, and a CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland). The column used was a

BR-5 ms (30 m × 0.25 mm I.D. × 0.25 μm film thickness) (Bruker Daltonics, Freemont, CA). Compounds were desorbed in the injector at 250ºC for 5 minutes. The oven temperature program was 40ºC (1 min) to 250ºC (10min) at 5ºC/min. The carrier gas was helium at 1 mL/min, with a constant flow. All mass spectra were acquired in the electron impact (EI) mode (ionization energy, 70 eV; source temperature, 180 °C). The analysis temperatures were as follows: trap, 170 °C; manifold, 50 °C; transfer line, 190 °C; and ion source, 210 °C.

2.2.13. Gallic acid o-quinone quantification – UPLC-Q-TOF-MS

After reaction, 3 mL of each samples of the kinetic analysis in model solutions (t=0; t=1h; t=24h) were derivatized overnight in closed vials with an OPD solution (0.05 M) to form stable quinoxalines.

The LC-ESI-UHR-QqTOF-MS analysis was performed on an UltiMate 3000 Dionex UHPLC (Thermo Scientific), coupled to a Ultra-High Resolution Qq-Time-Of-Flight (UHR-QqTOF) mass spectrometer with 50,000 Full-Sensitivity Resolution (FSR) (Impact II, Bruker Daltonics, Bremen, Germany).

Separation of metabolites was performed using an Acclaim RSLC 120 C18 column (100mm x 2.1 mm, 2,2um) (Dionex). Mobile phases were 0.1% aqueous formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The gradient started with 5% during increased to 95% in 7 min, which was kept constant for 2min and; returned to 5% B in 1 minute and maintained at 5% B for an additional 5 min at a flow rate of 0.25 mL/min. The injection volume was 1uL. Parameters for MS analysis was set using positive ionization mode with spectra acquired over a range from m/z 20 to 1000. The parameters were as follow: capillary voltage, 4.5kV; drying gas temperature, 200°C; drying gas flow, 8.0 L/min; nebulizing gas pressure, 2bar; collision RF, 300 Vpp; transfer time, 120us and prepulse storage, 4us. Post-acquisition internal mass calibration used sodium formate clusters with the sodium formate delivered by a syringe pump at the start of each chromatographic analysis.

High-resolution mass spectrometry was used to identify the adduct formed between the gallic acid quinone and the OPD (4-hydroxyphenazine-2-carboxylic acid). The elemental composition for the compound was confirmed according to accurate masse and isotope rate calculations designated as mSigma (Bruker Daltonics). The accurate mass measured was within 5mDa of the assigned elemental composition and mSigma values of < 20 provided confirmation. Gallic acid quinone were identified based on its accurate mass $[M+H]^+=$ 241.0619. Its concentration has been expressed in gallic acid equivalents in mg/L, for that samples without derivatization for times 0h, 1h and 24h were analyzed in the same acquisition conditions but in negative mode.

2.2.14. Phenolic Compounds and Others- UPLC-Q-TOF-MS

Prior to analysis samples were filtered with 0.2 μM PTFE filters and placed in 2mL vials.

Analysis of phenolic compounds were performed on an Ultimate 3000 Dionex UHPLC coupled to an ultra-high resolution Qq-time of flight (UHR-QqTOF) mass spectrometer (Impact II, Bruker Daltonics, Germany), according with the method described in (Monforte, Martins and Silva Ferreira, 2019).

Data were acquired in negative mode over a mass range of 50 to 1500 amu with scan duration of 0.3s in centroid mode. The MS parameters were: capillary voltage: 4.5 kV; drying gas temperature: 200°C; drying gas flow: 8 mL/min; nebulizing gas pressure: 2bar; collision RF:

300 Vpp; transfer time: 120 μs and prepulse storage: 4 μs. Mass calibration was performed by the external injection of a sodium formate solution.

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CHAPTER 3 STRECKER ALDEHYDES FORMATION IN WINE: NEW INSIGHTS INTO THE ROLE OF GALLIC ACID, GLUCOSE AND METALS IN PHENYLACETALDEHYDE FORMATION

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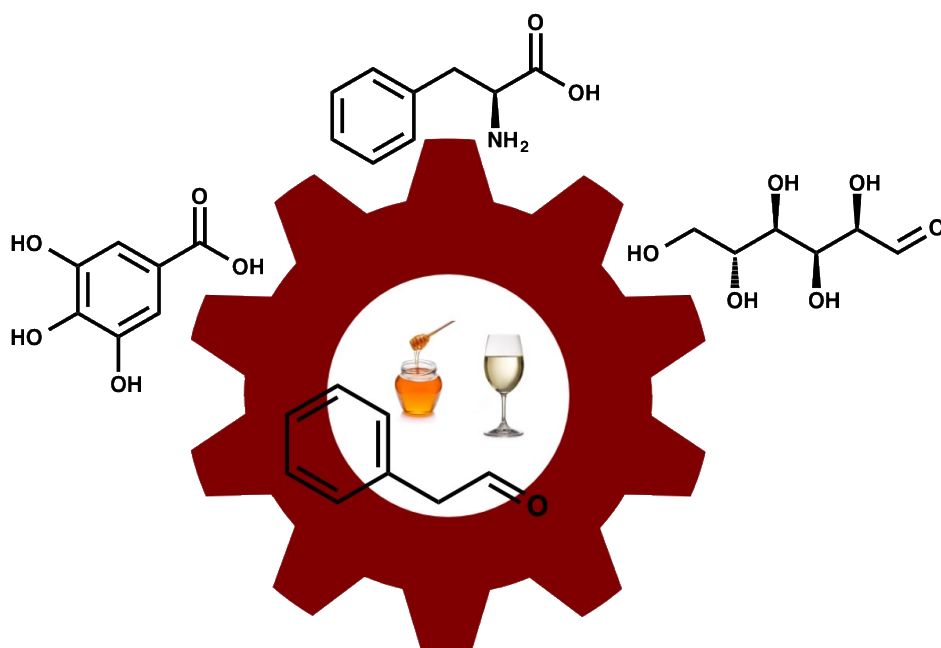
3.1. INTRODUCTION

In foods and in particular in wines, phenylacetaldehyde, a Strecker aldehyde, is reported to be particularly important, contributing to "honey" aroma notes associated to "premature oxidation (C. Silva Ferreira *et al.*, 2003; Bueno, Carrascón and Ferreira, 2016) Phenylacetaldehyde has been identified in several types of wines and reported to have an aroma threshold between 1.0 (Culleré, Cacho and Ferreira, 2007) and 25 µg/L (Silva Ferreira *et al.*, 2002).

Since Hodge review (Hodge, 1953) different studies have shown that besides reducing sugars, other dicarbonyls such as *o*-quinones formed by the oxidation of polyphenols (Rizzi, 2006) and lipids (Zamora, Mercedes León and J. Hidalgo, 2015)can also participate in Strecker degradation (SD) to produce flavour compounds.

The formation of Strecker aldehydes in wines is suggested to occur (i) by Strecker degradation of parent amino acid with α-dicarbonyl (Pripis-Nicolau *et al.*, 2000) or an *o*-quinone (Singleton, 1987; Nikolantonaki *et al.*, 2012) (ii) by the direct oxidation of the corresponding alcohol with a peroxidation mechanism (Jarauta, Cacho and Ferreira, 2005) or (iii) by the release of these compounds from them adducts with sulfur dioxide - the hydroxyalkylsulphonic acid (Bueno, Carrascón and Ferreira, 2016). Several intermediaries of this pathways have been identified in wines such as: hydroxyalkylsulphonic acids (de Azevedo *et al.*, 2007), *o*-quinones (Nikolantonaki *et al.*, 2012), as well as 3-deoxyosone (Oliveira *et al.*, 2016) an α-dicarbonyl generated by the degradation of the Amadori rearrangement through the enolization pathway.

In wine conditions Strecker aldehydes formation has been reported to be mainly due to the presence of metals and oxygen, during the phenolic oxidation. Metals will act as electron donors by adding a singlet electron to triplet oxygen (O_2). This transfer of an electron leads to a cascade of reactions that ends with a very reactive oxidant, the hydroxyl radical ($HO^\bullet$), which can abstract a hydrogen atom from organic compounds to produce water the final oxygen reduction product (Danilewicz, 2003; Waterhouse and Laurie, 2006). Metals will also be able to convert the phenolic compounds into *o*-quinones, which are highly reactive and can combine spontaneously with nucleophilic compounds such as amino acids resulting in the formation of aldehydes (Oliveira *et al.*, 2011). Rizzi (Rizzi, 2006) studied under phenolic oxidation conditions, the formation of PA at pH 7 with $K_3Fe(CN)_6$, phenylalanine

and several phenolic compounds. It was observed that PA was formed from phenolics under oxidative conditions, but only in the presence of a strong oxidant. More recently, Hidalgo *et al* (Delgado, Zamora and Hidalgo, 2015) evaluated the impact of phenolic compounds structure characteristics and their ability to degrade amino acids. In that study it was shown that polyphenols are capable of reacting with amino acids in the absence of metal ions independently of the pH, however the polyphenol needs to have two hydroxyl groups in *ortho* or *para* positions. However, in wine that is rich in polyphenols with vicinal hydroxyl structures such as gallic acid as well as transition metals such as copper (Cu) and iron (Fe), most studies in wine have shown that phenolic oxidation only occurs in the presence of metals (Danilewicz, 2003, 2013).

The interaction between Maillard reaction (MR) and phenolics oxidation (PO) has, to our knowledge, not yet been reported in wine conditions. Peterson et al, has investigated the role of catechin and epicatechin in preventing the formation of dicarbonyls and pyrazines (Totlani and Peterson, 2005), as well as flavour compounds (Schwambach and Peterson, 2006), proving that epicatechin is capable to form adducts with methyl glyoxal formed from the Maillard reaction (Totlani and Peterson, 2006). Recently, Wilker et al studied the impact of polyphenols in a solution of glucose/alanine (pH 5 and 7) at 80, 100 and 130°C in the formation of glucosone (Wilker, Heinrich and Kroh, 2015), and observed that the pro-oxidative effects of polyphenols depend on their concentration, pH and temperature of the model system. These studies focused on the impact of polyphenols in model systems or food matrices with pH's between 5 and 7 and never in hydro alcoholic solutions such as wine.

In order to better understand the impact of the interaction between Maillard reaction and phenolic oxidation in the formation of phenylacetaldehyde a systematic study in wine like model systems was conducted using a well-delivered design of experiments (DoE). Between several DoE techniques, full factorial design identifies experiments at every combination of factor levels. In the present work two full factorial designs have been studied focusing on the effect of: 1) pH and 2) temperature, when three factors: glucose, gallic acid and metals at two levels (present or absence) were varied while keeping phenylalanine constant. For the most relevant parameters extracted from the DoE, kinetics studies were also performed, and rate constants estimated for both wine model and real wine systems.

3.2. MATERIAL AND METHODS

3.2.1. Experimental Design

Two 2^4 full factorial designs were performed to identify the relationship existing between the response functions and variables as well as to determine those conditions that optimized the behavior of each response. Determination of responses was conducted as a duplicated completely randomized full factorial design. DoE 1: evaluating four factors (glucose, gallic acid and metals) at two levels (present or absence) at pH 3.4 and 7 and DoE 2: evaluating four factors (glucose, gallic acid and metals) at two levels (present or absence) at two temperatures (40ºC and 80ºC), as depicted in Figure 3.1.
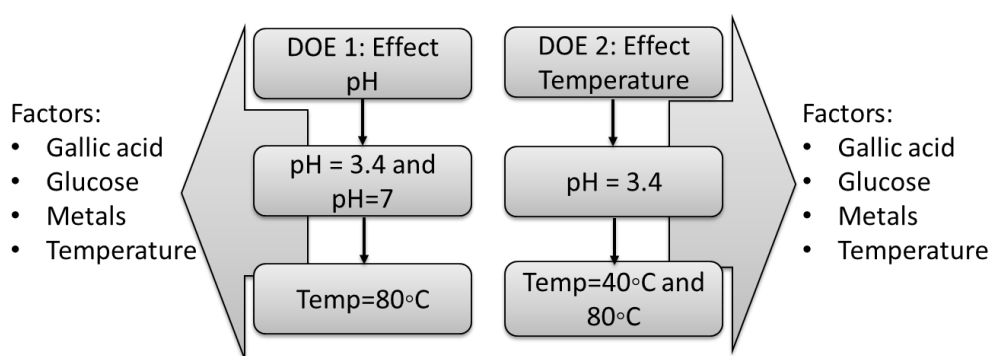
Figure 3.1 Full factorial design scheme applied in the experiment.

A total of 16 combinations were generated for each DOE's and performed in duplicated in random order. The models were built with JMP statistical software version 10.0.0 (SAS Institute Inc., Cary, NC, USA). Each response was analyzed by two-way analysis of variance (ANOVA) using the general linear model (GLM) procedure using JMP statistical software version 10.0.0 (SAS Institute Inc., Cary, NC, USA). The single and interactive effects on phenylacetaldehyde formation were determined to explore the significance of differences at *p-value < 0.05*. Graphics were created with the JMP statistical software version 10.0.0 (SAS Institute Inc., Cary, NC, USA).

3.2.2. Preparation of Model Systems and Storage Conditions

Two model systems comprising 12% (v/v) aqueous ethanol and tartaric acid (0.03M) with addition of phenylalanine (2.4 mM) buffered to pH 3.4 and 7 with NaOH (1M) were used for all experiments. The role of tartaric acid was not only to maintain acidity but in its role in coordinate preferentially to ferric ions to reduce the formal reduction potential of the Fe^{3+}/Fe^{2+} couple (Danilewicz, 2003). According with the DOE conditions, glucose (2.4 mM), gallic acid (2.4 mM) were added to the solution. The metal ions, copper (II) and iron (II), were added to the model system at concentrations of 6.3 µM and 0.1 mM respectively in the form of Cu (II) sulfate. pentahydrate and Fe (II) sulfate. heptahydrate. In the solutions without metals addition, a 50µM EDTA solution was also added. The solutions were prepared in 20mL vials. The vials were closed with an internal silicone septum and an external screw cap. An accelerated "browning" reaction scheme was employed. This involved heating the samples at 80oC or 40ºC depending on the DOE conditions. Dissolved oxygen was measured in the beginning of all experiments using a Fibox 3 LCD Trace, with Pst3 sensors (Presens GmbH, Germany). Concentration of dissolved oxygen for all the solutions were 9 mg/L. All samples were incubated at 80ºC or 40ºC for 24 hours, samples were prepared in duplicate.

3.2.3. Kinetics of Phenylacetaldehyde Formation in Model Solutions and in Real Wine

For the kinetic studies two equimolar (2.4mM) model systems were considered: i) containing phenylalanine and gallic acid (so called PO system) and ii) containing phenylalanine, gallic acid and glucose (so called PO + MR system), both with addition of metal ions. Solutions were prepared in wine model system comprising 12% (v/v) aqueous ethanol and tartaric acid (0.03 M). The metal ions, copper (II) and iron (II), has previously described were added at concentrations of 6.3 µM and 0.1 mM respectively. All solutions were adjusted to the pH of wine (pH 3.4) with NaOH (1M). In order to evaluate the impact of the presence of sugar in the phenylacetaldehyde formation in real wine systems, glucose 10 g/L has been added to a young, dry white wine, produced in the Douro region (North Portugal) (pH=3.4; SO2 free = 0.2 mM), following standard winemaking procedures, without any wood contact. Sample solutions were put into tightly closed glass tubes and placed in an air circulating, controlled oven at 40ºC. The samples were removed from the oven at different sampling times (1, 3, 5,

9, 21 and 24 hours). Dissolved oxygen was measured in the beginning of all experiments using a Fibox 3 LCD Trace, with Pst3 sensors (Presens GmbH, Germany). Concentrations of dissolved oxygen for all the solutions were 9 mg/L.

It was assumed that PA formation follows a pseudo-first-order reaction; that is:

$$C=C_0 \exp^{(-kt)}$$

where C is the concentration of the reactant (μM), C0 is the initial reactant concentration, k the reaction rate constant and t time (hours). Fit of the curves were optimized to the data by minimizing the sum of squares with the SOLVER add-in, Excel 2016 (Microsoft USA).

3.2.4. Phenylacetaldehyde Quantitation - GCMS

Phenylacetaldehyde was analyzed by headspace (HS) – solid phase micro extraction (SPME) using a SPME grey (DVB/CAR/PDMS) fiber (Supelco, Bellefonte, PA) and the volatiles were analyzed using a Varian 450 Gas Chromatograph, equipped with a mass spectral detector, Varian 240-MS and a CombiPAL auto sampler (CTC Analytics AG, Zwingen, Switzerland). The column used was a VF-WAXms (15m x 0.15mm, 0.15μm) (Varian). A 5 mL sample was spiked with 20 μL of 3-octanol (50 mg/L) as the internal standard. Anhydrous sodium sulfate (0.5 g) was added to increase ionic strength. Samples were pre-incubated in the CombiPAL oven at 40°C and 500 rpm for 2 min, then the fiber was exposed on the sample headspace for 10 minutes at 250 rpm and 40°C for extraction. The fiber was kept 10 mm from the bottom. Desorption of volatiles took place in the injector at 220°C for 15 minutes. The oven temperature was 40°C (1 min), and then increased 4 °C/min to 220 °C and held for 2.5 min (48.5 min). The carrier gas was Helium at 1 mL/min, with a constant flow. 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. All mass spectra were acquired in the electron impact (EI) mode (ionization energy, 70 eV; source temperature, 180°C). The analysis temperatures were trap 170°C, the manifold 50°C, transfer line 190°C and ion source 210°C. Compound identification was achieved by comparing retention times and mass spectra obtained from a sample containing pure, authentic standards. Compound quantitation was performed based on standard calibration curves (n=7) using m/z = 91 as

quantifier and m/z=120 as qualifier. LOD and LOQ of the method for PA are: 30 and 100 µg/L respectively. The recovery is 99.8% and the RSD was 2.5%.

3.2.5. *o*-Quinone Quantitation – LC-ESI-QqTOF-HRMS

After reaction, 3 mL of each samples of the kinetic analysis in model solutions (t=0; t=1h; t=24h) were derivatized overnight in closed vials with an OPD solution (0.05 M) to form stable quinoxalines.

These were further analyzed by LC-ESI-UHR-QqTOF-MS. The LC-ESI-UHR-QqTOF-MS analysis was performed on a UltiMate 3000 Dionex UHPLC (Thermo Scientific), coupled to a Ultra-High Resolution Qq-Time-Of-Flight (UHR-QqTOF) mass spectrometer with 50,000 Full-Sensitivity Resolution (FSR) (Impact II, Bruker Daltonics, Bremen, Germany).

Separation of metabolites was performed using an Acclaim RSLC 120 C18 column (100mm x 2.1 mm, 2,2um) (Dionex). Mobile phases were 0.1% aqueous formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The gradient started with 5% during increased to 95% in 7 min, which was kept constant for 2min and; returned to 5% B in 1 minute and maintained at 5% B for an additional 5 min at a flow rate of 0.25 mL/min. The injection volume was 1uL. Parameters for MS analysis was set using positive ionization mode with spectra acquired over a range from m/z 20 to 1000. The parameters were as follow: capillary voltage, 4.5kV; drying gas temperature, 200°C; drying gas flow, 8.0 L/min; nebulizing gas pressure, 2bar; collision RF, 300 Vpp; transfer time, 120us and prepulse storage, 4us. Post-acquisition internal mass calibration used sodium formate clusters with the sodium formate delivered by a syringe pump at the start of each chromatographic analysis.

High-resolution mass spectrometry was used to identify the adduct formed between the gallic acid quinone and the OPD (4-hydroxyphenazine-2-carboxylic acid). The elemental composition for the compound was confirmed according to accurate masse and isotope rate calculations designated as mSigma (Bruker Daltonics). The accurate mass measured was within 5mDa of the assigned elemental composition and mSigma values of < 20 provided confirmation. Gallic acid quinone were identified based on its accurate mass $[M+H]^+ =$ 241.0619. Its concentration has been expressed in gallic acid equivalents in mg/L, for that

samples without derivatization for times 0h, 1h and 24h were analyzed in the same acquisition conditions but in negative mode.

3.3. RESULTS AND DISCUSSION

The interactions between Maillard reaction (MR) and phenolic oxidation (PO), in phenylacetaldehyde (PA) formation in hydro alcoholic solutions, were studied using two full factorial designs where temperature and pH effects were studied separately. One DoE focused on the effect of pH (3.4 and 7) and the other on the effect of temperature (40°C and 80°C). In each DoE three factors: glucose, gallic acid and metals (Fe (II) and Cu (II)) at two levels (present or absence) were varied while keeping phenylalanine constant. All the experiments were performed in (12% ethanol) solutions with tartaric acid. F-test was conducted for the analysis of variance (ANOVA) to evaluate the statistical significance of each model.

For the two studied Doe's the coefficient of determination (R^2) was used to evaluate the fitness of the model. The R^2 of 0.97 for pH model and 0.98 for temperature model indicates that the predicted values obtained from each model are a good fit of the experimental data. The lack of fit compared the residual error to the pure error from duplicated experimental design points. In the full mode, the p-value for lack of fit is 0.204 and 0.154 for each model, which is greater than 0.05, indicating that the lack-of-fit is insignificant relative to the pure error. Also, the obtained F-value were 135.4 for the temperature and 48.4 for pH model, and the obtained *Prob > F-value of < 0.001* further indicated that the models were statistically significant for PA formation as shown in Figure 3.2 and 3.4.

3.3.1. DoE 1 "pH impact"

To evaluate the impact of reaction substrates on PA formation at different pH, wine pH (3.4) and pH 7, samples were heated at 80ºC. This temperature has been selected in order to promote the MR and avoid the degradation of gallic acid in other products excluding the respective *o*-quinone, at both pH's.

The ANOVA tests results are shown in Figure 3.2 and the results for PA formation for all combinations are depicted in Figure 3.3. As observed in Figure 3.2, any of the absolute value of the effects that extends beyond the reference line is potentially important. The effects were sorted from largest to smallest, and it was observed that gallic acid, pH and metals are the most important factors affecting the formation of PA. Systems with glucose, favoring potentially the Maillard reaction, showed an absolute value either close to the reference line or below it, indicating that these systems had lower impact on PA formation.

When comparing the results for PA formation for all combinations (Figure 3.3), pH seems to play an important role.
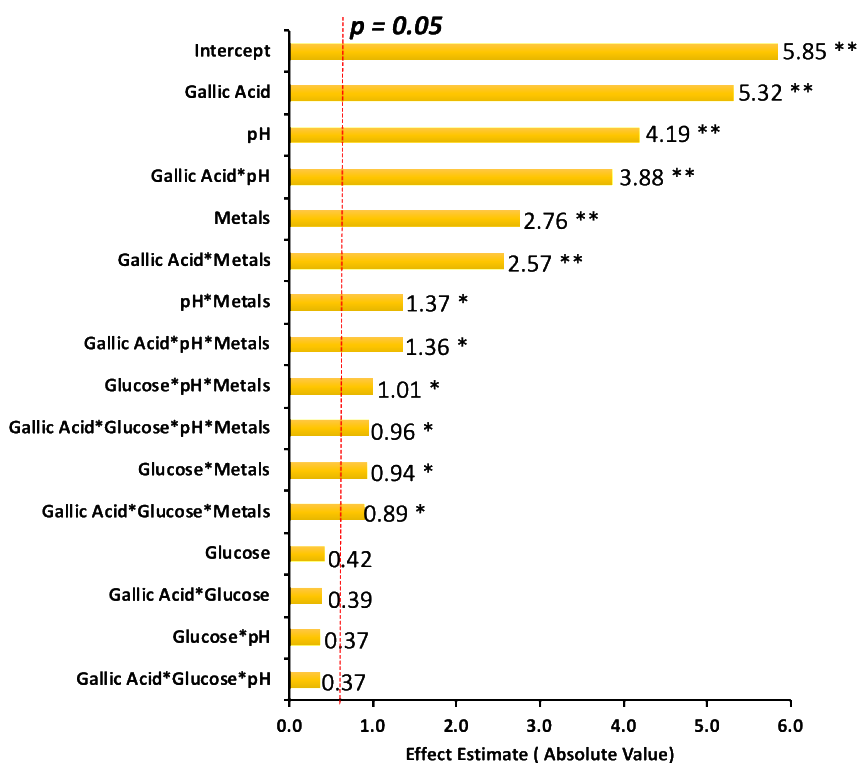
Figure 3.2 DoE 1 "impact of pH" Pareto chart of the effects (gallic acid, metals, glucose and pH) on PA formation for the two-level factorial design. ANOVA: **, significance with $p < 0.001$; *, significance with $p < 0.01$.
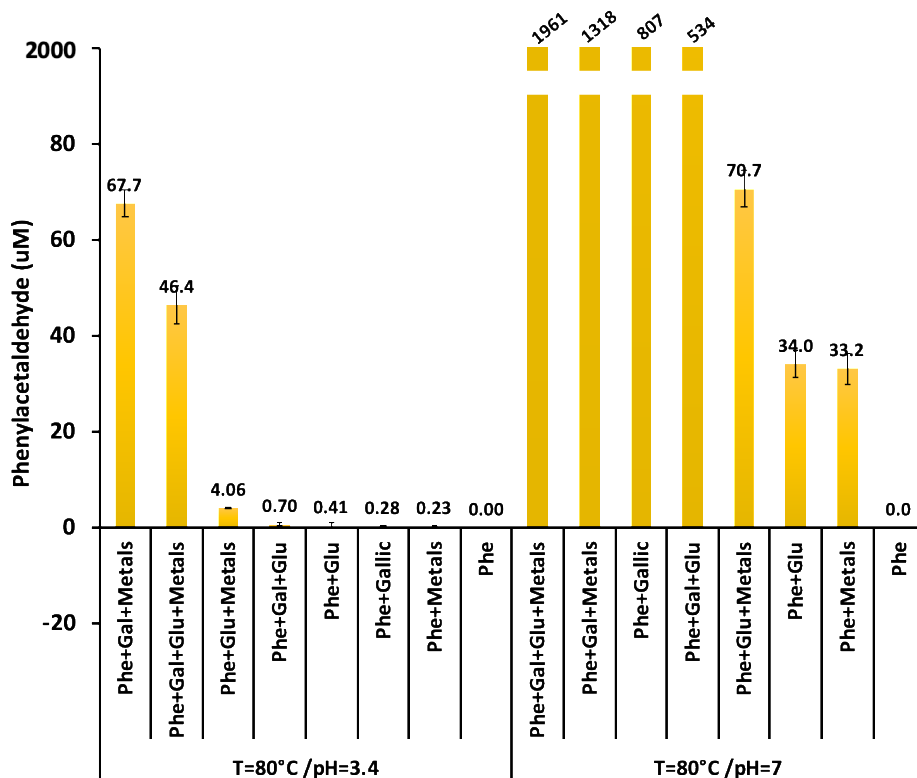
Figure 3.3 DoE 1 "impact of pH" Phenylacetaldehyde concentration for all the studied DoE combinations for two pH values (3.4 and 7) at 80ºC for 24 hours.

3.3.2. DoE 2 "Temperature impact"

In order to evaluate the impact of temperature on PA formation, by the selected factors, two temperatures were compared, 40ºC and 80ºC, at wine pH (3.4) The ANOVA tests results are shown in Figure 3.4.

The application of the full factorial design to the considered variables revealed that all the variables and interactions had significant influence on the response, where metals, temperature, gallic acid, and respective interactions were the most significant factors for PA formation. All systems containing glucose showed absolute values considerable closer to the reference. This is in line with previous studies where it was reported that oxygen cannot react directly with the polyphenols to oxidize them and the role of iron and copper are to act as catalysts in wine oxidation (Oliveira *et al.*, 2014). Moreover, the presence of glucose in the media mainly showed an impact on PA formation when in presence of gallic acid and

metals. At the studied wine pH an indication is given that glucose alone doesn't favor PA formation.
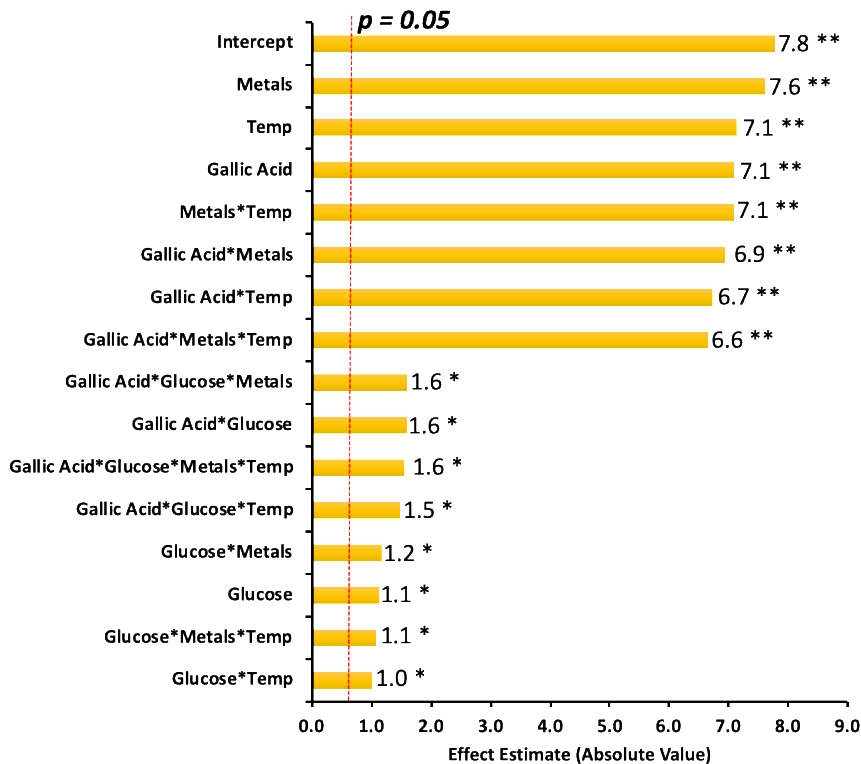
Figure 3.4 DoE 2 "impact of temperature" Pareto chart of the effects (gallic acid, metals, glucose and pH) on PA formation for the two-level factorial design. ANOVA: **, significance with $p < 0.001$; *, significance with $p < 0.01$. (B)

In fact, at pH < 5 the predominant degradation pathway of the MR proceeds by 3-deoxyosone, or the enol form of this intermediate, a pathway mainly linked with colour enhancement through the melanoidins formation and to a less extent for Strecker aldehydes (Martins and Van Boekel, 2005).

In Figure 3.5, DoE 2 results for all combinations after 24h at 40 and 80°C are depicted. It was observed that temperature has a clear impact on PA formation, and it is strongly dependent on gallic acid and metals. For both temperatures' higher amounts of phenylacetaldehyde were observed when metals were present. At 80°C, PA concentration is 8 times higher when the reaction was promoted with metals. In the absence of metals, PA concentration only increased by 4 times with the temperature increase. However, the

presence of metals, in the isolated oxidation system (phenylalanine + gallic acid), enhanced the PA content by 25 times (0.3 vs. 68 µM). In the MR (phenylalanine + glucose) system, PA formation was negligible indicating that this may not be a major pathway, via the Strecker degradation, contributing to PA formation. The presence of metals, though, in particular at 80°C, showed a considerable increase in PA formation in the MR systems, but still not as high as in the phenolic oxidation systems with gallic acid. From literature, metals are able to form complexes of various properties with MR products, as well as they can oxidize Amadori compounds and their derivatives, and catalyse further reactions of these compounds, such as the formation of deoxyosones, capable participate in the SD (Ramonaitytė *et al.*, 2009).

When comparing PA formation from phenolic oxidation (phenylalanine + gallic acid) and MR (phenylalanine + glucose) at 40°C, it was observed that PA formation was clearly promoted via the phenolic oxidation (PO) by a factor of twenty-eight-fold higher than in the isolated MR system (2.43 vs.0.1 µM). The same was observed in the systems studied at 80°C. The obtained results seem to indicate that in wine conditions the SD occurs preferentially via the PO reaction independently of the temperature (40°C or 80°C). The reaction of the amino acid with the *o*-quinone formed by the oxidation of the gallic acid seems to be favoured when compared with the Strecker degradation promoted by the reaction with α-dicarbonyls formed by MR between glucose and phenylalanine.

As observed in Figure 3.5, in the systems where the MR and the PO substrates were combined (phenylalanine + gallic acid + glucose + metals), the formation of PA was less than when compared with the oxidation system alone (phenylalanine + gallic acid + metals) for both temperatures (40°C and 80°C). It was observed that the presence of glucose together with gallic acid and metals had a negative effect on the formation of PA after long reaction times (24h at 80°C and 40°C), decreasing its formation approximately by half. In fact, in a separate study, it has been reported that sugars (glucose and fructose) can be either potent pro-oxidants in the presence of Cu^{2+}, or have antioxidant properties, like in the case of glucose (Wehmeier and Mooradian, 1994).
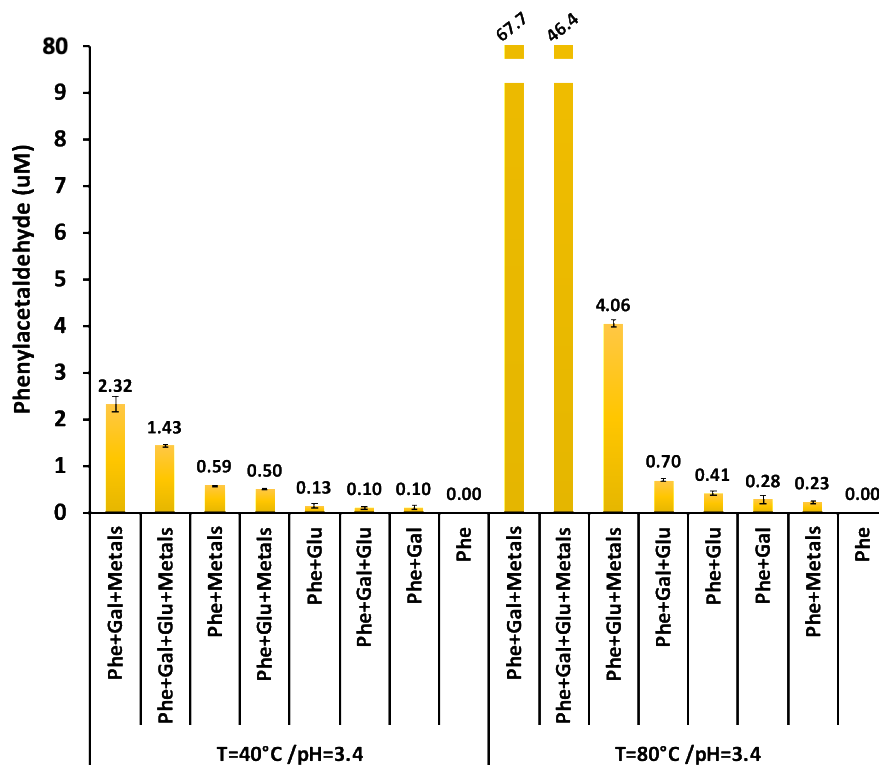
Figure 3.5 DoE 2 "impact of temperature" PA concentration for all the studied DoE combinations at wine pH (3.4) for 24 h of reaction at two temperatures (40 and 80ºC).

However, the inhibition impact of glucose on PA formation was only observed at pH 3.4 (Figure 3.3). At pH 7 the model solution with gallic acid + glucose + metals formed more PA than the one without glucose (1961 vs. 1318 μM) (Figure 3.3). Which suggest that the impact of glucose was related with the *o*-quinone that at pH 7 is more available through gallic acid that is in its deprotonated form and the reaction is less dependent on the cascade of ROS.

To elucidate the differentially strong impact of glucose on PA formation, a kinetic study has been performed and an important intermediary of the phenolic oxidation reaction: the quinone of gallic acid has been quantified. In the following section the results of the kinetic study for PA formation are presented taking into considerations sampling times of 1, 3, 5, 9, 21 and 24 hours.

3.3.3. Kinetic study in wine model systems

The influence of glucose on the gallic acid oxidation in the PA reaction rate was studied at 40°C in two wine model systems: 1) one with phenylalanine, gallic acid and metals and 2) the same as in 1 but with glucose added. A pseudo-first order for phenylacetaldehyde formation was observed for the two model systems (Figure 3.6). Analysis of variance on the reaction rate constants (k) showed highly significant effects of glucose addition (*p-value < 0.05*). In line with the DoE 1 results at wine pH, the mean formation of PA is significantly lower in the presence of glucose. The rate constant for PA formation in phenolic oxidation system (PO) (phenylalanine + gallic acid + metals) was 2 times higher when compared with the same model solution combined with the addition of glucose (MPO). The estimated rate constants were respectively 0.080 and 0.040 min^{-1}.

The formation of PA in the PO model is due to the formation of *o*-quinones, that will react with the amino acid to form the aldehyde. In order to understand this interaction of sugar in the rate of reaction, we decide to quantitate the gallic acid *o*-quinone by derivatization with OPD and analyzed in a LC-ESI-qTOF-HRMS (Table 3.1).
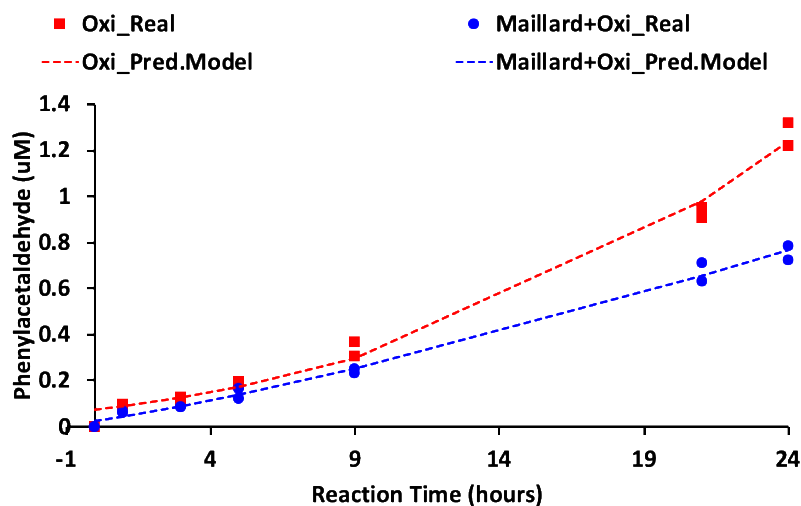
Figure 3.6 Phenylacetaldehyde formation in wine model solutions: phenylalanine+gallic acid+metals (□) and phenylalanine+gallic acid+glucose+metals (•) reacted at 40°C and pH 3.4.

Table 3.1 Accurate Mass Measurements of 4-hydroxyphenazine-2-carboxylic acid (compound formed between gallic acid o-quinone and OPD) as determined by LC-ESI-UHR-QqTOF-MS in MS/MS mode

Molecular ion / fragment ion	Elemental Composition	Calcd mass (m/z)	Measured mass (m/z)	abs. Error (mDa)	mSigma
$[M+H]^+$	$C_{13}H_9N_2O_3$	241.0608	241.0619	0.5	8.4
$[M+H - CO]^+$	$C_{12}H_9N_2O_2$	213.0659	213.0648	0.5	5.9
$[M+H - CH_2OH]^+$	$C_{12}H_7N_2O_2$	195.0553	195.0539	0.5	4.5
$[M+H - C_2O_2]^+$	$C_{11}H_9N_2O_2$	185.0709	185.0697	1.2	2
$[M+H - C_3H_3NO_3]^+$	$C_{10}H_6N$	140.0495	140.0488	0.7	4.3

In the phenolic oxidation model the concentration of o-quinone is the double of the model solution where the two reactions were promoted (phenylalanine + gallic acid + glucose + metals), Moreover in the PO the o-quinone concentration increased during time while in the solution with the addition of glucose the concentration of quinone decreased 25% after 24 hours. Apparently, the presence of the sugar in the solution had a direct impact in the o-quinone formation, as shown in Figure 3.7.
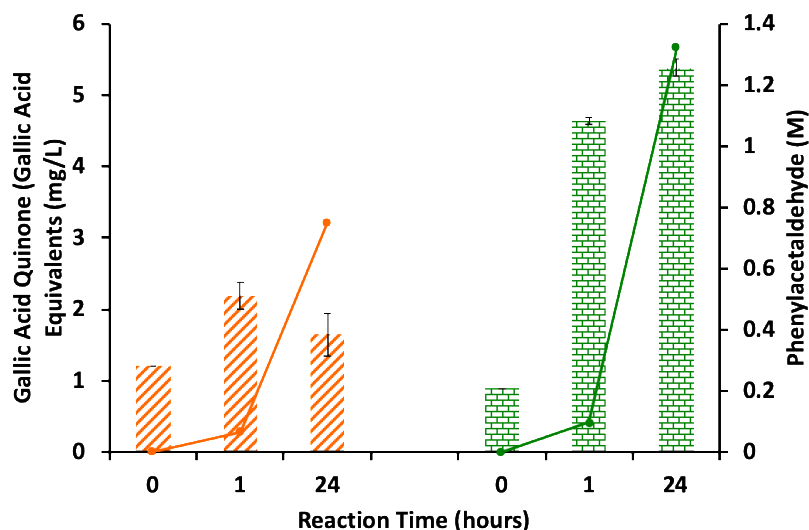
Figure 3.7 Phenylacetaldehyde formation (lines) and gallic acid quinone formation (bars) in wine model solutions for 0, 1 and 24h at 40ºC.

In fact, it has been reported that ketoses can sequester redox-active iron thus preventing the Fenton reaction (Spasojević *et al.*, 2009). Apparently, glucose, has a strong impact in the *o*-quinone formation with direct consequences in the aldehyde formation. In order to evaluate if in wine, glucose has the same effect, kinetic studies were also performed in a regular wine and the addition of sugar has been evaluated.

3.3.4. Kinetic study in real wine system

Results on a real wine system showed that the addition of glucose can have an inhibiting effect on the formation of PA (Figure 3.8). After 24 hours at 40ºC the concentration of PA was higher in the control wine than in the wine with added sugar, 0.74 ± 0.02 and 0.41 ± 0.02 µM, respectively. Furthermore, the addition of glucose had also an impact on the rate of reaction by decreasing the k value by 4 times when compared with the control (0.035 ± 0.023 vs. 0.134 ± 0.023 min-1). In line with the obtained results, Peinado et al,[29] indicated the capacity of sugars to participate in the stability of polyphenols in particular in the inhibition of the autoxidation of (+)-catechin by ferrous iron in a wine model system (pH = 4.0 tartrate buffer with 20% ethanol).
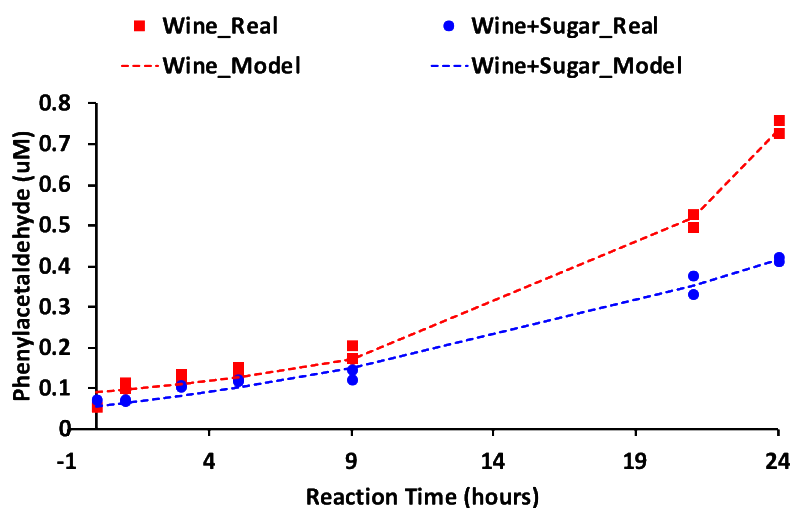
Figure 3.8 Phenylacetaldehyde formation in white wine: control (□) and wine with addition of 10 g/L of glucose (•) at 40ºC and pH 3.4.

3.4. CONCLUSION

The findings in the present study have given new insights to the understanding of the role of gallic acid, glucose as well as metals in the formation of PA. The DOE results seem to indicate that the presence of metals, is of higher relevance in promoting the Strecker degradation through the gallic acid oxidation pathway at lower pH's and different temperatures. Also, phenolic oxidation alone is the major pathway for phenylacetaldehyde formation, where the role of pH, metals and temperature are the most significant factors. This work has provided further evidence that the presence of glucose in combination with phenylalanine, gallic acid and metals inhibits the PA formation, in both model wine systems and wine itself. By gallic acid quinone quantitation an indication is given that glucose affects directly the concentration of the quinone, which suggest that in white wine glucose has an antioxidant effect by inhibiting *o*-quinones formation. Overall, the combination of the design of experiments to screen a broad experimental region combined with target experiments showed to be an efficient way to study the impact of reaction parameters on the formation of Strecker aldehydes, and key aroma compounds, in model-wine conditions. An approach that will be further applied in future studies.

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CHAPTER 4 APPLICABILITY OF PTR-TOF-MS TO STUDY THE KINETICS OF PHENYLACETALDEHYDE FORMATION IN MODEL SOLUTIONS IN DYNAMIC CONDITIONS

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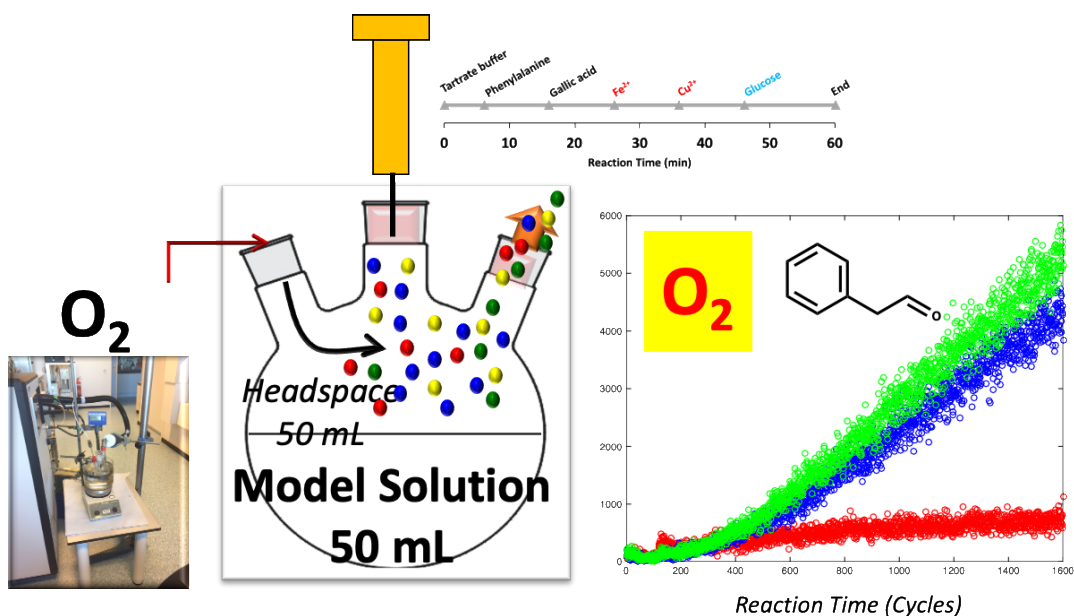
4.1. INTRODUCTION

Gas chromatography-based methods are the reference for the analysis of volatile organic compounds (VOCs), such as aldehydes. Nevertheless, this type of analytical technique is inherently slow due to the presence of, at least, one separation stage. This makes it unsuitable for characterizing the dynamic processes occurring during for example chemical reactions in real time, where acquisitions in short periods of time are needed in order to monitor compounds formation.

In recent years an increasing demand on the use of proton transfer reaction-mass spectrometry (PTR-MS) has been observed. The main advantages of the technique are the short response time and low detection limits, quite successful for on-line monitoring of VOCs and for the rapid fingerprinting of the headspace of complex samples, in particular, for food samples. Foods such as: honey (Kuś and van Ruth, 2015), milk (Zardin *et al.*, 2016), chocolate (Acierno *et al.*, no date), saffron (Nenadis *et al.*, 2016) and wine (Campbell-Sills *et al.*, 2016) were characterized in the last years regarding their volatile profile with PTR-MS.

PTRMS combines a soft and sensitive mode of chemical ionization based on proton transfer from H_3O^+ (Lindinger, Hansel and Jordan, 1998).The combination of the soft PTR ionization technique with a time-of-flight type mass spectrometer (PTR-TOF-MS) has been developed in recent years. In PTR-TOF-MS the ions are accelerated to a uniform energy by an electric field. The time of flight is directly related to the ions mass-to-charge ratio. Inherently, the whole mass spectrum can be measured in a single shot, within a fraction of a second.

The VOC signals acquired via PTR-MS are quantitative and can be assigned to specific compounds. In addition, PTR-MS data can be used to predict particular variables by chemometric methods. In summary, this equipment perfectly meets the requirements for upstream process monitoring by continuous, non-invasive measurement and quantification of the volatile metabolites.

This technique was applied for the first time to study dynamic changes in the headspace of glucose/proline Maillard reaction samples (Blank et al, Weurman, 2003). Thought we believe that this technique could be useful to study the kinetic dependence of several parameters in the Strecker degradation. In particular the presence of reaction substrates such

has sugars, phenolic compounds, metal ions and sulphur dioxide. As well as pH and temperature.

The present study concerns the experimental kinetic study for the Strecker degradation between gallic acid and phenylalanine and gallic acid, phenylalanine and glucose in the presence or absence of metal ions (iron and copper). The Strecker aldehyde formed in the reaction, phenylacetaldehyde was studied under dynamic conditions, in order to gather kinetic information regarding effects of pH, temperature and sulphur dioxide.

4.2. MATERIAL AND METHODS

4.2.1. Reagents and Standards

Chemicals were obtained from Sigma-Aldrich (tartaric acid, gallic acid, phenylalanine, glucose. Copper (II) sulphate pentahydrate and iron (II) sulphate heptahydrate were obtained from BDH (Poole, UK).

4.2.2. Preparation of model systems

Depending on the experiment conditions equimolar amounts of phenylalanine (2.4mM), gallic acid (2.4 mM) and glucose (2.4 mM) were added to a tartrate buffer at pH 3.4.

In the samples with metals additions, 0.4 ppm of Cu^{2+} in the form of copper sulphate pentahydrate, and 7.5 ppm of Fe^{2+} in the form of ferrous sulphate were added, as these concentrations are normally found in white wines (Danilewicz, 2007). Sulphur dioxide was added at 50 mg/L.

Phenylacetaldehyde released from the model solutions (50 mL) were monitored online by PTR-TOF-MS for 1 hour. Experiments were performed in a silicone bath at 80ºC in a three-neck reaction vessel, while the headspace (50 mL) was swept with oxygen (Figure 4.1). Vessel neck A was connected to the gas flushed to the sample, neck C was connected to the PTR-MS inlet and neck B was used to add to the reaction the substrates. This approach allowed us to study the impact of several substrates (gallic acid, metals, glucose, sulphur dioxide) in phenylacetaldehyde release profile.

Temperature effect were studied in the oxidation model (phenylalanine + gallic acid + Fe^{2+} & Cu^{2+}) at 40, 50, 60, 70 and 80ºC. Effect of pH was determined in the same model at 80ºC for pH 3.5, 5 and 7.
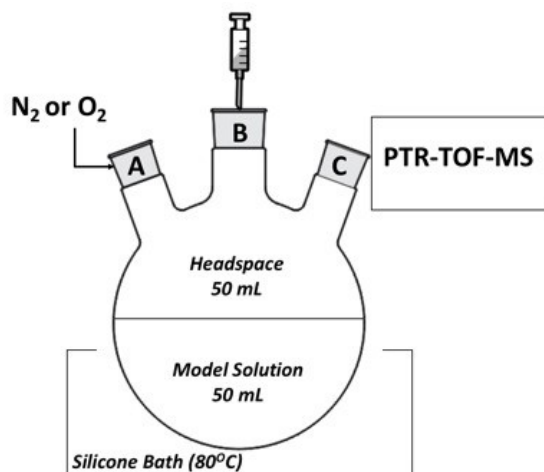
Figure 4.1 Scheme of the reaction vessel used for the analysis. A

4.2.3. Analytical procedure - PTR-TOF-MS Acquisition

A commercial PTR-TOF-MS 8000 (Ionicon Analytik GmbH, Innsbruck, Austria) instrument were used for measurements. The sampling time chosen was 0.1 ns for 60 minutes, resulting in 8000 cycles per sample. All measurements were carried out under inlet temperature of 125ºC, drift tube temperature of 90ºC, drift tube pressure of 3.8 mbar and drift voltage of 900 V with an electric field strength (E/N) of 131 Td (Townsend).

Mass axis calibration and calculation of peak areas were done using the software PTR-MS Viewer. In order to guarantee high mass accuracy throughout the analysis, the mass scale was calibrated following the peaks of known ions (H_3O^+, m/z=21.022; and NO^+). The raw data were subsequently corrected for transmission and peak areas converted into concentration (parts per billion by volume) using the primary ions H_3O^+ and $(H_2O)_2H^+$ measured by their respective ^{18}O isotopologues and using a common coefficient rate (k=2.10^{-9} cm^3/s).

Dead time correction, mass calibration, peak extraction and integration were performed using PTR-TOF DATA Analyzer software (v4.17). Three replicates were acquired for each condition and the spectra were averaged.

To assess for sensitivity, preliminary experiments were performed to control the linearity of the PTR-MS by injecting measured amounts of phenylacetaldehyde into the chamber. The compounds have been injected four times, alternated with blank injections to prevent carry-over effect between measurements. Phenylacetaldehyde was obtained by following m/z 121.065 corresponding to $C_8H_9O^+$.

4.2.4. Kinetic Measurements

Release kinetics was analysed by converting the curves of released to cumulative release vs. time. The cumulative release profiles were fitted to Weibull model as proposed by Liardin et al (Mateus *et al.*, 2007), using Excel for the calculation of the process rate constant k (min^{-1}) and the shape parameters (n). According to literature, Weibull model has greater accuracy than the first-order kinetics equation (Peleg, Corradini and Normand, 2004). According with the Weibull model n is the distribution shape factor. The estimation of the model parameters was performed by minimization of the sum of squares of residuals, by using Solver tool from Microsoft Excel 2011 and the fitting was evaluated using the coefficient of determination (R^2).

4.3. RESULTS AND DISCUSSION

Headspace sampling under dynamic conditions allowed the time of volatile generation to be observed during the Strecker degradation of phenylalanine. Phenylacetaldehyde release obtained, provided a valuable estimation of the impact of several factors in the kinetics of the reaction. Several factors were selected and studied in particular: temperature, pH, metals and sulphur dioxide additions.

Typically, the determination of this factors in the formation of specific compounds is performed by the normal kinetic approaches, consisting in the single point analysis taken from specific times. With PTR-MS it was possible to have a real time sampling point with PA release each 1 sec, which increase the resolution of the data obtained. Also, the injection

of increasing concentrations of the compound followed by blank injections showed that PTR-TOF-MS was sensible to the compound and no carry-over between measurements were observed in the blank sections.

In Figure 4.2 both release (A) and cumulative profile (C) of pphenylacetaldehyde formation is shown. In the release profile, PA is expressed in ppb per unit of time, while in the cumulative it is expressed in total concentration at that given time.
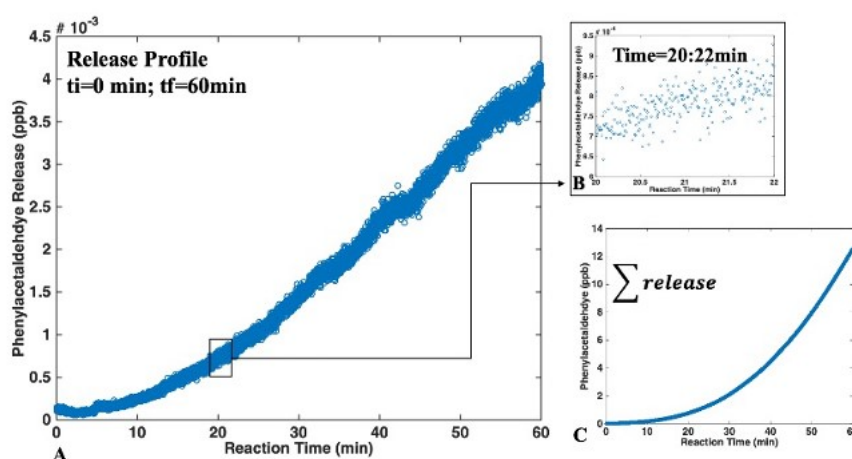
Figure 4.2 Phenylacetaldehyde release profile between 0 and 60 minutes (B) phenylacetaldehyde release between 20 to 22 minutes and (C) phenylacetaldehyde cumulative profile for 60 minutes of reaction. time at 80ºC (solution with phenylalanine, glucose, gallic acid, Fe^{2+} and Cu^{2+}, pH 3.4).

4.3.1. Impact of reaction substrates

The impact of reaction substrates was studied, in particular using two models: i) phenylalanine + gallic acid and ii) phenylalanine + gallic acid + glucose. The two models were studied with and without the addition of metals (Fe^{2+} and Cu^{2+}). Cumulative profiles for model system with phenylalanine and gallic acid are represented in Figure 4.3A. It is possible to observe that presence of metals has a significant impact in the formation of phenylacetaldehyde. Nevertheless, for both solutions PA formation was also observed in the absence of metal ions, even if in lower amounts.

The Weibull model was in good agreement with the experimental data ($r^2 > 0.95$), for all models and the root mean square values were small.

The estimated values of reaction rates (k) are represented in Figure 4.3B. The shape parameter represents the deviation of the phenylacetaldehyde formation from linearity. Higher shape parameters indicated more complexity to explain the formation of the compound.

The rate of reaction increases four times with metals addition (0.1 to 0.42) for the model with phenylalanine and gallic acid. Moreover, glucose addition decreases the rate of PA formation, confirming the results obtained in the previous chapter (Monforte, Martins and Silva Ferreira, 2018).
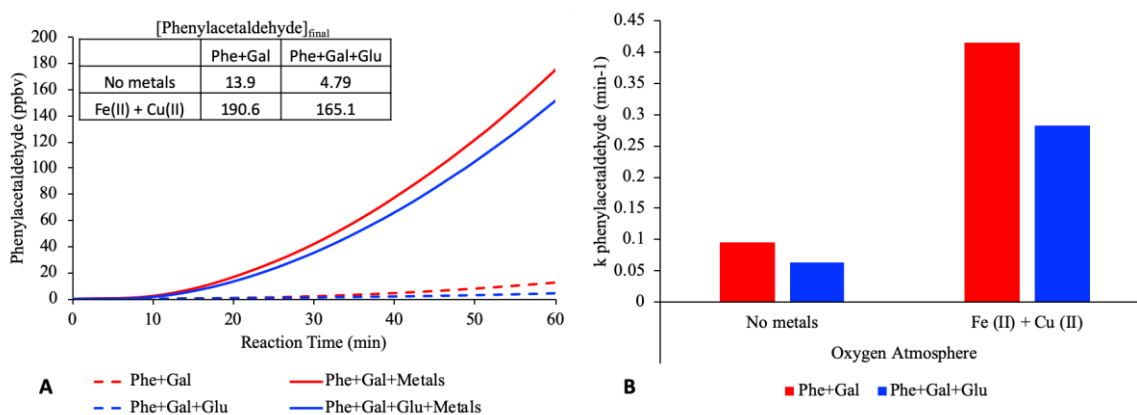
Figure 4.3 (A) Cumulative profiles of phenylacetaldehyde formation for the model solutions with (i) phenylalanine and gallic acid and (ii) phenylalanine, gallic acid and glucose. Both with and without the addition of Fe^{2+} and Cu^{2+}. (B) rate of phenylacetaldehyde formation (min^{-1}) obtained by Weibull model for the same conditions. Concentration of phenylacetaldehyde expressed in ppb.

To have more insights regarding the role of glucose, sequential additions of reactions substrates (gallic acid, glucose and metals) were performed to the model solution with phenylalanine according with the scheme represented in Figure 4.4. Results shown that reaction rate is highly affected by metals. When iron is added an increase in the release was observed. Yet the observed impact is dependent on glucose moment of addition: before metals addition (Figure 4.4A) or after metals addition (Figure 4.4B).

This observation suggests that glucose capacity of reducing the formation of the gallic acid
o-quinone and consequently decreasing PA formation could be related with its capacity to
chelate metal ions (Bersuder, Hole and Smith, 2001; Ruiz-Roca, Navarro and Seiquer,
2008).
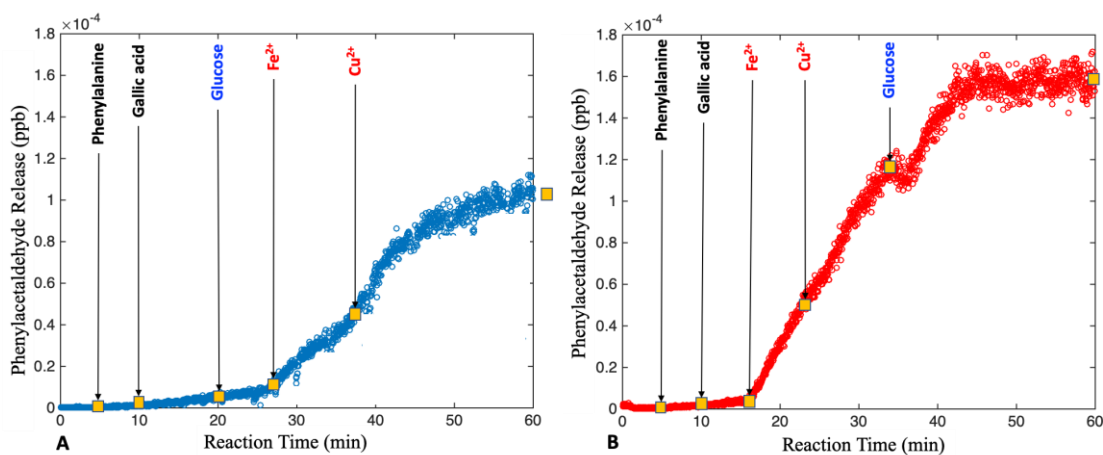
Figure 4.4 Phenylacetaldehyde release in dynamic conditions dependent on sequential
additions of substrates as indicated in the figure with an orange square across reaction time
(minutes).

4.3.2. Temperature and pH impact

In order to getter more information regarding the impact of extrinsic parameters such as temperature and pH on PA formation, solution was further studied at three pHs (pH 3.4, 5 and 7) and five temperatures (40, 50, 60, 70 and 80°C). Phenylacetaldehyde release kinetics were again fitted using the Weibull model. The results for the impact of pH and temperature are presented in Table 4.1 and Figure 4.5.

An increase in phenylacetaldehyde rate of formation was observed dependent on pH values: *pH 7 > pH 5 > pH 3.4*. An observation in line with previous studies. Several authors have reported that pH and temperature played a crucial role in Strecker degradation by increasing and/or decreasing its reaction rate. PA formation has been reported to be promoted by glucose or α-dicarbonyls (methyl glyoxal, glyoxal, 3-deoxyosone and glucosone) direct reaction with phenylalanine showing a maximum at pH 5 and a minimum at pH 3 & pH7. Yet at higher pH (7 and 9) the formation of phenylacetic acid was favoured (Hofmann, Münch and Schieberle, 2000).

Table 4.1 Rate of formation (k) and shape parameter (n) from the fitting of the Weibull model for the three pH (3.4, 5 and 7) and the five temperatures (40, 50, 60, 70 and 80°C).

		k (min^{-1})	n
pH **(T = 80°C)**	pH=3.4	0.30	0.56
	pH=5	0.32	0.56
	pH=7	0.36	0.56
Temperature (°C) **(pH=3.5)**	T=40°C	0.014	0.11
	T=50°C	0.033	0.24
	T=60°C	0.080	0.56
	T=70°C	0.174	0.56
	T=80°C	0.354	0.56

Conversely, when SD was promoted by the oxidation of a phenolic compound into an *o-quinone*, it was found that phenylacetaldehyde formation is more or less constant at pH values < 5 and decreased at higher pH values. The constant rate for pH < 5 could be depended

on the type of phenolic studied. In the referred study hydroquinone, 1,2,4-trihydroxybenzene
and benzoquinone were selected. According with literature at higher pH values, phenolic
hydroxyl in galloyl groups can be oxidized to form a quinonoid structure more easily (Hider,
Liu and Khodr, 2001). A similar result was obtained by comparing PA formation in the same
conditions at pH 3 and 7 (Monforte, Martins and Silva Ferreira, 2018).
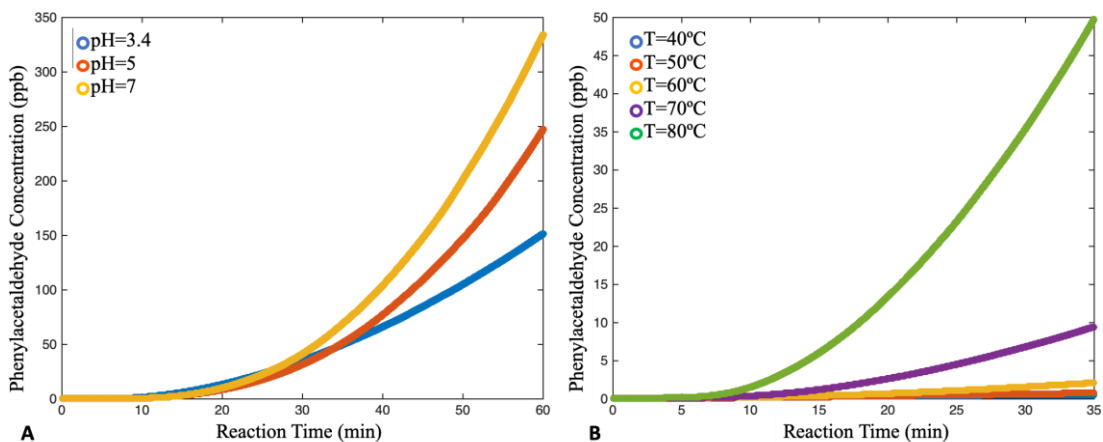
Figure 4.5 Cumulative profiles of phenylacetaldehyde formation for (A) pH (3.4, 5, and 7)
and (B) temperatures (40, 50, 60, 70 and 80ºC). Phenylacetaldehyde formation with different
pH was studied at 80oC, while temperature effect was studied at wine pH (3.4), (solution
with phenylalanine, glucose, gallic acid, Fe^{2+} and Cu^{2+}).

To study temperature effect on PA formation, oxidation was carried out separately at various
temperatures at pH 3.5. It can be observed that SD is highly favoured at high temperatures.
Arrhenius equation was fitted to experimental data (Figure 4.6) and activation energy of 73.5
± 0.8 kJ/mol was obtained with a coefficient of determination of 0.999.
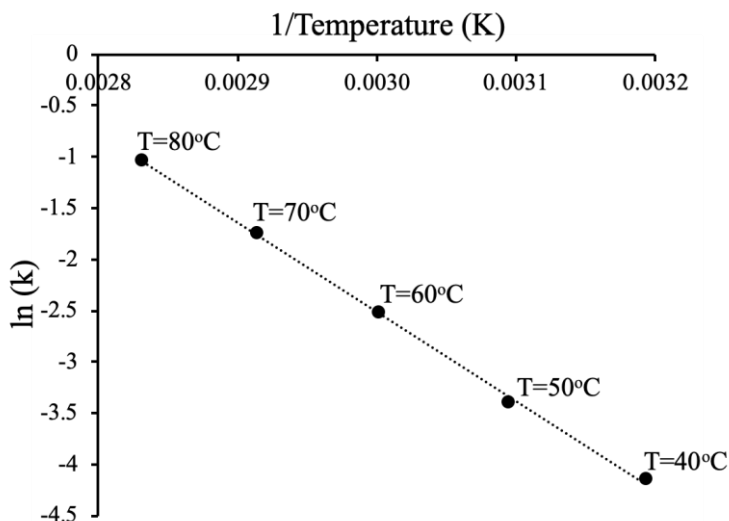
Figure 4.6 ln (k) vs. 1/T which illustrates the activation energy (Ea) where the slope is used
for the calculation of Ea.

Yet the obtained value of the activation energy is higher when compared with values
obtained by the study of PA formation exclusively from the Maillard reaction (21.5 kJ/mol)
(Chan, 2005) but is similar to values found in beer (76.7 kJ/mol), validating the impact of
temperature in conditions where phenolics are present (da Costa *et al.*, 2004).

Interestingly, the shape parameter (n) is higher with increase of temperature suggesting that
as temperature increased the mechanism of PA formation becomes more complex.
Mechanisms that seem to be occurring are: 1) the Maillard reaction, as temperature increases
SD promoted from Maillard reaction (glucose + phenylalanine) will contribute more to the
SD promoted by oxidation (quinone + phenylalanine); 2) Oxidation, at higher temperature
the oxidation of Amadori compound is promoted by the presence of metals (Hofmann and
Schieberle, 2000), leading to the higher formation of the aldehyde. These two facts seem to
explain the higher shape value as temperature increases.

4.3.3. Impact of Sulphur Dioxide

Sulphur dioxide plays an important role in oxidation and consequently on phenylacetaldehyde formation. The impact of sulphur dioxide in reducing PA formation has been reported in the literature as well as its capacity to bind aldehydes and produce adducts (Grant-Preece *et al.*, 2013; Bueno, Zapata and Ferreira, 2014).

Two sets of experiments were performed to study the impact of SO_2 in the formation of phenylacetaldehyde (i) addition of 50 mg/L SO_2 at time zero, (ii) SO_2 addition at time 20 minutes. In the second set, the impact of pH and SO_2 was studied.

The model solution used was with phenylalanine, gallic acid, glucose and metals. Phenylacetaldehyde release without SO_2 addition was higher in (0.0375) compared with the one with SO_2 (0.0058) (Figure 4.7A). This shown the impact of SO_2 in reducing PA formation (6 times). According with the literature sulphur dioxide has de ability to trap aldehydes as well as to block the *o*-quinones formation. In order to get more information regarding the role of SO_2, the reaction has been started with phenylalanine, gallic acid, glucose and metals and after 25 minutes SO_2 has been added (Figure 4.7B).
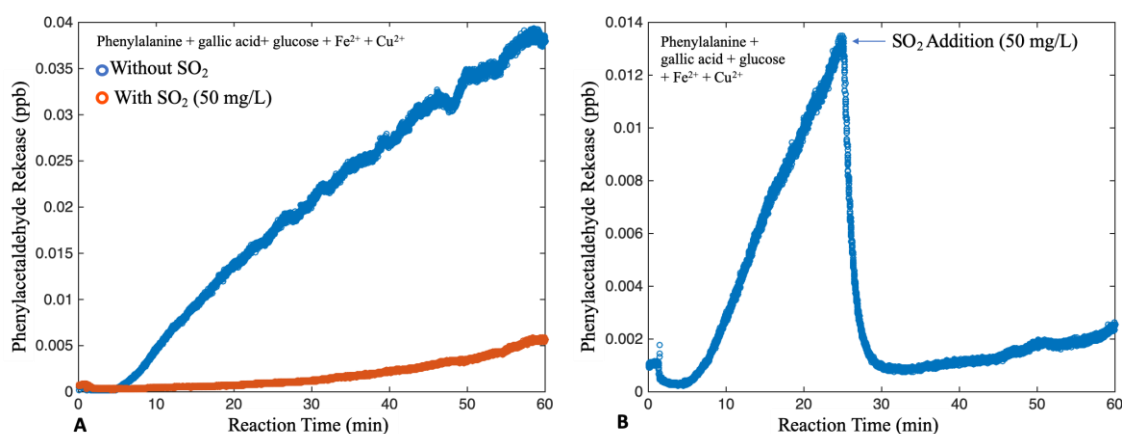
Figure 4.7 (A) Phenylacetaldehyde release profile in the model solution with and without addition of 50 mg/L SO2 and (B) Addition of 50 mg/L of SO_2 after 25 minutes of reaction (model solution: phenylalanine + gallic acid + glucose + Fe^{2+} + Cu^{2+}, pH=3.4 at 80ºC during 60minutes).

Results show the immediate consequence of SO_2 addition in decreasing dramatically PA release. However, a slightly increase in the release has been observed after 40 minutes of reaction, suggesting a reversibility in the PA trapping by SO_2. The reversibility of the binding of hydrogen sulphate could be promoted by addition of acetaldehyde (to bind), concentrated acid (to convert hydrogen sulphate to molecular sulphur dioxide) or hydrogen peroxide (to oxidize) are well known. Nevertheless, it has been observed in wine model conditions that the addition of the referred agents have a low impact on PA concentration after 1 hour of reaction. Still the impact of pH and temperature have also been studied. In Figure 4.8 it was possible to observe the release and cumulative profiles dependent on pH values (A and B) and temperatures (C and D). In Table 4.2 are indicated the phenylacetaldehyde release before and after SO_2 release as well as in the end of the reaction (60 min).

For temperature the impact is higher, before SO_2 addition and after SO_2 addition rate of PA release changes 4 times for 60 and 70ºC and 14 times for 80ºC. The effect of temperature in PA release after 40 minutes can be clearly observed in Figure 4.8C and in Table 4.2, for 60 and 70ºC the release is constant while for 80ºC an increase in the release was observed. In fact the stability of the complex bisulphite-phenylacetaldehyde depends on the temperature (de Azevedo *et al.*, 2007). According with the obtained results the stability of the adduct is more dependent on temperature.

Table 4.2 Phenylacetaldehyde release values before and after SO_2.

		Before SO_2 (t=20 min)	**After SO_2** (t=25 min)	**End** (t=60 min)
pH (T = 80ºC)	pH=3.4	1.4E-02	9.4E-04	2.2E-03
	pH=5	1.6E-02	1.1E-03	2.4E-03
	pH=7	2.3E-02	1.8E-03	2.7E-03
Temperature (pH=3.4)	T=60ºC	6.5E-04	1.5E-04	1.2E-04
	T=70ºC	2.8E-03	7.3E-04	4.6E-04
	T=80ºC	1.4E-02	9.9E-04	2.7E-03
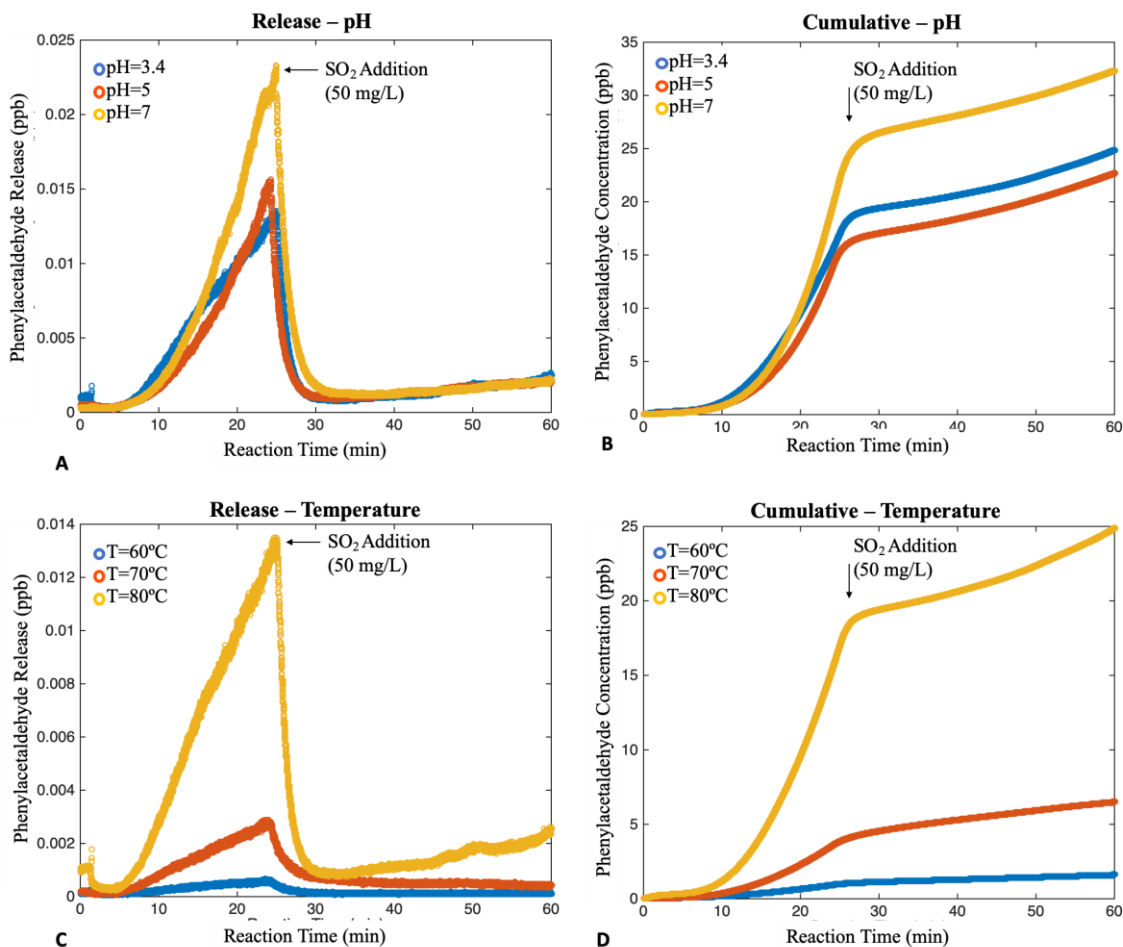
Figure 4.8 Impact of SO₂ addition after 20 minutes of reaction in the release and cumulative profiles of phenylacetaldehyde formation for (A) and (B) pH (3.5, 5, and 7) and (C) and (D) temperatures (60, 70 and 80ºC). Addition of 50 mg/L of SO₂ after 25 minutes of reaction (model solution: phenylalanine + gallic acid + glucose + Fe^{2+} + Cu^{2+}).

4.4. CONCLUSION

In conclusion, this study demonstrates the high potential of acquiring real time data particularly suitable to capture of subtle changes that the sampling method would probably not the able to take int account. Furthermore, the flexibility that this set up provides enables o number of combined effects that are simply out of reached on a classic kinetics DOE set-up. Therefore PTR-TOF-MS demonstrate its capabilities when one wants to study complex chemical reactions with impact on flavour properties such as Strecker degradation. Sequential additions shown that the impact of glucose in reduce phenylacetaldehyde formation could be related with interactions with metal ions (iron and copper).

4.5. REFERENCES

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CHAPTER 5 RESPONSE SURFACE METHODOLOGY: A TOOL TO MINIMIZE ALDEHYDES FORMATION AND OXYGEN CONSUMPTION IN WINE MODEL SYSTEMS.

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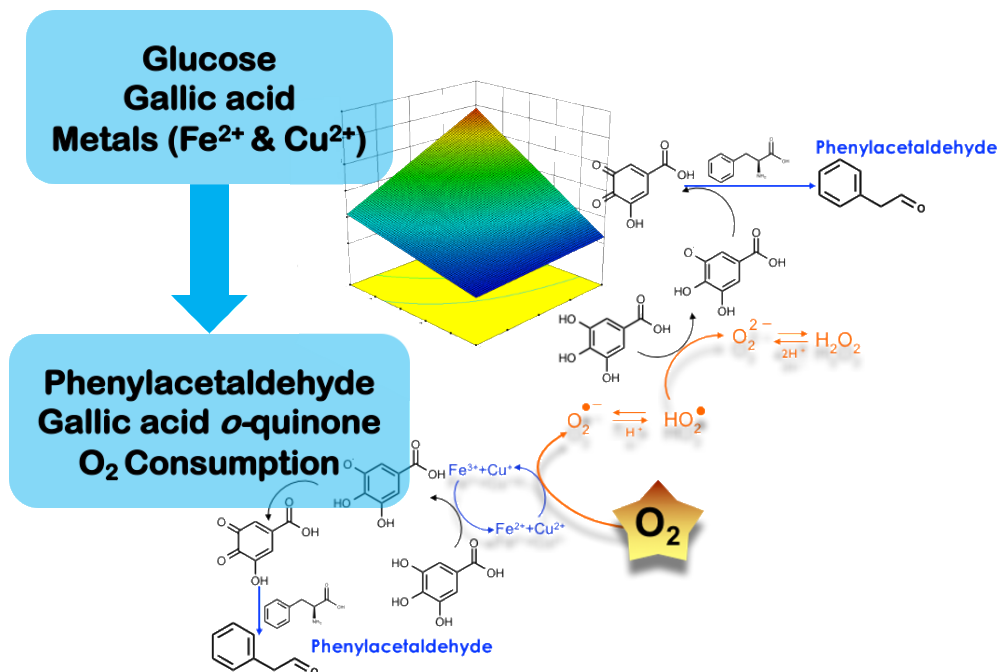
5.1. INTRODUCTION

Wine oxidation, a reaction that occurs during wine ageing, is a complex process involving several chemical species like oxygen, phenolics, metals, and amino acids (Singleton and Kramling, 1976; Pripis-Nicolau *et al.*, 2000; Danilewicz, 2003). Kinetically it depends on several parameters, like pH and temperature, and the connections between substrates is not fully understood due to the high degree of interaction between the mechanisms responsible for development of colour and flavours related to wine oxidation (Waterhouse and Laurie, 2006; Martins, Monforte and Silva Ferreira, 2013). Several volatile compounds are related with the oxidation of wines, in particular: aldehydes, furfurals and volatile thiols (Ugliano *et al.*, 2011; Ferreira *et al.*, 2014; Mayr *et al.*, 2015). Strecker aldehydes are well recognized as sensorial quality markers, typically as off-notes, when present in wines.

The most studied aldehydes responsible for white wines flavour deterioration are phenylacetaldehyde and methional (Escudero *et al.*, 2002; Silva Ferreira, Hogg and de Pinho, 2003). The formation of these compounds in wine has been related to the Strecker degradation that can occur due to the reaction of *o*-quinones with amino acids, where *o*-quinone is formed via the oxidation of phenolics, as well as to the reaction of dicarbonyls with amino acids, where the dicarbonyls are formed either by the sugars degradation, like the Maillard reaction, or by alcoholic and malolactic fermentation (Pripis-Nicolau *et al.*, 2000). Once formed the aldehydes are highly reactive molecules. These can react with compounds such as sulfur dioxide to form bisulphite adducts (de Azevedo *et al.*, 2007). These adducts are rather stable and are formed as a result of sulphite addition to wines, a common practice by producers to preserve wine sensorial characteristics during ageing and storage. However, in a recent study, it has been suggested that the hydrolysis of aldehydes-bisulfite adducts during storage can actually play a bigger role in SA formation, in particular for phenylacetaldehyde and methional, than the "novo" formation from substrates (Bueno, Carrascon and Ferreira, 2016; Bueno *et al.*, 2018). Additionally, in a separate study, it has also been shown that glucose can play an important role in reducing the aldehyde formation by decreasing the gallic acid *o*-quinone rate of formation in wine model solutions (Monforte, Martins and Silva Ferreira, 2018). The impact of phenolics in the Maillard reaction has already reported in model solutions and in foods, namely in reducing the amounts of glucosone, pyrazines, furfural and Strecker aldehydes (Totlani and Peterson, 2005; Schwambach and Peterson, 2006; Wilker, Heinrich and Kroh, 2015; Jansson *et al.*, 2017).

The aim of this new study is to apply, for the first time, response surface methodology to wine-like model systems to better understand Strecker aldehydes formation during the ageing process. The application of RSM is today largely disseminated due to its advantage to classic one-variable-at-a-time optimization, such as the generation of large amounts of information from a small number of experiments and the possibility of evaluating the interaction effect between the variables on the responses. This, mathematical technique is also useful for developing, improving and optimizing processes since it predicts the value of a dependent variable based on the controlled values of the independent variables (Yolmeh and Jafari, 2017). Three concentration levels of gallic acid, glucose and metals (Fe^{2+} and Cu^{2+}) were considered, and for each, it was determined the impact of substrates on the gallic acid o-quinone and phenylacetaldehyde formation, as well as on oxygen consumption. The developed regression equations allowed to determine what the optimal conditions were to minimize phenylacetaldehyde formation for the studied conditions. An important information, seen of great value, when trying to improve wines sensorial properties during shelf-life.

5.2. MATERIAL AND METHODS

5.2.1. Experimental Design

A design of experiments (DOE) was employed. It is a powerful tool for process investigation and optimization, by simultaneously considering many factors at different levels and their potential interactions. A three factor and a three-level face-centered composite design (FCCD) consisting of sixteen experimental runs was employed including 2 replicates at the center point (CP) and 2 replicates for the full factorial design. The design variables were the gallic acid concentration (X1, mM), glucose concentration (X2, mM) and metals (Fe^{2+} & Cu^{2+}) concentration (X3, mg/L). The selection of the factors concentration was for glucose and gallic acid based on previous results, whereas for the metals it was based on the average concentration of iron and cooper present in real wines (Danilewicz, 2016; Monforte, Martins and Silva Ferreira, 2018). The selected response variables (Y) were consumed oxygen, gallic acid o-quinone and phenylacetaldehyde concentration after 24 hours and 40ºC.

Given a response variable Y and three factors, X1, X2 and X3, the main purpose of RSM is to find the combination of factor levels to achieve the optimal response. Based on the

statistical analysis of the effects (main and interactions), those that were statistically significant were included in the developed models. Two types of models have been fitted to the experimental results:

i) the generalized second-order polynomial model, which describes the interaction between the different experimental design variables,

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i<j=1}^{3} \beta_{ij} X_i X_j + \varepsilon$$

and ii) polynomial function containing quadratic terms according with the equation presented below:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{3} \beta_{ij} X_i X_j + \varepsilon$$

where β_0 is the constant terms, β_i, β_{ii} and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, X_i and X_j are the independent variables and ε the residual associated to the experiments. The polynomial function allows to predict the concentration of the response Y based on specific concentrations of the selected factors (X1, X2 and X3).

The analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significance of all terms in the polynomial were judged statistically by computing the F-value at a probability (p) of 0.01 and 0.001. The regression coefficients were used to make statistical calculations. The statistical experimental design was generated, evaluated for the quality of fit and the constant and regression coefficients as well as response surfaces and contour plots were calculated and designed using the Design-Expert® software (Version 10, Stat-Ease Inc., Minneapolis, USA).

5.2.2. Preparation of model systems and storage conditions

Wine-like model solutions were prepared in 12% ethanol (v/v) with tartaric acid (0.03M) with the addition of phenylalanine (20mM) and the pH was adjusted to 3.4 by adding sodium

hydroxide (1M). Glucose and gallic acid were added as described by the DoE (Table 5.1). In the study the normal concentration of phenolic compounds in white wine (0.6 mM) was increased in order to increase the rate of reaction of phenylacetaldehyde formation.

The metal ions, Cu^{2+} and Fe^{2+}, were added to the model system at the concentrations indicated in Table 5.1, in the form of Cu (II)sulfate·$5H_2O$ and Fe (II)sulfate·$7H_2O$. In the solutions with no metal addition, a complexing agent (50 μM EDTA solution) was also added to prove the catalytic effect of metals in the reaction. The solutions were prepared in duplicate in 60 mL tightly screw capped glass vials. All vials also contained PSt3 oxygen sensors (Nomacorc S.A., Thimister-Clermont, Belgium). The flasks were filled completely avoiding any headspace. The initial concentration of dissolved oxygen measured for all samples was 8.5 ± 0.3 mg/L. Wines were stored in a dark incubator at 40ºC for 24 hours.

5.2.3. Dissolved Oxygen Measurements

The oxygen concentration was measured in the beginning and in the end of the experiment using a Fibox 3 LCD fiber optic oxygen transmitter, a polymer optical fiber and planar oxygen-sensitive spots (5 mm sensor spots PSt3).

A control sample (water) stored in the same conditions as the experimental samples were used to monitor if some oxygen ingress into the container, to be assured that oxygen entrance was not significant.

5.2.4. Phenylacetaldehyde quantitation – GC-MS

Phenylacetaldehyde was analyzed by a liquid-liquid extraction procedure with dicloromethane and analyzed by GC-MS according with (Silva Ferreira, Barbe and Bertrand, 2003).

5.2.5. Gallic Acid Quinone Quantification – LC-ESI-QqTOF-HRMS

Gallic acid *o*-quinone were quantified in its phenazine form by LC-ESI-QqTOF-HRMS according with the method described in (Monforte, Martins and Silva Ferreira, 2018). Gallic

acid *o*-quinone area has been normalized according to the highest area observed on the sample set expressed as a percentage.

5.3. RESULTS AND DISCUSSION

In the present study, response surface methodology has been applied to wine-like model systems with the aim to better understand Strecker aldehydes formation during wine's ageing process. Precursors concentration levels were varied and their impact on gallic acid *o*-quinone and phenylacetaldehyde formation, as well as on oxygen consumption, determined. In the following sections the obtained results will be discussed in detail, starting with statistical analysis showing the relevant parameters and the order of confidence obtained for the derived models.

5.3.1. Statistical Analysis

A face-centered composite design of experiments was employed where 16 experiments were performed in duplicate. The values obtained for oxygen (O_2) consumption, gallic acid *o*-quinone (GAQ) and phenylacetaldehyde formation are shown in Table 5.1.

The coefficient of equations obtained by fitting the experimental data for each response are summarized in Table 5.2 and 5.3. The obtained R^2 values indicate that the second-order polynomial model was more suitable for oxygen consumption and the full quadratic model was the more adequate to predict the gallic acid *o*-quinone and phenylacetaldehyde values. The regression coefficients for the derived models changed from 0.94, for oxygen consumption and phenylacetaldehyde formation, to 0.97, for GAQ formation. Both fits showed significant probability values ($p < 0.001$) and non-significant lack of fit values. The R^2 coefficient in this study ensured a satisfactory adjustment of the obtained models to the experimental data.

Table 5.1 Coded matrix of FCCD experimental design and the quantification of the three responses

	Factor				Responses		
Sample no.	Gallic Acid	Glucose	Metals		O_2 consumption (mg/L)	Gallic acid quinone (%)	Phenylacetaldehyde (μg/L)
1	-1	-1	-1		n.d.	5.13 ± 1.58	18.7 ± 0.45
2	-1	-1	1		0.60 ± 0.09	31.2 ± 0.20	177 ± 10.5
3	-1	0	0		0.03 ± 0.01	26.7 ± 2.76	146 ± 6.86
4	-1	1	-1		n.d.	n.d.	8.55 ± 1.36
5	-1	1	1		0.63 ± 0.13	18.0 ± 1.20	106 ± 1.77
6	0	-1	0		0.99 ± 0.07	50.1 ± 1.35	444 ± 62.8
7	0	0	-1		n.d.	n.d.	12.5 ± 8.80
8	0	0	1		1.65 ± 0.11	44.9 ± 0.92	358 ± 66.0
9	0	1	0		1.03 ± 0.04	33.3 ± 2.44	195 ± 7.18
10	1	-1	-1		0.05 ± 0.002	6.71 ± 0.47	33.8 ± 5.23
11	1	-1	1		2.72 ± 0.25	97.6 ± 3.39	716 ± 6.07
12	1	0	0		0.035 ± 0.002	26.7 ± 2.76	146 ± 6.86
13	1	1	-1		0.10 ± 0.009	4.91 ± 1.50	11.8 ± 0.56
14	1	1	1		1.91 ± 0.08	76.1 ± 1.82	620 ± 14.1
15	0	0	0		0.17 ± 0.03	43.7 ± 3.04	309 ± 8.34

Results expressed as mean ± standard deviation of 2 replicates

Gallic acid and Glucose: (-1): 2.4 mM, (0) 11.2 mM and (1) 20mM

Metals: (-1): 50μM EDTA (0): 7.5 mg/L Fe^{2+} and 0.4 mg/L Cu^{2+}, (1): 15 mg/L Fe^{2+} and 0.8 mg/L Cu^{2+}

5.3.2. Oxygen Consumption

For all the 32 samples measured, the oxygen consumption ranged from non-detected to 2.72 ± 0.25 mg/L (sample 11), being the lowest amounts observed when EDTA was present (sample 1, 4 and 7). This indicates that in the presence of EDTA, oxygen consumption is almost negligible. Previous studies reported in literature, that in the absence of metals, while

in the presence of a chelator such as EDTA, 1-hydroxyethyl radical can be formed resulting in an increase in oxygen consumption (Kreitman *et al.*, 2013). Yet in the present study this effect was only observed for higher concentration levels of gallic acid (20mM).

Overall, the maximum oxygen consumption was observed when metals and gallic acid were at the maximum concentration and glucose at the lowest concentration level.

Table 5.2 ANOVA for Response Surface Quadratic Model for consumed Oxygen

	Source	Sum of squares	df	F Value	p-value	Coefficient Estimate	Error
O_2 Consumption	**Model**	**15.05**	**9**	**59.69**	**<0.001**	**0.84**	**0.036**
	X1 - Gallic	**3.59**	**1**	**85.49**	**<0.001**	**0.42**	**0.046**
	X2 - Glucose	0.02	1	0.51	0.48	-0.033	0.046
	X3 - Metals	**9.06**	**1**	**215.13**	**<0.001**	**0.67**	**0.046**
	X1X2	0.005	1	0.14	0.71	-0.019	0.051
	X1X3	**2.35**	**1**	**55.94**	**<0.001**	**0.38**	**0.051**
	X2X3	0.02	1	0.55	0.47	-0.038	0.051
	X1^2	0.14	1	3.55	0.073	-0.16	0.085
	X2^2	0.07	1	1.77	0.20	0.11	0.085
	X3^3	0.02	1	0.54	0.47	-0.063	0.085
	Residual	1.05	25		*R^2=0.95*		

The experimental results obtained from the FCCD related to oxygen consumption combined with analysis of variance (ANOVA) allowed to examine the statistical significance of the three factors. The results are shown in Table 5.3. For oxygen consumption, the best model was achieved fitting a based second-order function. As shown in Table 5.3, O_2 consumption mainly depends on the metals (X3) and gallic acid (X1) as its linear effect was significant ($p<0.001$) (indicated). In addition, the interaction of both factors (X1X3) also had highly significant effect ($p<0.001$) on oxygen consumption. The obtained regression coefficients indicate that metals have the highest effective main term (0.67) followed by gallic acid concentration (0.42) and the interaction (0.38), meaning that these two factors are essential for oxygen consumption, and therefore oxidation, to occur in wine model systems.

The visualization of the predicted model, obtained by surface response plot and contour plot, is illustrated in Figure 5.1. By keeping glucose (a non-significant factor) at the average level for O_2 consumption (11.2 mM) it shows the profile of the second-order response plots for the optimization of gallic acid and metals, and investigate further their interactions, on oxygen consumption. As illustrated in Figure 5.1, quadratic effects do not have statistically significant meaning.
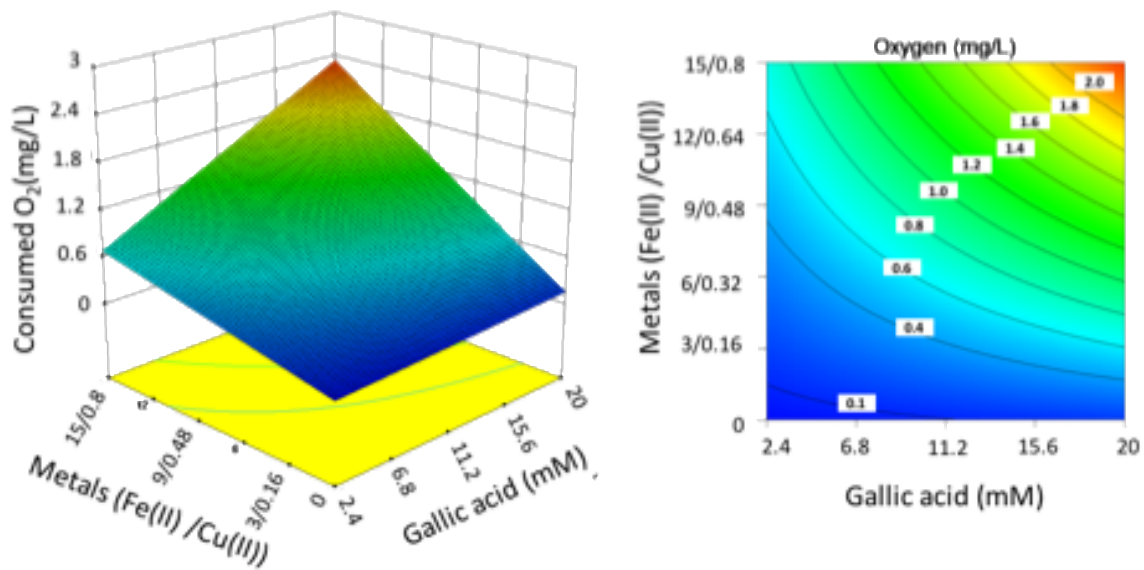
Figure 5.1 Response surface and contour plots for the effects of gallic acid (mM) and metals concentration (mg/L) on oxygen consumption in a wine-like model solution stored for 24 hours and 40ºC (glucose concentration = 11.2 mM).

Furthermore, a high correlation (but less than 0.9) was observed between O_2 consumption and phenylacetaldehyde formation, as illustrated in Figure 5.2A. In literature, it has been demonstrated that the oxidation of polyphenols depends on the iron redox cycling, and an increase in both polyphenol and metals will increase the consumption of oxygen (Danilewicz, 2007). Studies in wine-like model solutions also highlighted the key role of metals in the oxidation reactions and consequently in oxygen consumption (Danilewicz, 2013). Particularly the key role of free metals such as Cu^{2+} and Fe^{2+} in catalyzing Fenton-type reactions intermediate semi-quinone radical, which are further oxidized to o-quinones (Danilewicz, 2003).

These compounds can further react with several nucleophiles such as amino acids in the formation of Strecker aldehydes namely phenylacetaldehyde (Rizzi, 2006). These findings (Figure 5.2A) are in line with previous work which also demonstrated the high correlation between oxygen consumption and phenylacetaldehyde formation, in particular in wines and beer (Mayr *et al.*, 2015; Wietstock, Kunz and Methner, 2016). The involvement of oxygen in phenylacetaldehyde formation seems to be evident, however recent studies also showed that the initial O_2 uptake can only be due to an increase in the reaction with Fe^{2+} without any subsequent oxidation of the phenolic (Danilewicz, 2011).
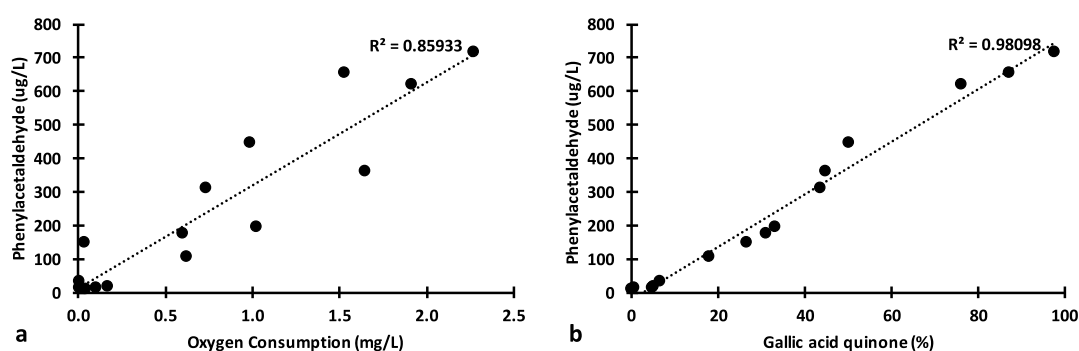
Figure 5.2 Relationship between oxygen consumption and phenylacetaldehyde formation (a) and phenylacetaldehyde and gallic acid quinone (b).

This could be a possible reason for the obtained correlation of 0.89 (less than 0.9) between O_2 consumption and phenylacetaldehyde formation. Oxygen may also be involved in other processes such as the Fenton reaction not implicated in phenylacetaldehyde formation, which may justify the not so high explained variance. Still a correlation of 0.98 was obtained between the GAQ and phenylacetaldehyde formation (Figure 5.2B), demonstrating that the formation of the aldehyde comes predominantly from the reaction of the *o*-quinone with phenylalanine in wine model systems, as will be discussed in the following section.

5.3.3. Gallic acid Quinone and Phenylacetaldehyde

A number of nucleophiles are present in wine systems, including amino acids, SO_2, glutathione, ascorbic acid amongst others. From these, the amino acids, in particular phenylalanine, show the lowest reactivity with the *o*-quinones (Nikolantonaki and

Waterhouse, 2012; Oliveira, Barros, *et al.*, 2016). Still it must be noted that quinone quantification reported in literature was not performed by measuring directly the molecule, but by using other techniques such as voltammetry and absorbance measurements at 400 nm (Makhotkina and Kilmartin, 2009; Nikolantonaki and Waterhouse, 2012).

Recently a few intermediate structures from the reaction of *o*-quinones with phenylalanine were identified by MS^n like for gallic and caffeic acids (Oliveira *et al.*, 2017). In this study gallic acid quinone was chemically confirmed by trapping as a phenazine derivatives (4-hydroxyphenazine-2-carboxylic acid). The molecule has been normalized according to the highest area observed on the sample set.

Phenylacetaldehyde concentration of FCCD samples range between 8.55 ± 1.36 (Sample 4) and 620 ± 14.1 μg/L (Sample 11) (Table 5.1), being the low amounts present when gallic and metals were at the minimum quantities. Besides, at low amounts of phenylacetaldehyde, no oxygen has been consumed nor quinone has been formed. An indication is given that in wine conditions metals are needed for SD, via quinone formation, to occur. When gallic is absent the aldehyde has been formed by the formation of dicarbonyls formed by the reaction of glucose and phenylalanine (Hofmann, Münch and Schieberle, 2000).

GAQ quinone formation was observed to a maximum of 6% even when EDTA where present (Sample 1 and 10). Although Recent studies demonstrated that quinones can be formed in the absence of added oxidants, being the only requisite the presence of two hydroxyl groups in ortho or para position, that is the case of gallic acid (Delgado, Zamora and Hidalgo, 2015). It must be emphasized that the reported observation was obtained when samples where submitted to temperatures close to 80ºC well above the normal storage condition and in the absence of SO_2. In the present study the temperature of 40°C was used. We recognize that these set up is not ideal to reproduce wine aging, nevertheless these conditions are common practice in wine studies, to allow shorter time frames still representative which allow us to generate replicates necessary to obtain statistically meaningful results (Martins, Monforte and Silva Ferreira, 2013).

Table 5.3 ANOVA for Response Surface Quadratic Model for Gallic acid *o*-quinone and phenylacetaldehyde formation.

	Source	Sum of squares	df	F Value	p-value	Coefficient Estimate	Error
Gallic acid Quinone	**Model**	**27281**	**9**	**94.6**	**<0.001**	**44.56**	**0.019**
	X1 - Gallic	**7168**	**1**	**223.79**	**<0.001**	**18.93**	**0.013**
	X2 - Glucose	**678**	**1**	**21.17**	**<0.001**	**-5.82**	**0.013**
	X3 - Metals	**12530**	**1**	**391.19**	**<0.001**	**25.03**	**0.013**
	X1X2	**6.34**	**1**	**0.2**	**<0.01**	**-0.63**	**0.014**
	X1X3	**3491**	**1**	**109**	**<0.001**	**14.77**	**0.014**
	X2X3	**192**	**1**	**5.98**	**<0.01**	**-3.46**	**0.014**
	X1^2	**628**	**1**	**19.6**	**<0.001**	**10.91**	**0.025**
	X2^2	55.40	1	1.73	0.202	-3.24	0.025
	X3^2	**2586**	**1**	**80.75**	**<0.001**	**-22.15**	**0.025**
	Residual	704.72	25		*R2=0.97*		
Phenylacetaldehyde	**Model**	**1.72×10^6**	**9**	**39.55**	**<0.001**	**334.87**	**23.33**
	X1 - Gallic	**5.20×10^5**	**1**	**107.21**	**<0.001**	**161.36**	**15.58**
	X2 - Glucose	**40072**	**1**	**8.25**	**<0.001**	**-44.76**	**15.58**
	X3 - Metals	**7.18×10^5**	**1**	**147.75**	**<0.001**	**189.42**	**15.58**
	X1X2	**3.59×10^2**	**1**	**0.074**	**<0.01**	**-0.47**	**17.42**
	X1X3	**2.68×10^5**	**1**	**55.18**	**<0.001**	**129.43**	**17.42**
	X2X3	**4469.51**	**1**	**0.92**	**<0.01**	**-16.81**	**17.42**
	X1^2	**25509.83**	**1**	**5.25**	**0.0319**	**69.56**	**30.35**
	X2^2	4.0×10^3	1	0.83	0.378	-27.61	30.35
	X3^2	**1.38×10^5**	**1**	**28.46**	**<0.001**	**-161.19**	**30.35**
	Residual	1.06×10^5	25		*R² = 0.94*		

Interestingly in samples where glucose was present at the maximum level and metals and gallic at minimum concentrations (sample 4), quinone has not been detected as well as for sample with factor 0 for gallic and glucose and minimum for metals (sample 7).

The high amount of aldehyde and *o*-quinone was found in sample 11, with maximum amount of gallic acid and metals and low amounts of glucose. An increase for the maximum concentration of glucose led to a decreased of PA to 620 ± 14 μg/L and reduced the *o*-quinone formation in 20%. These results confirm that glucose plays an important role in restraining phenylacetaldehyde formation by reducing the GAQ (Monforte, Martins and Silva Ferreira, 2018). Interestingly for the same samples, the presence of sugar decreases the consumption of oxygen from 2.72 ± 0.25 to 1.91 ± 0.08 mg/L, a 30% reduction. The capacity of Maillard reaction products to act as metal chelating agents is known (O'Brien and Morrissey, 1997). Their ability to behave like anionic polymers that are able to complex minerals, in particular with iron (Morales, Fernández-Fraguas and Jiménez-Pérez, 2005). This capacity of chelation can contribute to wine oxidation resistance. (Silva Ferreira *et al.*, 2002; Oliveira, Santos, *et al.*, 2016).

The ANOVA results for the sum of squares for the two quadratic models (phenylacetaldehyde and gallic acid quinone) after regression are shown in Table 5.3. The analysis indicates the most independent variables and some of the interactions are significant and contribute to aldehyde and GAQ formation. Based on the coefficients of the first order model terms (Table 5.3) phenylacetaldehyde and GAQ concentration increased with the increase of gallic and metals (X1 and X3) and decreased with glucose concentration (X2). Metals concentration is the most influential parameter with the highest coefficient followed by gallic and glucose concentration. Significant quadratic terms where obtained for gallic acid and metals. Interaction effect between (Gallic x Metals), where highly significant ($p<0.001$) and significant ($p<0.01$) for (Gallic x Glucose) and (Glucose x Metals).

The phenylacetaldehyde (Figure 5.3) has well as GAQ (data not shown) were fitted to several three-dimensional surfaces as a function of: i) metals and gallic acid; ii) metals and glucose and iii) gallic acid and glucose by using a combination of linear and quadratic terms, as well as interaction effects to construct polynomial equations for each response. Squared terms in the relevant equations for the fitted models represents the curvature of the surface.

Phenylacetaldehyde increased as a function of metals and gallic concentration and decreased with glucose concentration which is in the agreement with our previous work (Peinado, López de Lerma and Peinado, 2010; Monforte, Martins and Silva Ferreira, 2018).
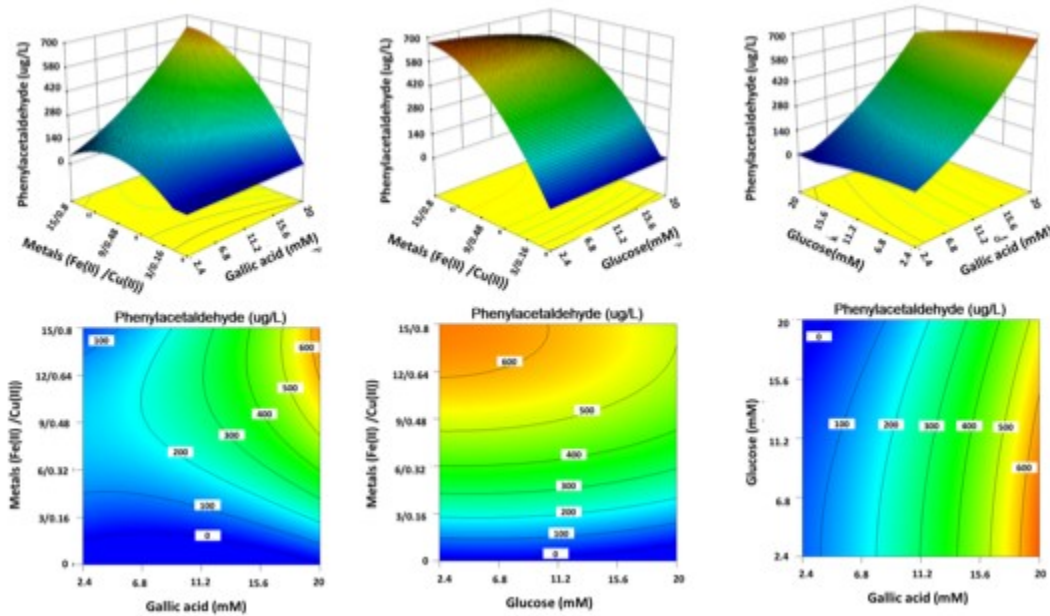
Figure 5.3 Response surface and contour plots fitted to experimental data points corresponding to phenylacetaldehyde formation as a function of metals, gallic acid and glucose concentration in a wine-like model solution stored for 24 hours and 40ºC.

5.4. CONCLUSION

In conclusion response surface methodology was effective in modelling the O_2 consumption, gallic acid quinone and phenylacetaldehyde formation based on the concentration of gallic acid, metals (Fe^{2+} and Cu^{2+}) and glucose in a wine model system. The regression equations developed can be used for prediction purposes. The best combination to minimize responses was achieved with lower amounts of gallic acid and metals and high amounts of glucose. It has been confirmed that glucose plays an important role in reducing the aldehyde formation in wine model systems, as well as it can reduce the formation of gallic acid quinone and the consumption of oxygen. Future studies are required to better understand the interaction between metals and glucose in the inhibition of Strecker aldehydes.

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CHAPTER 6 IMPACT OF PHENOLIC COMPOUNDS IN STRECKER ALDEHYDES FORMATION IN WINE MODEL SYSTEMS: TARGET AND UNTARGETED ANALYSIS

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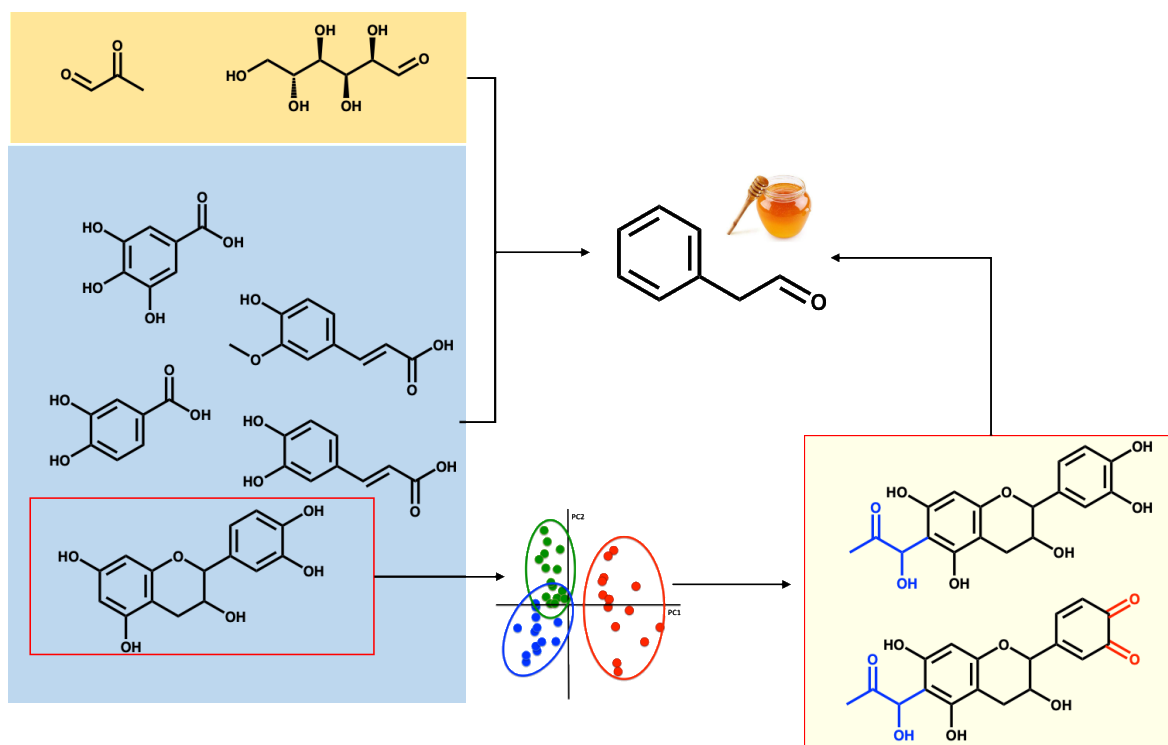
6.1. INTRODUCTION

Phenolic compounds are important contributors to food organoleptic properties. In literature, several studies have been reported showing the capacity of these compounds to produce Strecker Aldehydes (SA). (Saijō and Takeo, 1970; Mathew and Parpia, 1971) Phenolic compounds can be oxidized into *o*-quinones, that are capable to degrade amino acids leading to the formation of low olfactory perception SA.(Rizzi, 2006)

Rizzi (Rizzi, 2006) established that polyphenols (catechin, epicatechin, caffeic and chlorogenic acids) were able to degrade methionine and phenylalanine to form the respective aldehydes (methional and phenylacetaldehyde) *via* phenolic oxidation to *o*-quinone in the presence of metal ions (phosphate buffer, pH 7.17, stored at 22ºC). Delgado *et al.* (Delgado, Zamora and Hidalgo, 2015) contributed with more information related with structural characteristic of polyphenols to produce phenylacetaldehyde concluding that an hydroxy group in the -para or -ortho position promoted phenylacetaldehyde formation while the presence of two hydroxy groups at -meta position of the ring inhibited its formation.

Furthermore, other carbonyl compounds such as α-dicarbonyls can suffer Strecker degradation. In particular wine is rich in methyl glyoxal and diacetyl formed by microbial activity and wine ageing (Pripis-Nicolau *et al.*, 2000) and recently it was suggested that diacetyl could induce Strecker degradation of phenylalanine in wines. (Bueno *et al.*, 2018) Besides the reaction of carbonyl compounds with amino acids, other mechanisms can contribute for the formation of SA as indicated in Figure 6.1.
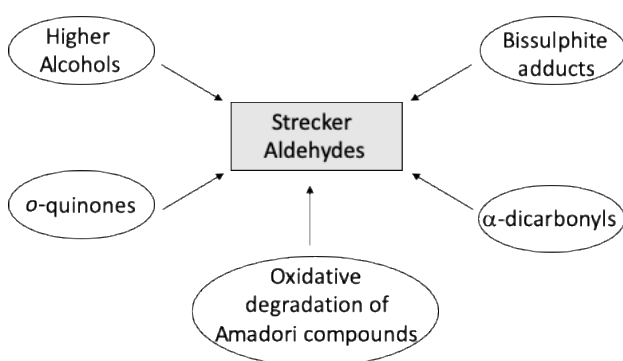
Figure 6.1 Possible routes for Strecker aldehydes formation in wine environment.

In particular, the hydrolysis of bisulphite adducts with SA (Bueno, Carrascón and Ferreira, 2016), higher alcohols oxidation (Escudero *et al.*, 2000) and the oxidative degradation of Amadori compounds. (Hofmann and Schieberle, 2000) All these mechanism are highly dependent of the presence of oxygen and metal ions. (Danilewicz, Seccombe and Whelan, 2008; Ugliano, 2013). Nevertheless, only a few studies were concerned about the contribution of several mechanisms for SA formation, and how they will active contribute to their formation.

The reactivity of phenolics to trap α-dicarbonyls has been studied in food model conditions. According to Zamora *et al.* (Zamora and Hidalgo, 2018) the reaction occurs between electron-rich polyphenols, such as the flavanol A-ring, and electrophilic carbonyl compounds.

Peterson and co-workers, (Totlani and Peterson, 2005, 2007; Noda and Peterson, 2007) demonstrated that flavan-3-ols (epicatechin, epigallocatechin and epigallocatechin gallate) were able to reduce the formation of glyoxal, methyl glyoxal, diacetyl and 3-deoxyosone in glucose/glycine model solutions with a direct consequence in the formation of aroma precursors. Nevertheless, all these studies were performed at high temperatures (> 100ºC).

Recently it has been demonstrated that in wine model solutions with phenylalanine, gallic acid and metal ions (iron and copper) the presence of glucose reduces the formation of PA due to a reduction in the formation of gallic acid *o*-quinone. (Monforte, Martins and Silva Ferreira, 2018)

All these studies demonstrate the complexity of the interaction between substrates for Strecker degradation. Clearly, more information related with this subject is crucial to better understand the chemical mechanism underlying the formation of flavour compounds and stability during food storage.

In that regard the aim of the present work was to study, in wine model conditions, the impact of 6 phenolic compounds that are associated with wine oxidation (3,4-dihydroxybenzoic acid, gallic acid, caffeic acid, ferulic acid, catechin and epicatechin) in phenylacetaldehyde formation with added metal ions (iron and copper). Also, to better understand the impact of the addition of glucose and methyl glyoxal in the aldehyde formation, as well as to investigate the kinetics of reaction intermediates between catechin and methyl glyoxal including the adducts and their respective *o*-quinones.

6.2. MATERIAL AND METHODS

6.2.1. Reagents and Standards

(+) catechin hydrate ($\geq$ 99%), (-) epicatechin hydrate ($\geq$ 98%), 3,4-dihydroxybenzoic acid ($\geq$ 97%), gallic acid monohydrate ($\geq$ 99%), ferulic acid ($\geq$ 99%) and coumaric acid ($\geq$ 98%) all obtained from Sigma-Aldrich (St. Louis, MO) as well as phenylalanine ($\geq$ 99%), methyl glyoxal (40%) and phenylacetaldehyde ($\geq$ 99%). d-Glucose ($\geq$ 99%) were purchased from Alfa Aesar (Massachusetts, US). Tartaric acid, sodium hydroxide, Cu (II) sulfate.$5H_2O$, Fe (II) sulfate.$7H_2O$ and formic acid were obtained from Merck (Darmstadt, Germany). Ethanol, methanol, acetonitrile and isopropanol (all LC-MS grade) were acquired from Fischer Scientific (UK).

6.2.2. Model Reactions – samples preparation

Six phenolic compounds were selected: catechin, epicatechin, caffeic acid, ferulic acid, gallic, acid and 3,4-dihydroxybenzoic acid (3,4-DHB) due to their presence and reactivity in wine.

The experimental conditions were selected based on a previous article. (Monforte, Martins and Silva Ferreira, 2018) Briefly, equimolar solutions (2.4 mM) of phenylalanine with i) addition of selected phenolic (control), ii) addition of glucose and iii) addition of methyl glyoxal were prepared in a wine model solution (12% ethanol with 4 g/L of tartaric acid, pH = 3.6 adjusted with NaOH (4M)).

The metals Fe^{2+} and Cu^{2+} were added to all model systems at concentrations of 0.1 mM and 6.3 μM in the form of $FeSO_4.7H_2O$ and $CuSO_4.5H_2O$ respectively. The solutions were prepared in 20 mL glass flasks, closed with an internal PTFE septum and an external screw cap, 20% of the vial volume were left with air. Samples were stored, in the dark, for 24 hours at 80ºC. The chosen temperature is not the normal temperature of wine storage. The reasoning for its selection was mainly to consider previous published work(Monforte, Martins and Silva Ferreira, 2018), and to minimise the reaction time while maximising the knowledge built. Also, SA formation as function of phenolic compounds is seen of relevance

to other food products that may undergo the selected temperature. The initial concentration of dissolved oxygen in all samples was 8.7 ± 0.4 mg/L.

6.2.3. Kinetic study of Catechin reactions

In parallel, three kinetic studies were also conducted. Equimolar solutions (2.4 mM) were prepared in wine model solutions in duplicate: i) phenylalanine + catechin + metals, ii) phenylalanine + catechin + glucose + metals, iii) phenylalanine + catechin + methyl glyoxal + metals. The metals Fe^{2+} and Cu^{2+} were added to all model systems at concentrations of 0.1 mM and 6.3 μM in the form of $FeSO_4.7H_2O$ and $CuSO_4.5H_2O$ respectively. The solutions were prepared in 20 mL vials, closed with an internal PTFE septum and an external screw cap, 20% of the vial volume were left with air. Samples were heated at 80°C for 2, 4, 6, 8 and 24 hours. At each time sample was taken and cooled in ice water and then stored at -20°C.

6.2.4. Dissolved oxygen measurements

In order to assure approximately the same amount of dissolved oxygen in all the flasks, measurements were performed using a Fibox 3 LCD optic oxygen transmitter with an optic fiber and planar oxygen-sensitive spots placed in the middle of each flask (5 mm sensor spot PSt3).

6.2.5. Phenylacetaldehyde Quantification by GC-MS

Phenylacetaldehyde was determined by headspace-solid phase micro extraction using a SPME fiber (DVB/CAR/PDMS), briefly 5 mL of model solution were spiked with 50 μL of 3-octanol (50 mg/L). Samples were pre-incubated in the CombiPAL oven at 40°C and 500 rpm for 2 min, the fiber was exposed after during 10 minutes at 250 rpm for extraction. Desorption of the volatiles in the injector were performed in the injector at 220°C for 15 minutes. The data acquisition conditions used were the same as previously reported (Monforte, Martins and Silva Ferreira, 2018).

Phenylacetaldehyde was calibrated by using a calibration curve between 0.01 and 1 mM (five points) prepared in a 12% ethanol with 4 g/L of tartaric acid, pH = 3.6 adjusted with NaOH (4M). The selected ion for phenylacetaldehyde was *m/z* 91. The standard curve was built by plotting the normalized are between the compounds and the internal standard against the concentration. The recovery is 99% and the RSD was 2.5%.

6.2.6. High Resolution LC-MS

The LC-ESI-UHR-QqTOF-MS (Impact II, Bruker) analysis was performed on an UltiMate 3000 Dionex UHPLC (Thermo Scientific), coupled to an ultrahigh- resolution Qq-time-of-flight (UHR-QqTOF) mass spectrometer. Data were acquired in negative mode. Separation of metabolites was performed using an Acclaim RSLC 120 C18 column (100 mm × 2.1 mm, 2.2 μm) (Dionex). Mobile phases were 0.1% aqueous formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The gradient started with 5% and increased to 95% in 7 min, which was kept constant for 2 min and then returned to 5% B in 1 min and maintained at 5% B for an additional 5 min at a flow rate of 0.25 mL/min. The injection volume was 5 μL. Parameters for MS analysis were set using positive ionization mode with spectra acquired over a range from m/z 20 to 1000. The selected parameters were as follows: capillary voltage, 4.5 kV; drying gas temperature, 200 °C; drying gas flow, 8.0 L/min; nebulizing gas pressure, 2 bar; collision RF, 300 Vpp; transfer time, 120 μs; and prepulse storage, 4 μs. Post-acquisition internal mass calibration used sodium formate clusters with the sodium formate delivered by a syringe pump at the start of each chromatographic analysis.

6.2.7. Data Analysis

UPLC-MS data were analysed by applying MZmine 2.3.3 (Pluskal *et al.*, 2010), for mass detection, chromatogram deconvolution and alignment. A table were generated by using aligned retention times, mass data *m/z* and peak area, further it was handled by MATLAB (version 7.0.4 R14, MathWorks Inc.) for multivariate data analysis using PLStoolbox (Eigenvector Technologies, Manson, Washington, USA). The qualitative metabolite profiling was performed using Principal Component Analysis (PCA).

6.3. RESULTS AND DISCUSSION

6.3.1. Impact of phenolic compounds in phenylacetaldehyde formation

The ability of 6 phenolic compounds (gallic acid, caffeic acid, ferulic acid, 3,4-dihydroxybenzoic acid, catechin and epicatechin) to convert phenylalanine into phenylacetaldehyde was investigated in the presence of glucose or methyl glyoxal in wine model solutions stored at 80ºC for 24 hours. Glucose was selected due to the fact that its presence together with the amino acid promotes the Maillard reaction (Hofmann, Münch and Schieberle, 2000), at the studied conditions (pH and temperature). Also, as recently reported, glucose presence seems to inhibit PA formation , in particular when in combination with gallic acid (Monforte, Martins and Silva Ferreira, 2018). Methyl glyoxal was selected as representative of α-dicarbonyls, due to its effectiveness in generating phenylacetaldehyde in model solutions, in particular at lowers pHs (Hofmann, Münch and Schieberle, 2000).

The obtained results (Table 6.1) showed that Strecker degradation is affected, as expected, by the type of substrates present in the model solution. The higher concentrations of phenylacetaldehyde (0.48 – 1.05 mM) were obtained in the solutions where methyl glyoxal was added, and lower concentrations when glucose was present (0.06 - 0.25 mM).

In the reference solution (phenylalanine + metals) it was observed the formation of 0.07 mM of PA, a possible explanation for this fact is the presence of oxygen and transition metal ions results in the formation of hydroxyl radicals by the Fenton reaction. These radicals are highly reactive capable and capable to oxidize tartaric acid in diketosuccinic acid, a possible substrate for SD. (Fenton, 1905) Also, the oxidation of phenylalanine in phenylacetaldehyde was already described in beer model solutions and correlated with the formation of hydroxyl and ethoxy radicals (Wietstock and Methner, 2013).

In control samples, PA formation was statistical different from the no phenol added samples for gallic acid, 3,4-dihydroxybenzoic acid and caffeic acid. These molecules are characterized by having a benzene ring, with an acid group and ortho-hydroxyl groups capable of generation 1,2-quinones. Other studies demonstrated that two hydroxyl groups are needed for phenylacetaldehyde formation, which explains the fact that ferulic acid was not different from the control (Delgado, Zamora and Hidalgo, 2015). Moreover, the presence of a conjugated carbon-carbon double bond (caffeic acid) increased PA by 70% with respect to absence of phenol.

Table 6.1 Formation of phenylacetaldehyde in tested models of i) phenylalnine with phenol (Control), ii) phenylalanine with phenol and glucose (Glucose Add.) and iii) phenylalanine with phenolic and MG (MG Add.)

Phenolic	Control	Glucose Add.	MG Add.
No phenolic added	0.07 ± 0.004 *a*	0.12 ± 0.021 *a,b*	0.63 ± 0.014 *b*
Catechin	0.10 ± 0.006 *a*	0.06 ± 0.007 *a*	0.62 ± 0.023 *a*
Epicatechin	0.13 ± 0.003 *a*	0.06 ± 0.006 *a*	0.58 ± 0.022 *a*
3,4-DHB	0.31 ± 0.032 *b,c*	0.19 ± 0.029 *c,d*	0.71 ± 0.016 *b,c*
Gallic acid	0.33 ± 0.012 *c*	0.25 ± 0.004 *d*	0.64 ± 0.058 *b*
Caffeic acid	0.26 ± 0.029 *b*	0.15 ± 0.011 *b,c*	0.77 ± 0.008 *c*
Ferulic acid	0.11 ± 0.009 *a*	0.06 ± 0.009 *a*	1.05 ± 0.018 *d*

[a] *Phenylacetaldehyde expressed in. Values with the same letter in the same column are not significantly different ($p < 0.05$).*

Complex phenolic compounds with several aromatic rings such as catechin and epicatechin, only increased PA formation in 25% and 42% respectively. Similar results were obtained in a recent study that demonstrated that phenolics with two hydroxyl groups in *ortho* or *para* position enhances PA formation while the presence of hydroxyl groups in position *meta* of other aromatic rings can inhibit PA formation (Delgado, Zamora and Hidalgo, 2015).

Comparing reference (Control) samples with samples where glucose was added, the formation of PA decreased for all phenolic compounds. Still in the sugar control sample (without phenolic addition) PA increased 40% in comparison with the control sample (no phenolic and no glucose). This observation suggests that the Maillard reaction is occurring. Consequently, the formation of phenylacetaldehyde occurs through the generation of α-dicarbonyls generated by the degradation of the Amadori rearrangement through the enolization pathway or by the direct oxidation of Amadori compound due to the presence of metal ions. (Hofmann and Schieberle, 2000)

Nevertheless, if a phenolic compound was present together with glucose, a decrease of PA was observed in comparison with the respective reference samples without glucose. This decrease is higher for flavan-3-ols, epicatechin and catechin (102% and 65%) and for cinnamic acids (ferulic and caffeic acids). Lower inhibition (56% and 34%) were observed for hydroxybenzoic acids (gallic acid and 3,4-DHB acid).

A possible explanation for PA decrease when glucose is present, can be the fact that metals have an enhancing effect on the oxidation steps of Maillard reaction, and in particular the direct oxidation of glucose or the Amadori product can result in the formation of D-glucosone. At neutral pH, it has been observed that phenolic compounds can have a pro-oxidative effect and increase D-glucosone formation. (Haase *et al.*, 2017) However, our results shown no statistically significant differences between the sugar control (no polyphenol added) and samples where phenols were added together with glucose (data not shown).

The higher concentrations of PA were observed for samples where methyl glyoxal was present. Remarkably the contribution of methyl glyoxal for the formation of the SA is higher compared with the reaction of o-quinones reaction with phenylalanine. Recently it was suggested that dicarbonyls, and in particular, diacetyl is an important precursor for the formation of Strecker aldehydes. (Bueno *et al.*, 2018)

Different trends in PA formation were observed dependent on the type of phenolic selected. Increase in PA formation was observed for 3,4-DHB, caffeic and ferulic acids (11%, 17% and 40% respectively). While a decrease of 30% and 21% in PA was observed for catechin and epicatechin. These results suggest that the trapping capacity of phenolics is dependent on their structure. In fact it was reported that the polyhydroxy benzene ring configuration typical of flavan-3-ols, is considered a key parameter that has influence on the reactivity of phenolic compounds towards the suppression of Maillard intermediaries such as α-dicarbonyls. (Totlani and Peterson, 2006)

Our results may indicate that this ability of flavan-3-ols to trap methyl glyoxal, has a direct consequence in reducing PA formation.

To elucidate the impact of the studied substrates in the formation of phenylacetaldehyde, a kinetic study has been performed using catechins, a flavan-3-ol as the phenolic source.

Catechin was selected due to its reactivity in wine as well as the fact that its presence together with MG reduced the formation of PA.

6.3.2. Catechin Kinetic studies

As observed in the previous section, in the presence of catechin both in together with glucose or methyl glyoxal PA concentration clearly decreased and catechin has been considered an important compound in wine oxidation. (Oliveira *et al.*, 2016)

To better understand the formation of reaction intermediates three model systems were studied: (1) phenylalanine, catechin and metals, (2) the same as in system 1 but with the addition of glucose and (3) the same as in system 1 but with the addition of methyl glyoxal. Samples were stored at 80ºC and sampling was performed at 2, 4, 6, 8 and 24 hours.

To evaluate similarities and differences between models, untarget analysis was performed. Given that, all MS features detected do not correspond to relevant information related with intrinsic variability of sample, one-way ANOVA with *p-value < 0.05* were applied using MATLAB to detect significant differences between groups. From the 4654 MS features aligned and filtered through data processing, 1311 were considered statistically significant (*p-value < 0.05*). Figure 6.2A shows Venn Diagrams of the candidate features (*p < 0.05*) retained by the three model solutions. From the 1311 m/z's, 472 were in common in the three sets of model solutions. MG model solutions were the most complex, with 579 *m/z's* exclusively markers, while reference and glucose addition sets were more similar sharing 241 *m/z's* markers. Nevertheless, glucose addition results in 13 exclusive *m/z's*.

Multivariate PCA statistics of normalized peak areas from all samples, gives a measure of the degree of separation between the three model solutions (Figure 6.2B). The MS features comprising the first component are responsible for the separation from the set where MG were added of the other two model solutions, while PC2 is related with glucose addition separation from control samples set.
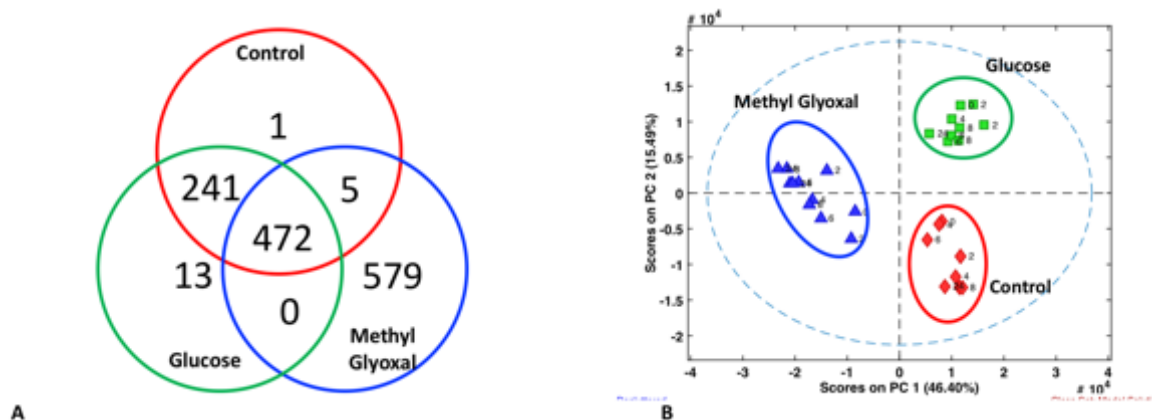
Figure 6.2 (A) Venn Diagram illustrating the number of common and model specific m/z's extracted from MZmine data of the three model solutions stored during 24 hours at 80C. (B) PCA score plot (PC1 vs. PC2) from the UPLC-MS data table obtained in MZmine.

From the loadings inspection (data not shown) different catechin reaction intermediates were identified and selected as target molecules for contextualization in particular catechin, catechin *o*-quinone (CatQ), catechin-MG adducts (CatMG) and the respective *o*-quinones (CatMGQ) (Figure 6.3).

In all model solutions two common peaks were observed in the chromatogram. These corresponded to catechin (molecular ion peak at *m/z* 289.0717) and the respective *o*-quinone (molecular ion peak at *m/z* 287.0717).

When MG was added, two other peaks appeared, which corresponded to the mono-MG adduct of catechin (molecular ion peak at *m/z* 361.0929) and the respective *o*-quinone (molecular ion peak at m/z 359.0920). The findings were confirmed by the presence of a fragment corresponding to the loss of the MG unit (fragment *m/z* 289.07176). These results are in line with literature where MG trapping by phenolics can occur at the two unsubstituted carbons in the A ring (Totlani and Peterson, 2005).
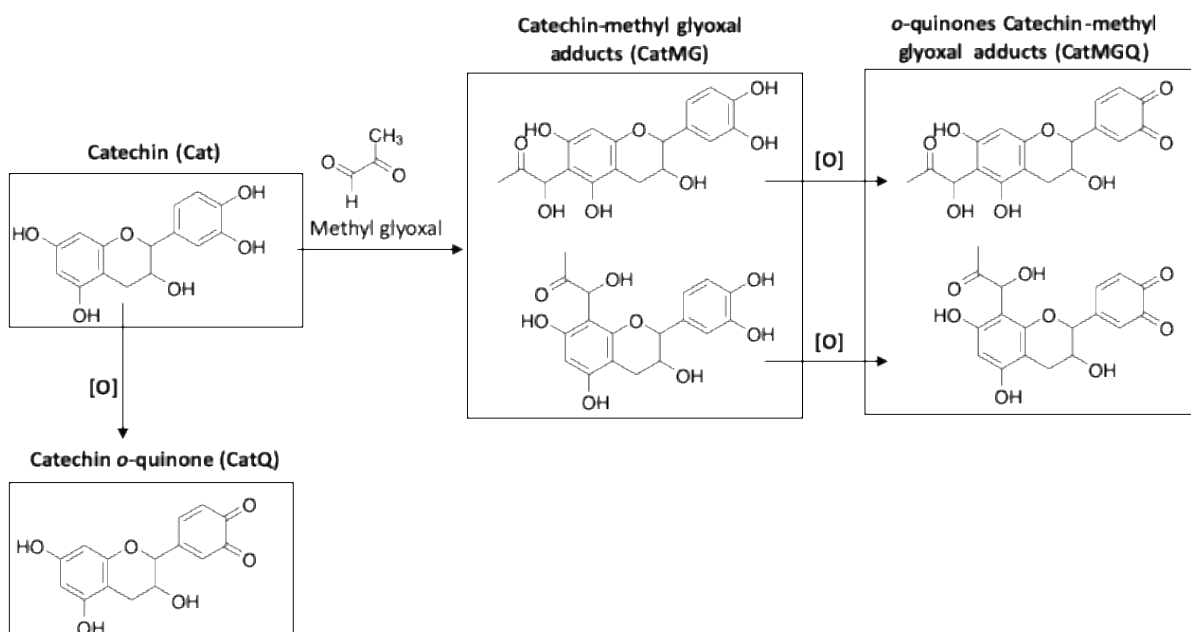
Figure 6.3 Structures of catechin and its methyl glyoxal adducts and their respective *o*-quinones.

Figure 6.4 showed that catechin (A) is consumed during time in all model solutions while CatQ is formed (Figure 6.4B). Demonstrating that catechin is being oxidized in their respective *o*-quinone. The higher catechin consumption was observed in samples where MG was present (Figure 6.4A - blue bars). Interestingly the higher consumption is not related with the formation of the respective *o*-quinone, whose concentration is superior in the reference samples (Figure 6.4B – red bars). The addition of MG reduced the formation of CatQ by 80%. The addition of glucose also led to a higher catechin consumption and it seems to be related with the formation of the respective *o*-quinone (Figure 6.4B – green bars). A similar observation has been recently reported when glucose was added to a model solution containing gallic acid and phenylalanine (Monforte, Martins and Silva Ferreira, 2018).

Flavan-3-ols quinones are formed on the B-ring of the molecules during oxidation, this structure has the capacity to react with the amino acid by a Michael addition, resulting in the formation of aldehydes in foods. Recently it was however demonstrated that catechin is capable to form non-volatile adducts with amino acid capable to modify the antioxidant properties of the phenolic, through the reestablishment of the oxidation state of B ring (Guerra and Yaylayan, 2014). On the other side the interaction of ring A of catechin with α-

dicarbonyls is well established (Totlani and Peterson, 2006). Nevertheless, the search for possible *o*-quinones of the later compounds is still unknown.

In Figure 6.5, it is represented the behaviour of the adduct between catechin and MG (CatMG) and the respective *o*-quinone (CatMGQ).

Interestingly, both compounds increased until 6 hours of reaction and after decreased. Similar behaviour was observed for α-dicarbonyls (3-deoxyosone and glyoxal) in thermally treated glucose/phenylalanine (Hofmann and Schieberle, 2000). These results suggest that phenylacetaldehyde formation could happen due to the oxidation of the CatMG in the respective *o*-quinone (CatMGQ).
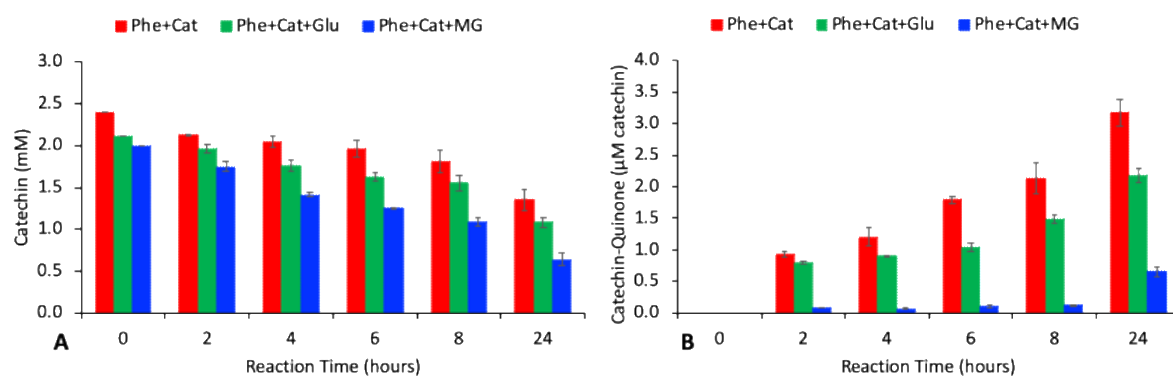
Figure 6.4 Time course of the consumption of catechin (A) and the formation of CatQ (B) in the three model solutions. (Phe: phenylalanine, Cat: catechin, Glu: glucose, MG: methyl glyoxal.) (mean ± standard deviation)
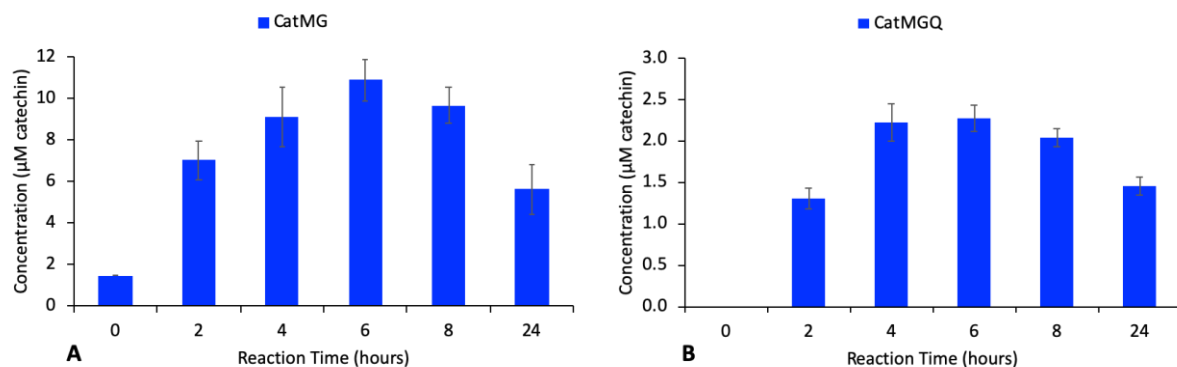
Figure 6.5 Time course of the formation of CatMG (A) and CatMGQ (B) in model solutions with phenylalanine, catechin, metals and methyl glyoxal (mean standard ± deviation of 2 replicates).

6.4 CONCLUSION

In conclusion it has been shown that phenolic compounds are involved in the Strecker degradation through the reaction of the *o*-quinones with the amino acid or due to their capacity to trap Maillard intermediates such as MG. Moreover, their impact is dependent on their structure, as well as on the substrates present for the reaction. The formation of phenolic-MG adducts can result in an increase of the oxidation status of the product due to the formation of adducts *o*-quinones.

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CHAPTER 7 DISCRIMINATION OF WINE "OXIDATION-STATUS" BASED ON UNTARGET PEAK PICKING APPROACH WITH MULTI-CLASS TARGET COUPLED WITH MACHINE LEARNING ALGORITHMS

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7.1. INTRODUCTION

In wine, shelf life is closely related with changes in the sensory products of the product. In particular sensorial characteristics can be compromised by the browning phenomenon that is due to complex chemical reactions resulting for example in white wines into the detrimental change in colour aroma and taste of the product.

The sensorial impact of "premature ageing" in white wines leads to the loss of freshness and fruitiness and to the development of off-flavours. The impact of the negative aromas contributes to an increase in the notes of: "honey-like", "farm-feed", "hay", "boiled-potato", "cooked vegetables" and "woody-like". Some of these sensorial notes were correlated with specific molecules such as phenylacetaldehyde and methional for the "honey-like" and the "boiled-potato" respectively (Escudero *et al.*, 2000; Silva Ferreira *et al.*, 2002). Nevertheless, other molecules were considered markers of white wine deterioration, besides Strecker aldehydes such as: aliphatic aldehydes such as trans-2-nonenal (Ferreira *et al.*, 1997), sotolon (Lavigne *et al.*, 2008), TDN (Silva Ferreira *et al.*, 2003) , *cis-* and *trans-* dioxanes and dioxolanes (Silva Ferreira, Barbe and Bertrand, 2002a; Peterson, Gambuti and Waterhouse, 2015) and the formation/deterioration of sulfur-containing compounds (Ugliano *et al.*, 2012).

The major drawbacks of the acquisition of a "oxidative fingerprint" suitable to be used as an indicator of oxidative-status of wine samples are: i) the oxidation markers quantification requires different sample preparation/acquisition methods, ii) the method/package software to perform data processing implicates changes in the data structure, iii) the absence of a classifier suitable to be used as supervision for samples classification.

Several studies were performed in order to attempt to establish a chemical "oxidative fingerprint" by using several methodologies such as: voltammetry (Martins *et al.*, 2008), GC-FID (Jacobson, Monforte and Ferreira, 2013), GC-MS (Mayr *et al.*, 2015; Monforte, Jacobson and Silva Ferreira, 2015; Pinto *et al.*, 2018) and UPLC-Q-TOF-MS (Arapitsas *et al.*, 2012; Roullier-Gall *et al.*, 2016).

The favoured detector to monitor volatile chemical changes is GC-MS. Normally GC-MS data processing requires alignment, peak picking, deconvolution and integration. In order to pre-process the data for metabolomic studies several platforms are available (Niu *et al.*, 2014), XCMS and MZmine2 have become the most widely used free software's to process

untargeted metabolomics (D. Myers *et al.*, 2017). Nevertheless, the platforms require the establishment of several parameters in order to extract with confidence the data without the risk of create false peaks. Moreover, the obtained data after pre-processing is a peak list consisting in retention times concatenated with an *m/z*, meaning the mass spectra structure is lost. Other packages such as PARADISe, based in PARAFAC have the advantage of correctly determine the mass spectrum of the individual compounds with the disadvantage of being time consuming and requires the establishment of intervals (Johnsen *et al.*, 2017).

Taking the last-mentioned point normally to study wine oxidation several classifiers were tested in order to extract more information regarding wine oxidation. Browning (A420) has been used to establish 3 classes (control, least oxidized and most oxidized) and HS-SPME-GC-MS and NMR data was analysed in order to extract molecules related with the established classes by OPLS-DA (Pinto *et al.*, 2018).

The aim of this study is to compare samples classification using target molecules/parameters with a GC-MS fingerprint in order to establish a pipeline for the classification of white wine oxidation. Wine storage time and target oxidation markers (phenylacetaldehyde, methional, absorbance at 420 nm, acetaldehyde, furfural, TDN and *cis*-1,3-dioxane) were selected as supervisors to find relationships between the oxidative status of the samples, their chemical parameters and the discriminant compounds extracted using PLS-DA and Random Forest. Moreover, a pipeline has been developed and put in a friendly graphical user interface in order to facilitate the extraction and validation of marker compounds of oxidation process based on multi-class establishment.

7.2. MATERIAL AND METHODS

7.2.1. Reagents and Standards

All chemicals used in this study were of the highest purity available grade, and purchased from Sigma-Aldrich (St. Louis, MO).

7.2.2. White Wine Samples

Twenty-four dry white wines were collected from seven producers from 4 regions of Portugal (Lisbon, Douro, Dão and Alentejo). Amongst them, 4 samples from C, 2 from DB, 4 from DQ, 2 from MS, 1 from TA, five from T and 6 from DV. The storage time includes wines with 2, 3, 4, 5, 6, 7, 8, 9,10, 14, 17, 23, 24 and 37 years old. Before bottling the wines had no contact with wood. Wines were stored in cellar conditions with natural corks.

Each sample was codified referring to its brand together with the storage time (wine DV stored for two years was codified as DV_2y).

7.2.3. Wine Characterization

Immediately after opening the bottles, dissolved oxygen was measured using a WTW 340 oxygen probe. Analysis carried out after included pH, absorbances at 280nm (total phenolics) and 420nm (browning index) and acetaldehyde. Acetaldehyde was determined according with the method described by Silva Ferreira et al (Silva Ferreira, Barbe and Bertrand, 2003).

7.2.4. GC-MS Analysis

Volatile compounds were extracted by a liquid-liquid procedure according with the method described in (Silva Ferreira, Avila and Guedes de Pinho, 2005). Briefly a 5 g of anhydrous sodium sulphate and 50 μL of internal standard (3-octanol) (500 mg/L) was added to a total of 50 mL of wine. Samples were extracted twice with dichloromethane (5 mL) using a magnetic stir bar for 5 minutes per extraction and 2 mL of the organic phase were concentrated under a nitrogen stream 4 times. The extract was injected (2 μL) into the GC-MS (Varian 450 gas chromatograph, coupled to a Varian 240-MS mass spectrometer). Chromatographic conditions were the following, column BP-21 (50m x 0.25mm x 0.25μm) fused silica; oven temperature 40°C for 1 minute at a rate of 2°C/min to 220°C.Retention indices for each peak was calculated by the injection of a series of alkanes (C3-C30).

7.2.5. Data Treatment and Analysis

All raw chromatograms were exported as netCDF-files. Data files were imported, and all the pre-processing steps were performed in MATLAB version 8.4 (R2014b) (The MathWorks Inc., Natick, MA, USA) using built-in functions.

The steps for analysis include:

i)   Data importation;

ii)   Definition of regions in m/z and time domains;

iii)   Alignment;

iv)   Peak apex's extraction.

Critical steps include data alignment and definition of thresholds for extraction of peak apexes, to avoid lose relevant information or consideration of noise in the variables extracted.

The graphical user interface shown in Figure 7.1, combines specific functions and graphics to facilitate interpretation and visualization of data. According with Figure 7.1, each section is described above.
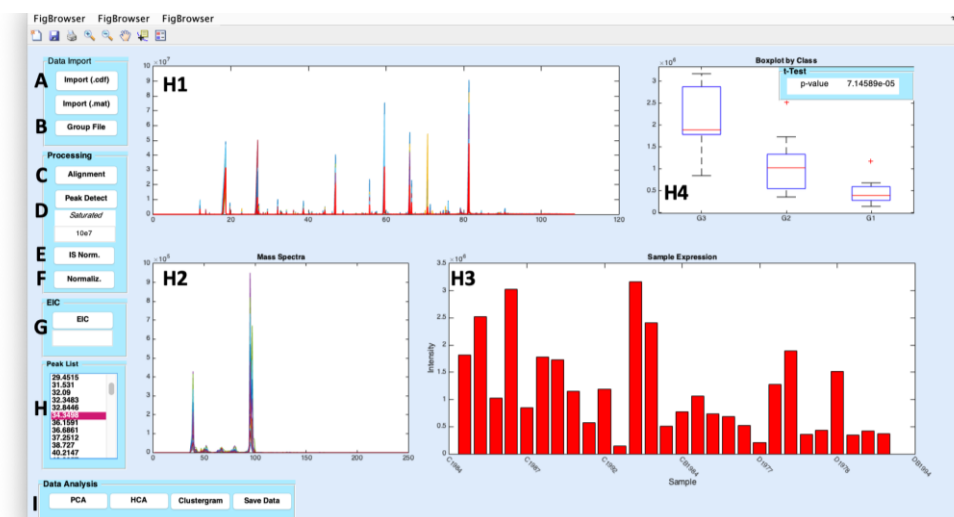
Figure 7.1 Graphical User Interface for GC-MS data analysis.

A: Data Importation of netCDF-files (built in function). Allows the user to select *m/z* range for analysis. TIC is projected in graphic H1.

B: Import an Excel file (.xlsx) with pre-defined classes (optional).

C: Before modeling, chromatographic data could be aligned using the iCoshift algorithm (Figure 7.2) (Tomasi, Savorani and Engelsen, 2011). In the dataset three steps for alignment were performed, i) 7 intervals, ii) 74 intervals and iii) 36 intervals.
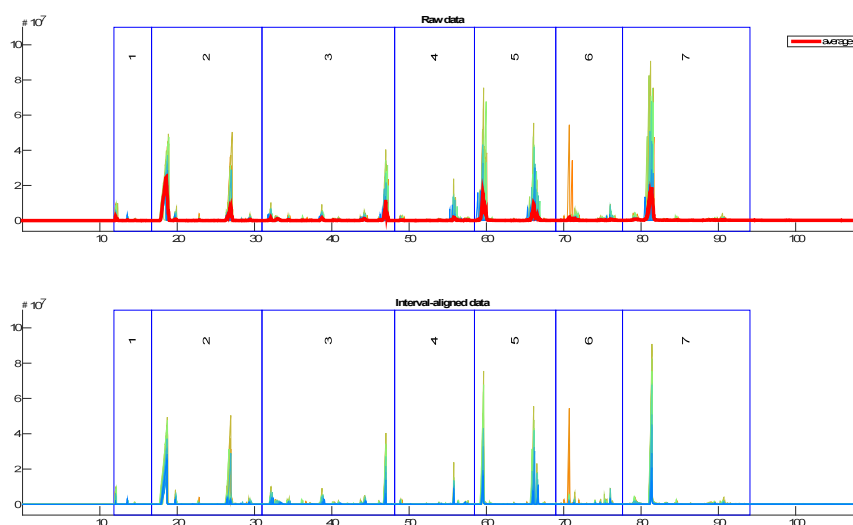
Figure 7.2 Example of iCoshift for the first 7 intervals alignment (step C).

D. Peak apexes recognition and integration are performed based on two thresholds, one for baseline and other a cut-off to exclude peaks out of the linear zone of response of GC-MS. The consideration of a peak is a variable that is either larger than its two neighbouring variables. For the analysed dataset threshold was set as 10000 counts. In Figure 7.3 is shown the reliability of the algorithm for peaks detection.
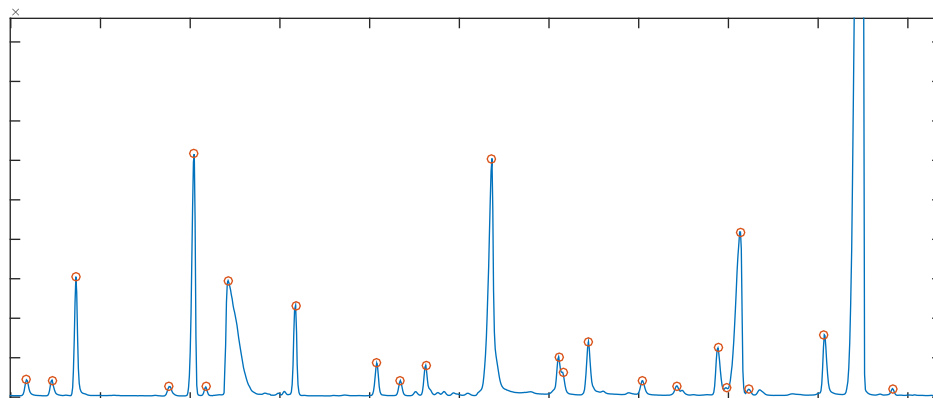
Figure 7.3 Peaks extraction (step D) for a specific interval (28 to 48 minutes).

E. Internal Standard normalization allows to the user to select a determined retention time and normalize all the extracted peaks by the area of the selected compound.

F. Normalize the extracted variables by unit-variance.

G. Projection of all samples TIC for a single *m/z*.

H. List of retention times of the extracted apexes. Selection of RT is linked to graphic H2. H3 and H4.

H2. Mass spectrums for the selected retention time for all samples.

H3. Expression of the are in all samples.

H4. Boxplot of selected variable based on the classes defined in **B**, as well as the *p-value* for the respective variable.

I. Statistical analyses in the data matrix (samples x peaks) for fast inspection of the data classification.

Multivariate Data Analysis

Principal component analysis (PCA) and hierarchical cluster analysis (HCA) was first performed as unsupervised clustering to identify the similarity or the differences between

sample profiles. Grouping, trends and outliers were revealed from the scatter plot and the dendrogram, respectively.

Partial least squares regression (PLS-R) was carried out to model as a function of the wine age, two models were constructed from target and untarget datasets.

To identify subsets of chemical compounds associated with specific sample classes according with Table 7.1 (LOW or HIGH) two supervised classification methods were used: partial least squares- discriminant analysis (PLS-DA) and random forests (RF).

PLS-DA is based on PLS-R of a set of categorical variables (classes). RF is a machine learning technique used for the classification and estimation of variable importance based on multiple decision trees.

Feature selection was performed based on variable importance for projection (VIP) coefficient from PLS-DA and mean decrease accuracy provided by RF. For feature validation univariate analysis tests were performed in SPSS 17.0 for Windows, in particular Wilcoxon Mann-Whitney Test, with *p-value* < 0.01 set as the level of statistical significance.

The areas under the receiver operating characteristic curves (ROC) were employed to assess the significance of the biomarker.

All the metabolomic analysis included PCA, PLS-DA, RF, hierarchical clustering and ROC were performed in MetaboAnalyst (Chong *et al.*, 2018) and in MATLAB version 8.4 (R2014b) (The MathWorks Inc., Natick, MA, USA).

Table 7.1 Table with the 8 defined classes for supervised analysis (PLS-DA and RF).

	Age	PA	A420	Acetaldehyde	2-furfural	Methional	cis-1,3-dioxane	TDN
DV_2y	*YOUNG*	H	L	L	L	L	L	L
DV_3y	*YOUNG*	L	L	L	L	L	L	L
DV_4y	*YOUNG*	L	H	L	L	L	L	L
DV_8y	OLD	H	H	H	H	L	L	H
DV_9y	OLD	L	L	L	L	L	L	H
DV_10y	OLD	L	L	L	L	H	L	L
T_2y	*YOUNG*	L	L	H	L	L	L	H
T_4y	*YOUNG*	L	L	H	L	L	H	H
T_5y	*YOUNG*	L	L	H	L	L	H	L
T_7y	*YOUNG*	L	L	L	L	H	H	H
T_8y	OLD	H	H	H	L	H	H	H
DQ_4y	*YOUNG*	L	L	L	L	L	L	L
DQ_5y	*YOUNG*	L	L	L	L	L	L	L
DQ_6y	*YOUNG*	L	L	L	L	L	L	L
DQ_7y	*YOUNG*	L	L	L	L	L	L	H
C_9y	OLD	L	H	H	L	L	H	L
C_14y	OLD	H	H	H	H	H	H	H
C_17y	OLD	L	H	H	H	H	L	L
CC_17y	OLD	H	H	H	H	H	H	H
DB_7y	*YOUNG*	H	L	L	L	H	L	H
DB_23y	OLD	L	H	H	H	H	H	L
MS_24y	OLD	L	H	H	H	H	L	L
MS_37y	OLD	H	H	H	H	H	H	L
TA_7y	*YOUNG*	L	L	L	H	L	L	H

L: Low level ; H: High level

7.3. RESULTS AND DISCUSSION

7.3.1. Target Metabolite Analysis

The results from the chemical analyses performed immediately after open the bottles (dissolved oxygen, pH and absorbance at 280nm) are shown in Table 7.2. It is indicated the minimum, maximum and average with standard deviation for all types of wines (independently of the indicated age).

Dissolved oxygen ranges from 0.35 and 1.54 in all the samples and no correlation were observed with wines aged. Values were not significantly when comparing samples from the same wine type ($p=0.14$). Yet, pH and phenolics index were significantly different between wines ($p<0.001$) but no correlation with each other was observed. Values for pH and phenolics index range from $2.9 - 3.51$ and $0.21 - 0.85$, respectively.

Table 7.2 Chemical analyses of bottled wines grouped by brand.

Wines	pH			Phenolics (Abs=280 nm)			Dissolved O$_2$ (mg/L)		
	Min	*Max*	*Av. ± Std*	*Min*	*Max*	*Av. ± Std*	*Min*	*Max*	*Av. ± Std*
C (n=4)	3.07	3.32	**3.07 ± 0.11[ab]**	0.41	0.85	**0.63 ± 0.24[b]**	0.50	0.90	**0.7 ± 0.2 [a]**
DB (n=2)	3.17	3.29	**3.23 ± 0.06[abc]**	0.27	0.52	**0.37 ± 0.13[ab]**	0.51	1.06	**0.8 ± 0.3 [a]**
DQ (n=4)	3.30	3.49	**3.42 ± 0.08[c]**	0.28	0.37	**0.32 ± 0.04[a]**	0.60	0.82	**0.7± 0.1 [a]**
MS (n=2)	2.97	3.08	**3.03 ± 0.07[a]**	0.41	0.42	**0.41 ± 0.01[ab]**	0.65	0.73	**0.7 ± 0.1 [a]**
TA (n=1)	3.10	3.10	**3.10 ± 0.00[ab]**	0.28	0.28	**0.28 ± 0.00[a]**	0.55	0.55	**0.6 ± 0.0 [a]**
T (n=5)	3.05	3.27	**3.14 ± 0.07[ab]**	0.21	0.32	**0.28 ± 0.04[a]**	0.35	1.09	**0.8 ± 0.2 [a]**
DV (n=6)	3.15	3.51	**3.30 ± 0.15[bc]**	0.25	0.31	**0.31 ± 0.06[a]**	0.88	1.54	**1.1 ± 0.3 [a]**

Av. ± Std[a]: average ± standard deviation, Values with the same letter in the same column are not significantly different (p < 0.05), n=number of samples.

The knowledge regarding chemical markers of white wine oxidation establish the roles of browning (absorbance at 420 nm) and several molecules such as: phenylacetaldehyde, methional, furfural, cis and trans dioxanes and dioxolanes in white wine deterioration.

The mentioned oxidation markers were quantified in order to evaluate oxidation levels of each wine. Temporal age of the wines was used to define two classes YOUNG (wines aged between 2 and 7 years old) and OLD (aged for more than 8 years old).

The heat map commonly used for unsupervised clustering was constructed for the target molecules. The parallel heatmap visualization (Figure 7.4A) using Ward's method in computational systems analysis for all the samples showed distinct segregation between the two groups. Nevertheless, samples DV_10y, DV_9y and C_9y are clustered together with YOUNG groups and sample DB_7y with OLD group. Meaning that target molecules are not sufficient to serve as a fingerprint of samples oxidation during time.

To test the impact of some molecules in wine grouping boxplots were constructed (Figure 7.4B). In some cases, outlier samples regarding for example phenylacetaldehyde were observed. Three groups of variables were clustered together: i) *cis-* and *trans-* dioxanes and dioxolanes, ii) furfurals (2-furfural and 5-methylfurfural), acetaldehyde and A420 and iii) the two Strecker aldehydes (phenylacetaldehyde and methional) and TDN.

It was found that best correlations with storage time for: 2-furfural (0.83, $p<0.01$), acetaldehyde (0.76, $p<0.01$), A420 (0.73, $p<0.01$) and cis-1,3-dioxane (0.63, $p<0.01$). Nevertheless, correlations above 0.5 with no significative value ($p>0.05$) were obtained for methional (0.37), phenylacetaldehyde (0.18) and TDN (-0.1). These observations were in agreement with the results of the heat map in Figure 7.4A. According with, samples belonging to the group with high A420, showed higher levels of 2-furfural, acetaldehyde and 5-methylfurfural. Acetaldehyde increases during ageing, due to the oxidation of ethanol and due to the cleavage of the acetaldehyde-SO_2 bound. Yet the compound could be involved at the same time in condensation reactions with phenolic compounds and in the formation of volatile compounds such as heterocyclic acetals. In fact, a correlation of 0.73 ($p<0.01$) was obtained between acetaldehyde and *cis*-1,3-dioxane, formed by the reaction with glycerol and very susceptible to oxygen levels (Silva Ferreira, Barbe and Bertrand, 2002b).

The impact of furfural as indicator of oxidation has been established for fortified wines, such as white fortified wines, Madeira and Port wine and related with the wood contact and/or with the reaction of the pentoses due to Maillard reaction (wines with high amount of sugar) (Cutzach, Chatonnet and Dubourdieu, 2000; Pereira *et al.*, 2011; Martins, Monforte and Silva Ferreira, 2013)
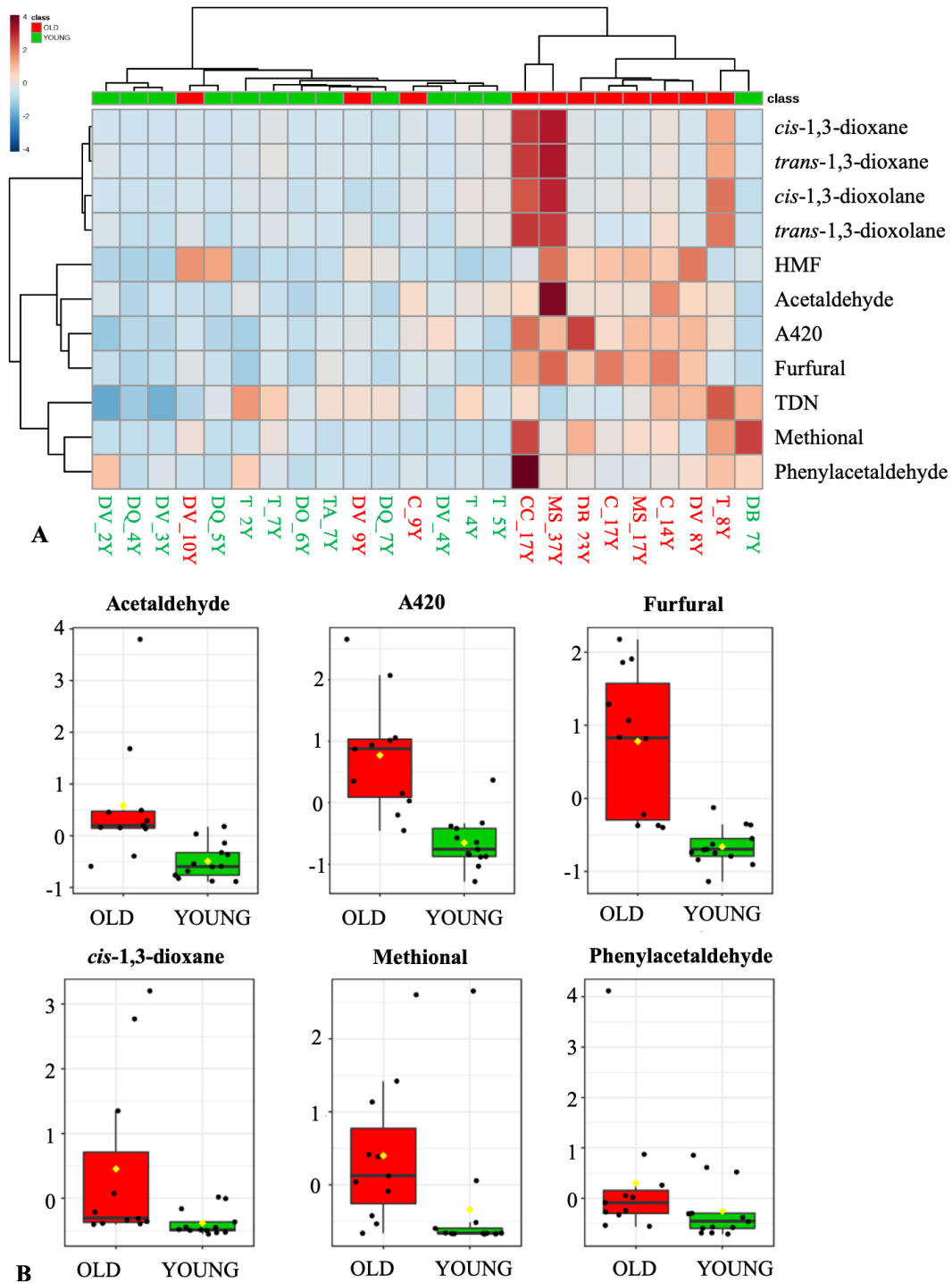
Figure 7.4 Heat map visualization for the target molecules quantified in the samples (rows: chemical compounds; columns: samples; colour key indicates molecule expression value, blue: lowest, red: highest. Box plot of the most discriminant molecules based on the two groups: YOUNG and OLD.

7.3.2. Unsupervised Metabolite Profiling

Target results as mentioned and in particular age vector used as classification vector were insufficient to describe the chemical fingerprint of samples oxidation. The metabolomic pipeline developed was applied to all GC-MS chromatograms, the processing time (step A to step H) for 24 samples was 3 minutes involving all the steps needed to extract a peak table. After data processing was obtained a two-dimensional matrix, including normalized peak areas. Overall before inspection 197 retention times were extracted and after filtering procedures based on raw mass spectra, 132 retention times were considered in each sample profile.

PCA was first used to investigate general interrelation between groups, including clustering and outliers among samples. In Figure 7.5, a comparison between target (A) and untarget (B) PCA score plot is shown. The principal components 1 and 2 explained 56.4 % and 16.3 % of the total variance, respectively for target molecules and 43.7 % and 8.7 % for untarget analysis. Based on PCA score plot results a better separation between OLD and YOUNG samples was observed for the untarget dataset, indicating that more molecules not including in the target analysis could have better discriminative power.

In order to quantitatively evaluate the predictive ability of each dataset, two PLS-R models were constructed to predict the age of samples using target and untarget datasets. Root-mean-square error of cross validation (RMSECV) value was used to determine the accuracy of the models. R^2Y and Q^2Y was used as indication of how well a model fitted the data. A higher R_2Y value in a model indicates a better model fit. Q_2Y values within the range of 0.5–1.0 are considered to indicate good predictability. Taking the calibration model of the target molecules under 2 factors the RMSECV and R^2Y and Q^2Y of the calibration set were 4.7 years, 0.83 and 0.66, respectively. Similarly, was developed a model for the untarget molecules under 6 factors. The RMSECV was 3.5 years, the R^2Y 0.99 and Q^2Y was 0.80. All the three indicators shown that untarget dataset have better predictive abilities regarding white wines indicated age.
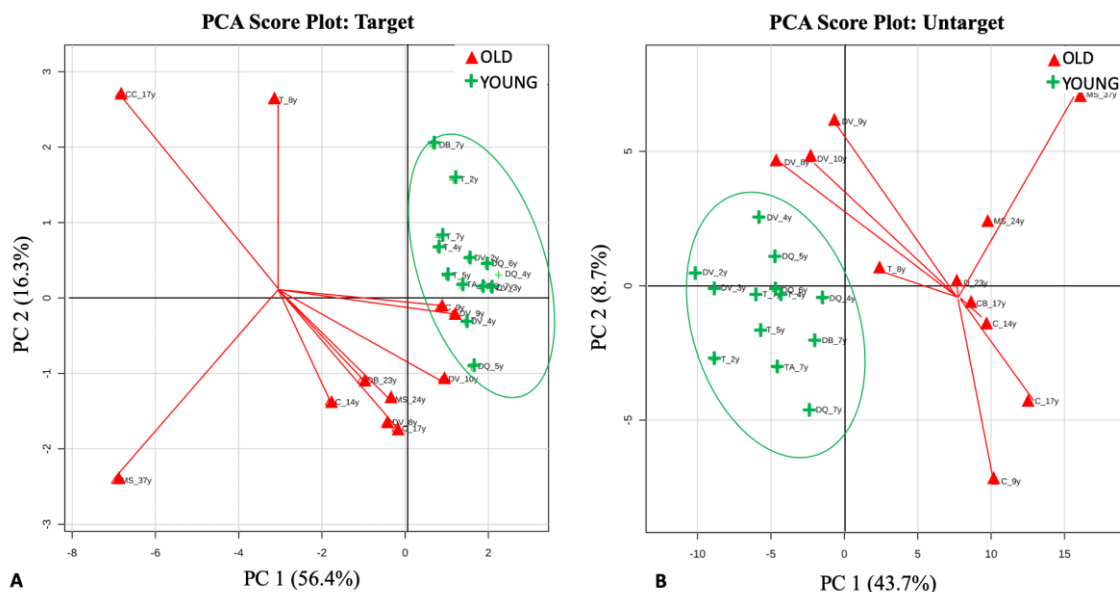
Figure 7.5 PCA score plots of A) Target Analysis (11 variables) and B) Untarget Analysis (132 GC-MS variables). Red symbols (wines with ages between 8 and 37 years) and green symbols (wines with ages between 2 and 7 years old).

7.3.3. Supervised Metabolite Profiling

For the supervised metabolite profiling 8 classes were considered as indicated in Table 7.1. The reason to select 8 classes based in target compounds recognized has oxidation markers is to have an "holistic vision" of the system regarding both, wine age (time) and chemical oxidation markers.

PLS-DA was used firstly has a projection tool in order to observe for samples classifications according with a given class, results are shown in Figure 7.6. According with the model's parameters, the better classification results was obtained for: age, A420, methional, furfural and acetaldehyde.

Low Q^2Y for phenylacetaldehyde, TDN and cis-1,3-dioxane demonstrate the lack of predictive power for the three compounds. Due to the fact that one of the main disadvantages of PLS-DA is the data overfitting, random forest was performed to model the data. Better results were obtained in particular for *cis*-1,3-dioxane.
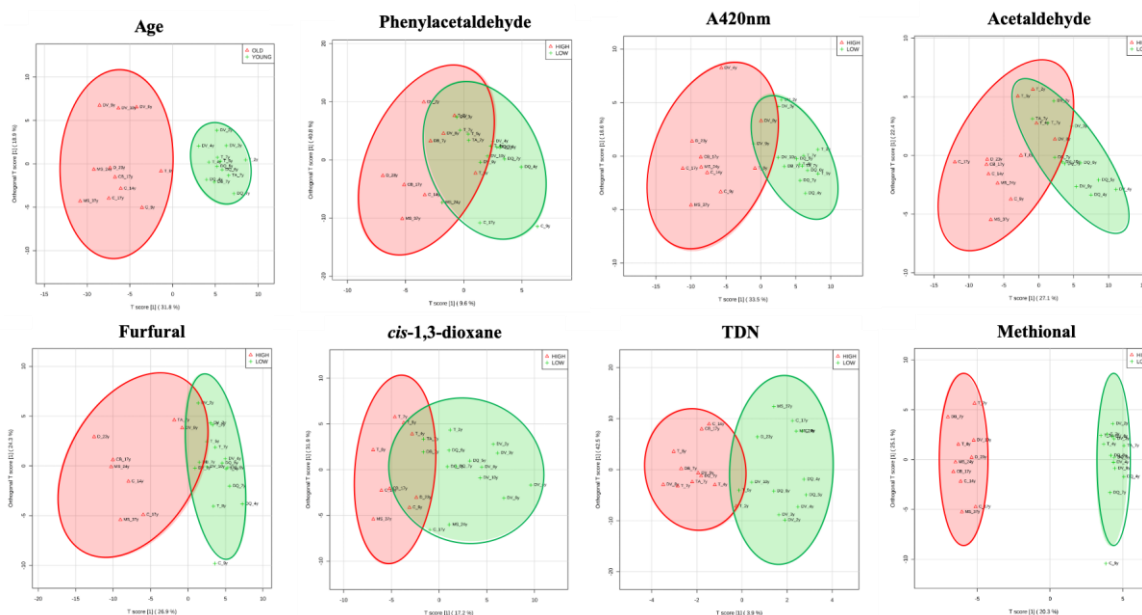
Figure 7.6 Orthogonal projection of latent structure-discriminant analysis (OPLS-DA) score plots in the analysis of the 132 GC-MS peaks in the 8 selected classes.

Mean decrease accuracy (MDA) provided by random forest was merged to VIPs for features selection. It was established that those variables with a VIP coefficient higher than 1 can be considered important (Chong and Jun, 2005).

For each class variables were extracted based on the VIP (>1), MDA (>0.005), *p-value* (<0.05) and *q-value* (<0.05). No biomarkers following the rules were found for phenylacetaldehyde and TDN, confirming the lack of predictive ability for the two molecules. For the other class vectors (age, acetaldehyde A420, methional, furfural and *cis*-1,3-dioxane), 52 candidates were extracted.

Table 7.3 Extracted molecules (Level 1 and Level 2) with the 4 rules: q-value, area under the curve (AUC) and fold changes (FC)

	#	Class	RT (min)	RI	q-value	AUC	FC	Identification
Level 1	1	Age	60.7	1981	<0.01	0.84	5	Unknown (m/z 159, 141, 113)
	2	Acetaldehyde	34.0	1497	<0.01	0.91	5	2,4,5-trimethyl-1,3-dioxolane
	3	Furfural	52.2	1828	<0.05	0.86	35	Benzofuran
	4	Furfural	53.6	1854	<0.05	0.89	3	Ethylphenyl acetate
	5	Furfural	60.5	1978	<0.01	0.94	3	3-(2,3,6,-trimethyl-1-cyclohexen-1-yl)-2-propenal
	6	Abs420	54.2	1864	<0.01	0.94	12	2-phenylethylacetate
	7	Abs420	40.3	1612	<0.01	0.87	2	Isoamyl lactate
Level 2	8	Methional Furfural	31.5	1452	<0.01 / <0.05	0.86 / 0.89	4	Ethyl-2-hydroxy-3-methylbutanoate
	9	Methional Furfural	34.3	1503	<0.01 / <0.01	0.93 / 1	3 / 3	2-furfural
	10	Furfural Age	34.1	1499	<0.001 / <0.001	0.96 / 0.96	3	*cis*-linalool oxide
	11	Furfural Age	84.6	2416	<0.01 / <0.01	0.93 / 1	8	Ethyl citrate
	12	Furfural Abs420	32.1	1463	<0.05 / <0.01	0.89 / 0.88	-4	Ethyl octanoate
	13	Methional Age	62.9	2023	<0.05 / <0.01	0.87 / 0.93	6	Unknown (m/z 131, 157, 103, 85)
	14	Methional Acetaldehyde	54.7	1874	<0.01 / <0.01	0.94 / 0.93	11	cis-1,3-dioxolane
	15	Acetaldehyde Abs420	78.6	2307	<0.05 / <0.05	0.84 / 0.86	4	Unknown (m/z 207,104 , 133)
	16	cis-1,3-dioxane Acetaldehyde	36.2	1536	<0.05 / <0.01	0.9 / 0.92	17	cis-1,3-dioxane
	17	cis-1,3-dioxane Acetaldehyde	42.9	1658	<0.05 / <0.05	0.92 / 0.85	7	trans-1,3-dioxolane

Table 7.4 Extracted molecules (Level 1 and Level 2) with the 4 rules: q-value, area under the curve (AUC) and fold changes (FC)

	#	Class	RT (min)	RI	q-value	AUC	FC	Identification
Level 3	18	Methional	20.2	1246	<0.05	0.88	3	2,2-dimethyl-5-(methylpropenyl)-tetrahydrofuran
		Furfural			<0.01	0.93		
		Age			<0.01	0.993		
	19	Methional	89.9	2512	<0.01	0.89	2	Unknown (m/z 257, 203, 129)
		Acetaldehyde			<0.05	0.8		
		Abs420			<0.01	0.86		
	20	Furfural	32.3	1467	<0.001	0.99	3	*trans*-linalool oxide
		Age			<0.001	0.944		
		Abs420			<0.01	0.93		
Level 4	21	Furfural	37.0	1552	<0.05	0.83	3	Ethyl-2-hydroxy-4-methylpentanoate
		Methional			<0.01	0.9		
		Acetaldehyde			<0.05	0.86		
		Abs420			<0.01	0.89		
	22	Methional	42.1	1644	<0.05	0.85	4	Unknown (*m/z* 119 / 73)
		Age			<0.001	1		
		Acetaldehyde			<0.05	0.83		
		Abs420			<0.01	0.9		
Level 5	23	Methional	43.0	1660	<0.01	0.94	5	Ethyl levulinate
		Furfural			<0.05	0.89		
		Age			<0.001	0.95		
		Acetaldehyde			<0.01	0.93		
		Abs420			<0.01	0.95		

7.3.4. Multi-class metabolite performance

The 52 metabolites extracted using the four filters (*p*-value, *q*-value, VIP and MDA) were selected for algorithm training. ROC analysis has been applied to test the diagnostic ability of biomarkers in metabolomics studies (Broadhurst and Kell, 2006). The generated AUC values are used to quantify each model diagnostic power. Models with AUCs greater than 0.7 were considered to be significantly relevant for the classification.

From the univariate ROCs for each biomarkers a total of 23 molecules were selected for identification (Table 7.3 and Table 7.4). Tables were divided in 5 levels dependent on the number of classes where the candidate was found to be relevant. For example, 1 compound founded to be correlated with three classes such as #20 belongs to level 3. The validation of the proposed methodology relies on the identification of relevant compounds capable to be contextualized in white wines reactions occurring during storage.

To better understand the significance of the extracted molecules, network based on the correlation with a given class and the MDA were constructed (Figure 7.7). Network links (edges) were weighted according to their mean decrease accuracy extracted with random forest. Nodes colour were set based on the fold changes (FC) of each molecule comparing the linked class.

Several esters were found to be relevant for the established classes. Normally the contribution of esters to the fruity aroma of wines decrease during aging due to hydrolysis, which was observed for ethyl octanoate. Nevertheless, other esters were identified and increased in wines stored for long periods of time in particular: ethyl-2-hydroxy-4-methylpentanoate (E2H4MP, FC=3), ethyl-2-hydroxy-3-methylbutanoate (E2HIV,FC=4), ethyl phenyl acetate (FC=3), 2-phenylethyl acetate (FC=12), ethyl levulinate (FC=5) and ethyl citrate (FC=8).
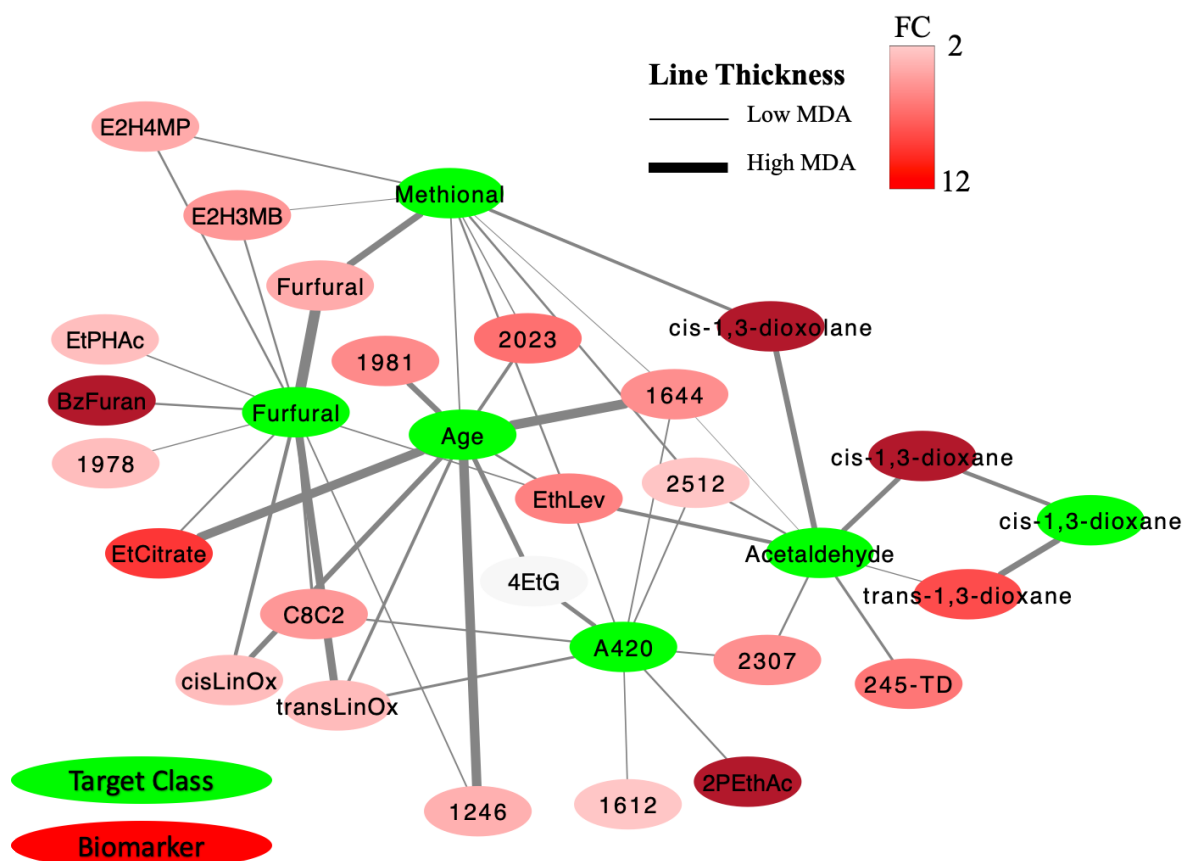
Figure 7.7 Correlation network based on mean decrease accuracy values (thickness of edges) for each class with Area Under Curve higher than 0.7. Each node represents the retention index of the compound and the colour of the node the fold changes (FC) between the group of old and young samples.

Ethyl hydroxylated esters (E2H4MP and E2H3MB) were firstly isolated and identified as contributors with fruity aroma in aged wines (Madeira and Sherry wines) (Campo, Cacho and Ferreira, 2006). Later it was found that E2H3MP was associated with "blackberry aroma", for both forms S and R and the last form was found to be higher in old white wines that did not express that type of aroma (Lytra *et al.*, 2012). While E2H3MB was found to have aromas such as candy, with strawberry, pineapple, and kiwifruit notes for the R form whereas the S form was characterized by "red fruits", "pineapple" and "green apple" descriptors. Nevertheless quantitation in white and red wines and spike studies shown that this ester cannot have a direct impact on fruity aroma modulation (Gammacurta *et al.*, 2018).

According with the available knowledge the formation of this esters is due to the reaction of the correspondent acid with ethanol, yet amounts in white range from non-detectable to 200 and µg/L for the acid of E2H3MB and 700 µg/l for the acid of E2H3MP (Gracia-Moreno, Lopez and Ferreira, 2015).

Our results shown a linear correlation of this compounds with the oxidation status of the analysed wines, with a linear increase during ageing time and a clear separation between more and less oxidized wines (Figure 7.8). Further studies are needed in order to understand the sensorial contribution of this compounds to the "oxidized character" of white wines.

Correlated exclusively with furfural were identified two compounds: benzofuran and ethyl phenyl acetate. Benzofuran changed dramatically when comparing high and low oxidation levels based on furfural class (FC=35). This compound was identified has an ageing marker of Nebbiolo wines (Bordiga *et al.*, 2014) and in aged Chardonnay wines submitted to hyperoxygenation (Jesús Cejudo-Bastante *et al.*, 2011).
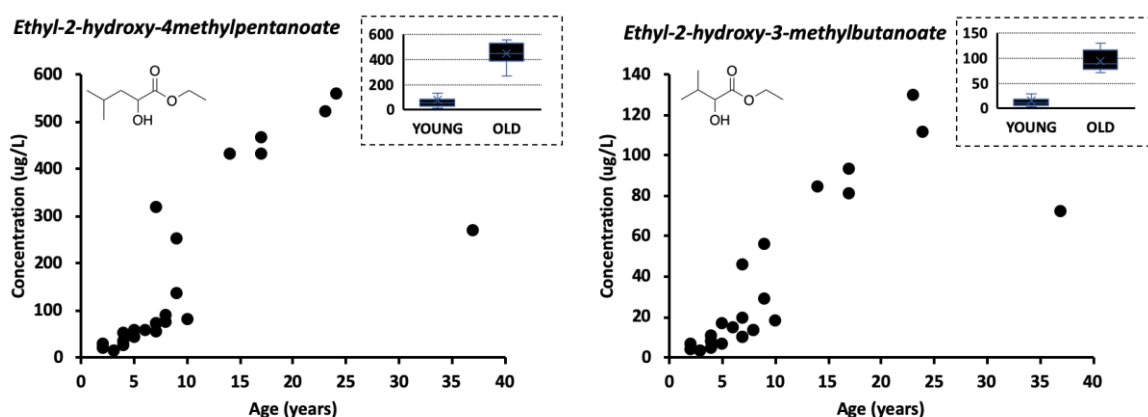
Figure 7.8 Time expression of ethyl-2-hydroxy-4-methylpentanoate and ethyl-2-hydroxy-3-methylbutanoate.

The good correlation of ethyl phenyl acetate with oxidation could explain the lack of correlation of phenylacetaldehyde with age time or another compound in the dataset. During time the aldehyde could be oxidized in phenylacetic acid capable to suffer esterification to the ester. Besides the ester could gave to the wines a strong "honey-like" character, suggesting that for longer periods of time other compounds besides the recognized marker phenylacetaldehyde could contribute to the "honey-like" off-flavour (Tat *et al.*, 2007).

Correlated with age and furfural, cis- and trans- linalool oxides were identified (FC=3). These compounds are monoterpenes oxides and are formed from cyclization and oxidation of linalool(Genovese *et al.*, 2007). Acetaldehyde is linked to the respective acetals from the reaction with glycerol (cis-1,3-dioxane, trans-1,3-dioxane and trans-1,3-dioxolane) and with 2,3-butanediol (2,4,5-trimethyl-1,3-dioxolane). These particular acetals were already identified as increasing during ageing and sensible to oxygen in wines (Ferreira *et al.*, 1997; Silva Ferreira, Barbe and Bertrand, 2002b).

7.4 CONCLUSION

In conclusion, the proposed multi-class methodology combined with PLS-DA and machine learning techniques (random forest) prove to be an efficient way of classifying wines according with the oxidation state. Furthermore, the strategy employed extract new markers of white wines deterioration during aging, contributing to new knowledge regarding the evaluation of volatile changes occurring during wine oxidation.

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CHAPTER 8 CHANGES IN WHITE WINE COMPOSITION AFTER TREATMENT WITH CATIONIC EXCHANGE RESIN: IMPACT ON WINE OXIDATION AFTER 8 YEARS OF BOTTLE STORAGE

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8.1. INTRODUCTION

During storage, several modifications occur in wine that affect its taste and aroma. These modifications are related with oxidation. In the particular in the case of white wine, the excessive exposure to oxygen impart detrimental sensorial effects. The consequences of oxidation in sensorial terms are the formation of molecules responsible for an increase in browning (Singleton, 1987), as well as of molecules with sensorial impact (Figure 8.1). These molecules were reported in several studies and include Strecker aldehydes (Escudero, Cacho and Ferreira, 2000; Silva Ferreira *et al.*, 2003) , (E)-2-alkenals, sotolon, benzaldehyde, furfural and hexanal (Escudero *et al.*, 2002).

In terms of mechanism, oxygen in nature is in its unreactive and stable form (triplet oxygen) and is not capable to react with organic molecules. Still it is capable to accept a single electron produced by the oxidation of a catalyst, that in the case of wine is iron (Waterhouse and Laurie, 2006). The transfer of electrons will generate a cascade of reactive oxygen species catalyzed by metals such as: iron and copper. This cascade will lead to the oxidation of phenolic compounds resulting in the formation of *o*-quinones from the oxidation of phenolic compounds and hydrogen peroxide as the main byproduct (Danilewicz, 2003). Besides, SO_2 can react with quinones, either to reduce them back to catechols or by a nucleophilic reaction to produce catechol sulfonate preventing oxidation reactions to proceed and resulting in the triggering of the Fenton reaction.

When considering wine oxidation in the proposed scheme, the rate of oxidation will depend on Fe, Cu, polyphenol, oxygen and sulfite concentrations (Figure 8.1). In bottle aged wines, the random nature of the problem turns it difficult to understand the underlying mechanism involved (Karbowiak *et al.*, 2009). The oxygen absorption capacity of wines during bottle storage is affected by several technological parameters such as temperature (Martins *et al.*, 2013), oxygen (Lopes *et al.*, 2009), and light (C. Clark *et al.*, 2011).

Strecker aldehydes, in particular methional and phenylacetaldehyde are probably the most studied volatile molecules, due to their sensory impact and because their formation is highly dependent on the dissolved oxygen levels (Silva Ferreira *et al.*, 2002). The formation of these molecules is not yet fully understood. Several routes for their formation have been reported namely: (i) Strecker degradation of the parent amino acid via α-dicarbonyls or *o*-quinones, (ii) release from adducts between aldehyde-SO_2 and (iii) oxidation of the

respective alcohol. Moreover it was shown that glucose can inhibit phenylacetaldehyde formation in white wines(Monforte, Martins and Silva Ferreira, 2018).
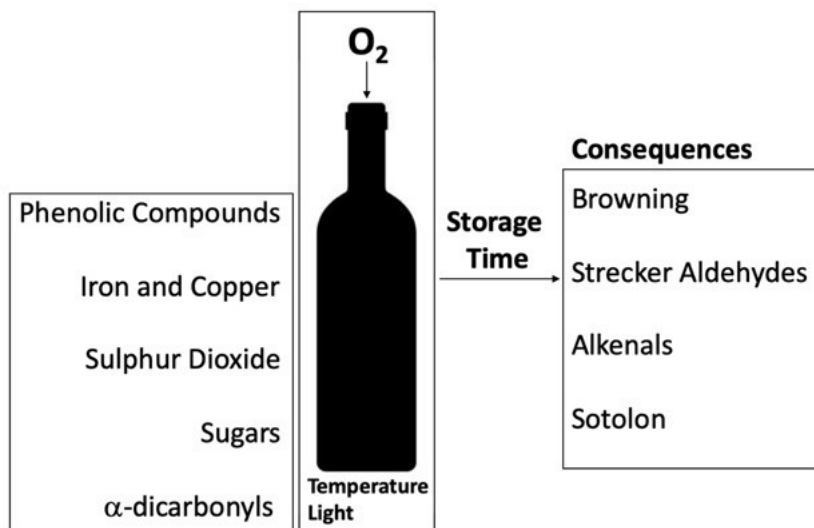
Figure 8.1 Main substrates and consequences of wine oxidation during bottle storage time.

Strecker degradation promoted by the reaction of *o*-quinones with phenylalanine and methionine was shown to be unfavorable due to the low reactivity of the amino acid, when compared with other nucleophiles (Nikolantonaki *et al.*, 2012). Nevertheless the contribution of α-dicarbonyls, in particular methyl glyoxal for the formation of phenylacetaldehyde in wine conditions were recently demonstrated to be more reactive when compared with SD promoted by *o*-quinones (Monforte, Martins and Silva Ferreira, 2019). Moreover in red wines it was found a high correlation between diacetyl and PA formation, suggesting that diacetyl could induce SD of phenylalanine (Bueno *et al.*, 2018).

Aldehydes are highly reactive molecules capable to interact with other molecules such as SO_2, resulting in the formation of α-hydroxyalkyl sulfonates in wine conditions (de Azevedo *et al.*, 2007). It was recently demonstrated that these compounds (SA bound to SO_2) could be cleaved during oxidation releasing the SA, caused by the equilibrium shifts due to depletion of SO_2 (Bueno *et al.*, 2016).

The impact of wine composition in oxygen and SO_2 consumption rates were recently studied, where 8 white wines were submitted to consecutive air-saturation cycles. It was shown that

oxygen consumption were proportional to copper, quercetin and kaempferol contents and negatively proportional to hydroxycinnamic acids (Carrascón *et al.*, 2017).

The most common ways to study wine oxidation are by the analysis of (i) wines stored for different period of time, (ii) the same wine stored in different containers or (iii) wines submitted to different conditions of oxygen and temperature to monitor changes during short periods of time (< 12 months).

In this study three white wines were submitted to a cation exchange resin treatment in different proportions and stored for 8 years. The main consequences of this treatment reported so far are the changes in pH (Walker *et al.*, 2002), monomeric fraction of proanthocyanins (Ibeas, Correia and Jordão, 2015), retention of cations such as iron, copper, calcium and potassium , lower polyphenolic compounds concentration as well as reduction of susceptibility to browning (Benítez, Castro and Barroso, 2002). Regarding wine sensory quality attributes positive and negative effects were also reported in wines submitted to cation exchange resin treatment. Nevertheless, this treatment has the add value of prevent tartrate wine precipitation, reduce pH and remove metal ions (Mira *et al.*, 2006).

Our aim is to perturb the wine composition of the same type of wine with the cationic resin and evaluate after storage time the impact of composition in the "oxidation-status" (phenylacetaldehyde has a "proxy" for oxidation). Forty-seven parameters divided in (i) substrates for wine oxidation and (ii) markers of wine oxidation were determined.

8.2. MATERIAL AND METHODS

8.2.1. Reagents and Standards

All chemicals used in this study were of the highest purity available grade, and purchased from Sigma-Aldrich (St. Louis, MO).

8.2.2. Wine Samples

A total of 42 samples of bottled white wine from three different regions in Portugal stored for 8 years were analysed. Wines were sourced from: Minho (WA), Douro (WB) and Dão (WC) regions.

In order to study the impact of metals a cation exchange resin was used. The regeneration of the resin was performed with HCl (30% v/v) with a flow rate of 5L/hour and the clearing phase using bi-distilled water (flow rate of 10 L/hour).

Wines were divided into 7 lots for each treatment (15 L each). One lot was representative of the control wine (untreated) and other represent the eluted wine (100%). The remaining five containing cation exchanged wine, were mixed in different proportions (5%, 10%, 15%, 20%, 25%) with the untreated wine. Each wine was sealed in three green glass bottles (0.750 L) with natural corks and stored in cellar conditions for 8 years. Two replicates for each wine were analysed.

8.2.3. Chemical Characterization

Wine samples characterization was performed in the beginning of the experiment (2011) and after 8 years of storage. In particular measurements of pH, free and total SO_2 concentration (iodine method) and colour determination (absorbances at 420, 520 and 620 nm of the undiluted samples using plastic 1 cm cells).

8.2.4. Element determination

Elements (Cu, Fe, Mn, Mg, B, Na, Cd, P, Ca, Zn and K) were determined using a Perkin Elmer Optima 7000 DV ICP-OES. The wine samples were diluted ten times in nitric acid (5%). Calibrations were performed in wine model solution (10% ethanol with 4 g/L of tartaric acid and pH=3.2). The dilution was validated with spike recovery studies.

8.2.5. Voltammetry Measurements

Electrochemical analysis was performed with Nomasense Polyscan electrochemical analyser (Nomacorc, Belgium) using disposable screen-printed sensors in a three-electrode arrangement. Two indices were obtained: easily oxidized compounds (EasyOx) and total oxidizable compounds correlated to Folin Ciocalteau (PhenOx).

8.2.6. Total acetaldehyde quantification

The determination of acetaldehyde concentration was performed by gas chromatography coupled to a flame ionization detector according with the method previously described (Barbe *et al.*, 2000).

8.2.7. Aldehydes and α-dicarbonyls quantification

The analysis of Strecker aldehydes, alkanals and α-dicarbonyls was performed according (Moreira *et al.*, 2019). Wine (2mL) was placed in a 20 mL vial, 2 g/L of PFBHA was added. Sample was incubated at 40°C for 10 minutes and molecules were extracted with PDMS/DVB fiber for 20min at the same temperature. Compounds were analyzed using a Varian 240-MS, and a CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland). The column used was a

BR-5 ms (30 m × 0.25 mm I.D. × 0.25 μm film thickness) (Bruker Daltonics, Freemont, CA). Compounds were desorbed in the injector at 250°C for 5 minutes. The oven temperature program was 40°C (1 min) to 250°C (10min) at 5°C/min. The carrier gas was helium at 1 mL/min, with a constant flow. All mass spectra were acquired in the electron impact (EI)

mode (ionization energy, 70 eV; source temperature, 180 °C). The analysis temperatures were as follows: trap, 170 °C; manifold, 50 °C; transfer line, 190 °C; and ion source, 210 °C.

8.2.8. Phenolic compounds quantification

Prior to analysis samples were filtered with 0.2 μM PTFE filters and placed in 2mL vials. Analysis of phenolic compounds were performed on an Ultimate 3000 Dionex UHPLC coupled to an ultra-high resolution Qq-time of flight (UHR-QqTOF) mass spectrometer (Impact II, Bruker Daltonics, Germany), according with the method described previously(Monforte, Martins and Silva Ferreira, 2019). Data were acquired in negative mode over a mass range of 50 to 1500 amu with scan duration of 0.3s in centroid mode. The MS parameters were: capillary voltage: 4.5 kV; drying gas temperature: 200°C; drying gas flow: 8 mL/min; nebulizing gas pressure: 2bar; collision RF: 300 Vpp; transfer time: 120 μs and prepulse storage: 4 μs. Mass calibration was performed by the external injection of a sodium formate solution.

8.2.9. Data analysis

The statistical significances of the variables were tested by Tukey's multiple t-test of one-way ANOVA using SPSS 12.0 software (SPSS Inc., Chicago, USA). The multivariate data was processed by MetaboAnalyst 4.0 (http://www.metaboanalyst.ca/) for heatmap cluster analysis, heatmap correlation analysis and Partial Least Square Discriminant Analysis (PLS-DA)(Chong *et al.*, 2018). Before statistical analysis data was transformed through a generalized logarithm transformation and scaled by means of autoscaling procedure. Phenylacetaldehyde markers extracted by PLS-DA were determined based on variable important for projection (VIP) values and p-values. The validation of the extracted markers was determined based on area under the curve (AUC) obtained with ROC plots.

8.3. RESULTS AND DISCUSSION

8.3.1. Wine Characterization

This study of white wine storage was carried out after 8 years. Before the storage, samples were taken and analysed for chemical parameters with oenological meaning, in particular free and total SO_2 and pH for the control wines (0%). The indicated parameters are shown in Table 8.1, as well as the iron, copper and absorbance at 420nm measured after 8 years.

Wine pH was found to be lower in WA and higher in WB and WC and no changes were observed after storage. An increase in the total SO_2 and a decrease in the free SO_2 was observed for all wines. Meaning that bound SO_2 increased during aging time, which can happen due to the capacity of SO_2 to bind with several compounds. The percentage of bound form is higher for WC (56%) when compared with WA (29%) and WB (28%). The same trend was observed for the decrease of free SO_2. Also, higher A420nm (i.e. browning) was observed for the same wine type, while no significant differences were observed for WA and WB.

Table 8.1 Wine characterization before (2011) and after storage (2019) of the three wines control samples (0%).

Wine	Year	pH	Total SO2 (mg/L)	Free SO2 (mg/L)	Fe (mg/L)	Cu (mg/L)	Abs 420 nm
Verde (WA)	2011	3.02	125	26			
	2019	3.06	158	19	2.5	0.053	0.13
Douro (WB)	2011	3.25	142	26			
	2019	3.25	175	15	1.4	0.059	0.12
Dao (WC)	2011	3.36	113	41			
	2019	3.37	176	12	0.3	0.092	0.3

Regarding iron and copper, concentrations of Fe were found to be higher for WA (2.5 mg/L) followed by WB (1.4 mg/L) and WC (0.3 mg/L). Copper concentrations are well below iron concentrations as expected and are higher in WC (0.092 mg/L) and lower in WA and WB (no statistical difference).

Wines with low amounts of iron (WC) were the most oxidized samples. These observation is not in agreement with the observation that wine oxidation should increase with increased concentrations of iron (Cacho *et al.*, 1995). Still, in the referred study, added iron concentrations, to the same wine, varied between 3.8 and 23 mg/L.. In the present study the concentrations measured in the aged wines were above 3 mg/L. Also, the three wine types studied showed different composition, like in the phenolics concentration, which could contribute for the observed differences. The compounds most affected by the cation resin treatment after 8 years of bottle storage, comparison between all lots (0, 5, 10,15, 20, 25 and 100%) and excluding the total eluted wine (0, 5, 10,15, 20 and 25%) was performed for each wine (Table 8.2). The impact of treatment on wines composition after storage is wine type dependent, except for pH. According with the results WC is the most affected while WA and WB were similarly affected, except for Mn, Fe, Mg and Ca which are significantly different in WA. All elements, except Cu were different between lots for WC.

Glucose, catechin, 5-methylfurfural are affected only in the total eluted wines (100%) for the three wines. Free and Total SO_2, epicatechin, quercetin, phenox and easyox are significant different between lots only for WC.

Changes in A420, acetaldehyde, furfural, 5-hidroxymethylfurfural, octanal, nonanal and decanal were observed for WC.

Table 8.2 Summary of ANOVA Table p-values for the three wine types: impact of lots with and without the total eluted wine.

Variables	WA		WB		WC	
	0 - 100%	0 - 25%	0 - 100%	0 - 25%	0 - 100%	0 - 25%
pH	$p < 0.001$	p < 0.001	p < 0.001	p < 0.001	p < 0.001	p < 0.001
Glucose	$p < 0.001$	ns	p < 0.001	ns	p < 0.001	ns
Free SO2	ns	ns	ns	ns	p < 0.01	p < 0.01
Total SO2	ns	ns	ns	ns	p < 0.01	p < 0.05
Catechin	$p < 0.01$	ns	p < 0.01	ns	p < 0.01	ns
Epicatechin	ns	ns	ns	ns	p < 0.01	ns
Protocatechuic	$p < 0.01$	ns	p < 0.01	ns	ns	ns
Quercetin	ns	ns	ns	ns	p < 0.01	ns
Phenox	ns	ns	ns	ns	p < 0.01	ns
Easyox	ns	ns	ns	ns	p < 0.01	p < 0.05
Zn	ns	ns	ns	ns	p < 0.001	p < 0.001
Cd	ns	ns	ns	ns	p < 0.001	p < 0.05
P	ns	ns	ns	ns	p < 0.001	p < 0.05
B	ns	ns	ns	ns	p < 0.001	p < 0.01
Mn	p < 0.001	ns	ns	ns	p < 0.001	p < 0.05
Fe	p < 0.001	ns	ns	ns	p < 0.001	ns
Mg	p < 0.001	ns	p < 0.001	ns	p < 0.001	p < 0.01
Ca	p < 0.001	ns	ns	ns	p < 0.001	p < 0.01
Cu	ns	ns	ns	ns	ns	p < 0.05
Na	ns	ns	ns	ns	p < 0.001	p < 0.05
K	ns	ns	ns	ns	p < 0.001	ns
420nm	ns	ns	ns	ns	p < 0.01	p < 0.05
Acetaldehyde	ns	ns	ns	ns	p < 0.01	p < 0.05
Furfural	ns	ns	ns	ns	p < 0.01	p < 0.05
5-HMF	ns	ns	ns	ns	p < 0.01	ns
5-MF	p < 0.001	ns	p < 0.001	ns	p < 0.01	p < 0.05
Octanal	ns	ns	ns	ns	p < 0.001	p < 0.001
Nonanal	ns	ns	ns	ns	p < 0.001	p < 0.001
Decanal	ns	ns	ns	ns	p < 0.001	p < 0.001

ns: not significance

0 – 100% : ANOVA comparison between 0, 5, 10, 15, 20, 25 and 100% lots

0 – 25% : ANOVA comparison between 0, 5, 10, 15, 20 and 25% lots

8.3.2. Unsupervised Approach

The target sample set includes thirty-seven samples and forty-seven variables in particular: free and total SO_2, pH, the two electrochemical parameters (easyOx and phenOx), absorbance at 420 nm (A420), sugars (glucose and fructose), ethanol, acetic acid, phenolic compounds (caffeic acid, coumaric acid, protocatechuic acid, epicatechin, catechin and quercetin), Strecker aldehydes (isobutanal, 2- and 3-methyl-1-butanal, phenylacetaldehyde and methional), α-dicarbonyls (methyl glyoxal and diacetyl), acetaldehyde, alkanals (pentanal, hexanal, heptanal, octanal, nonanal and decanal), furanic compounds (2-furfural, 5-methylfurfural and 5-hydroxymethylfurfural), *cis*-1,3-dioxane, benzaldehyde and metal ions (Cu, Fe, Mn, Mg, B, Na, Cd, P, Ca, Zn and K).

Significant differences were for some samples observed between replicates of the same wine (same lot) stored in a different container. By considering the proposed wine oxidation scheme, rate of oxidation should depend on Fe, Cu, polyphenol, oxygen and SO_2 concentration (Danilewicz, 2007). However, in bottled wine, especially in wines stored for long periods of time, oxidation depends on the O_2 rate through the cork. An observed value for O_2 ingress rate of approximately 0.3 mg/L/month for natural cork in the 2-12 month period after bottling has been reported (Lopes *et al.*, 2009). This fact could contribute to the heterogeneity observed for the same wine stored under the same conditions in different bottles (example: WA_10%_1 and WA_10%_2).

The heat map in Figure 8.2A, illustrates the dendrogram for the 25 most relevant variables regarding wine type (*p-value* < 0.01), n the vertical axis and wine samples averaged by type of wine on the horizontal axis. Three cluster of variables coloured according to the wine type were observed:

- Blue cluster is related to the WC and includes phenolic compounds (protocatechuic acid, coumaric acid, catechin and epicatechin) and the voltammetry indices (phenox and easyox), methional, A420nm, ethanol and copper.
- Red cluster related to WA with high mounts of diacetyl, Fe, sugars and furfural.
- Greed cluster related to WB with strecker aldehydes (2- and 3-methylbutanal and phenylacetaldehyde) together with: acetaldehyde, *cis*-1,3-dioxane, benzaldehyde and quercetin.

In order to reduce the high scope of the molecules and to look further for correlations regarding browning and Strecker aldehydes, namely phenylacetaldehyde, a heat map matrix correlation was employed to clarify the potential relationships among the target parameters (Figure 8.2B). Accordingly, 47 parameters were clustered based on their Pearson correlation coefficients, which were indicated on the plot with different colours (red: high positive correlation and blue: high negative correlation). Two groups of samples were therefore distinguished:

- Group A includes two subgroups CA and CB (white squares). Cluster A (CA), puts together elements (Ca, Zn, K, Mn and Mg) and pH, this cluster is related with the parameters most affected by the percentage of resin. While cluster B (CB) includes A420, copper, phenolic compounds, voltammetry indices and ethanol.

- Group B includes acetaldehyde, dicarbonyls, Strecker aldehydes, alkanals, furanic compounds, sugars, total and free SO_2, caffeic acid, quercetin and elements (Fe, Cd, P, B, Na).

A strong and positive correlation was observed between browning (A420) with catechin ($r^2=0.77$, p <0.01), epicatechin ($r^2=0.72$, p <0.01), coumaric acid ($r^2=0.5$, p <0.01) and protocatechuic acid ($r^2=0.8$, p <0.01), easyox ($r^2=0.66$, p <0.01) and phenox ($r^2=0.76$, p <0.01) indexes and copper ($r^2=0.63$, p <0.01) (Figure 8.2B). Meaning that wines with higher browning degree (A420) have high levels of the referred phenolics and they are easily oxidized, given the good correlation with easyOx and phenOx. The results suggest that phenolic compounds are the major substances to cause browning in aged wines, and the order of magnitude is dependent on their concentration. The results are in line with literature where, in particular the role of flavan-3-ols in wine colour formation has been established. Also, catechin has been considered the principal browning agent in white wines due to the high correlation observed with A420 (Sioumis *et al.*, 2006). Moreover flavanols can react with non-flavonoid quinones, where the electron transfer reaction is faster than the nucleophilic reaction, leading to the formation of new flavonoid coloured quinones capable to stimulating wine browning (Ma and Waterhouse, 2018).
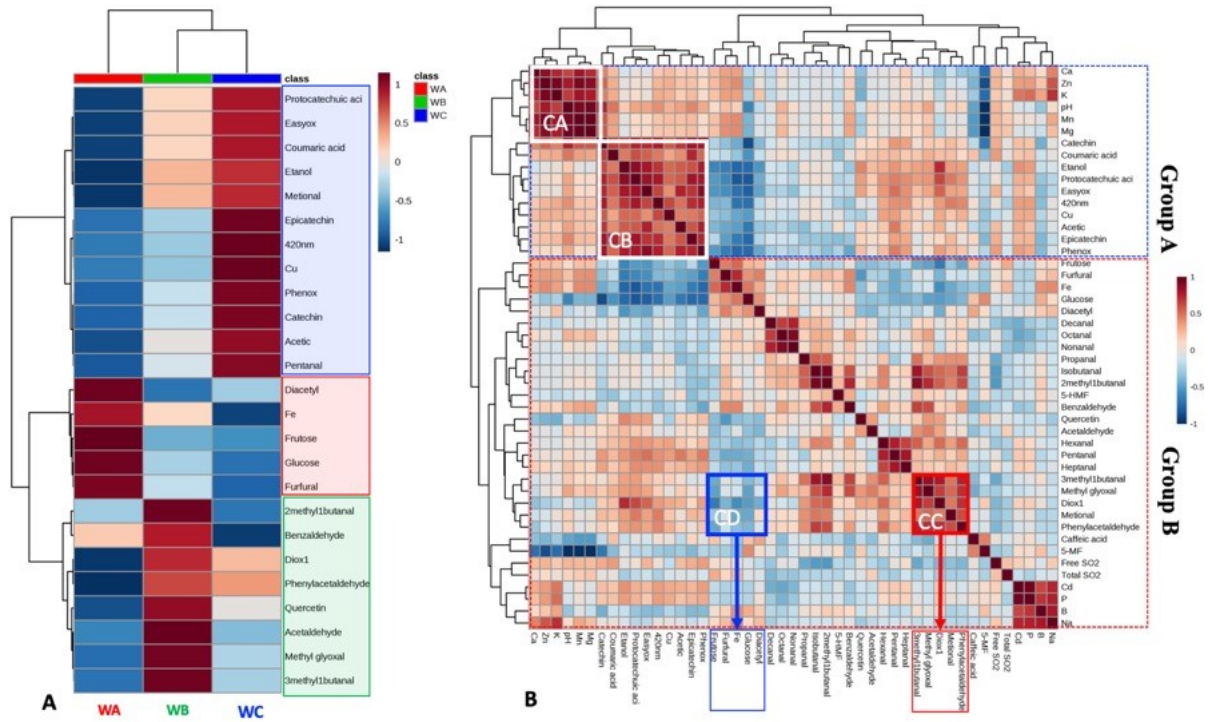
Figure 8.2 (A) Heat map constructed based on the relevant metabolites (p-value < 0.01) for the differentiation of WA, WB and WC. (B) Heatmap matrix correlations ($-1 \leqslant R^2 \leqslant 1$ corresponding to blue (negative correlation) to red (positive correlation) range colours). Yellow squares shown compounds with positive and negative correlations with phenylacetaldehyde.

The correlation between A420 with copper ($r^2=0.63$, p <0.01), could be related with the oxygen consumption rate due to the fact that the presence of copper is sensitive at low concentration (0.05-0.15 mg/L) in aiding the oxidation of Fe (II) to Fe (III) (Danilewicz and Wallbridge, 2010). High correlations copper and OCR was already reported in white wines stored under consecutive air saturations(Carrascón *et al.*, 2017). Moreover copper could promote an enhancement of the colour through the formation of xanthylium pigments (yellow) from the oxidative polymerisation of the flavan-3-ols with glyoxylic acid (C. Clark, D. Prenzler and R. Scollary, 2003). In this study, positive correlation was found between copper and catechin ($r^2=0.6$, p <0.01) and epicatechin. ($r^2=0.6$, p <0.01).

In group B, several clusters were observed. Of particular interest are two clusters CC (red square) and CD (blue square), both related with phenylacetaldehyde. Phenylacetaldehyde was chosen as known oxidation marker of white wine, due to its sensorial impact. Positive

correlations were found between PA and 3-methylbutanal (r^2=0.76, p <0.01), methional (r^2=0.62, p <0.01), *cis*-1,3-dioxane (r^2=0.7, p <0.01) and methyl glyoxal (r^2=0.75, p <0.01).

The correlation between the three SA (PA, methional and 3-methylbutanal) is explained by the common formation reactions, in particular (i) Strecker degradation of the parent amino acid *via* α-dicarbonyl or *o*-quinone, ii) oxidation of the correspondent alcohol or iii) cleavage of aldehyde bisulfite adducts. A significant increase in the concentration of these 3 aldehydes were observed in 14 years old white wine (Mayr *et al.*, 2015).

For the first time is was shown a high correlation between SA and methyl glyoxal, i.e. samples with high concentration of methylglyoxal produced more SA. This observation suggests that α-dicarbonyls are important contributors for SD to occur during wine storage. In red wines it was found recently a negative correlation between diacetyl and the accumulation of SA (Bueno *et al.*, 2018). Moreover it was demonstrated in wine model solutions that the contribution of MG for the formation of PA was higher compared with the reaction of *o*-quinones with the amino acid (Monforte, Martins and Silva Ferreira, 2019). Confirmed as well by the low reactivity observed between *o*-quinones and amino acids (phenylalanine and methionine) when compared with other nucleophiles (Nikolantonaki *et al.*, 2012).

Negative correlations were found between PA and glucose (r^2=0.57, p <0.01), fructose (r^2=0.67, p <0.01), iron (r^2=0.57, p <0.01). Meaning that samples with high amounts of sugars have less SA. This fact confirms the observations in wine model solutions and in real wines that glucose can inhibit SA formation, in particular phenylacetaldehyde formation (Monforte, Martins and Silva Ferreira, 2018).

The negative correlation between iron and SA, can be explained by the amounts of iron found in samples (0.2-2.5 mg/L) and due to the fact that wine oxidation be dependent on the amount of Fe (II) and Fe(III) as well as their ratio (Danilewicz, 2016).

It was observed a positive correlation of furfural with glucose (r^2=0.63, p <0.01) and iron (r^2=0.73, p <0.01) (Figure 8.3). The formation of this compound in aged bottled white wines was proposed to be due to the degradation of carbohydrates in particular glucose which validates their correlation. Moreover iron could be a catalyser of the reaction by accelerating sugars degradation, and in several model solutions it was shown that metals can increase furfural formation (Gökmen and Şenyuva, 2007).
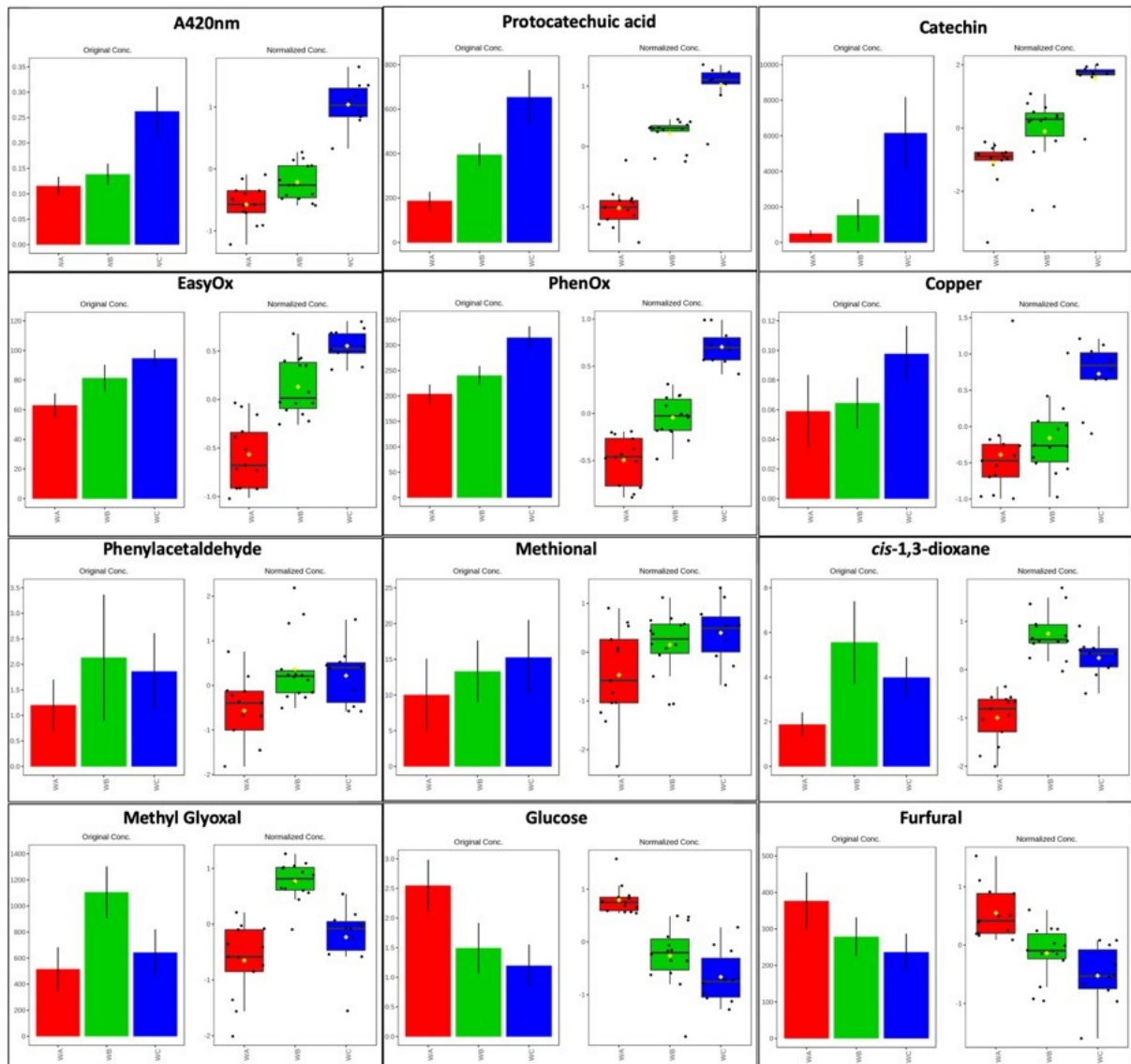
Figure 8.3 Bar plot of original values (mean ± standard deviation) and combined box-and-whisker and dot plot of normalized intensity for A420, protocatechuic acid, catechin, easyox, phenox, copper, phenylacetaldehyde, methional, cis-1,3-dioxane, methyl glyoxal, glucose and furfural. Red: WA; Green: WB; Blue: WC. Bar plot concentrations expressed in µg/L for all compounds except for glucose (mg/l).

8.3.3. Supervised Approach by Phenylacetaldehyde

In order to getter more information regarding SA formation in wines in particular PA, and due to the fact that no significant differences were observed between wine type (Figure 8.3), three classes were established based on PA concentration: L1 (< 1ug/L), L2 (<1<2 ug/L) and L3 (>2 ug/L) (Figure 8.4A). Partial least-squares discriminant analysis (PLS-DA) was then used for classes separation regarding the established categories. Separation was observed in the PLS-DA score plot with a slight overlap between classes (Figure 8.4B). Nevertheless, the PLS-DA model had an accuracy of 0.67 and R^2 and Q^2 of 0.82 and 0.52 respectively, values above the acceptability threshold of 0.5.
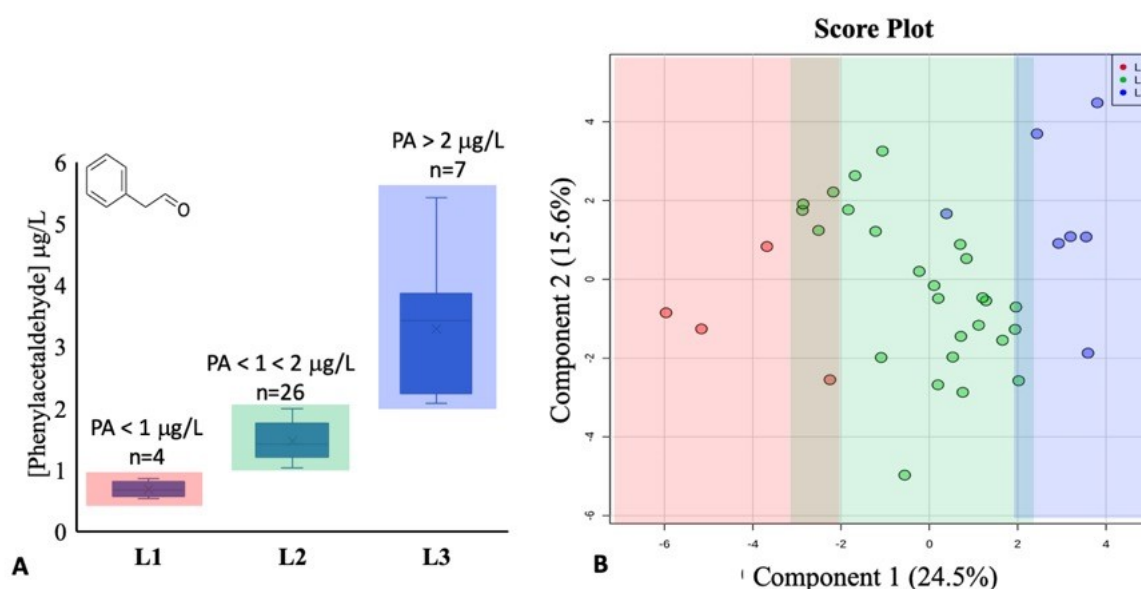
Figure 8.4 (A) Boxplot of phenylacetaldehyde classes (L1, L2 and L3) and (B) 2D PLS-DA score plot, the colour indicated in each point correspond to the groups of the boxplot.

According to the Variable Importance in the Projection model (VIP), *p-value* and PLS-DA-based ROC curves nine parameters contributed to PA classification (Table 8.3). The AUC values were calculated based on classes L1 and L3. Variables with the major influence on the PA classes were SA (2- and 3-methylbutanal, isobutanal, methional), hexanal, *cis*-1,3-dioxane, A420, methyl glyoxal and glucose.

Hexanal is a product of the oxidative degradation of fatty acids, and has been related in oxidized white has contributors to 'liquor' and 'pungent' sensory descriptors (Escudero *et*

al., 2002). Correlation of this compound with PA was never reported. Cis-1,3-dioxane, is one of the acetals of acetaldehyde and glycerol, this type of compounds are markers of oxidation in wines (Silva Ferreira, Barbe and Bertrand, 2002).

As observed above in the correlation heat map, the calculated parameters (VIP, p-value and AUC) confirm the relevance of methyl glyoxal and glucose in Strecker aldehydes formation and Figure 8.5 shows the impact of MG and glucose in the three established groups for PA, validating the result.

We report here a characterization of several groups of molecules/parameters in bottle aged wines and its correlation with their oxidative state (i.e. phenylacetaldehyde formation).

Table 8.3 Differential parameters and their VIP, p-value, AUC and mean ± standard deviation for each class (L1, L2 and L3).

Compound	VIP	*p-value*	AUC (L1 vs. L3)	Mean ± standard deviation		
				L1	L2	L3
3-methylbutanal	2.1	< 0.001	0.99	22 ± 10	45 ± 18	79 ± 46
cis-1,3-dioxane	2.0	< 0.001	0.93	1.6 ± 0.8	3.6 ± 1.3	5.9 ± 2.8
Methional	1.9	< 0.001	0.99	6.5 ± 3.7	12 ± 4	17 ± 5
Methyl glyoxal	1.8	< 0.001	0.99	337 ± 119	780 ± 277	995 ± 326
Hexanal	1.5	< 0.001	0.93	5.3 ± 1.6	6.9 ± 1.8	10.6 ± 4.2
Isobutanal	1.5	< 0.001	0.86	70 ± 40	103 ± 30	142 ± 66
A420	1.4	< 0.001	0.98	0.09 ± 0.01	0.16 ± 0.06	0.2 ± 0.07
2-methylbutanal	1.4	< 0.01	0.86	25 ± 12	37 ± 9	48 ± 18
Glucose	1.3	< 0.01	0.99	2.9 ± 0.7	1.6 ± 0.6	1.6 ± 0.5
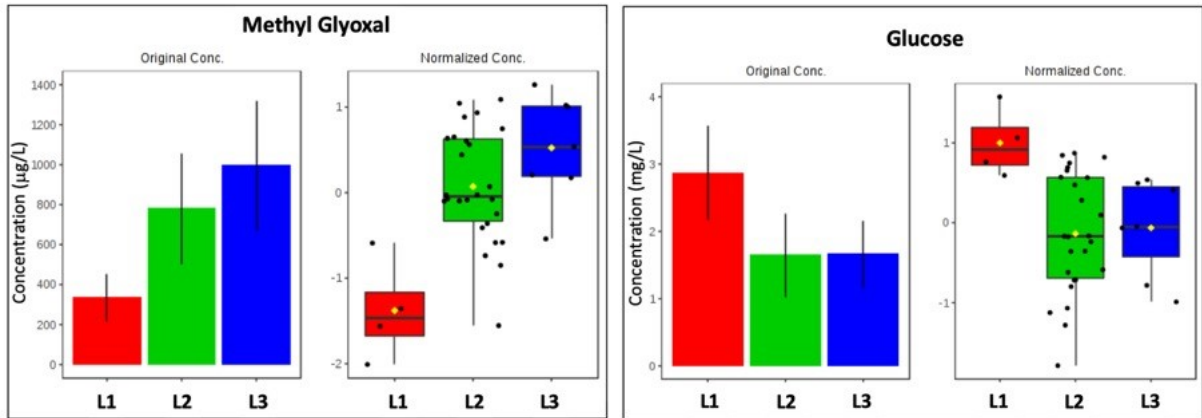
Figure 8.5 Concentration bar plot and traditional boxplot for methyl glyoxal and glucose for the three groups based on PA concentration L1, L2 and L3.

8.4. CONCLUSION

In conclusion by assessing the 47 parameters a comprehensive understanding of the evolution of oxidation patterns after 8 years of storage was established. The three types of wines showed different trends. It was demonstrated that phenolic compounds (catechin, epicatechin, protocatechuic acid and coumaric acid) and copper contributed mainly to the browning, while methyl glyoxal and glucose have a positive and negative impact respectively in the formation of Strecker aldehydes, and off-note generation in aged white wines.

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CHAPTER 9 CONCLUSIONS

9. CONCLUSIONS

The main objectives of this thesis work were to:

- o To identify and quantify the main factors with impact in the formation of Strecker Aldehydes.
- o To reveal relevant kinetic data and determine the storage conditions dependence of the estimated parameters.
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- o To develop an array of detectors and respective acquisition parameters capable to detect the systems changes over storage time and join together all the information (data fusion) – "Omic Sensor".
- o To conduct computational analysis and use multivariate analysis techniques to extract biomarkers related with each mechanism.

The combination of human and technical resources has made it possible to complete the majority of these objectives over the time available to us. We propose in this final part to summarize the results obtained for each problem and to identify the possible axes to continue this study.

Chapter 3. Strecker aldehydes formation in wine: new insights into the role of gallic acid, glucose and metals in phenylacetaldehyde formation

Strecker degradation (SD) leading to the formation of phenylacetaldehyde (PA) was studied in wine systems. New insights were gained by using two full factorial designs focusing on the effect of: 1) pH and 2) temperature. In each DoE three factors: glucose, gallic acid and metals at two levels (present or absence) were varied while phenylalanine was kept constant. The obtained results gave a clear indication, with statistical significance, that in wine conditions, the SD occurs in the presence of metals preferentially via the phenolic oxidation independently of the temperature (40°C or 80°C). The reaction of the amino acid with the *o*-quinone formed by the oxidation of the gallic acid seems to be favored when compared with the SD promoted by the reaction with α-dicarbonyls formed by MR between glucose and phenylalanine. In fact, kinetic results showed that the presence of glucose, had an inhibitory

effect on PA rate of formation. PA formation was 4 times higher in the control wine when compared to the same wine but with 10 g/L of glucose added. By gallic acid quinone quantitation it is shown that glucose affects directly the concentration of the quinone. decreasing the rate of quinone formation. This highlights the role of sugar on the o-quinones concentration and consequently on the impact on Strecker aldehydes formation, a promising new perspective regarding wine shelf-life understanding.

Chapter 4. Applicability of PTR-TOF-MS to study the kinetics of phenylacetaldehyde formation in model solutions in dynamic conditions

This chapter shows key possibilities in modelling the kinetics of phenylacetaldehyde, taking into account the most relevant factors found in Chapter 3. The molecule formation was evaluated as a function of sugar, phenolic compounds, metals and sulphur dioxide. The release kinetics were measured online by proton transfer reaction-mass spectrometry (PTR-MS). Phenylacetaldehyde formation was fitted using Weibull models and an activation energy of 73 kJ/mol estimated. Also, a confirmation that glucose can inhibit the aldehyde formation was demonstrated, and the sequential additions in real time showed that the inhibition level was dependent on metal ions presence. Moreover, for the first time it was observed in real time the capacity of SO_2 to bind with phenylacetaldehyde, and by trapping it, lowering its release. Finally, the impact of pH and temperature in the stability of the formed adducts and underling release mechanism is also elucidated.

Chapter 5. Response Surface Methodology: a tool to minimize aldehydes formation and oxygen consumption in wine model system.

To model the formation of phenylacetaldehyde and create a tool to predict is formation based on several conditions response surface methodology was applied to study the effect of precursors on o-quinone and phenylacetaldehyde formation in wine model systems stored at 40ºC for 24 hours. The results confirmed that glucose plays an important role in reducing aldehyde formation by inhibiting the formation of o-quinone. The regression equations showed that oxygen consumption followed a 2nd polynomial equation whereas phenylacetaldehyde and o-quinone best fit with a polynomial function containing quadratic terms. These behaviors indicate that different pathways are involved in the respective aldehyde formation and oxygen consumption. RSM revealed to be a powerful tool to better understand key chemical reactions. By considering a number of factors, individually and in combinations, the derived equations predicted that the best combination to minimize

phenylacetaldehyde was achieved for high glucose levels and low amounts of gallic acid and metals. An important information, seen of great value, when trying to improve wines sensorial properties during shelf-life.

Chapter 6. Impact of Phenolic Compounds in Strecker Aldehydes Formation in Wine Model Systems: Target and Untargeted Analysis

In this chapter the Strecker degradation of phenylalanine has been extended to several phenolic compounds and methyl glyoxal was evaluated as a relevant substrate. The study was performed in in phenolic/phenylalanine wine model systems. Six phenolic compounds (3,4-dihydroxybenzoic acid, gallic acid, caffeic acid, ferulic acid, catechin and epicatechin) were compared in the formation of phenylacetaldehyde when in the presence of glucose or methyl glyoxal. The addition of glucose reduced the formation of the Strecker aldehyde, independently of the phenolic compound. The addition of methyl glyoxal, on the other hand, increased phenylacetaldehyde formation for the hydroxybenzoic acids, and decreased for the flavan-3-ols, confirming their capacity to trap the dicarbonyl compound.

As target phenolic, catechin was chosen to perform kinetic studies to further understand the reaction intermediates involved in the mechanism of phenylacetaldehyde formation in particular: catechin-*o*-quinone and catechin-methylglyoxal adduct. The addition of glucose and methyl glyoxal increased the consumption of catechin while a reduction in the respective *o*-quinone was observed, suggesting that these substrates have an impact in other reactions involving catechin. In that regards for the first time it was demonstrated that the catechin-MG adduct was capable to oxidize and form a new *o*-quinone, contributing for wine instability promoted by oxidation reactions.

Chapter 7. Discrimination of wine "oxidation-status" based on untarget peak picking approach with multi-class target coupled with machine learning algorithms.

In the previous chapters it has been demonstrated that several factors could affect the formation of Strecker aldehydes in wine model conditions. Nevertheless, in real wines the complexity of the several chemical reactions involved turns the capacity of white wine deterioration prediction and consequently the capacity to avoid extremely difficult.

In these chapter the chemical information regarding the volatile fraction affected by oxidation has been studied and several molecules were considered key markers of "oxidative spoilage".

In order to have a holistic view of the chemical profile we propose an untarget methodology based on machine learning algorithms capable to classify wines according to their "oxidative-status". Instead of the normal approach using one class for classification in this work eight classes were selected based on target oxidation markers for the extraction of relevant compounds. Two approaches for feature selection were used: VIPS from OPLS-DA and mean decrease accuracy from random forest. Furthermore, *p-values* and *q-values* were set as molecules filter. These 4 parameters rule outlines 51 molecules correlated with 5 classes, from which 23 were selected has having higher sensitivities (AUC > 0.85). Compounds such as benzofuran, cis- and trans- linalool oxides, acetals of acetaldehyde and glycerol and acetaldehyde with 2,3-butanediol, between others were found to have good predictive abilities in which regards wine oxidation state.

For the first time to our knowledge hydroxy esters ethyl-2-hydroxy-3-methylbutanal and ethyl-2-hydroxy-4-methylpentanal were found to be correlated with oxidation markers (furfural and methional) and consequently to be discriminant of the wine-oxidation status. Correlation networks based on mean decrease accuracy of the 23 compounds shown that oxidation status of wines affected several hubs of target compounds.

Chapter 8. Changes in white wine composition after treatment with cationic exchange resin: impact on wine oxidation after 8 years of bottle storage.

In this chapter we take into account the complexity of study different wines stored for different periods of time, as well as the impact of wine composition in deterioration over storage time.

In order to study the same wine affected by a particular perturbation, samples from 3 wine types were treated with a cationic exchange resin (7 lots) and stored for 8 years (47 samples). Forty-seven parameters were determined, including (1) important substrates with impact in white wine oxidation and (2) markers of oxidation. From group 1, sugars, elements, phenolic compounds, α-dicarbonyls and SO_2 and from group 2, browning (A420), acetaldehyde, alkanals, furanic compounds were quantified.

Results regarding the cationic exchange resin impact after storage shown that is dependent on wine composition.

Good correlations with browning were obtained for wines with higher concentration of phenolic compounds (flavan-3-ols, protocatechuic and coumaric acids) and copper. While aromatic degradation related with the formation of Strecker aldehydes was positive correlated with methyl glyoxal and negatively correlated with iron and glucose.

PLS-DA was performed against three classes established based on phenylacetaldehyde formation, and results confirm that methyl glyoxal is a substrate for phenylalanine Strecker degradation and the presence of glucose can reduce the formation of the aldehyde after long periods of storage.

In conclusion, all the studies demonstrated the chemical complexity involved in the formation of Strecker aldehydes in wine. In particular the interaction between reactions substrates and their impact in the kinetics of phenylacetaldehyde formation. The application of a chemiomics pipeline shown in the last three chapters the advantages of having a holistic perspective of the changes occurring during wine storage.