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CONTRIBUTION OF WINE MICROORGANISMS TO THE AROMA COMPOSITION OF WINE AND ITS SENSORY IMPACT

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PhD Thesis by

Inês Pereira Biscaia de Oliveira

January 2019

Supervisors:

Dr. Vicente Ferreira González

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Laae
Laboratorio de Análisis de Aroma y Enología

MicroWine
A Marie Curie Initial Training Network

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Instituto Universitario de Investigación Mixto Agroalimentario de Aragón
Universidad Zaragoza

Contribution of wine microorganisms to the aroma composition of wine and its sensory impact

Departamento de Química Analítica
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by

Inês Pereira Biscaia de Oliveira

Dissertation presented for the degree of
Doctor of Philosophy in Analytical Chemistry

January 2019

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D. VICENTE FERREIRA GONZÁLEZ, Catedrático del Departamento de Química Analítica y D. ULRICH FISCHER, director en el Instituto DLR-Rheinpfalz, Alemania

CERTIFICAN:

Que la presente memoria, titulada "Contribution of wine microorganisms to the aroma composition of wine and its sensory impact" correspondiente al plan de investigación aprobado por la Comisión de Doctorado del Departamento de Química Analítica y presentada para optar al grado de doctora en Ciencia Analítica en Química, ha sido realizada bajo nuestra dirección por Dª. Inês Pereira Biscaia de Oliveira, autorizando su presentación para proseguir los trámites oportunos y proceder a su calificación por el tribunal correspondiente.

Zaragoza, 17 enero de 2019.

Fdo. Dr. Vicente Ferreira González Fdo. Dr. Ulrich Fischer

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Aos meus pais, avós e Tiago

Vossa mercê tem razão
E é ingratidão falar mal do vinho.
E a provar o que digo
Vamos, meu amigo,
A mais um copinho!

"Eu já fui", reponde o vinho,
"folha solta a brincar ao vento,
Fui raio de sol no firmamento
Que trouxe à uva, doce carinho.

Ainda guardo o calor do sol
E assim eu até dou vida
Aumento o valor seja de quem for
Na boa conta, peso e medida

Amália Rodriguez In Oiça lá ó senhor vinho

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Presentation

Presentation

This thesis was integrated in the Initial Training Network (ITN) Marie Curie "Microwine" which intended to build a network of 15 PhD to study the action of microorganism on grapevine and vineyard, on winemaking and on wine preservation. The network encouraged straight collaboration between individual projects to take advantage of as many scientific tools as possible, to further understand wine environment and its dynamics.

This thesis was hosted at the Laboratorio de Análisis del Aroma y Enología (LAAE), having as main collaborator partner DLR-Rheinpfalz in Germany.

The work is divided into three sections addressing three different topics regarding wine aroma formation. The first two sections were performed using model fermentations and the third section was performed with real wine and in straight collaboration with the group in DLR-Rheinpfalz.

Firstly, the specific goals of each section are presented.

Secondly, the bibliographic introduction, covering the main topics which will be further discussed throughout the thesis, includes a brief definition of wine aroma, a description of the main aroma precursors in grape, characterization of important metabolic routes active during alcoholic fermentation and chemical processes that yield an aroma compounds during wine aging. Differences between spontaneous and inoculated fermentations are also described as well as the role of different yeast genera in wine aroma variability.

Each section contains a smaller and redirected introduction and description of the different methodologies used, the results and respective discussion and individual conclusions.

The first section addresses the origin of aldehydes linked with wine oxidation during wine storage, assessing the effects of yeast, the levels of metal cations, such as zinc, and must supplementation with SO_2.

The second section focused on the hierarchy of factors affecting the aroma profile of Riesling and Garnacha wines: yeast from different genera, glycosidic precursors and time.

The third section has focused on Riesling aroma profile, exploring the changes introduced by different wine appellations and vineyard versus cellar microbiome on the wine chemical composition and its sensory impact.

Finally, general conclusions, industrial relevance and future perspectives are given at the end of the Thesis.

Global tables with quantitative data as well as some statistical studies performed to better understand the data, but not crucial for the development of the different chapters are presented in supplementary data.

Esta tesis está integrada en el programa Microwine, una de las Marie Curie Initial Training Network, formado por 15 PhD y cuyo objetivo principal ha sido estudiar la actividad microbiana en la vid, en los viñedos, durante la producción del vino y también durante la crianza. Esta red ha incentivado la colaboración entre los diferentes proyectos individuales aprovechando de esta forma, la amplia oferta de herramientas científicas con el objetivo de dar respuesta a las cuestiones que todavía existen sobre el mundo del vino

Esta tesis ha sido llevada a cabo en el Laboratorio de Análisis del Aroma y Enología (LAAE) colaborando también con el grupo de investigación del Instituto DLR-Rheinpfalz en Alemania.

Este trabajo está dividido en tres secciones que han abordado diferentes temas relacionados con la formación del aroma en el vino. Las dos primeras secciones han sido realizadas con vino modelo y la tercera con vino real en colaboración directa con el grupo de investigación del DLR-Rheinpfalz.

En primer lugar, se presentan los objetivos específicos de cada sección.

A continuación, en la introducción bibliográfica, se abordan los temas más importantes para la discusión de los resultados a lo largo de la tesis. Entre ellos, se hace una breve descripción de lo que es el aroma del vino, los principales precursores aromáticos en la uva, se describen importantes rutas metabólicas activas durante la fermentación alcohólica y algunos procesos químicos que contribuyen a la formación y modificación del aroma durante la crianza. Además, se describen las principales diferencias entre fermentaciones espontánea e inoculadas, así como también la contribución de diferentes géneros de levaduras en la variabilidad aromática del vino.

Cada sección comienza con una introducción dirigida al tema que se va a abordar, se describen los métodos utilizados y se presentan y discuten los resultados obtenidos. Finalmente, se resumen las principales conclusiones.

En la primera sección se estudia el origen de la formación de aldehídos asociados con la oxidación del vino durante la crianza, investigado el efecto causado por diferentes cepas de levaduras, el nivel de cationes metálicos como el zinc y la adición de SO_2 al mosto.

La segunda sección se centra en establecer la importancia que la utilización de diferentes tipos de levaduras, precursores glicosídicos y el tiempo de envejecimiento tienen sobre la formación del perfil aromático de vinos de Riesling y garnacha.

La tercera sección se centra en el perfil aromático del Riesling explorando los cambios en la composición química y en el plano sensorial producidos por

diferentes regiones vitivinícolas y por la población microbiana de los viñedos frente a la de las bodegas.
Finalmente, las conclusiones generales, los aspectos relevantes para la industria vitivinícola y las perspectivas futuras se presentan al final de la tesis.

Las tablas con los resultados de cuantificación generales y los análisis realizados para comprender mejor el conjunto de datos, pero que no han sido necesarios para la discusión de los mismos, se presentan en el material suplementario.

Introduction

Introduction

1. Wine flavour and its perception

Flavour of foods has long been described as the complex interaction of odour, aroma, taste and mouthfeel. Odour comprises the perception of volatiles directly by ortho-nasal via while the aroma entails the perception of volatiles which are ingested and detected by retro-nasal via. Major factors differentiating odour and aroma are volatilization temperature and the distinctive mass transfer conditions between the product and the product spread in buccal mucosa. Taste is well known by its attributes sweet, sour, salty, bitter and umami sensed by tongue receptors and mouthfeel describes the tactile sensation produced by food (Baert et al., 2012).

It is not an easy task to define flavour since it depends on some aspects which are specific to each individual, such as the sensitivities of their olfactory and taste receptors and their degree of experience. The flavour is related not only with the presence of taste and odour-active molecules in the product and to their concentration profile, but also with the way in which those molecules interact with the matrix, to the possible existence of different species in equilibrium and to their specific ability to break the wine aroma buffer. Furthermore, perceptual interactions between the individual perceptions elicited by each odorant will also affect the way they are perceived by human senses (Ferreira, 2010).

All the aroma-sensory attributes found in wine are caused by one or more molecules which were present in sufficient concentration to surpass the wine aroma buffer. The wine aroma buffer refers to the specific sensory properties of the mixture of 27 compounds from different chemical families found in all wines and alcoholic beverages at the concentrations produced in a normal alcoholic fermentation. These molecules are the main secondary products of alcoholic

fermentation and are responsible for important processes of aroma suppression, particularly of fruity and woody notes (de-la-Fuente-Blanco et al., 2016). The composition of the wine buffer can slightly change since it depends on yeast metabolism and other oenological practices, but, all in all, its sensory profile does not change much and is described as "vinous" (Ferreira et al., 2019).

An aroma vector is defined as "a perceptual unit constituted by one or several molecules with similar aroma descriptors, which altogether and in an integrated form, are responsible for a specific set of sensory features of a type of products; wine in our case" (Ferreira et al., 2019).

Wine flavour is one of the most complex and difficult to characterize and manage, since there is a huge variability associated with its formation. Factors like grape variety, vine management, sanitary conditions, location and soil type, microorganisms involved in fermentation, technological choices of wine making practices, additives, wood barrels and wine preservation are only a few examples of important sources of aroma variability (Fischer et al., 1999; Robinson et al., 2014).

1.1 Wine aroma genesis

Wine volatiles can be classified according to their chemical structure, their odour into aroma families, or according to their contribution to a specific aroma vector. However, in this work we are more interested in a classification according to their genesis into the following categories:

1. Aroma compounds derived from specific precursors in the grape
2. Fermentative aroma compounds from unspecific precursors:
    a. Related to the grape variety
    b. Unrelated to the grape variety
3. Aroma compounds formed or extracted during aging

The most important grape varieties for winemaking have a rather neutral aroma character, however, they contain a series of specific aroma precursors, which after a more or less complex chemical process including hydrolysis, enzymatic cleavage or spontaneous chemical rearrangement, render the odorant. Compounds in this category are easily identified as varietal aroma compounds because chemically they have been built by the grape. Specific aroma precursors are glycosidic precursors and cysteinylated and glutathionylated precursors being the former responsible for the formation of terpenes, norisoprenoids and volatile phenols and the later for polyfunctional mercaptans (Ferreira, 2010). A third compound in this category is the amino acid S-methyl methionine, the precursor of dimethyl sulphide (DMS) (Landaud et al., 2008).

However, grapes also contain a more or less specific profile of nutrients which will determine yeast metabolism and hence, also the fermentative aroma profile. Those grape components influencing yeast metabolism can be regarded as unspecific aroma precursors related to the grape variety. These compounds are most often classified as fermentative, since structurally they have been built by the yeast and not by the grape, however, they may have a very important role in the identity of the specific aroma of the variety.

Important non-specific grape precursors are amino acids which will lead to the formation of compounds like higher alcohols, branched acids, their ethyl esters and the acetate esters of higher alcohols. The specific profile of compounds of these chemical families formed during fermentation is strongly linked to the specific grape amino acid profile, however its formation occurs due to alcoholic and/or malolactic fermentation (Ferreira, 2010; Hernández-Orte et al., 2002; Swiegers et al., 2005).

There are of course, other fermentative compounds formed from unspecific aroma precursors not related with the grape variety. Ethanol is the most important in this category, but hydrogen sulphide (H_2S), whose levels are

strongly determined by the residues of elemental sulphur sprayed to the vine, can be also classified in this category (Jiranek et al., 1995; Mendes-Ferreira et al., 2009).

Finally, aged-related aroma is formed by molecules extracted from the wood such as whiskylactones, or formed by oxidation of different precursors, such as strecker aldehydes or sotolon, or formed by the reaction of wine components, such as furfurylthiol which is formed by reaction between H_2S and furfural. Little amounts of H_2S or Methanethiol (MeSH) formed by slow catalytical decomposition of S-amino acids also belong to this category (Ferreira, 2010; Loscos et al., 2009).

1.2 Grape variety-related non-specific precursors

The main non-specific grape precursors are grape amino acids (Albers et al., 1996; Hernández-Orte et al., 2002). Other compounds which may be regarded as unspecific precursors are grape lipids, notably phytosterols. A very recent report demonstrates that the wine aroma signature strongly depends on the presence and type of these compounds (Fairbairn, 2018). However, and to the best of our knowledge, there are no further clues about the aroma compounds related to the presence of those compounds, although it can be hypothesized that they are fatty acids and their corresponding esters.

Amino acids are the most well-known grape elements related with odorants. Early studies revealed that levels of higher alcohols, their acetates, branched acids and their ethyl esters were linked to the grape variety with which the wine was made (Ferreira et al., 2000). Later, it was demonstrated that the fermentation of synthetic musts containing the characteristic amino acid profiles of each variety, effectively produced specific aroma profiles containing all these aroma compounds (Hernández-Orte et al., 2002).

1.3 Glycosidic precursors

Glycosylation is a common transport and detoxification plant mechanism and thus has been described in several plant species (Sarry and Günata, 2004; Winterhalter and Skouroumounis, 1997). Glycosidic precursors are formed by units containing a β-$_D$-glucose moiety (sugar moiety) and an aglycone which will produce the volatile molecule (Winterhalter and Skouroumounis, 1997). Common sugar moieties in grapes are α-L-arabinofuranosyl-β-D-glucopyranoside, α-L-rhamnopyranosyl-β-D-glucopyranoside, β-D-xylopyranosyl-β-D-glucopyranoside, β-D-apiofuranosyl-β-D-glucopyranoside, and β-D-glucopyranoside-β-D-glucopyranoside. Regarding the aglycone, the diversity is immense and includes terpenes, C_{13}-norisoprenoids, C_6-alcohols, volatile phenols and benzyl derivatives (Liu et al., 2017; Wilson et al., 1984; Winterhalter and Rouseff, 2001; Winterhalter and Skouroumounis, 1997).

Glycosidic precursors contribute to the varietal expression of wine aroma and are a huge source of aroma variability, due to the high number of factors affecting the composition of this fraction and also to high number of factors affecting the odorants finally produced from it.

These precursors were initially identified and studied in the aromatic variety Muscat liking monoterpenes like linalool and geraniol with its varietal character. Their importance was further confirmed in other varieties like Gewürztraminer and Riesling (Günata et al., 1985; Strauss et al., 1986). Other relevant aroma compounds like β-damascenone, cis-rose oxide, 1,1,6-trimethyl-1,2-dyhydronaphthalene (TDN) or 4-vinylphenol were further identified in Riesling, Gewürztraminer and other grape varieties and were also linked to glycosidic precursors (Parker et al., 2017; Sefton et al., 2011; Winterhalter and Rouseff, 2001; Winterhalter and Skouroumounis, 1997).

Some of these precursors are able to produce a free odorant by direct hydrolysis of the aglycone (Strauss et al., 1986; Wilson et al., 1984), however other compounds can have multiple intermediary precursors, generally non-volatile, which by a series of rearrangements will ultimately originate a free volatile. One of the most studied cases are the compounds derived from carotenoids (Winterhalter and Rouseff, 2001).

However, normal glycosylation mechanism that occur in grapevines (Winterhalter and Skouroumounis, 1997), are not the only source of glycosidic precursors. The plant is also able to glycosylate volatile molecules existing in the external environment being the best-known case from smoke. The appearance of smoke-taint in wines after large wildfires close to vineyards was attributed to the glycosylation of volatile phenols, such as guaiacol and 4-methylguaiacol, present in the atmosphere. The study conducted by Kennison et al., 2008 comparing grape juice and corresponding final wine from vineyards exposed to smoke and unsmoked grapevines, has shown that volatile phenols present in smoke were accumulated as non-volatile grape glycosidic precursors in exposed grapes, but they were only revealed as free odorants in final wine after must fermentation, reaching levels resulting in consumer rejection. Further analysis on bottled aged wine showed that levels continued to increase indicating that these molecules kept on being hydrolysed from the glycosides by slow but spontaneous chemical hydrolysis.

1.4 Cysteinylated and glutathionylated precursors

Cysteinylated and glutathionylated precursors were first discovered in Sauvignon Blanc and are responsible for the formation of the potent varietal polyfunctional mercaptans in wine: 4-mercapto-4-methyl-2-pentanone (4M4M2P), 4-mercapto-4-methyl-2-pentanol (4M4M2POH), 3-

mercaptohexanol (3MH) and 3-mercaptohexyl acetate (Darriet et al., 1993; Peña-Gallego et al., 2012). These compounds are present in wine in ranges of ng/L but have an extreme odorant potential of tropical fruits, guava, passion fruit, grapefruit or boxtree. Depending on the concentration, they can also be perceived as sweat or onion (Ferreira and San Juan, 2012; Mateo-Vivaracho et al., 2010; Peña-Gallego et al., 2012).

1.5 Effects of external additives on wine aroma

In addition to grape composition, also external additives can influence the formation of aroma compounds during alcoholic fermentation. These elements include metal cations often added as fertilizers or pesticides, SO_2 added to preserve wine quality or oak barrels to mention only a few examples. These elements integrate wine production process and can highly affect the microbiome activity by modulating their enzymatic response signal by the presence of certain metal cations (De Nicola et al., 2009) or by having antiseptic action as in the case of SO_2. Oak barrels can contribute highly to the extraction of wood molecules such as lactones, however they were also linked with the appearance of off-flavours by spoilage yeast activity (Ferreira, 2010; Malfeito-Ferreira, 2011; Ribéreau-Gayon et al., 2006).

Zinc, for instance, is present in vineyards and consequently in grape must. It has a wide range of sources from water pipes to pesticides (De Nicola et al., 2009; Hopfer et al., 2015). Yeast cells regulate the uptake of zinc by membrane transporters and the activation or inhibition of several yeast metabolic pathways are regulated by the zinc external concentration through a specific metal-responsive regulatory protein (De Smidt et al., 2008). This cation is crucial for the development of yeast strains, since it integrates the active-site of 6 important

classes of enzymes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (De Nicola et al., 2009).

SO_2 is commonly used in winemaking due to its antiseptic and antioxidant properties. When added to the must in sufficient concentration, SO_2 destroys most of wild spontaneous yeast and bacteria, preventing spontaneous fermentation (Henick-Kling et al., 1998). Later on, during storage, this compound is also added to the wine for preventing the proliferation of spoilage microorganism like acetic acid bacteria or *Brettanomyces spp.*, thus, avoiding the formation of off-flavours like vinegar and horse sweat, respectively (Malfeito-Ferreira, 2011; Ribéreau-Gayon et al., 2006).

Additionally, SO_2 is highly reactive with oxygen and carbonyl compounds, thus it prevents direct oxidation by binding with dissolved oxygen and by inhibiting oxidation enzymes. On an indirect manner, it prevents typical oxidation flavours in wine by binding to aldehydes such as acetaldehyde (Ferreira et al., 2015; Ribéreau-Gayon et al., 2006).

2. Wine aroma formation from specific and non-specific precursors during alcoholic fermentation

Fermentations have a crucial role on the formation of wine aroma. On one hand, microorganisms are directly responsible for the formation of many aroma compounds found transversely in all wines (fermentative compounds), but they are also important modulators in the production of varietal flavours from specific precursors. The formation of these last compounds is not, however, fully understood, despite large research conducted to date.

2.1 Spontaneous versus inoculation fermentations

In nature, several yeast genera have been isolated both from vineyards and wineries, which demonstrates the existence of a complex microbiome in both locations (Barata et al., 2012; Fleet, 2003). *S. cerevisiae* has been described as the "wine yeast" due to its high fermentative vigour and resistance to other competitor microorganism and even antiseptics like SO_2 (Fleet, 2003; Henick-Kling et al., 1998; Jolly et al., 2014). However, other yeast genera, mostly non-*Saccharomyces* yeasts, were shown to be active during the latent phase of normal alcoholic fermentations after which *S. cerevisiae* takes over, carrying most part of the alcoholic fermentation (Barata et al., 2012). However, not all the yeast active were considered positive and rather were often associated with the formation of off-flavours like acetic acid and ethyl acetate (Jolly et al., 2014). For this reason, for years, traditional winemaking has preferred to use commercial dried yeast products of isolated *S. cerevisiae* or *S. bayanus* strains. The addition of a large number of cells to the grape must ensures that any spontaneous microorganisms are surpassed, obtaining a controlled fermentation and avoiding stuck or problematic fermentations.

This approached ensures indeed higher control over fermentative processes, but has been demonstrated to lead to standardization of wines and thus, new studies have been done to assess which non-*Saccharomyces* genera could lead to positive traits in wine (Henick-Kling et al., 1998; Hernández-Orte et al., 2008; Jolly et al., 2014; Padilla et al., 2016).

During alcoholic fermentation, *S. cerevisiae* is not only responsible for the conversion of glucose and fructose into ethanol and CO_2, but also for the formation of important volatiles due to the secondary yeast metabolism. The wine metabolome is dependent on the pool of grape precursors and on the efficiency with which they are converted into volatile molecules during alcoholic fermentation. The formation of volatile compounds through yeast

metabolism is obviously dependent on their enzymatic activity and hence, on their genetic pool, being this the reason why the intervention of non-*Saccharomyces* yeast in winemaking can be an important source of aroma variability (Hernández-Orte et al., 2008; Loscos et al., 2007; Padilla et al., 2016). Currently, in line with the desire to obtain controlled fermentations, avoiding the formation of off-flavours but obtaining complex wines and maintaining its varietal character, several starter cultures of non-*Saccharomyces* yeast strains have been developed and protocols for sequential or for co-inoculation of non-*Saccharomyces* and *S. cerevisiae* strains have been implemented in the winemaking industry (Benito et al., 2015; Gobbi et al., 2013; Zott et al., 2011).

2.2 Yeast metabolism during alcoholic fermentation

The two major metabolic pathways of *S. cerevisiae* during alcoholic fermentation are glycolysis and amino acid catabolism, as shown in Figure 1 as numbers 1 and 2, respectively.

During these pathways several enzymes are required and some of the reactions are actually reversible, being the regulatory mechanisms determined by external factors such as sugar concentration, presence of metal cations or cell requirements like NAD(P)H or restoring the redox balance (Hirst and Richter, 2016; Ribéreau-Gayon et al., 2006). During glycolysis several of the major compounds in wine are formed: ethanol, glycerol and acetaldehyde. The first compound has high importance on the volatilization of other odorants, the second contributes for a rounder mouthfeel and the third, depending on its concentration, may contribute to wine aroma, being usually linked to oxidative processes contributing with overripe and oxidized apple notes (Styger et al., 2011; Swiegers et al., 2005).

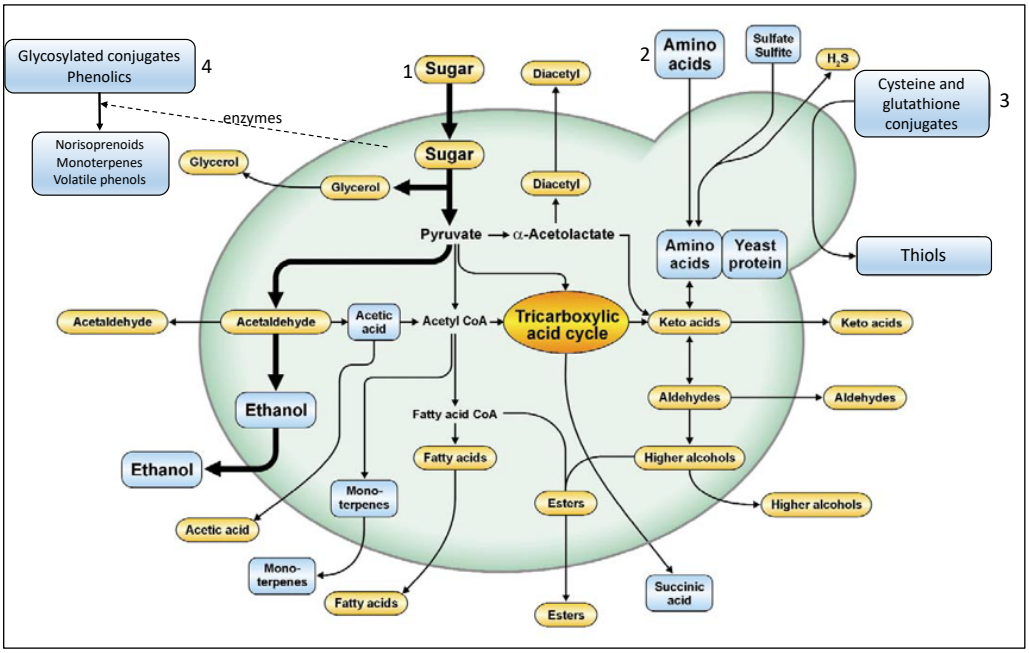

Figure 1 Aroma volatiles produced due to yeast metabolism from and non-specific precursors (1 and 2) and secretion of yeast enzymes hydrolysing specific grape precursors (3 and 4) during alcoholic fermentation by S. cerevisiae adapted from Swiegers et al., 2005.

Furthermore, yeast strains use nitrogen sources to ultimately, produce fusel alcohols, having as intermediary molecules acids and aldehydes. The esterification of fusel alcohols with acetic acid produces fruity esters which highly contribute to the final profile of wine. Here, the ability of each strain to carry these metabolic pathways is determinant for the volatile composition, however the grape pool of non-specific precursors is also crucial (Hernandez-Orte et al., 2006; Styger et al., 2011). During amino acid catabolism or Ehrlich pathway (Figure 2), several enzymes are involved and some of them have been identified, such as seven different alcohol dehydrogenases encoded by seven genes. Other enzymes are also involved, such as aminotransferases, transaminases and esterases (Ehrlich, 1904; Hazelwood et al., 2008; Ribéreau-Gayon et al., 2006).

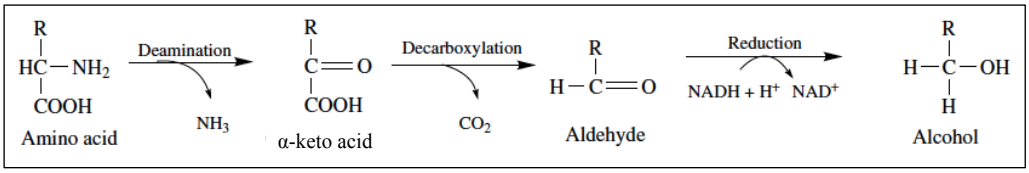

Figure 2 Reactions of the Ehrlich pathway leading to fusel alcohols from grape amino acids In Ribéreau-Gayon et al., 2006)

Several of the compounds formed at relatively high levels in wine are related to the catabolism of the branched-chain amino acids valine, leucine and isoleucine. Isobutanol, isoamyl alcohol and 2-methyl butanol and isobutyric, isovaleric and 3-methylbutyric acids are products of this catabolic pathway. The redox requirements of the yeast cell determine if the aldehyde is further reduced to a higher alcohol, using NADH or oxidized to the volatile acid producing NADH. Both compounds have relevance in wine aroma.

The aromatic amino acids phenylalanine and tyrosine are also metabolized by yeast through Ehrlich pathway originating 2-phenylethyl alcohol and tyrosol, respectively. Methionine is similarly metabolized yielding methionol. In these last cases, however, the fraction of aldehyde oxidized to the corresponding acid (phenylacetic acid, p-hydroxyphenyl acetic and 3-(methylthio)propanoic acid) seems to be comparatively much smaller. Methionine is a sulphur containing amino acid and, in parallel to the normal Ehrlich pathway, it can originate methanethiol and α-ketobutyrate due to the action of a demethiolase (Hazelwood et al., 2008; Perpète et al., 2006). Moreover it can also yield cysteine via oxaloacetate, which is highly linked with the formation of volatile sulphur compounds such as hydrogen sulphide (Moreira et al., 2002).

Nonetheless, amino acids are not the single source of the compounds mentioned above, since they can also be produced through different anabolic pathways. Branched amino acids are formed from pyruvate formed in glycolysis (Eden et al., 2001; Hazelwood et al., 2008; Styger et al., 2011).

As mentioned, aldehydes are intermediaries in the formation of ethanol through glycolysis, but also in Ehrlich pathway. Acetaldehyde is the major carbonyl compound found in wine and during glycolysis is converted to ethanol by alcohol dehydrogenase enzymes oxidizing NADH to NAD^+ and restoring the cell redox balance. Alternatively, acetaldehyde can be oxidized to acetic acid as an intermediary in the pyruvate dehydrogenase bypass generating acetyl-CoA required for the lipid or amino acid metabolisms, among others (Peddie, 1990; Swiegers et al., 2005). Similar mechanisms to maintain cell redox balance occur in the amino acid catabolism to form branched acids and higher alcohols.

The esterification catalysed by alcohol acetyltransferases (AAT) of higher alcohols, or of ethanol with acetic acid originate acetate esters through an energy consuming reaction requiring acetyl-CoA. This reaction is highly strain dependent since several genes encoding the required enzyme have been identified in *S. cerevisiae* (Peddie, 1990; Swiegers and Pretorius, 2005). The major acetate produced is ethyl acetate, with a strong glue odour, but isoamyl acetate and phenylethyl acetate can be also present at relatively high levels in wine contributing with banana and rose like descriptors, respectively (Styger et al., 2011).

Medium chain fatty acids are produced by yeast in order to maintain the cell energy net generating acetyl CoA, which is quickly consumed at the beginning of alcoholic fermentation. As cell metabolism reduces there is an excess of fatty acids, thus esterification with ethanol occurs producing fatty acid ethyl esters via also acyl coenzyme A (Peddie, 1990; Saerens et al., 2008). This reaction is not fully known, however, data indicates that the reaction is catalysed by alcohol acyltransferases (Saerens, 2006).

Acetate esters are excreted from the yeast cell by simple diffusion while the ethyl esters require active membrane transportation (Peddie, 1990; Saerens et al., 2008).

The exact role of ester formation is not fully known and some studies even suggest that they have no relevance for yeast cell. However, a likely hypothesis is that they are formed to keep cell energy balance and to detoxify the cells of fatty acids (Peddie, 1990; Saerens et al., 2008, 2010).
Since the formation of esters is a reversible process, the overall formation of esters is dependent on the balance between esters, acids and alcohols as well as of the presence of ester-synthesizing enzymes and of esterases, which hydrolyse esters (Peddie, 1990; Saerens et al., 2010).

2.3 Release of aroma compounds from glycosidic precursors

The release of aroma volatiles from glycosidic precursors can occur directly or can require several intermediates and steps. Depending on the case, the production of the aroma volatile can occur due to normal acid hydrolysis, due to yeast enzymatic activity or can even require the concourse of both processes (Waterhouse et al., 2016).

2.3.1 Release and formation of monoterpenes

Monoterpenes are normal constituents of the grape glycosidic fraction. The three most important odorants derived from such fraction are linalool, geraniol and c-rose oxide. For the formation of the aroma volatile, the glycosidic bond has to be cleaved to release the aglycone. In some cases, the aglycone itself is the odorant, or alternatively, can be an intermediary precursor molecule, which by further reaction or rearrangement, will yield the odorant.
The ability of yeast to break the glycosidic bound is highly strain dependent (Gunata et al., 1988). Several yeast strains haven been screened for their ability to hydrolyse different aromatic glycosides and for their β-glycosidase activities, including some less common enzymes such as α-arabinofuranosidase, α-

rhamnosidase and β-xylosidase. Not all yeast genera encode these enzymes and β-D-glycosidase is the most general and the one found at highest levels in all cases.

The sugar forming the glycoside, can be simply glucose or can be a disaccharide. In the first case, the hydrolysis will be carried out by a single enzyme, but in the second case, the consecutive action of two enzymes will be required (Gunata et al., 1988). The aglycone will be only released enzymatically when linked to β-D-glucose (Winterhalter and Skouroumounis, 1997).

In general, non-*Saccharomyces* yeasts can show higher activities of this enzyme compared to *S. cerevisiae*, which has led to the hypothesis that these yeasts would be more efficient producing aroma from non-volatile grape conjugates (Anfang et al., 2009; Barata et al., 2012; Padilla et al., 2016).

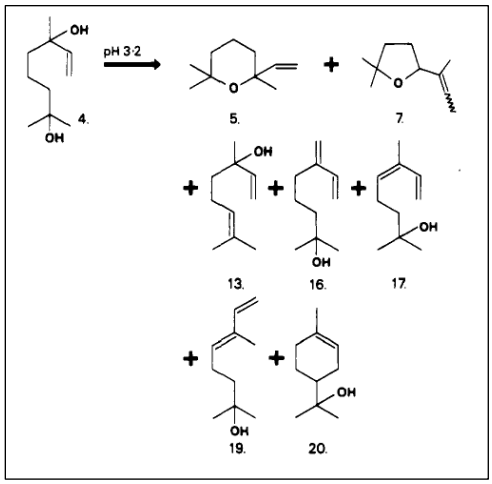

Figure 3 Products of enediol (hydroxylated linalool) due to spontaneous chemical rearrangements by acid catalysis at wine pH In Williams et al., 1980)

While some glycosidic precursors yield directly the aroma molecule after the cleavage of the glycosidic bond, some others give different molecules which only after spontaneous chemical rearrangement at wine pH become the molecule. An example is shown in the previous Figure 3 taken from works carried out by Australian researchers (Strauss et al., 1988; Williams et al., 1980).

But, acid catalysis is also responsible for the transformation of relevant aroma volatiles, such as geraniol, which after being released from the glycoside, will spontaneously transform into the much less odorant α-terpineol, as shown in Figure 4.

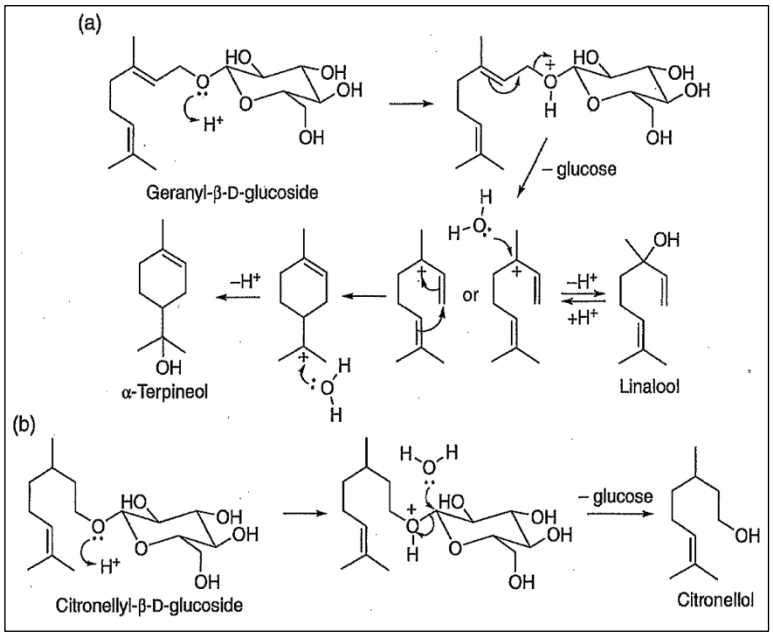

Figure 4 Formation of monoterpenes from glycosidic precursors due to enzymatic or acidic hydrolysis (a) and (b) followed by chemical rearrangements to form other monoterpene molecules due to acidic environment (a) adapted from Waterhouse et al., 2016.

2.3.2 Release and formation of norisoprenoids

The most important aroma compounds derived from norisoprenoids are β-damascenone, β-ionone and TDN. Some other aroma molecules with less aromatic relevance are α-ionone, Riesling acetal and vitispiranes. All these molecules are chemically ketones, hydrocarbons or ethers, meaning that they cannot be part of aglycones, which by nature have to have a hydroxyl group (alcohols or carboxylic acids). This implies that the formation of these aroma

molecules will, necessarily, take place after different reactions other than the cleavage of the glycosidic precursors. For instance, one possible mechanism for β-damascenone formation in wine has been shown to be from the carotenoid neoxanthin, requiring oxidative cleavage, followed by enzymatic reduction and finally acid hydrolysis in order to become volatile (Winterhalter and Rouseff, 2001).

While all norisoprenoids derive from carotenoid breakdown, two possible formation routes are possible (figure 5):

1. the direct degradation from carotenoids present in the grape must or in the wine, as seems to be the case of β-ionone (Winterhalter and Rouseff, 2001)
2. the cleavage and further rearrangement of non-volatile glycosides of C_{13}-norisoprenoids intermediates formed from carotenoids (β-damascenone, TDN, vitispirane or Riesling acetal (Winterhalter, 1991; Winterhalter and Rouseff, 2001).

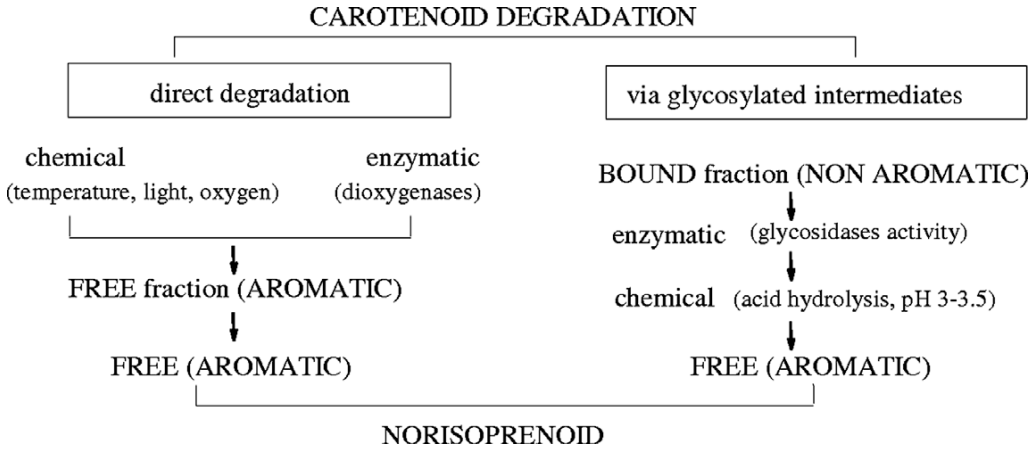

Figure 5 Possible pathways to form volatile norisoprenoids compounds from carotenoid initial precursors involving multiple glycosylated intermediates and/or chemical, enzymatic or acid hydrolysis In Mendes-Pinto, 2009.

One of the difficulties is that for all the important odorants, there are multiple precursors, whose structures are not well known. This was first observed by Peter Winterhalter in 1991 for TDN, vitispirane and Riesling acetal, but seems to be valid also for β-damascenone. To complicate more things, the same precursors can yield different molecules, and some of them, such as Riesling acetal, can be intermediates in the production of TDN (Gök, 2015).

2.3.3 Release and formation of volatile phenols

Volatile phenols such as guaiacol, vanillin, cresols and eugenol can be extracted from wood (oak barrels, wood chips, etc) contributing with smoky, sweet or clove flavours to wine (Kennison et al., 2008). Small amounts of these volatiles are also present under the form of glycosides in grapes, so that these compounds can be also released by enzymatic or acid hydrolysis.

Moreover, grapes contain a large variety of phenolic compounds including phenolic acids, flavonoids, anthocyanins or tannins (Kheir et al., 2013). Major differences exist between white and grape varieties, which precisely provides colour differences in wine. However, some of these phenolic compounds are actual aroma precursors of volatile phenols, such as vinyl phenols, which can be also found as glycosidic precursors. Vinyl phenols are more important in white wines since the biochemical reactions for the transformation of phenolic acids are inhibited by red wine components (Basha et al., 2004; Chatonnet et al., 1993).

Phenolic acids in grape are divided into two groups benzoic and cinnamic acids and are present in larger amount in red grapes. Cinnamic acids exist in grape berries under different forms, since in addition to the free molecules, they can also be as esters of tartaric acid and as glycosides (Basha et al., 2004; Kheir et al., 2013). *S. cerevisiae* is able to form vinylphenols during alcoholic

fermentation from cinnamic acids, in particular ferulic and *p*-coumaric acid catalysed by the enzyme cinnamate decarboxylase (Chatonnet et al., 1993).
The conversion of vinylphenols into ethyl phenols is rare in microorganisms and large amounts of these compounds have been linked with spoilage yeast *Brettanomyces spp.* (Kheir et al., 2013).

3. Selection of non-Saccharomyces yeast strains

Non-*Saccharomyces* yeasts are rarely used as single inoculums since they are inhibited by high ethanol levels and their sugar metabolism is not as efficient as that of *S. cerevisiae*. In fact, non-*Saccharomyces* yeasts have been in the past associated with sluggish or stuck fermentations with an increased risk of spoilage by competitor microorganisms such as acetic acid bacteria (Fleet, 2003). These non-*Saccharomyces* are then used as mixed inoculum or following sequential inoculation procedures in which the final part of the fermentation is carried out by *S. cerevisiae*.
In both cases, non-*Saccharomyces* yeasts can act as potential enhancers of wine aroma due to their potentially different enzymatic activity compared to *S. cerevisiae,* namely by their specific β-glucosidase and β-lyase activities (Mendes Ferreira et al., 2001; Zott et al., 2011), but there are many other aroma compounds whose levels are modulated by the presence of these yeasts. The modulation may take via different mechanisms not yet clear, such a direct interaction between yeast strains or competition. The reduction of nutrients, the early amino acid intake or even limitations in dissolved oxygen could be important modulators of yeast metabolism (Clemente-Jimenez et al., 2005; Fleet, 2003; Kapsopoulou et al., 2007; Moreno et al., 1991). On the other hand, some strains of non-*Saccharomyces* with abilities to secrete lipolytic enzymes to

the media, may degrade grape lipids into free fatty acids, compounds which can inhibit the growth of *S. cerevisiae (Escribano et al., 2017)*.

Three commercial strains, isolated from wine environments, are often used in wine making and have shown particular traits.

3.1 Torulaspora delbrueckii

T. delbrueckii was one of the first non-*Saccharomyces* commercial strains. It is described as having medium fermentative power and to be a low producer of acetic acid. When co-inoculated with *S. cerevisiae,* it was shown to improve the sensory description of wine, however it is not clear how this was accomplished, since researchers described that co-inoculation induced the decrease of isoamyl acetate, of fatty acids with known role on fruity aromas, such as hexanoic acid and also of vinyl phenols (Azzolini et al., 2014; Jolly et al., 2014). Researchers have also suggested that this strain has relevant β-glycosidase and carbon-sulfur lyase activities (Escribano et al., 2017; Padilla et al., 2016).

3.2 Pichia kluyveri

P. kluyveri was first associated with a higher release of volatile thiols (Anfang et al., 2009), but it has been also linked with the formation of acetate esters (Viana et al., 2008). Comparing to other yeast strains, this yeast has a quite specific metabolic characteristics including an oxidative metabolism characterized by the formation of biofilms (Barata et al., 2012).

3.3 Lachancea thermotolerans

This yeast was first described by its ability to produce lactic acid and thus, contribute for the wine roundness (Kapsopoulou et al., 2007; Varela and Borneman, 2017). Besides, sensory descriptours such as spiciness where found to increase in wines fermented with this yeast (Gobbi et al., 2013).

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Objectives

The main goal of this Thesis is to contribute to a better understanding of the roles played by yeasts on the formation of wine aroma. The following specific objectives have been defined:

1. To study the possible fermentative origin of Strecker aldehydes and, in particular, to test previous hypothesis about the potential implication of Zn levels an of SO_2 on the formation of these important aroma compounds.
2. To study the effects associated with the presence of non-*Saccharomyces* yeasts in sequential inoculation procedures on the formation and evolution with time of wine aroma, in particular, in the varietal aroma derived from glycosidic precursors extracted from Garnacha and Riesling varieties.
3. To assess the sensory and GC-olfactometric profiles of young Riesling wines (*Großes Gewächs* Riesling wines)
4. To assess the relative weights introduced by the microbiota diversity from the vineyard and from the cellar in the aroma composition of Riesling wines.

Section I

Strecker aldehydes are normal by-products of alcoholic fermentation linked to yeast sulphite metabolism

1. Introduction

Strecker aldehydes (SAs), namely 2-methylpropanal, 3-methylbutanal, 2-methylbutanal, methional and phenylacetaldehyde, are powerful aroma molecules playing relevant roles in the flavour of wine and beer. In beer, 3-methylbutanal and 2-methylbutanal were first proposed as responsible for the malt flavour, note characteristic of some alcohol-free beers (Beal and Mottram, 1994). Other authors later demonstrated that methional was in fact more relevant in such off-odour (Perpete and Collin, 1999a). Furthermore, the implication of methional and phenylacetaldehyde in some negative odour characteristics of oxidized/aged beer was demonstrated in 2004 (Soares da Costa et al., 2004) and confirmed in more recent studies (Saison et al., 2010; Wietstock et al., 2016). In the case of wine, the involvement of methional in the "cooked vegetables" note of oxidized wines was stablished in 2000 (Escudero et al., 2000) and phenylacetaldehyde was identified by GC-O (GC-Olfactometry) as one of the key odorants of oxidized white wines in 2003 (Silva Ferreira et al., 2003). The main roles played by these compounds in the odour notes of oxidized wines were further confirmed in 2007 (Cullere et al., 2007).

These ubiquitous and powerful smelling molecules are chemically or biochemically related to the so called Strecker amino acids: valine, leucine, isoleucine, methionine and phenylalanine. The oxidative deamination of these amino acids in the presence of tea polyphenols to form the corresponding aldehydes was observed as soon as 1954 and was confirmed in the 70's when Japanese researchers demonstrated that the degradation involved the reaction of the amino acid with a quinone derived from a flavanol undergoing oxidation (Saijō and Takeo, 1970a). The chemical routes leading to the formation of these aldehydes in oxidation-related processes have been relatively well established

(Baert et al., 2012; Bueno et al., 2018; Grant-Preece et al., 2013; Rizzi, 2006; Wietstock et al., 2016).

The fermentative origin of these compounds was first suggested also by (Saijō and Takeo, 1970b) who observed that supplementing fermenting tea leaves with phenylalanine resulted in phenylacetaldehyde formation. Nowadays, it is known that SAs are normal intermediates of the Ehrlich pathway in the amino acid metabolism, but the current believes establish that they are mostly reduced to the corresponding alcohols, so that levels of SAs in fresh beer or wines are thought to be negligible. However, there are some evidences pointing out that in certain conditions, yeast cannot reduce all the SAs. This was first observed in cold fermentation conditions for the production of alcohol-free beer (Perpete and Collin, 2000a). Such inability was tentatively attributed to the presence of sulphite or flavonoids (Perpete and Collin, 2000b). Other researchers further confirmed that refermentation of aged beer reduced but was not able to completely eliminate SAs and that residual levels were strain dependent (Saison et al., 2010). I.e., these works show that researchers have been long aware of the fact that aldehydes can form stable and reversible non-volatile adducts with SO_2 (Baert et al., 2012; de Azevedo et al., 2007), that such adducts could limit the efficiency of yeast reductases, and that adducts could also play some role in the ulterior development of oxidized notes. In spite of this evidence, the potential importance of alcoholic fermentation as a relevant source of SAs remains unexplored.

Recently, we developed an analytical method able to measure free aldehydes and to estimate the bonded fraction (Bueno et al., 2014). Using such methodology, it was possible to confirm that non-oxidized wines may contain a large pool of SAs under the form of sulphite adducts. These adducts are progressively cleaved

during the first stages of wine oxidation as free SO_2 is depleted, concomitantly releasing the free forms of the aldehydes (Bueno et al., 2016). The production of SAs from oxidative degradation of amino acids was found to take place only when levels of free SO_2 become smaller than 4 mg/L, suggesting that oxidative odour notes developed by some wines during aging could be in fact due to the simple release of sulphite adducts, and not to the oxidation of amino acids, alcohols or other precursors. Furthermore, PLS modelling suggested that SAs present in normal non-oxidized wines as sulphite adducts, could have been formed in fermentation as a consequence of a failure in the action of alcohol dehydrogenases, possibly induced by a lack of zinc, and likely by the aldehyde-protecting action of SO_2 (Bueno et al., 2016).

In order to confirm those evidences, the research presented in this paper, studies the formation of SAs in alcoholic fermentation using synthetic media resembling grape must. The major objectives are to assess the effects of the strain of yeast and of the levels of zinc and SO_2 of the initial must on the levels of SAs formed in the alcoholic fermentation.

2. Methodology

2.1 Reagents and standards

Sodium metabisulfite (97%), zinc chloride (97%), hydrogen peroxide 3% stabilized w/v, indicator 4.4 mixed (methyl red-methylene blue), sodium hydroxide 0.01 mol/L, ortho phosphoric acid (85%) of VINIKIT line were obtained from Panreac (Barcelona, Spain).

Dichloromethane (DCM), ethanol and methanol (≥ 99%) with Distol-Pesticide residue grade were supplied by Merck (Darmstadt, Germany). Glyoxal solution 40 wt. % in H_2O was purchased from Sigma-Aldrich (Madrid, Spain).

The internal standards methyl 2-methylbutyrate (≥ 99%) and 2-butanol (≥ 99%) were obtained from Merck, while 4-methyl-2-pentanol (99%), 4-hydroxi-4-methyl-2-pentanone (99%), ethyl heptanoate (99%) 2-octanol (99.5%) and heptanoic acid (99 %) were purchased from Sigma Aldrich. Water was purified using Milli-Q® system from Millipore (Merck).

2.2 Culture conditions:

2.2.1 Synthetic must composition

Synthetic must: the synthetic must resembling grape must was adapted from Bely, Sablayrolles, & Barre, 1990 and had the following composition: Oligoelements: $MnCl_2.4H_2O$ 4.7 mg/L, $Co(NO_3)_2 \cdot 6H_2O$ 0.49 mg/L, $NaMoO_4 \cdot 2H_2O$ 0.19 mg/L, $CuCl_2$ 0.54 mg/L, KIO_3 1.29 mg/L, H_3BO_3 1 mg/L, $SO_4Mg \cdot 7H_2O$ 0.2 g/L, KH_2PO_4 2 g/L, $CaCl_2 \cdot 2H_2O$ 0.155 g/L; Acids: malic acid 0.3 g/L, tartaric acid 3 g/L, citric acid 0.3 g/L, with pH adjusted to 3.5 with HCl; Vitamins -all supplied from Merck (≥ 98%): pyridoxine hydrochloride 1 mg/L, nicotinic acid 1 mg/L, calcium pantothenate 1 mg/L, thiamine hydrochloride 1 mg/L, p-aminobenzoic acid 1 mg/L, riboflavin 0.2 mg/L, folic acid 0.2 mg/L,

biotin 0.04 mg/L; myo-inositol (≥ 99%) 0.3 g/L, ergosterol (≥ 75%) 15 mg/L; Sugars were from Panreac Applichem (Spain): glucose 100 g/L; fructose 100 g/L; tween 80® 0.05 % (v/v) (Sigma-Aldrich); nitrogen source: $(NH_4)_2HPO_4$ 0.2199 g/L; amino acids (Merck) (mg/L): GABA 44.37, alanine 58.51, tyrosine 14.34, valine 17.73, isoleucine 14.43, leucine 13.42, aspartate 34.82, glutamic acid 61.83, glutamine 104.83, serine 21.21, glycine 1.11, histidine 109.2, threonine 18.8, arginine 199.5, proline 241.46, methionine 29.85, phenylalanine 11.15, lysine 3.33.

Zinc was added from a stock solution of $ZnCl_2$, 162 mg/L.

SO_2 was added from a freshly prepared $Na_2S_2O_5$ solution, 5000 mg/L.

2.2.2 Yeast culture and fermentation set-up

Yeast culture: three commercial *Saccharomyces cerevisiae* yeast strains were selected: L1 - Q23 (Lallemand), L2 - Merit (Chr. Hansen); L3 - Fermicru AR2 (Oenobrands). The yeast cells were hydrated for 1 hour at 35°C followed by the addition of synthetic must to activate them at the same temperature for around 30 min to 1hour. The fermenters were inoculated with 10^6 cells/ml.

Must manipulation: the synthetic must was sterilized by means of sterile cellulose nitrate membrane filters with 0.45 µm pores (Albet, A&S Filter Co., Ltd) after its preparation and pH adjustment. All media manipulations were made inside a vertical laminar flow chamber PV-100 (Telstar, S.A), to ensure working under aseptic conditions. Small volumes were sterilized using syringe filters with 0.2 µm HT Tuffryn® membrane from PALL (New York, USA). All glassware was sterilized using an autoclave AES-28 from Raypa (Barcelona, Spain).

Fermentation set-up: The first experimental set-up was performed without SO_2 and the concentrations of zinc tested were 10 mg/L, 5 mg/L and 1 mg/L. In the

second set-up all samples contained 30 mg/L of SO_2 and the concentrations of zinc were 10 mg/L, 1 mg/L and 0 mg/L. Two biological replicates containing 150 mL of the must were used for each condition.

Fermentation: Fermentations were carried out in 250 mL blue cap glass flasks (Ilmabor TGI, Germany) closed with airlock valves and were kept at constant temperature of 20°C. The progress of fermentation was monitored by daily control of the weight. Fermentation was considered finished when the loss of weight between two consecutive days was smaller than 0.1 g.

Once fermentation was considered finished, the fermenters were sealed and sonicated for 15 minutes and were then introduced in an anoxic chamber from Jacomex (Dagneux, France) where they were let to sediment for 5 hours and were then aliquoted. Aldehyde and SO_2 analysis were performed on the following hours, while the aliquots for the remaining aroma compounds were preserved in vials in the fridge.

2.3 Analytical methods

2.3.1 Classical oenological characterization

The wines were characterized according to their general enological parameters using the recommended methodologies by OIV (International Organization of Vine and Wine): reducing sugars, total acidity and volatile acidity (International Organisation of vine and wine, 2011).

pH was measured using a pHmeter. Wine total acidity was measured by titration with NaOH 0.1 N. Volatile acidity was titration with NaOH 0.02 M of the volatile fraction obtained by steam distillation. Residual sugars were calculated using the Fehling procedure based on oxidation of reducing sugars with CuII in alkaline media by boiling the solution. Excess of cupper not oxidized by the

sugars, oxidases iodine added as KI. The solution is then titrated with sodium thiosulfate using starch as indicator to determine I_2.

Free sulphur dioxide was determined by HeadSpace Gas Chromatography Mass Spectrometry (HS-GC-MS) using a GCMS-QP2010 from Shimadzu (Kyoto, Japan) as described in the literature (Carrascón et al., 2017). A DB-WAX column was used (30 m x 0.25 mm i.d x 0.25 μm film thickness) from J&W Scientific (Agilent Technologies, Santa Clara, CA USA). This method is based on the displacement of SO_2 equilibrium forms with orthophosphoric acid (85%), in which 4.5 mL of wine with 20 μL of 2-cloroethanol (internal standard) are carfully capped in a 10 mL headspace vial. Just before the analysis the sample is acidified with 500 μL of orthophosphoric acid (85%).

Total SO_2 was analysed using the aspiration/ titration method described by Rankine and recommended by the OIV (International Organisation of vine and wine, 2011). 3 mL of the hydrogen peroxide 3% (p/v) with 3 drops of methyl red-methylene blue indicator and 2-3 drops of NaOH 0.01 M, so that the solution turns from purple to green, are prepared in a heart-shape flask with a bubbler. The 10 mL of wine are transferred to a round flask with 5 mL of H_3PO_4 at 20% are secured in the water vacuum system with a bubbler tube. The round flask is heated and the aspiration system is activated for 15 minutes. Total SO_2 is measured by titration with NaOH 0.01 M.

2.3.2 Quantification of total aldehydes

Total aldehydes were analysed using a previously described method (Bueno et al., 2014). Aldehyde-sulphite adducts (hydroxyalkylsulfonates) were previously cleaved by incubating the wine in strict anoxic conditions with 6 g/L of glyoxal at 50°C during 6 hours. Released aldehydes are further analysed by HeadSpace Solid Phase MicroExtraction Gas Chromatography Mass Spectrometry (HS-

SPME-GC-MS). The extraction fibre was a PDMS/DVB, the chromatographic column was a SPB-1 Sulfur column (30 m x 0.32 mm i.d x 4 µm film thickness) from Supelco, and the GC-MS system was a Shimadzu QP-2010 equipped with a quadrupole mass spectrometer working in SIM mode, as described (Bueno et al., 2014).

2.3.3 Analysis of major volatile compounds

Analyses of higher alcohols, volatile fatty acids and major esters (See supplementary data) was carried out by performing a liquid-liquid microextraction with dichloromethane (DCM). In a screw-capped centrifuge tube 4.1 g of $(NH4)_2SO_4$, 3 ml of wine (with previous addition of 37 µl of the internal standards solution to 5 ml of wine using a volumetric flask), 7 ml of Milli-Q water and 250 µl of DCM. The tubes are agitated horizontally for 90 minutes and afterwards centrifuged for 10 minutes at 2500 r.p.m. The aqueous phase formed is discarded and the organic phase is collected with a syringe. The organic phase is further analyzed by Gas-Chromatography with Flame Ionization Detection (GC-FID). 2 µl of sample was injected at 250ºC with a split ratio of 1/20. The column was kept initially at 40ºC for 5 minutes and then temperature starts increasing until 102ºC at 4ºC/min, then at 2ºC/min until 112, then at 3ºC/min until 125ºC and hold for 5 minutes. Temperature increases until 160ºC at 3ºC/min and finally until 200ºC at 6ºC/min and hold for 30 minutes. The H_2 flow was 2.2 ml/min, as previously described (Ortega et al., 2001).

2.4 Data treatment

Data from the two batches of fermentations, with and without external SO_2, were processed separately for checking statistical significance. Data included some

ratios between the levels of two molecules involved in a same metabolic pathway: aldehydes (in μg/L) per alcohol unit (mg/L); aldehydes (in μg/L) per acid unit (mg/L); medium chain fatty acid esters (mol/L) per corresponding fatty acid unit (mol/L).

A 2-way ANOVA was applied to each data set to assess whether the levels of SAs, major volatiles and the aforementioned ratios were related to the yeast strain and/or to the levels of zinc, as well as their interactions. ANOVAs were carried using XLSTAT (Addinsoft, 2018 version).

In order to assess the significance of the addition of external SO_2 to the must as well as of the final SO_2 content of the wines, simple correlations between initial or final levels of SO_2 and levels of volatiles were calculated.

The whole set of compositional data, including data from the two batches of fermentations, was processed by Principal Component Analysis using R. The relative effects of the different factors were assessed by representing the centroids of each group together with the 95% probability ellipses.

3. Results and discussion

Previous reports have shown that sulphite adducts of SAs formed during fermentation could be responsible for increases in the levels of SAs observed during wine aging and have further suggested that the formation of those adducts was related to the SO_2 and Zn contents of the grape must (Bueno et al., 2016). Accordingly, two sets of synthetic musts containing all the required nutritional elements and different levels of Zn and SO_2 were fermented by three different yeast strains. Immediately after fermentation, fermented media were transferred to an anoxic chamber to avoid any contact with oxygen. Sulphite adducts were cleaved by incubation with glyoxal and released SAs were analysed by HS-SPME-GC-MS. Major volatile metabolites of fermentation were determined by GC-FID.

Fermentations were carried out in two independent experiments so that in order to check the statistical significance of the factors, data were processed separately via 2-way ANOVA or correlation analysis (see methods). The statistical significance exerted by the factors in the ANOVA models and the significance of the correlation coefficients are summarized in Table 1. The mean levels of aroma compounds significantly affected by yeast strain or Zn levels are given in Tables 2 and 3, respectively. Additional data treatments are given as supplementary material.

Results of the Principal Component Analysis carried out with the whole data set are summarized in Figure 6. The figure shows the representation of the experimental samples in the plane formed by the first two principal components, which retains 58% of the original variance. Each one of the four plots in the figure highlights the centroids of the different groups of samples with their corresponding 95% probability ellipses: the first plot for yeast strains, the second

for Zn levels, the third for reproducibility of biological replicates and the fourth for SO_2 levels. A simple look at the plots reveals that replicates were very consistent and that by far, the most influential factor is the yeast strain. This is further confirmed by results of the 2-way ANOVA given in Table 1: the strain of yeast significantly affects the levels of nearly all aroma molecules analysed, strongly determining the distribution of samples in the PC plot. Figure 6 and Table 1 also suggest that levels of SO_2 initially added to the media may play an important role on the levels of several of the studied metabolites (Figure 6 and Table 1). However, because of the experimental design, initial SO_2 effects could be confounded with batch effects, so that SO_2 effects will be later discussed. Table 1 also reveals that, contrarily to previous hypotheses, the direct effects of Zn levels on SAs were not as evident as expected, and only in the set of fermentations with SO_2 the levels of 3-methylbutanal and phenylacetaldehyde were significantly affected. Nonetheless, the ratios aldehyde/ alcohol of these two aldehydes as well as that of methional were influenced by Zn levels in the same experiment. Most unexpectedly, Zn levels exerted a strong influence on the levels of ethyl esters of fatty acids and in ethyl ester/ fatty acid ratios.

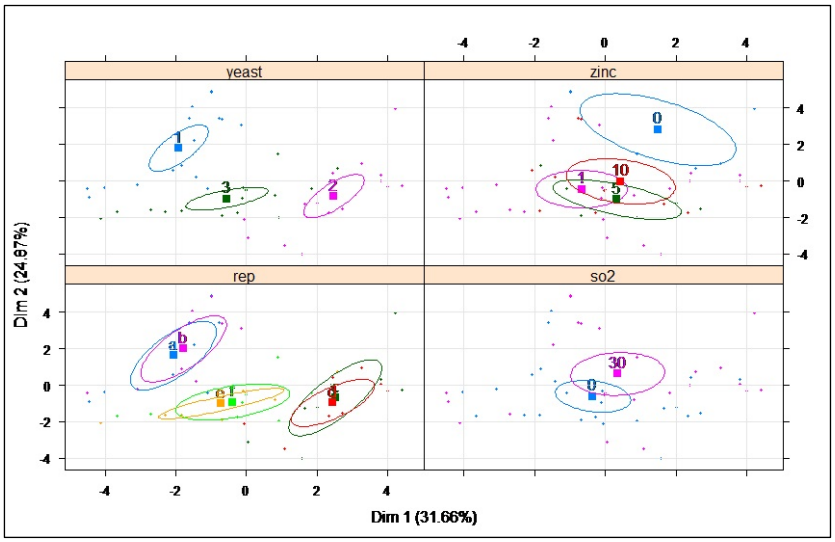

Figure 6 ***PCA analysis:*** *major effects in the plane formed by the two first Principal Components showing 58% of variance highlighting how are the samples segregated according to the different factors under regard in this study yeast, zinc and SO_2 addition), as well as all the biological replicates of each yeast.*

3.1 Strecker aldehydes are normal fermentative compounds

A first remarkable observation is that all wines contained significant amounts of SAs whose single origin can be fermentation, since they were not added in the original unfermented synthetic musts, and wines were carefully protected from oxygen after fermentation. The maximum averages values found in the experiments were 7.2, 30.4, 2.7, 28.8 and 18.6 µg/L of 2-methylpropanal, 3-methylbutanal, 2-methylbutanal, methional and phenylacetaldehyde, respectively.

These levels are in the low-average range of total aldehyde found in whites and rosé wines, but leaving aside 2-methylbutanal, are well above the corresponding odour thresholds (Bueno et al., 2016). These results confirm that fermentation is a normal source of SAs in wine and that the chemical oxidation of alcohols or amino acids is not strictly required to explain the presence of SAs in wines.

Table 1 Summary of the significance of the effects played by the factors yeast and zinc as well as their interaction on the levels of total SO_2, SAs and major fermentation volatiles assessed by two way-ANOVAs carried out in the two data sets; addition of external SO_2 and the levels of total SO_2 found after fermentation given by significance of their correlation with compounds and ratios.

	2-way ANOVA						correlation	
	without SO_2			with SO_2			added	final
	yeast	zinc	yeast*zinc	yeast	zinc	yeast*zinc	SO_2	SO_2
total SO_2	0.000	0.033	-	0.000	-	0.040	0.000	0.002
aldehydes								
2-methylpropanal	-	-	-	0.003	-	-	-	-
3-methylbutanal	-	-	-	0.011	0.002	0.011	0.018	0.000
2-methylbutanal	-	-	-	0.003	-	-	-	-
methional	0.000	-	-	-	-	-	0.01	-
phenylacetaldehyde	0.007	-	-	0.000	0.001	0.000	0.000	0.000
fusel alcohols								
isobutanol	0.000	-	-	0.000	0.014	0.047	0.006	0.000
isoamyl alcohol	0.000	-	-	0.000	0.025	-	0.001	0.000
methionol	0.001	-	-	0.000	-	-	-	-
2-phenylethanol	0.002	0.010	-	0.000	0.010	0.009	0.000	-
iso-acids								
2-methylpropanoic acid	0.000	-	-	0.000	-	-	-	0.000
3-methylbutanoic acid	0.000	0.010	-	0.000	-	-	-	0.000
aldehyde/alcohol ratio								
2-methylpropanal/ isobutanol	-	-	-	0.003	-	-	-	0.000
3-methylbutanal/ isoamyl alcohol	0.019	-	-	0.001	0.007	0.041	-	0.000
methional/ methionol	0.002	-	-	0.001	0.021	-	0.002	-
phenylacetaldehyde / 2-phenylethanol	0.001	-	-	0.000	0.000	0.000	0.000	0.000
aldehyde/acid ratio								
2-methylpropanal/ 2-methylpropanoic acid	-	-	-	0.049	-	-	-	0.000
3-methylbutanal/ 3-methylbutanoic acid	-	-	-	0.000	0.002	0.003	-	0.000
isobutanol/2-methylpropanoic acid	0.000	-	-	-	0.042	-	0.006	-
isoamyl alcohol/ 3-methylbutanoic acid	0.008	-	-	-	0.043	-	-	-
2-methylbutanal/ isovaleraldeyde	0.000	-	0.046	0.015	-	-	0.004	0.003
medium chain fatty acids								
hexanoic acid	0.000	-	0.019	0.000	0.008	-	0.019	-
octanoic acid	0.000	0.027	-	0.000	-	-	-	-
decanoic acid	0.001	-	0.029	0.009	-	-	-	-
esters								
isoamyl acetate	0.000	-	-	0.000	-	-	0.000	-
ethyl hexanoate	-	0.000	-	-	0.000	-	0.004	-
ethyl octanoate	0.001	0.000	0.006	0.023	0.000	-	-	-
ethyl decanoate	0.000	0.000	0.001	0.032	0.001	-	0.001	-
ester/acid ratios								
ethyl hexanoate/ hexanoic acid	0.010	0.000	-	-	0.000	-	0.000	-
ethyl octanoate/ octanoic acid	-	0.000	-	-	0.000	-	-	-
ethyl decanoate/ decanoic acid	-	0.000	-	-	0.000	-	0.000	-

The ability of *S. cerevisiae* to form and excrete SAs during cold contact fermentation in the production of low alcoholic beers was demonstrated time ago (Perpete and Collin, 2000a). Similar conclusions were reached studying the reduction of aldehydes by re-fermentation (Saison et al., 2010). It is, however, believed that the formation of aldehydes by fermenting yeast in beer is most likely limited and of scarce importance, at least in comparison with other sources linked to wort production (Baert et al., 2012). The experiments carried out by Saison et al., 2010, much in accordance with all observations regarding the ability of yeast to reduce aldehydes during fermentation (Peppard and Halsey, 1981), suggest that SAs produced during malting and boiling will be reduced to the corresponding alcohols during fermentation, but that there is a fraction of SAs which will remain and is dependent on the yeast strain and wort composition. Likewise, this work hereby confirms that, during alcoholic fermentation of grape must, a fraction of SAs at sensory relevant levels remains in the wine and this is highly depend on the yeast strain, as seen in Table 1.

Regarding the particular effects of each strain on levels of SAs, data in Table 2 reveal that levels formed are specific for each case. L1 produces in general smaller levels, reaching smallest values for 2-methylpropanal and 2-methylbutanal, but levels of phenylacetaldehyde produced by this strain were relatively large. On the other hand, L3 produced maxima values of 3-methylbutanal and phenylacetaldehyde, but levels of methional were close to those produced by L1. In contrast, L2 produced maxima levels of methional and minima of phenylacetaldehyde. This complex pattern of dependence would have been expected, since these aldehydes are produced during the synthesis of amino acids and are further reduced to alcohols by a complex and heterogeneous enzymatic system which has to restore the cell redox cycle (Peppard and Halsey, 1981). This pool is integrated by alcohol dehydrogenases (ADHs), aldehyde

dehydrogenases and aldoketoreductases using either NAD(H) of NADP(H) as cofactors (Perpete and Collin, 1999b; Van Iersel et al., 1997).

Attending to the previous discussion, levels of SAs retained in the wines after fermentation, should then be related to the amount of higher alcohols produced by the strain, to the selectivity and effectivity of the ADH-system of the strain, and eventually, to the differential level of any molecular species able to protect the aldehyde from reduction or oxidation, such as SO_2 (Perpete and Collin, 2000b). The fact that the ratios between the levels of aldehyde and those of the corresponding alcohol are also significantly related to the yeast strain (Tables 1 and 2), supports that the specific ability of each strain to reduce these aldehydes exerts a major role on the final level of remaining aldehydes. Wines made with L3 have maxima aldehyde/ alcohol ratios (except methional/ methionol) and very low levels of higher alcohols (except methionol). On the contrary, wines made with L2 contained maxima levels of higher alcohols (except 2-phenylethanol) and lowest aldehyde/alcohol ratios (except methional/methionol), suggesting that this strain reduces aldehydes to alcohols efficiently.

*Table 2 Average levels of total SO_2, aroma compounds and of some relevant ratios attending to the yeast strain. Concentration data are in mg/L except aldehydes which are in μg/L. Cases showing significant interactions yeast x zinc are marked with • or * for the experiments without or with SO_2, respectively. Significant differences attending to Duncan test are indicated with letters [a-c] being "[a]" the lowest average value.*

	L1	L2	L3
total SO_2*	22.1 ± 2[b]	11 ± 1[a]	32.8 ± 3[c]
aldehydes			
2-methylpropanal	3.6 ± 0.4[a]	7.2 ± 0.7[b]	6.1 ± 0.8[b]
3-methylbutanal*	23.8 ± 2.1[a]	23 ± 1.8[a]	30.4 ± 3.2[b]
2-methylbutanal	1.7 ± 0.1[a]	2.6 ± 0.2[b]	2.7 ± 0.3[b]
methional	23.7 ± 0.6[a]	28.8 ± 0.7[b]	24.7 ± 1.4[a]
phenylacetaldehyde*	14.2 ± 2.9[b]	8.2 ± 1.5[a]	18.6 ± 4.3[c]
fusel alcohols			
isobutanol*	13.9 ± 0.5[a]	41.1 ± 2.3[c]	18.1 ± 0.9[b]
isoamyl alcohol	163 ± 6[b]	242 ± 9[c]	135 ± 10[a]
methionol	4 ± 0.2[a]	6.1 ± 0.2[b]	6.1 ± 0.3[b]
2-phenylethanol*	27.1 ±1.5[c]	23 ± 0.9[b]	20 ± 1.5[a]
iso-acids			
2-methylpropanoic acid	0.97 ± 0.0[a]	2.2 ± 0.1[c]	1.2 ± 0.1[b]
3-methylbutanoic acid	1.2 ± 0.0[a]	1.9 ± 0.1[b]	1.2 ± 0.1[a]
aldehyde/alcohol ratio			
isobutiraldehyde/ isobutanol	0.26 ± 0.0[ab]	0.2 ± 0.0[a]	0.4 ± 0.0[c]
3-methylbutanal/ isoamyl alcohol*	0.15 ± 0.01[a]	0.1 ± 0.0[a]	0.2 ± 0.0[b]
methional/ methionol	6.3 ± 0.5[c]	4.8 ± 0.2[b]	4.2 ± 0.2[a]
phenylacetaldehyde/ 2-phenylethanol*	0.5 ± 0.1[a]	0.4 ± 0.1[a]	0.88[b]
2-methylpropanal/ 2-methylpropanoic acid	-	-	-
3-methylbutanal/ 3-methylbutanoic acid*	20.6 ± 1.8[b]	12.3 ± 1.1[a]	26.5 ± 3.8[c]
isobutanol/ 2-methylpropanoic acid	14.8 ± 1[a]	18.9 ± 1[b]	15.4 ± 1.1[a]
isoamyl alcohol/ 3-methylbutanoic acid	141 ± 7[b]	129 ± 6[ab]	112.5 ± 8.5[a]
2-methylbutanal/ 3-methylbutanal•	0.1 ± 0.0[a]	0.1 ± 0.0[b]	0.1 ± 0.0[a]
medium chain fatty acids			
hexanoic acid•	1.9 ± 0.0[c]	1.2± 0.0[b]	1 ± 0.1[a]
octanoic acid	3.7 ± 0.2[b]	2.4 ± 0.1[a]	2.3 ± 0.1[a]
decanoic acid•	0.9 ± 0.1[c]	0.6 ± 0.0[b]	0.4 ± 0.0[a]
esters			
isoamyl acetate	0.3 ± 0.0[b]	0.5 ± 0.0[c]	0.2 ± 0.0[a]
ethyl hexanoate	-	-	-
ethyl octanoate•	3.7 ± 0.2[b]	2.4 ± 0.1[a]	2.3 ± 0.1[a]
ethyl decanoate•	0.3 ± 0.1[b]	0.2 ± 0.0[b]	0.1 ± 0.0[a]
ratios			
ethyl hexanoate/ hexanoic acid	14.5 ± 2.4[a]	23 ± 5.9[b]	23.6 ± 5.4[b]
ethyl octanoate/ octanoic acid	-	-	-
ethyl decanoate/ decanoic acid	-	-	-

3.2 Role of SO_2

It is worth mentioning that the final levels of total SO_2 found in the fermenting media were in fact more related with the yeast strain than with the initial level of SO_2 added to the must, as can be seen in Table 2 and in Figure 7. Table 2 shows that the average levels of SO_2 remaining in samples fermented with L3 were the highest, with 32.8 mg/L, whereas those remaining in samples fermented with L2 were the lowest, with 11.03 mg/L. Figure 7 further illustrates that all samples fermented with L2 had final levels of total SO_2 below 15 mg/L, even if the initial must contained 30 mg/L of this antioxidant. On the other hand, samples fermented with L3 without external SO_2 contained 20-25 mg/L at the end of fermentation, and the external addition brought about an extra increase of nearly 20 mg/L. It is obvious that each yeast strain metabolizes SO_2 differently, using it either as source of sulphur or, on the contrary, producing it from other sulphur sources, most likely to take advantage of its toxic effect on competing microorganisms. In fact, the resistance to SO_2 is a genetically determined characteristic of yeasts linked with an interesting molecular mechanism only observed in wine strains (Perez-Ortin et al., 2002) which has received some attention for its potential industrial interest (Divol et al., 2012). Moreover, Nadai et al., 2016 have recently found that strains showing higher resistance to SO_2, produced higher levels of SO_2 in comparison to those sensitive to this molecule, which suggests that SO_2 resistance and production are related. Furthermore, resistant strains were shown to have much higher basal gene expression level of SSU1, the gene considered the main responsible for sulphite tolerance by regulating the transport of this molecule through the plasmatic membrane (Avram and Bakalinsky, 1997), and in some strains, also of those genes related to sulphur metabolism.

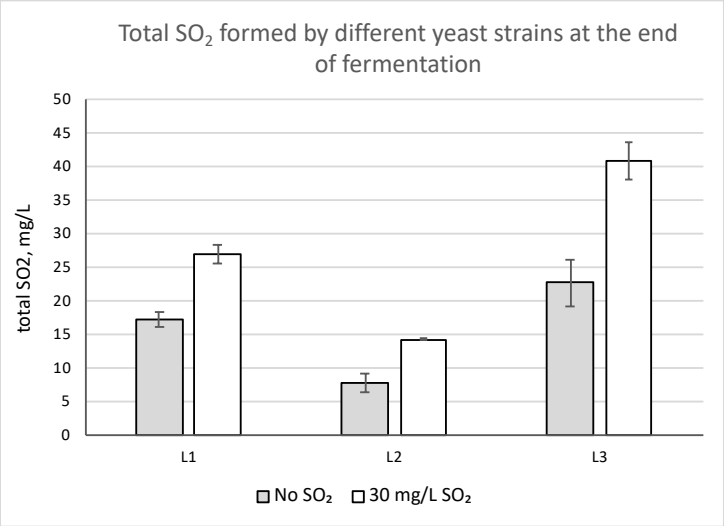

Figure 7 – ***Effects of yeast and of external SO_2 on the final levels of SO_2:*** *Average final SO_2 levels found in samples fermented with three types of strain and with or without addition of external SO_2 (30 mg/L). Error bars are standard errors of the mean.*

In any case, those data suggest that each strain of yeast has to contain a certain level of SO_2 within the cell, and it can be postulated that such internal SO_2 level will be correlated with the final level of SO_2 remaining in the media after fermentation. As one of the most obvious reasons limiting the efficiency of yeast reductases would be that part of the aldehydes were protected by SO_2, it can be further postulated that the ratios aldehyde/ alcohol and aldehyde/ acid should be significantly correlated to the final level of SO_2 remaining in the media after fermentation. This seems to be the case, as shown in Table 1 and particularly in Figure 8, which highlights three examples. In this Figure, the average ratios of the different samples (means of two biological replicates) are segregated by yeast and represented versus the final content in total SO_2 of the fermented media. Figure 8a corresponds to phenylacetaldehyde/ 2-phenylethanol ratio; Figure 8b to 3-methylbutanal/ 3-methylbutanoic acid ratio and Figure 8c to methional/ methionol ratio. It can be observed that in the first two cases the three strains followed a similar dependency, so that the fraction of aldehyde remaining

per unit of alcohol (Figure 8a) or acid formed (Figure 8b) is directly proportional to the level of SO_2, being the proportionality constant roughly independent of the strain of yeast. However, in the third case, the proportionality constant between the fraction of aldehyde remaining per unit of alcohol formed in yeast 3 is much smaller than those of yeast strains 1 and 2. This explains why the overall correlation coefficient between these parameters was not significant for methional (Table 1). As previously mentioned, this would be consistent with the hypothesis that SO_2 present within the yeast cell (in both cytoplasm and mitochondria) is the main factor determining the final levels of SAs after fermentation. The strong strain dependency could, therefore, be primarily due to the yeast intrinsic metabolism of SO_2.

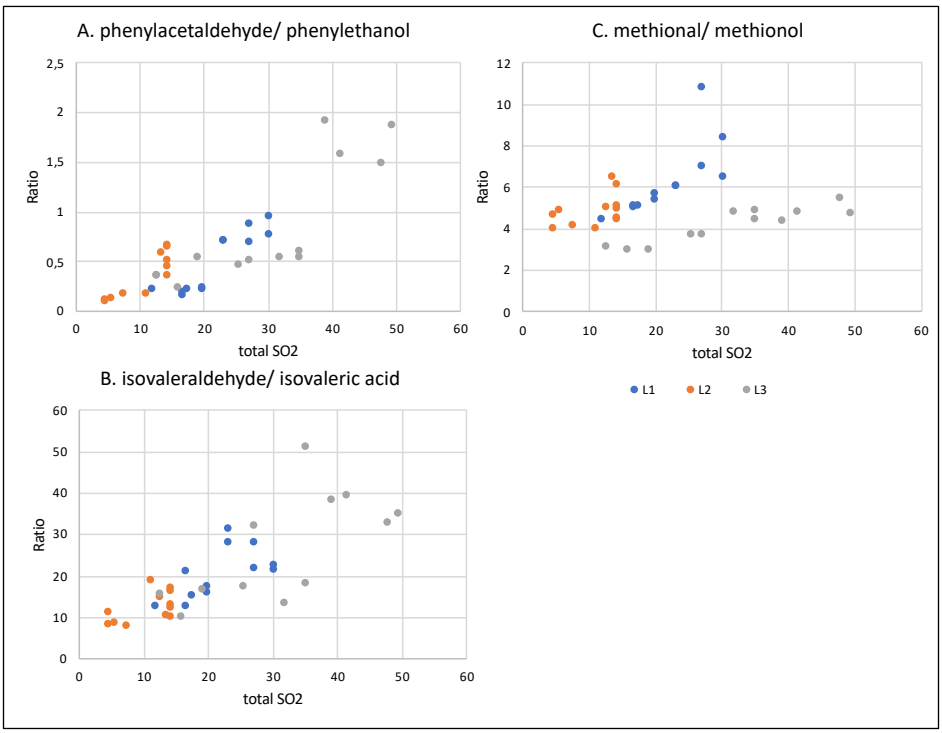

Figure 8 ***Aldehyde/alcohol or aldehyde/acid ratios and final SO_2 content:*** *Plots showing the relationship between important aldehyde/alcohol or aldehyde/acid ratios and the final SO_2 levels found in samples fermented with three different yeast strains. A) phenylacetaldehyde/2-phenylethanol ratio; B) 3-methylbutanal/3-methylbutanoic acid ratio; C) methional/ methionol ratio*

3.3 Role of Zn on SA formation

As previously mentioned, the effects of Zn on the formation of SAs seems to be secondary (Table 1) only being significant in the cases of 3-methylbutanal and phenylacetaldehyde in the experiment in which the 0 level of zinc was considered. As shown in Table 3, the levels of those aldehydes were minimum when levels of Zn are 1 mg/L, becoming maxima at 10 and 0 mg/L. As in this experiment, levels of isobutanol, isoamyl alcohol and 2-phenylethanol were significantly smaller in conditions of Zn starvation, some of the ratios aldehyde/ alcohol become at Zn starvation significantly higher than those observed at 1 mg/L. The same is observed in the aldehyde/ acid ratio 3-methylbutanal/ 3-methylbutanoic acid. Alcohol/ acid ratios were also significantly affected by Zn levels, significantly increasing with Zn levels (Table 3). These results suggest Zn levels may have an effect on the ADH activities of yeast which may have an indirect effect on SAs formation. In fact, Zn is an essential component of many dehydrogenases (De Smidt et al., 2008) which contain a zinc-containing active site (Persson et al., 1993). Our data suggest that the overall ADH efficiency of yeasts largely decreases in conditions of complete zinc starvation. These observations are consistent with the known fact that under low zinc condition, a regulatory metal-responsive protein alters several *S. cerevisiae* metabolic pathways including repressing some of the genes that express yeast ADH (Eide, 2009).

3.4 Fatty acids and their ethyl esters

As expected, levels of fatty acids and of their ethyl esters were significantly influenced by the yeast strain, as shown in Table 1. As seen in Table 2, yeast strain L1 produced maxima levels of the three acids and of two of the ethyl esters, while L3 produced in most cases the smallest levels. Effects were not

significant for ethyl hexanoate, for which the esterification ratio in L1 was minimum.

However, the most remarkable effect on levels of fatty acids, their ethyl esters and their esterification rates are played by Zn content. As seen in Tables 1 and 3, Zn levels have a significant, but not very important effect on the absolute levels of hexanoic and octanoic acids, but quite intense effects on the levels of esters and on the esterification ratios. As seen in Table 3, levels of ethyl hexanoate, octanoate and decanoate reached maxima values in conditions of Zn starvation or of low Zn levels in the first experiment. Maximum levels were more than 3-4 times higher than the minimum levels. The effects on the esterification ratios followed a similar trend, with maxima levels in conditions of Zn starvation or of low Zn levels.

To the best of our knowledge, the relevant effects of Zn on ethyl esters and particularly, on esterification ratios, have not been previously reported. By using genomic and transcriptomic analysis it has been known for a time that the activities of all the enzymes of the cytidine diphosphate diacylglycerol (CDP-DAG) pathway, the major route in the synthesis of phospholipids, are decreased in Zn-limited cells (Iwanyshyn et al., 2004) while a different set of enzymes related to an alternative route of synthesis known as Kennedy pathway, displays more activity (Kersting and Carman, 2006; Soto and Carman, 2008). I.e., it is well established that in low Zn conditions there is a metabolic remodelling in the synthesis of phospholipids (Eide, 2009), and therefore it should not be surprising that the levels of fatty acids and their ethyl esters, which are by-products of such synthesis, change.

*Table 3 Average levels of total SO_2, aroma compounds and of some relevant ratios attending to the Zn levels of the must in the two experiments. Concentration data are in mg/L except aldehydes which are in μg/L. Cases showing significant interactions Zn x yeast are marked with • or * for the experiments without or with SO_2, respectively. Significant differences attending to Duncan test are indicated with letters [a-c] being "[a]" the lowest average value.*

	No SO_2			30ppm SO_2		
	10 mg/L	5 mg/L	1 mg/L	10 mg/L	1 mg/L	0 mg/L
total SO_2*	20.3 ± 3.7[b]	13.6 ± 3.5[a]	13.8 ± 2.5[a]	-	-	-
aldehydes						
2-methylpropanal	-	-	-	-	-	-
3-methylbutanal*	-	-	-	33 ± 1.8[b]	24.5 ± 1.6[a]	32.8 ± 3.4[b]
2-methylbutanal	-	-	-	-	-	-
methional	-	-	-	-	-	-
phenylacetaldehyde*	-	-	-	25.5 ± 5[b]	17 ± 2[a]	24.1 ± 4.9[b]
fusel alcohols						
isobutanol*	-	-	-	30 ± 8[b]	26.6 ± 5.4[b]	21.6 ± 4.8[a]
isoamyl alcohol	-	-	-	207 ± 29[b]	202 ± 16[b]	163 ±17[a]
methionol	-	-	-	-	-	-
2-phenylethanol*	17.7 ± 2[a]	20.6 ± 1.4[ab]	23.1 ± 1.2[b]	27.2 ± 2.7[b]	27.9 ± 1.4[b]	23.8 ± 1[a]
iso-acids						
2-methylpropanoic acid	-	-	-	-	-	-
3-methylbutanoic acid	1.2 ± 0.1[a]	1.5 ± 0.1[b]	1.5 ± 0.2[b]	-	-	-
aldehyde/ alcohol ratio						
2-methylpropanal/isobutanol	-	-	-	-	-	-
3-methylbutanal/isoamyl alcohol*	-	-	-	0.2 ± 0.0[b]	0.1 ± 0.0[a]	0.2 ± 0.0[b]
methional/ methionol	-	-	-	5.1 ± 0.3[a]	5.6 ± 0.5[a]	6.7 ± 1[b]
phenylacetaldehyde/2-phenylethanol*	-	-	-	1 ± 0.3[b]	0.6 ± 0.0[a]	1 ± 0.2[b]
aldehyde/ acid ratio						
2-methylpropanal/2-methylpropanoic acid	-	-	-	-	-	-
3-methylbutanal/3-methylbutanoic acid*	-	-	-	26.9 ± 4.2[b]	17.3 ± 2.8[a]	23.6 ± 4.4[b]
isobutanol/2-methylpropanoic acid	-	-	-	20.4 ± 1.4[b]	18.8 ± 1.9[ab]	13.6 ± 1.2[a]
isoamyl alcohol/3-methylbutanoic acid	-	-	-	151 ± 8[b]	136 ± 13[ab]	108 ± 8[a]
2-methylbutanal/3-methylbutanal•	-	-	-	-	-	-
medium chain fatty acids						
hexanoic acid•	-	-	-	1.5 ± 0.2[b]	1.4 ± 0.2[ab]	1.3 ± 0.2[a]
octanoic acid	2.7 ± 0.2[b]	2.3 ± 0.2[a]	2.9 ± 0.4[b]	-	-	-
decanoic acid•	-	-	-	-	-	-
esters						
isoamyl acetate	-	-	-	-	-	-
ethyl hexanoate	0.1 ± 0.0[a]	0.1 ± 0.0[a]	0.4 ± 0.0[b]	0.2 ± 0.0[a]	0.2 ± 0.0[a]	0.7 ± 0.1[b]
ethyl octanoate•	0.2 ± 0.0[a]	0.1 ± 0.0[a]	0.5 ± 0.1[b]	0.3 ± 0.1[a]	0.3 ± 0.1[a]	1 ± 0.1[b]
ethyl decanoate•	0.1 ± 0.0[a]	0.1 ± 0.0[a]	0.3 ± 0.1[b]	0.1 ± 0.0[a]	0.1 ± 0.0[a]	0.4 ± 0.1[b]
ratios						
ethyl hexanoate/hexanoic acid	8.7 ± 0.8[a]	9.2 ± 0.6[a]	31.9 ± 4[b]	12.7 ± 1.1[a]	12.4 ± 1[a]	47.5 ± 7.7[b]
ethyl octanoate/octanoic acid	5.0 ± 0.7[a]	4.8 ± 0.4[a]	14.8 ± 0.7[b]	8.2 ± 1.1[a]	8.2 ± 1.2[a]	29.3 ± 3.6[b]
ethyl decanoate/decanoic acid	15.7 ± 1.5[a]	11.7 ± 0.8[a]	44.8 ± 5.2[b]	11.4 ± 1.2[a]	12.7 ± 0.6[a]	42 ± 3.8[b]

4. Conclusions

The finding that *Saccharomyces cerevisiae* is able to produce SO_2-bonded forms of SAs at levels which may be sensory-relevant, can have a strong industrial relevance. Since in a recent paper (Bueno et al., 2016) it was demonstrated that SO_2-bonded forms of SAs can be completely released during wine oxidation as free SO_2 levels decrease, this implies that wine oxidative self-life may be, in fact, primarily determined from alcoholic fermentation. Even though SAs can be additionally formed by metal catalysed Strecker degradation of amino acids during oxidation (Bueno et al., 2018), the aromatic degradation of wine could happen before. Furthermore, this study presents evidence that SAs accumulation is related to the specific yeast strains metabolism of SO_2, which would determine the intracellular levels of this molecule, which, in turn, would determine the fraction of aldehyde not oxidized or reduced by the yeast ADHs through Ehrlich pathway. External metal cations such as Zn are hereby shown to have a secondary effect on the formation of SAs. However, low Zn levels led to an increment in the levels of wine fruity esters. Overall, findings in this paper open the possibility to expand wine self-life by re-engineering alcoholic fermentation to minimize SAs formation and controlling must composition to favour formation of positive esters.

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Section II

Roles of yeast on the formation and evolution of the aroma of Riesling and Garnacha wines

Introduction and Methodology

Chapter 1 Effects of sequential fermentation with different non-*Saccharomyces* on the formation and further evolution of Riesling aroma

Chapter 2 The roles of yeasts on the formation and evolution of Garnacha wine aroma

Chapter 3 Observations, questions and conclusions derived from the comparison between varieties

Introduction and Methodology

1. Section II – Introduction

For long, the aroma of wine has been known to have different origins and has been traditionally divided into grape-derived compounds, fermentative or aged related compounds. Nonetheless, aroma formation is an interactive and complex process, which implies that these three groups are not necessarily segregated. In fact, grape composition deeply modulates the activity of microorganisms carrying out fermentation so that fermentative profiles can be strongly dependent on the variety of grape (Ferreira et al., 1996; Hernández-Orte et al., 2002)). Similarly, the development and evolution of wine aroma with time and hence, its aging potential is related to a complex array of chemical processes acting on compounds derived from the grape or formed during fermentation (Ferreira and San Juan, 2012).

The majority of aroma compounds in neutral grapes are present as non-volatile precursors. Many of them are conjugates of an aroma molecule and a non-volatile and water-soluble molecule, such as a glycoside. They represent a fundamental source of precursors of varietal aroma compounds in wine. Glycosidic precursors are formed by one or more sugar moieties (glycones) linked to an aglycone which can originate a volatile odorant upon release. To date, several aglycones have been identified and vary from straight chain alcohols to terpenoids, shikimic acid metabolites or norisoprenoids. Glycosidic precursors are linked to the formation of important varietal compounds such as linalool, geraniol, β-damascenone, α-ionone and β-ionone or 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) (Hjelmeland and Ebeler, 2015; Winterhalter and Rouseff, 2001; Winterhalter and Skouroumounis, 1997; Zoecklein et al., 1999). Studies with glycosidic precursors were first carried out with aromatic varieties such as Muscat, but also with Riesling for its high content in several of the

compounds previously mentioned, and for the particular interest aroused by the development with time of descriptors such as floral, citrus or kerosene (Fischer, 2007; Simpson, 1978; Simpson and Miller, 1983; Winterhalter et al., 1990; Zoecklein et al., 1999). On the other hand, little is known about the relevance of glycosidic precursors to form the aroma of red varieties, specially Garnacha. This variety has been described with black fruit, chocolate and even flowery notes however the compounds involved in the formation of these descriptors are not fully known (Lopez et al., 2004).

In order to form an odorant, the aglycone has to be released from the glycoside either by slow acid hydrolysis at wine pH or by enzymatic hydrolysis. This last can be carried out by enzymes from the plant, but it takes mainly place by the action of the different microorganisms carrying out fermentation. Alcohols and monoterpenes have been identified as compounds that could be directly released from glycosidic precursors (Waterhouse et al., 2016; Williams et al., 1980, 1993). Nonetheless, some relevant aroma compounds are not formed by straight hydrolysis of the glycosidic bound between the sugar and the aglycone but are formed after further spontaneous chemical rearrangements of the aglycone. This is the case of some relevant aroma compounds derived from carotenoids like norisoprenoids (Fischer, 2007; Mendes-Pinto, 2009; Waterhouse et al., 2016; Winterhalter et al., 1990; Winterhalter and Rouseff, 2001; Winterhalter and Skouroumounis, 1997).

Difficulties arise since the hydrolysis of one particular precursor can originate different compounds and one specific odorant can be often formed from different precursors. This makes that linking aroma compounds with specific precursors is rather difficult task (Waldmann and Winterhalter, 1992; Winterhalter and Skouroumounis, 1997; Zoecklein et al., 1999). Moreover, there is also evidence that most glycosides are not hydrolysed during winemaking or that they are

hydrolysed yielding non-volatile compounds like polyols, which by further chemical rearrangement will yield the aroma molecule (Williams et al., 1980; Zoecklein et al., 1999). This implies that fermentation can have a quite complex set of effects on the aroma potential of wine, and that many of these effects will not be identified but after long time. Furthermore, those effects can be further influenced by storage conditions, oxygen contact and presence of lees (Zoecklein et al., 1998, 1999), which adds more difficulties in the rationalization of the effects of fermentation on varietal aroma. The efficiency of the hydrolysis due to enzymatic activity is highly strain dependent, since the glycosidase activities of different strains can differ both in intensity and in the range of active substrates. Additional activities can be found in non-*Saccharomyces* genera, which has leaded to the development of fermentations combining cultures of non-*Saccharomyces* and *Saccharomyces cerevisiae* sequentially inoculated. These strategies have been shown to modulate aroma formation and to have potential to produce wines of higher quality and complexity, which has been attributed to the different abilities to release volatiles from grape precursors (Benito et al., 2015; Escribano et al., 2017; Padilla et al., 2016). However, few studies have actually been carried out with extensive analysis of the aroma compounds formed, and few less have taken into consideration the effects of aging, hence there are yet many open questions regarding the role of yeast in wine aroma formation and evolution, especially in what concerns their action on grape derived precursors.

2. Goals

The present set of studies intends to bring some light into the roles played by yeasts, in the formation and development of varietal and fermentative aroma of wine. For this, a specific research involving sequential fermentations with different yeast strains, synthetic must containing real fractions of precursors from two grape varieties and different aging times has been carried out. The aims of the study are:

1. To determine the hierarchy of factors (yeast, precursors, time) affecting wine aroma profile.
2. To assess the specific effects linked to the presence of aroma precursors on wine aroma and on its evolution with time.
3. To assess the effects of yeast on varietal and fermentative aromas and on the evolution of aroma with time.
4. To derive general practical conclusions about the possibilities to modulate wine aroma using sequential fermentations.

3. Section II – Methodology

3.1 Reagents and standards

Dichloromethane (DCM), ethanol and methanol (≥ 99%) Disto-Pesticide residue grade were supplied by Merck (Darmstadt, Germany). Milli-Q® system from Millipore (Merck, Germany).

2-butanol (≥ 99%), 4-methyl-2-pentanol (99%), 4-hydroxi-4-methyl-2-pentanone (99%), ethyl heptanoate (99%) and heptanoic acid (99%) were used as internal standards for major compounds analysis and 2-octanol (99.5%), 3-octanone (99%) and 3,4-dimethylphenol (99%) were used as internal standards for minor and trace compounds analysis and were purchased from Merck.

The chemical standards used in this study were supplied by Merck with purity ≥ 98%. TDN was synthesised by Synchem UG & Co with a purity of 80%.

An alkane solution in dichloromethane (C7-C28) was used to calculate approximate linear retention index of analytes.

3.2 Glycosidic precursors extraction

3.2.1 Grape processing

The glycosidic precursor fractions were obtained during harvest 2016 and approximately 23 Kg of grapes were obtained for each variety. Riesling grapes were obtained in Neustadt an der Weinstrasse, Germany and Garnacha grapes were given by *Bodegas Román* from D.O. Campo de Borja, Spain.

The grapes were crushed by feet and cold macerated for 24 hours in the case of Riesling and 48 hours in the case of Garnacha, in the presence of Lafazym®CL (Laffort, France). The differences of maceration time relate with the fact that white and red winemaking have originally different maceration periods, and thus this approximates real winemaking to the small-scale experiments. To protect

the grapes of spontaneous microorganisms and reduce oxidation, 60 mg/L of SO_2 were added.

Due to access to different winery material, Riesling grapes were pressed using an Europress (Scharfenberger, Germany) and then cleared by flotation with N_2 and divided into three 5L batches with addition of 80 mg/L of SO_2 in each. Garnacha grapes were pressed using a manual hydraulic press and then cleared by sedimentation. The cleared must was divided into two 5L batches, since the must yield was lower, with addition of 80 mg/L of SO_2 in each.

3.2.2 Glycosidic extraction using a SPE based method

The extraction of each must batch was performed with 5 g of Lichrolut-EN resins. The resins were washed and preconditioned by packing them in 60 ml cartridges with polyethylene frits (Supelco), they were then rinsed with 45 ml of dichloromethane, 45 ml of methanol and 54 ml of Milli-Q® water. The activated resins were then let freely in the must with constant agitation during 48 hours, in a cool room. After extraction, the resins were recovered using paper filter with 150 mm pore (Macherey-Nagel) rinsed with Milli-Q® water, repacked in the cartridges and dried in a VAC ELUT station (Varian). The resin was washed with 45 ml of dichloromethane to remove grape aroma compounds and were further eluted with 90 ml of ethyl acetate with 10% methanol (v/v). The resins were afterwards re-activated and the extraction process was repeated adding 50 mg/L of SO_2 to each must batch.

The eluted fractions were mixed and dried from its solvent using a rotavapor R-215 coupled with a heating bath B-491, from Buchi (Switzerland) and N_2 flow. Prior to usage, the glycosidic fractions were re-dissolved with 70ml of ethanol. The volume of precursor fraction added to each small fermenter was calculated

following the proportion that total re-dissolved fraction corresponds to the total volume of clean must obtained from each variety.

3.3 Synthetic must fermentation

3.3.1 Synthetic must composition

A complex synthetic must was prepared and adjusted to 3,5 pH and then filtered by means of sterile cellulose nitrate membrane filters with 0.45 µm pores (Albet, A&S Filter Co., Ltd) inside a vertical laminar flow chamber PV-100 (Telstar, S.A) to ensure that all manipulations were done under aseptic conditions. All glassware was sterilized using an autoclave AES-28 from Raypa (Barcelona, Spain).

Must composition was adapted from Bely et al., 1990 and had the following composition: oligoelements: $MnCl_2.4H_2O$ 4.7 mg/L, $Co(NO_3)_2 \cdot 6H_2O$ 0.49 mg/L, $NaMoO_4 \cdot 2H_2O$ 0.19 mg/L, $CuCl_2$ 0.54 mg/L, KIO_3 1.29 mg/L, H_3BO_3 1 mg/L; malic acid 0.3 g/l; tartaric acid 3 g/L; $SO_4Mg \cdot 7H_2O$ 0.2 g/L; KH_2PO_4 2 g/L; $CaCl_2 \cdot 2H_2O$ 0.155 g/L; citric acid 0.3 g/L; vitamins from Merck (≥ 98%): pyridoxine hydrochloride 1 mg/L, nicotinic acid 1 mg/L, calcium panthothenate 1 mg/L, thiamine hydrochloride 1 mg/L, ρ-aminobenzoic acid 1 mg/l, riboflavin 0.2 mg/L, folic acid 0.2 mg/L, biotin 0.04 mg/L. Myo-inositol 0.3 g/L; ergosterol 15 mg/L. Sugars were obtain from Panreac Applichem (Spain): glucose 100 g/L; fructose 100 g/L. Tween80® 0.05% (v/v) (Merck). Nitrogen source: $(NH_4)_2HPO_4$ 219.9 mg/L; amino acids (Merck) (mg/L): GABA 44.37, alanine 58.51, tyrosine 14.34, valine 17.73, isoleucine 14.43, leucine 13.42, aspartate 34.82, glutamic acid 61.83, glutamine 104.83, serine 21.21, glycine 1.11, histidine 109.2, threonine 18.8, arginine 199.5, proline 241.46, methionine 29.85, phenylalanine 11.15, lysine 3.33.

3.3.2 Yeast culture

Four commercial yeasts were selected from Chr. Hansen (Denmark) Viniflora® product line to ferment synthetic must: *Saccharomyces cerevisiae* Merit, *Pichia kluyveri* FrootZen™, *Torulaspora delbrueckii* Prelude™ and *Lachancea thermotolerans* Concerto™.

With the exception of *Pichia kluyveri* (FrootZen™), which is commercially available for direct inoculation, the remaining strains are dried products. As fermentations with the different varieties were not carried out at the same time, after product opening and usage for the first experimental setup, the yeast cells were preserved at -80ºC. For that, all the dried yeasts were cultured and pure colonies were grown in yeast extract peptone dextrose medium (YEPD). The yeast cells were aliquoted (10^7 CFU/ml) and preserved with glycerol at -80°C

The inoculations for Garnacha fermentations were made from the dried products following the product instructions: 1 g of dried yeast from a recently open package was hydrated in 20 ml of water for 1 hour using a water bath at 35°C to maintain he temperature. Afterwards, 20 ml of synthetic must was added to pre-activate the cells at the same temperature for 30 minutes. The fermenters were inoculated with 10^6 cells/ml.

For Riesling experimental fermentations inoculations were made from the frozen preserved cells: each aliquot was centrifugated and the pellet was washed with phosphate buffer solution. A clean pellet was used to inoculate each fermenter with 10^6 CFU/ml.

3.3.3 Fermentation set-up and accelerated aging

Two series of 8 fermenters containing 350 ml of synthetic must were prepared using 500 ml blue cap glass flasks (Ilmabor TGI, Germany). One series was spiked with the glycosidic precursor fraction and the second one was used as

must control. The four yeast strains selected were used to ferment both series, with glycosidic precursors and controls, following the same inoculation protocol. Two biological replicates of each experiment were prepared. Fermentations with only *S. cerevisiae* were used as microbiologic controls while all non-*Saccharomyces* were sequentially inoculated with *S. cerevisiae* after 4 days of individual fermentation[1]. The fermenters were closed with airlock valves and fermentations were carried at 20°C. Fermentation progress was followed by weighing the fermenters daily until the weight loss between two consecutive measurements was smaller than 0.1 g.

Once fermentation was finished the fermenters were centrifuged and aliquoted for the different analysis inside an anoxic chamber Jacomex (Dagneux, France). Additionally, the wines were aliquoted into air tight Wine In Tubes (WIT, Bordeaux, France) bagged in high density plastic bags containing oxygen scavengers AnaeroGen™ (Thermo Scientific, USA) and aged in a 50°C laboratory heater (J.P. Selecta, Spain). Samples were collected and analysed at the end of fermentation and after 1, 2 and 5 weeks of accelerated aging.

3.3.4 Unfermented controls

Unfermented controls were included to assess the effect of acid hydrolysis on the release and formation of volatile compounds[2].

Synthetic wine with 12% ethanol, 3.5 g/L of tartaric acid and Milli-Q® water was adjusted to pH 3.5. Eight WIT were prepared inside the anoxic chamber: four WIT were capped with only synthetic wine and the in remaining four WIT synthetic wine was spiked with precursors fraction. The WIT were further

[1] Although a sequential inoculation protocol was followed for all the wines fermented with a non-*Saccharomyces* yeast, for the data analysis only the name of the non-*Saccharomyces* strains was used as reference to the wines

[2] Unfermented controls are referred in the figures and tables as AH.

bagged as described for the accelerated aging experiments. All eight WIT were kept in the same incubators as the fermented incubators (21ºC) and then the accelerated aging samples (50ºC). Two tubes, one with only synthetic wine and one spiked with precursors, were taken after fermentation and after 1, 2 and 5 weeks at 50ºC.

3.4 Analytical methods

Classical oenological characterization and major volatile compounds were analysed as described in Section I.

3.4.1 Analysis of minor and trace volatile compounds

In order to analyze specific acetate esters and ethyl esters, terpenes, norisoprenoids, lactones, volatile phenols and vanillin derivatives present at low levels (0.1-1000 μg/L) a SPE (Solid Phase Microextraction) extraction was applied using the methodology described by Lopez et al., 2002 with some modifications. The three internal standards 2-octanol, 3-octanone and 3,4-dimethylphenol were added to 15 ml of wine followed by the extraction by SPE using 65 mg cartridges with Lichrolut-EN resin previously activated with 2 ml of DCM, methanol and hydro-alcoholic solution with 12% ethanol. After the wine has been passed through the cartridge, the resin is washed with a solution of water with 30% methanol (v/v) and 1% of $NaHCO_3$ (p/v). After cartridge drying, 600 μl were eluted with DCM containing 5% methanol (v/v).

3.4.2 Chromatographic method

A new chromatographic method was developed to analyze these compounds.

A QP2010 gas chromatograph equipped with a quadrupole mass spectrometer detector from Shimadzu (Japan) was used. The column was a DB-WAXetr from

Agilent (USA), 30m x 0,25mm with 0,5μm film thickness, preceded by a 2m x 0.25mm medium-polar uncoated precolumn. The carrier gas was He at 1.26 mL/min. The chromatographic oven was initially at 40°C for 5min, the temperature increased with a rate of 1°C/min until 65°C and then the temperature was raised to 220°C at 2°C/min and hold for 50 min. A SPL injector (split/splitless) was used at a temperature of 250°C. The injection was carried out in splitless mode; 2μL of sample was injected using a pressure pulse to ensure a column flow of 4.50 mL/min during 1.5 min, time at which the split valve was opened. The temperature of the ion source was kept at 220°C and the interface at 230°C. The mass analyser was set in single ion monitoring mode (SIM) and the complete list of m/z ratios selected for each compound as well as their retention time are shown in Table 4. Identification was made using chemical standard compounds injected in SCAN mode and confirming MS data with NIST data base. Quantification was done by interpolating the SI-normalized peak area in the straight lines built by repeated analysis of calibrated solutions containing at least three different concentration levels of each compound. The calibrated solutions were carefully prepared so that minimal levels of some compounds were compensated with maxima levels of others, so that the total mass of volatiles in the solution remained approximately constant. In all cases, the ions selected were those providing maxima selectivity and sensitivity. A minimum of two ions were selected, in order to have an additional criterion of identity. In our conditions, furaneol, because of its high polarity, did not produce any well-defined peak at the required concentration levels and could not be quantified.

Table 4 Mass spectra ions selected to quantify minor and trace compounds using GC-MS.

Compounds	RT	*m/z*
Ethyl esters and acetates		
Ethyl isobutyrate	7.5	71[a], 116
Ethyl 2-methylbutyrate	12.0	57[a], 102
Ethyl 3-methylbutyrate	13.10	88[a], 115, 70
Ethyl 4-methylpentanoate	24.15	88[a], 101
Ethyl cyclohexanoate	45.55	83[a], 101, 156
Isobutyl acetate	9.71	56[a], 73
Phenylethyl acetate	79.90	91[a]
Norisoprenoids		
Rose oxide	39.73/ 40.93	139[a], 154
Vitispirane*	52.8/ 53.08	192[a], 93, 121, 171
Riesling acetal*	59.9	138[a], 125, 133
β-damascenone	79.86	69[a], 190
α-ionone	72,4	121[a], 93, 192
β-ionone	77.08	177[a], 192
1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN)	66.48	157[a], 142, 172
Monoterpenes		
Linalool	55.01	71[a], 93, 121
α-terpineol	64.05	93[a], 121, 136
Geraniol	72.63	69[a], 123
β-citronellol	68.13	69[a], 81, 123
Lactones		
δ-nonalactone	81.81	85[a], 100
δ-decalactone	87.36	85[a], 100
Whiskylactone	74.56/ 78.18	99[a], 114
Cinnamates		
Ethyl dihydrocinnamate	74.54	178[a], 133
Ethyl cinnamate	86.80	131[a], 176
Volatile phenols		
Guaiacol	73.5	109[a], 124
o-cresol	81.16	108[a], 79
m-cresol	85.35	108[a], 79
4-ethylguaiacol	82.17	137[a], 152
Eugenol	88.73	164[a], 149
E-isoeugenol	96.83	164[a], 149
4-ethylphenol	89.33	107[a], 122
4-propylguaiacol	85.96	137[a], 166
4-vinylguaiacol	90.14	150[a], 135
4-vinylphenol	99.04	120[a], 91
2.6-dimethoxyphenol	93.27	154[a], 139
4-allyl-2,6-dimethoxyphenol	104.87	194[a], 119
Vanillin derivates		
Vanillin	105.85	151[a], 152, 123
Acetovanillone	108.83	166[a], 123
Syringaldehyde	127.15	182[a], 181, 167

*Compounds tentatively quantified using alkanes to determine the retention index;
[a] Quantitative fragments m/z

For Vitispirane and Riesling acetal a commercial standard was not available thus, their identification was made using *m/z* and retention index from bibliography references (Loscos et al., 2007) in SCAN mode as well as injection of alkanes to calculate retention index in a DB-wax column.

3.5 Data treatment

Relative areas were obtained by dividing the ion peak area of the analyte by the area of the corresponding internal standard. Those areas were transformed into concentrations by interpolation in the calibration graphs built by the analysis of calibrated samples. Data processing was made using Microsoft Excel Visual Basic for application (VBA) simple coding.

Analysis of variance (ANOVA) was made on the compounds with area above the limit of quantification, assessing the factors presence of precursor fraction, yeast strain and accelerated aging time as well as the binary interactions (presence of precursors x yeast strain and yeast strain x aging time). Principal Component Analysis and Scatter plots were used to analyse the data. These analyses were performed using XLSTAT (Addinsoft, 2018 version).

The complete data set for each grape variety was also analyzed by Principal Component Analysis (PCA) to assess the hierarchy of factors affecting the aroma formation.

All graphics were made using Microsoft Excel, 2016 version.

The data obtained for each variety were analysed individually in chapters I and II and a further comparative analysis was performed in Chapter III.

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Chapter 1

Effects of sequential inoculation with different non-Saccharomyces on the formation and further evolution of Riesling wine aroma

Chapter 1 - Effects of sequential fermentation with different non-*Saccharomyces* on the formation and evolution of Riesling aroma

1. Results and discussion

The experimental approach followed in this work makes it possible to identify the aroma compounds that are exclusively formed from components present in the grape glycosidic precursor fraction, differencing them from those which are formed exclusively due to yeast metabolism. The former will be only found in samples containing precursors, fermented or non-fermented -acid-hydrolysis controls. The latter will be found in all the fermented samples, regardless of the presence of glycosidic precursor fraction.

The approach also allows the identification of differential effects of yeasts on the formation of varietal compounds, first by comparing fermented samples with the unfermented controls containing just precursors from which, aroma compounds are formed by acid hydrolysis and second by comparing the samples fermented with different yeasts.

Finally, the approach adds a time variable, since wines have been submitted to accelerated aging allowing the identification of different aging patterns linked to the presence of yeast.

The wines were firstly characterized according to their classical oenological parameters and then a comparative analysis of the volatile composition among the different controls and wines spiked with glycosidic precursors was made.

1.1 Classic oenological parameters of final synthetic wines

Important oenological parameters are presented in Table 5.

All fermentations have reached dryness and the pH was very similar in all samples (3.37 ± 0.09). Important differences were observed on the natural formation of SO_2 by yeast, being the control fermentations made exclusively with *S. cerevisiae* those with the highest levels (45 mg/l), while those made with *T. delbrueckii* the ones with the lowest levels. The highest level observed was 45 mg/L. Samples fermented with *T. delbrueckii* and, particularly, with *L. thermotolerans* have lower levels of acetic acid (0.27 ± 0.05) compared to *S. cerevisiae* and *P. kluyveri* (0.47 ± 0.06). There are no major differences in classical oenological parameters for pairs of samples with (PR-samples) or without (CTL-samples) glycosidic precursors fermented with the same yeasts. Hence, the presence of the glyosidic precursor fraction did not cause any relevant major impact on the yeast physiological activity.

Table 5 Classical oenological parameters of recently fermented synthetic wine given as average between the two biological replicates. Control samples without precursors fraction are indicated as CTL and wines spiked with Riesling precursors fraction are indicated with PR.

	Total SO_2	Volatile acidity	pH	Total acidity	Residual sugars
CTL *S. cerevisiae*	37.6 ± 2.4	0.51 ± 0.07	3.32 ± 0.06	5.06 ± 0.11	0.30 ± 0.1
PR *S. cerevisiae*	44.8 ± 0.0	0.48 ± 0.04	3.36 ± 0.05	5.03 ± 0.08	0.30 ± 0.2
CTL *P. kluyveri*	25.6 ± 1.6	0.49 ± 0.02	3.52 ± 0.03	4.76 ± 0.04	0.5 ± 0.2
PR *P. kluyveri*	31.2 ± 7.2	0.40 ± 0.0	3.56 ± 0.04	4.95 ± 0.08	0.20 ± 0.1
CTL *L. thermotolerans*	20.8 ± 1.6	0.22 ± 0.0	3.46 ± 0.02	4.65 ± 0.08	0.20 ± 0.1
PR *L. thermotolerans*	17.6 ± 1.6	0.26 ± 0.0	3.49 ± 0.01	4.58 ± 0.0	0.05 ± 0.05
CTL *T. delbrueckii*	28.0 ± 4.0	0.35 ± 0.02	3.52 ± 0.01	4.91 ± 0.11	0.15 ± 0.05
PR *T. delbrueckii*	28.8 ± 1.6	0.24 ± 0.02	3.58 ± 0.01	4.80 ± 0.23	0.20 ± 0.0

1.2 Overview of the aroma composition of final wines

Out of the 69 volatile compounds targeted in the quantitative analysis, 41 were found at levels above detection limits and were quantified. Riesling acetal and vitispirane, for which there was no chemical standard available were given just as the relative area to the internal standard 3-octanone. Furthermore, four out of the 69 compounds were not quantified due to chromatographic problems: ethyl succinate, isovaleric acid and ethyl butyrate had co-elution problems with other components present in the samples and furaneol, due to its high polarity, was not well separated using the current method. The remaining targeted compounds were definitively not present in the samples at levels above the detection limits of the method.

Twenty-six volatile compounds were present at quantifiable levels exclusively in all fermented samples, regardless of the addition of glycosidic precursors, but they were not present in the unfermented controls. Among these 26 compounds there were:

- alcohols (isobutanol, butanol, hexanol, isoamyl alcohol, methionol and β-phenylethanol);
- ethyl esters (ethyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl lactate, ethyl isobutyrate, ethyl 2-methylbutyrate and ethyl isovalerate);
- acetates (isoamyl acetate, isobutyl acetate, phenylethyl acetate);
- lactones (γ-nonalactone γ-decalactone, γ -butyrolactone);
- acids (acetic acid, butyric acid, isobutyric acid, hexanoic acid, octanoic acid and decanoic acid).

These volatiles will be regarded as fermentative compounds.

The remaining 15 volatiles were identified only in the samples spiked with the fraction with glycosidic precursors; both in fermented synthetic wines as well as in unfermented controls mainly after some aging:

- 4 norisoprenoids (1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN), β-damascenone, vitispirane and Riesling acetal);
- 3 terpenoids (linalool, geraniol and α-terpineol);
- 2 volatile phenols (4-vinylguaiacol and 4-vinylphenol);
- 2 vanillin derivatives (vanillin and acetovanillone).

These volatiles are genuine varietal aroma compounds.

The experimental set-up also makes it possible to assess the effects of non-*Saccharomyces* yeasts in both fermentative and varietal aroma fractions of wines. As aroma compounds are also susceptible to great changes during storage, all samples were subjected to anoxic aging so that the effects of yeasts could be assessed on more realistic basis.

Principal Component Analysis (PCA) was carried out on the dataset containing aroma compounds from all fermented samples in order to evaluate the hierarchy of the three effects under study (presence of precursors, yeast strain and aging) on the modulation of aroma composition. Unfermented controls were excluded of this study. Results are shown in Figure 9.

The plots shown in this figure summarize the PCA analysis representing the projection of variables loadings (top plot) and sample scores (bottom plot) in the plane formed by the two first principal components which retain 51.55% of the original variance. The effects of all three factors are well patent in this figure, being the presence of precursors and, particularly, the yeast strain the most determinant.

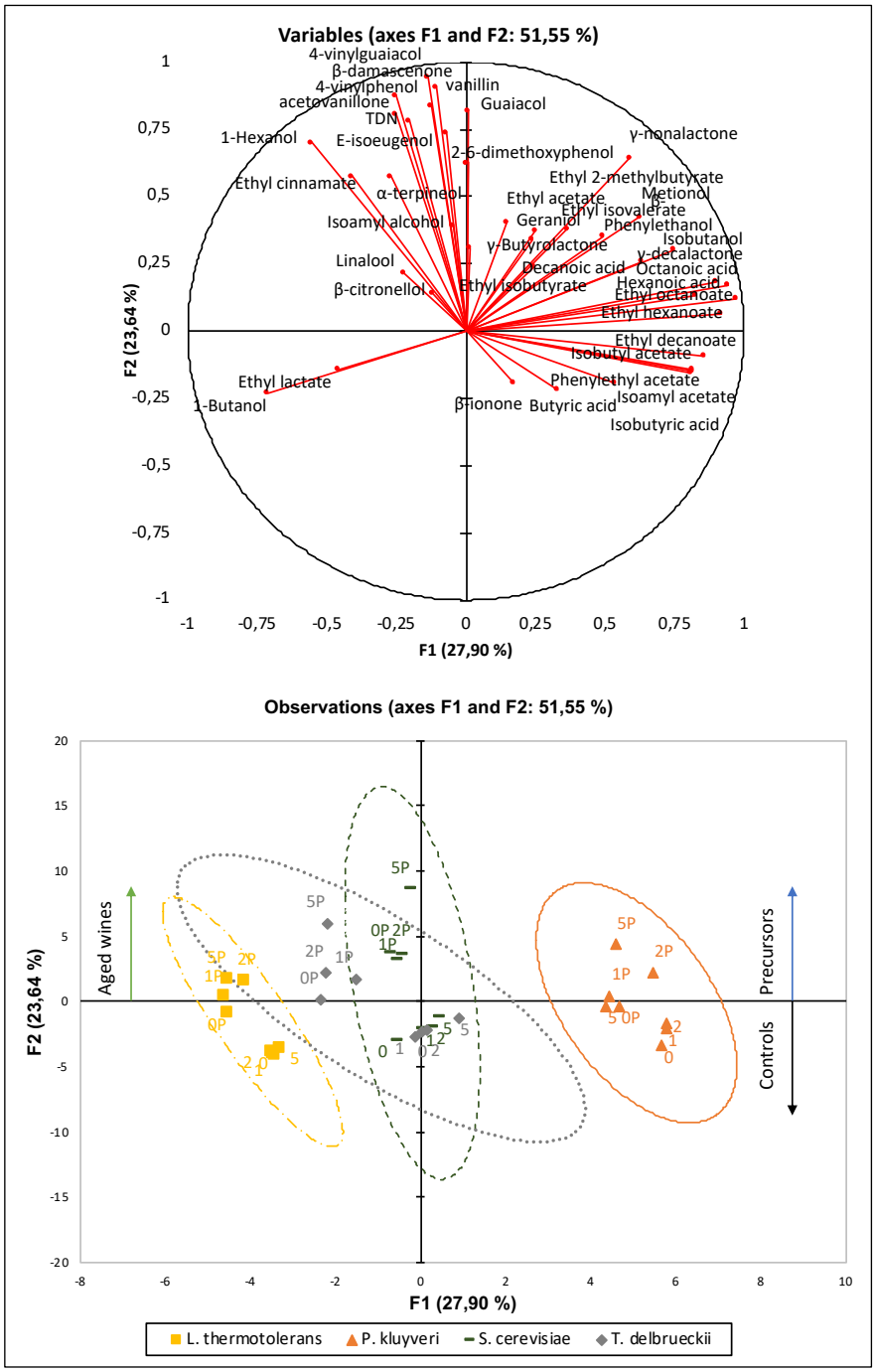

Figure 9 ***Principal component analysis carried out on the complete aroma volatiles data set*** *quantified in Riesling from all fermented samples. The plot shows the projection of variables (top plot) or samples (bottom) -average of two biological replicates- in the plane formed by the first two components, which retained 51.55 % of the original variance. Numbers in the samples refer to the weeks of anoxic storage at 50°C. P, indicates samples fermented with precursors.*

The sample scores plot clearly show that samples are arranged in four clusters corresponding to each yeast genera along the First Component. Samples fermented with *P. kluyveri* are isolated on the positive side of F1 in strict opposition to *L. thermotolerans* which is represented on the negative side of the same axis. Following, the presence or absence of glycosidic precursors in the must also has a visible influence in the whole data set, since all samples where glycosidic precursors were spiked are on the positive side of F2 while all control samples without precursors are on the negative part of this axis. Yet, the segregation of samples according to the presence of glycosidic precursors occurs within each yeast strain cluster. The effect of aging time can also be observed on both components, with all samples showing displacement tendencies towards the positive-left part of this component with aging.

As expected, all varietal compounds have positive and high loadings in the second component which is also indicative of their relevance in the aging process. Moreover, most alcohols, acetates, fatty acids and their ethyl esters have higher scores in the first component which suggests they have higher importance in differences between yeast strains.

1.3 Fermentative compounds in Riesling

All fermentative aroma compounds found in all the fermented samples, regardless of the presence of precursors and aging time, were analysed by PCA. Only unfermented controls were excluded from this study. Results are summarized in Figure 10. As can be seen, the major factor determining the position of the samples in the plane formed by the two first components (retaining 62.5% of the original variance) is the yeast strain. Samples fermented with *P. kluyveri* are the most distinctive, with large scores in F1;

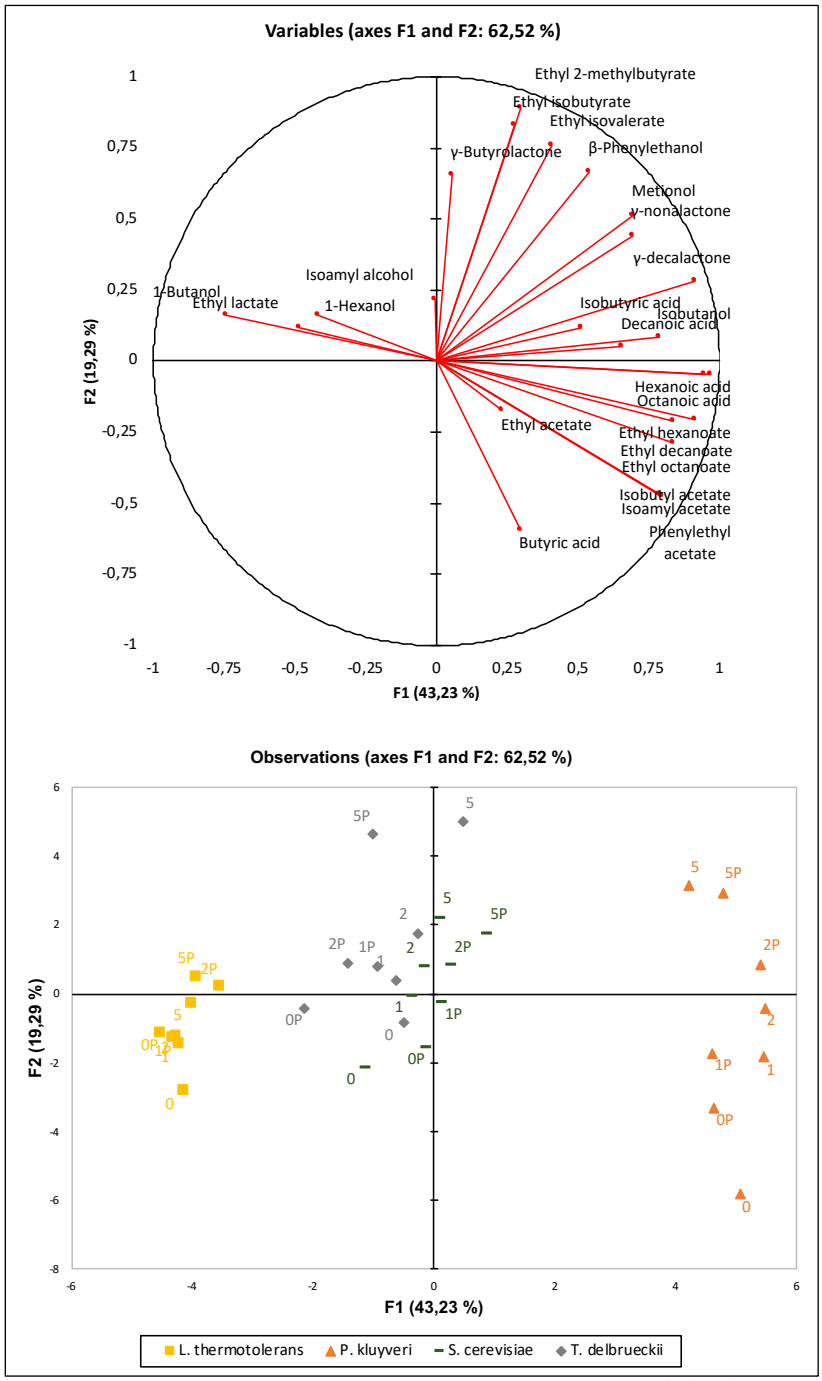

Figure 10 ***Principal component analysis on fermentative compounds*** *quantified in fermented samples with or without the precursor fraction of Riesling grapes. The plot shows the projection of variables (top plot) or samples (bottom) -average of two biological replicates- in the plane formed by the first two components, which retained 62.5 % of the original variance. Numbers in the samples refer to the weeks of anoxic storage at 50°C. P, indicates samples fermented with precursors.*

samples made only with *S. cerevisiae* are found in the middle of the plane and not clearly separated from those made with *T. delbrueckii,* which are at their left side. Finally, all samples fermented with *L. Thermotolerans* have negative scores in the first component. The influence of aging time is also seen in the figure, with aged samples having higher scores in the second component. However, the presence of precursors has no clear effect in the representation, meaning that the contents in fermentative volatiles are not highly affected by the presence of precursors in the fermenting must.

The variable loading plot, given in the upper part of the figure, shows that nearly all components have positive loadings in the first component, which is particularly correlated with volatile fatty acids, their ethyl esters and with the acetates of fusel alcohols. Only 1-butanol, ethyl lactate and 1-hexanol, keep a negative correlation with the first component.

This, certainly, indicates that samples fermented with *P. kluyveri* have the highest levels of most volatile compounds, notably of fatty acids, their ethyl esters and of the acetates of higher alcohols.

As for the second component, it is positively correlated with the ethyl esters of branched acids, with γ-butyrolactone and β-phenylethanol and negatively correlated with the acetates of fusel alcohols and with butyric acid.

Figures 11 to 13 include a selection of plots showing the evolution with time of the different compounds in the wines fermented with different yeasts, in order to facilitate the interpretation of results.

Figure 11, gives the plots with the evolution of acetates and other esters and acids. As can be seen, isoamyl acetate and phenylethyl acetate are found at much higher levels in samples fermented with *P. kluyveri.* It is also obvious that levels of acetates are slightly, but significantly, higher in samples not containing

precursors, suggesting that the acetyl transferase activity has been negatively influenced by the fraction of precursors. Nevertheless, recently fermented samples contain more than 2.5 mg/L of phenylethyl acetate, an amount exceeding, by far, the odour threshold of this compound. Levels of isoamyl acetate are, however, not particularly large. Both compounds follow a decreasing trend with time, since the acid-alcohol/ester equilibrium is displaced towards the dissociated form. Yet, levels of phenylethyl acetate after 5 weeks of aging are high enough to have high sensory implications.

Hexanoic and decanoic acids, as well as their corresponding ethyl esters are also illustrated in Figure 11. Also, in this case, it is evident that samples fermented by *P. kluyveri* have the highest levels, although differences are not as marked as for acetates. Nonetheless, levels followed the order *P. kluyveri* < *Saccharomyces* < *T. delbrueckii* < *L. Thermotolerans*. Levels of the ethyl esters are not particularly high, but this can be partly attributed to the large evaporation rate of these compounds when fermentation is carried out in small volumes. Nevertheless, levels of the corresponding fatty acids are normal-high, promoting the ethyl esters content to remain constant with time, contrarily to the case of acetates.

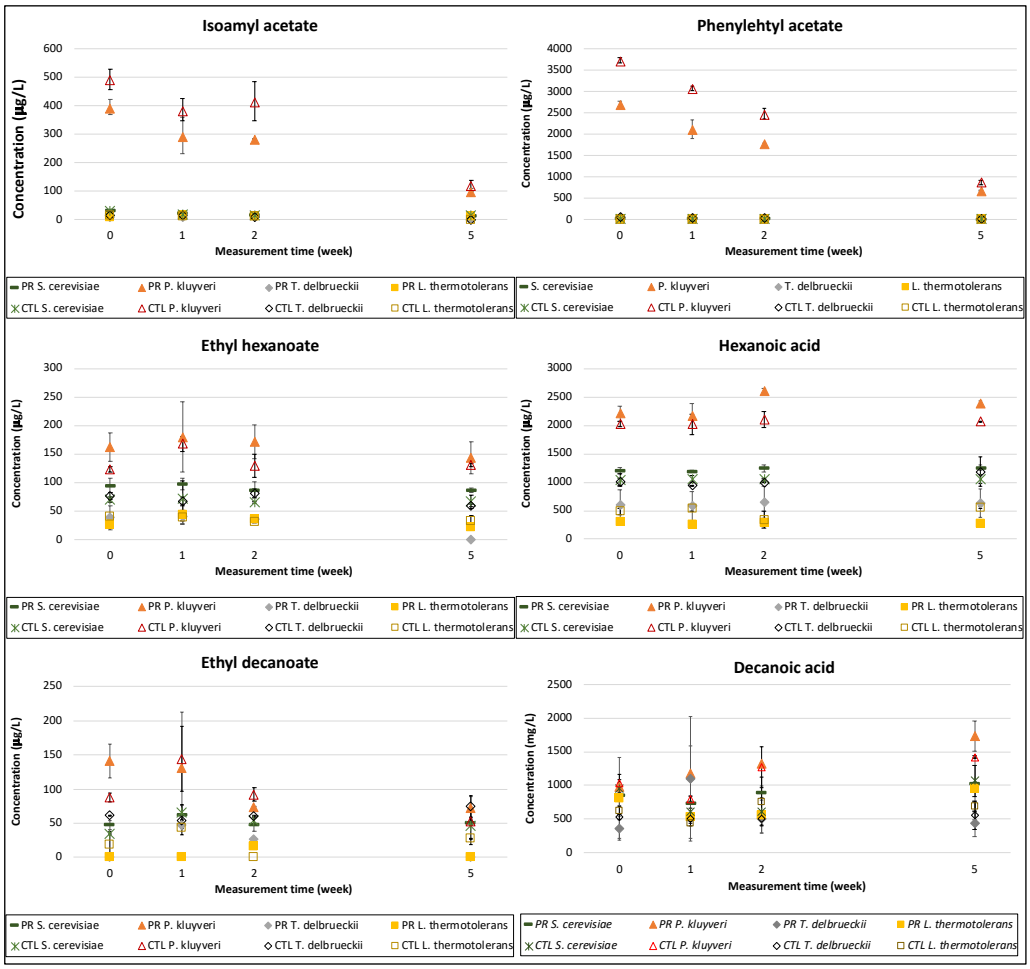

Figure 11 ***Yeast and aging effects on acetate esters, fatty acids and they ethyl esters*** *– evolutions with time of two acetates, two ethyl esters and their corresponding fatty acids (µg/L) according to the presence (PR) or absence (CTL) of precursors and to the yeast genera that carried out fermentation; samples were taken after fermentation – 0 and 1, 2 and 5 weeks of accelerated aging. Acetates and ethyl esters show decreasing tendencies while acids show increasing tendencies with time. P. kluyveri outstands in the production of esters and of hexanoic acid.*

The most relevant higher alcohols in wine can be seen in Figure 12. In all cases, yeast strains had a significant effect, but the outcome is compound dependent. In the case of isoamyl alcohol, wines fermented with *S. cerevisiae* contained significantly higher levels, while wines fermented with *P. kluyveri* had the

smallest levels. On the contrary, for methionol and β-phenylethanol the levels follow the order *P. kluyveri* > *T. delbrueckii* > *S. cerevisiae* > *L. Thermotolerans;* In the case of isobutanol, the pattern for all yeast is similar to methionol and β-phenylethanol with the exception of *S. cerevisiae* which can produce equivalent levels to those of *P. kluyveri.* Levels of these compounds remain fairly stable during aging, as can be seen in the figure.

Regarding ethyl esters of branched acids, these compounds are formed by slow esterification of their corresponding acids. Accordingly, levels of the ethyl esters are close to 0 in the recently fermented samples, increasing with time, as shown in Figure 13. Levels of isobutyric acid are fairly stable with time. Regarding yeast aptitude to form these two compounds, the order was *T. delbrueckii* > *P. kluyveri* > *S. cerevisiae* > *L. Thermotolerans* and, as observed in the case of acetates, samples fermented without precursors have higher contents than those spiked with Riesling precursors. Fermentative compounds are by-products of yeast secondary metabolisms. Several aroma compounds are formed in routes related to yeast amino acids metabolism and a second group to yeast lipid metabolism. A third group, the acetates, are related with both metabolic routes since their production is formed by acetyl-CoA transferases acting on higher alcohols which are produced during the synthesis of amino acids. Thus, it is evident that these routes are highly strain-dependent and are also influenced by the interaction between non-*Saccharomyces* and *S. cerevisiae* (Fleet, 2003). In fact, and as shown in Figure 9 and 10, despite that all wines were sequentially inoculated with *S. cerevisiae*, non-*Saccharomyces* introduced a major source of aroma variability on all main metabolic outcomes.

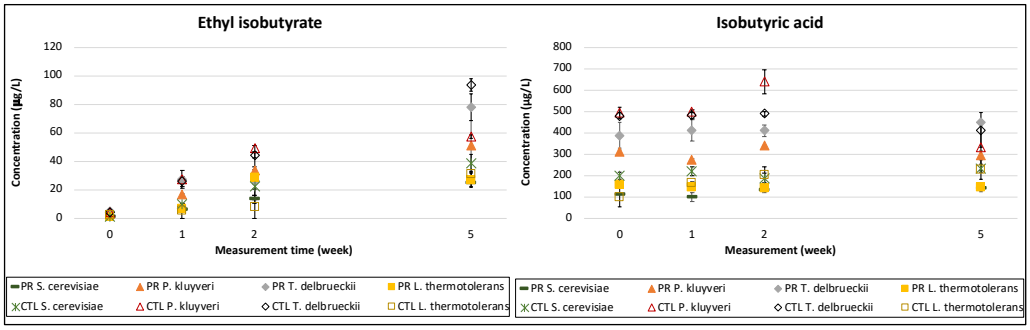

Figure 12 ***Yeast and aging effects on branched acids and ethyl esters -*** *one of the branched acids and its corresponding ethyl ester evolutions with time (µg/L) according to the presence (PR) or absence (CTL) of precursors and to the yeast genera that carried out fermentation; samples were taken after fermentation and after 1, 2 and 5 weeks of accelerated aging. Each yeast strains shows similar formation patterns for both compounds. The ester increases continuously and the acid is stable with time.*

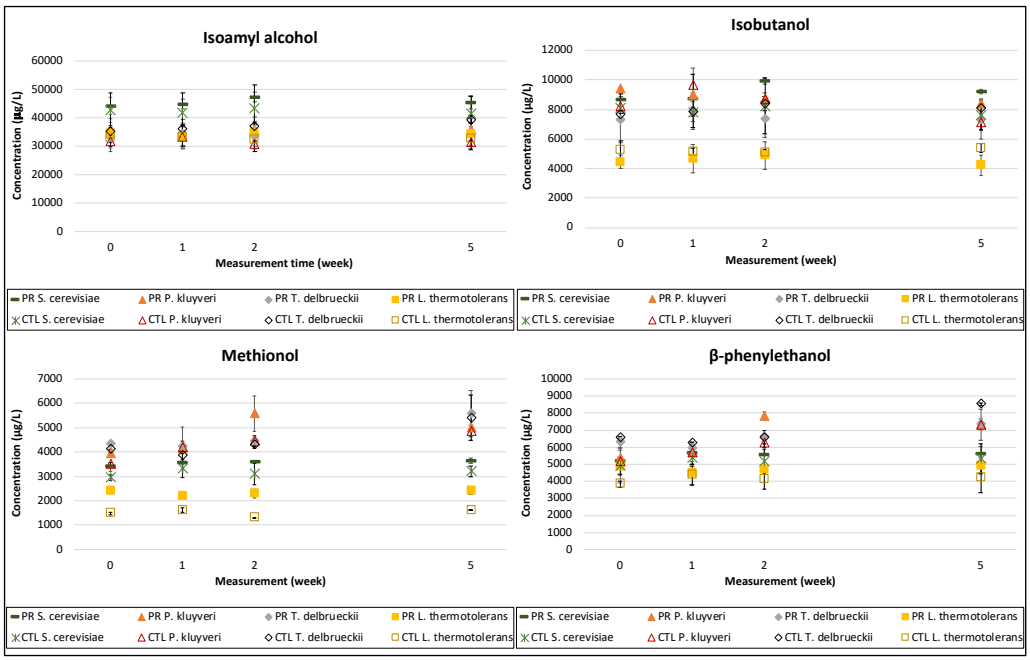

Figure 13 ***Yeast and aging effects on fusel alcohols*** *- major fusel alcohols evolution with time (µg/L) according to the presence (PR) or absence (CTL) of precursors and to the yeast genera that carried out fermentation; samples were taken after fermentation and after 1, 2 and 5 weeks of accelerated aging. L. thermotolerans produces lowest levels of these compounds. Fusel alcohols are quite stable during time.*

The effects of *P. kluyveri* on the levels of fatty acids, their ethyl esters and particularly on the acetates of higher alcohols, clearly show that these metabolic routes have been much promoted in the presence of this yeast, although *S. cerevisiae* has carried out most part of the fermentation (Padilla et al., 2016). On the contrary, the presence of *L. thermotolerans* has the opposite effect, strongly limiting the number of esters, acetates and fatty acids produced during fermentation. On the other hand, *L. thermotolerans* has an outstanding capacity to produce ethyl lactate as can be seen in the variables plot of Figure 9. That is most likely linked to this yeast reported aptitude to form lactic acid during alcoholic fermentation (Benito et al., 2015; Gobbi et al., 2013; Kapsopoulou et al., 2007). Samples fermented with *T. delbrueckii* on its side, show fermentative volatile profiles closer to those of *S. cerevisiae*. Remarkably, the three non-*Saccharomyces* resulted in wines with smaller levels of isoamyl alcohol compared with fermentations carried entirely out by *S. cerevisiae.* This reduction may have sensory relevance since this compound is a strong suppressor of wine fruity and woody notes (de-la-Fuente-Blanco et al., 2016). Furthermore, these data suggest that sequential inoculation of non-*Saccharomyces* yeast strains leads to wines with higher aroma complexity (Escribano et al., 2017; Jolly et al., 2014). Indeed, all samples where non-*Saccharomyces* yeast were inoculated had final aroma content fairly different from *S. cerevisiae* revealing that this methodology has repercussions on the final wine profile. The fact that wines fermented with *P. kluyveri* have overall higher ester content and above their odour threshold is likely to result in more fruity and flowery-like wines. On the other hand, *L. thermotolerans* strongly limits the levels of fermentative odorants formed, suggesting that it can be an important modulator of wine tactile properties, due to its ability to produce lactic acid and its derivatives (Benito et al., 2015; Swiegers et al., 2005) and also that is able to produce wines in which non-fermentative notes will be more easily perceived.

1.4 Varietal compounds in Riesling

Figure 14 summarizes the PCA carried out on varietal aroma compounds found in the data set. Only samples containing precursors, fermented or not, were included in the analysis. The two first components retain nearly a 60% of the original variance. As can be seen in the plot, the factor most influential in the location of samples in the plane is aging time, followed by fermentation and the yeast strain. The freshly fermented samples have the most negative scores in the first component, with a single exception for *S. cerevisiae.*

On the contrary, samples with 5 weeks of accelerated aging have highest scores in the first component. Additionally, unfermented samples have the highest scores of the second component contrarily to samples fermented with *S. cerevisiae* with equivalent aging times, which have the smallest scores on the same axis. The plot suggests that in this case, samples fermented with *S. cerevisiae* are the most different to the pure varietal aroma obtained by simple acid hydrolysis, while the intervention of non-*Saccharomyces* yeasts creates varietal wine profiles more similar to those observed by simple acid hydrolysis. A look at the sample loading plot of Figure 6, reveals that aging is related to geraniol and linalool decreases and with TDN and vinylphenols increases. Moreover, the existence of fermentation leads to increased levels of acetovanillone and minima of linalool, α-terpineol and β-damascenone.

Figures 15-17 represent the evolution with time of varietal compounds in the wines fermented with the different yeast strains throughout time. Looking at the different plots, it should be noted that in some relevant cases, notably those of linalool, α-terpineol, Riesling acetal, β-damascenone and geraniol, unfermented controls contained always higher levels than fermented samples (Figures 15 and 16).

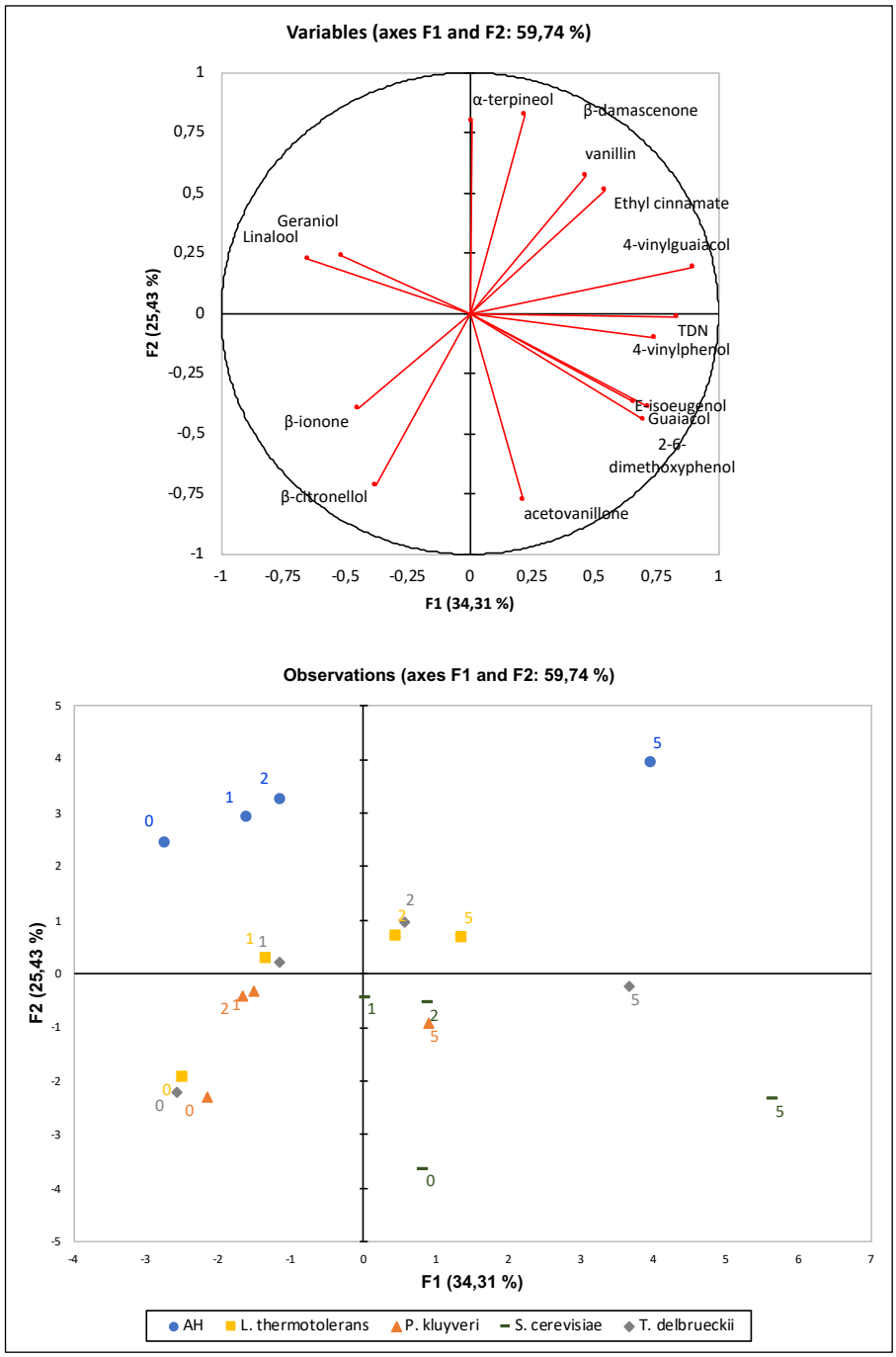

Figure 14 ***Principal Component Analysis on varietal compounds*** *quantified in fermented samples with Riesling precursor fraction, as well as unfermented controls spiked with precursors. The plot shows the projection of variables (top plot) or samples (bottom) showing the two biological replicates- in the plane formed by the first two components, which retained 59.74 % of the original variance. Numbers in the samples refer to the weeks of anoxic storage at 50°C.*

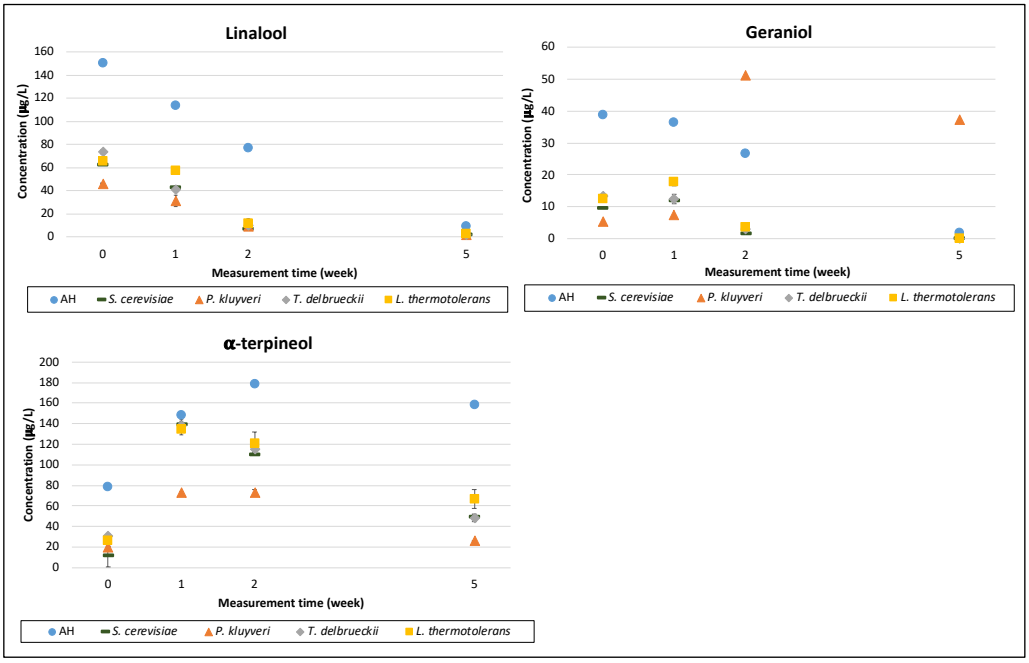

Figure 15 Evolution with time of the levels of the main monoterpenes, linalool, geraniol and α-terpineol, in fermented samples and unfermented controls containing precursors extracted from Riesling grapes. Samples were taken at the end of fermentation (0) and after 1, 2 and 5 weeks of accelerated aging. Wines fermented exclusively with S. cerevisiae were used as controls and fermentations with non-Saccharomyces strains of P. kluyveri. T. delbrueckii and L. thermotolerans were sequentially inoculated with S. cerevisiae. AH (acid hydrolysis) was used as unfermented control of synthetic wine spiked with the glycosidic precursor fraction. Data of geraniol content in wines fermented by P. Kluyveri are given by a single sample.

This pattern was not really expected and suggests that a large portion of these compounds' precursors were not really glycosides, but different polyols, which by simple rearrangement in acid media, yielded the aroma compounds within the few weeks between the preparation of the synthetic musts and the time of analysis after fermentation. Although this might affect equally the fermented and unfermented samples, since the fraction is the same, the volatile compounds already present during fermentation will inevitably be partially co-evaporated with CO_2 produced during fermentation, which helps explaining why levels in fermented samples are consistently smaller.

The different cases will be briefly analysed and discussed.

Linalool and geraniol are quite unstable compounds at wine pH and have a general tendency to decrease during aging (Figure 15). The decreasing rate is mitigated since new molecules released from precursors replace the decomposed ones. As aforementioned, the lower levels in fermented controls could be attributed to the partial evaporation of early formed aroma compounds during fermentation.

Differences between yeast strains were only moderately significant (see supplementary data) showing that wines fermented with *P. kluyveri* seem to have the lowest content of linalool, α-terpineol and geraniol. The action of such strain is not limited to a low efficiency in the hydrolysis of the precursors, but to the fact that precursors were probably transformed into different compounds. Otherwise, a slower rate of decrease should have been observed. Data also show that wines from *L. thermotolerans* have significantly higher levels of the three aroma compounds after 1 week of aging. This suggests that either the enzymes excreted by this strain during fermentation or upon cell autolysis were still active during accelerated aging, or that enzymes from this strain were particularly efficient at avoiding transforming precursors into molecules different to the targeted odorants.

Most surprisingly, levels of geraniol were found to increase in *P. kluyveri* wines after 2 weeks of aging. Levels in these wines were above 50 μg/L, the highest of this compound observed. This peculiar result needs further experimental checking, since the analytical results of one biological replicate for geraniol was lost.

The trend followed by α-terpineol is different, since the compound increases to a maximum level before starting to decrease, which is consistent with the fact

that this compound is an intermediate in the decomposition of the other monoterpenes.

Figure 16 shows the plots corresponding to some norisoprenoids. Riesling acetal and β-damascenone follow similar trends, characterized by a slight increase during the first 3 time points and then a plateau or a little decrease. This is consistent with the higher stability of these aroma compounds, in comparison to linalool and geraniol. It should be observed that β-damascenone has been demonstrated to be reactive towards SO_2 (Capone and Jeffery, 2011), but in spite of this, levels are relatively stable.

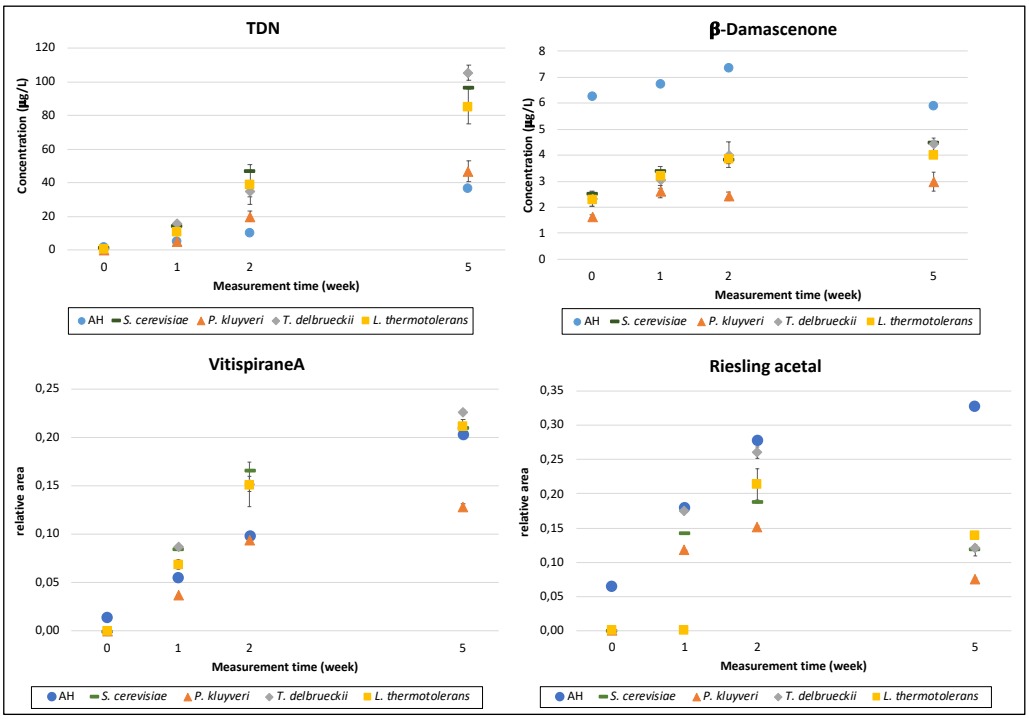

Figure 16 ***Evolution with aging time of the main norisoprenoids****: TDN, β-damascenone, one vitispirane and Riesling acetal present in fermented samples or unfermented controls both containing precursors extracted from Riesling grapes. Samples were taken at the end of fermentation (0) and after 1, 2 and 5 weeks of accelerated aging. Wines fermented exclusively with S. cerevisiae were used as controls and fermentations with non-Saccharomyces strains of P. kluyveri, T. delbrueckii and L. thermotolerans were sequentially inoculated with S. cerevisiae. AH (acid hydrolysis) was used as unfermented control of synthetic wine spiked with the glycosidic precursor fraction.*

As can be seen, levels of this important aroma compound are not much affected by the yeast strain, although is noteworthy that samples fermented with *P. kluyveri* contain significantly smaller levels of β-damascenone. The evolution of TDN as well as vitispirane is completely different. TDN is completely absent initially, but its levels increase continuously with time, following an approximately linear trend, as seen in the plot. The fact that it is not present either in the unfermented controls or in the fermented samples in the first time point, suggest that TDN does not exist as aglycone of any glycoside, but that it is formed by chemical rearrangement of different aglycones through complex chemical processes that take long time. The fact that yeast exerts a most evident positive effect on its formation rate, suggests that there is a pool of glycosides cleaved by the action of yeasts, whose aglycones yield TDN by further rearrangement. As the levels of aglycones increases, so does TDN formation rate. Giving that this aroma molecule can impart a kerosene-like sensory note which in some circumstances can be considered an off-odour, results clearly suggest that the intensity of such off-odour can be easily regulated by an adequate selection of yeast strain. As for monoterpenes and β-damascenone, samples fermented with *P. kluyveri* had smaller levels of TDN.

Similar trends regarding yeast strains and aging evolution were observed for vitispirane. The fact that the action of *P. kluyveri* occurs prior to *S. cerevisiae* inoculation, which, in pure inoculum, produced maxima levels of this molecule, together with the systematically smaller levels of monoterpenes and of the remaining norisoprenoids found in *P. kluyveri* samples, raises the hypothesis that this yeast partially metabolises carotenoids and monoterpenes, which could be consistent to the observed increases in geraniol.

The evolution of 4-vinylguaiacol and 4-vinylphenol can be seen in Figure 17. These compounds show quite complex trends, consequence of the larger number of possible precursors and formation pathways in wines. Three different trends

were found, the one followed by unfermented controls, a second one followed by pure *S. cerevisiae* culture and a third one followed by sequential-inoculation cultures.

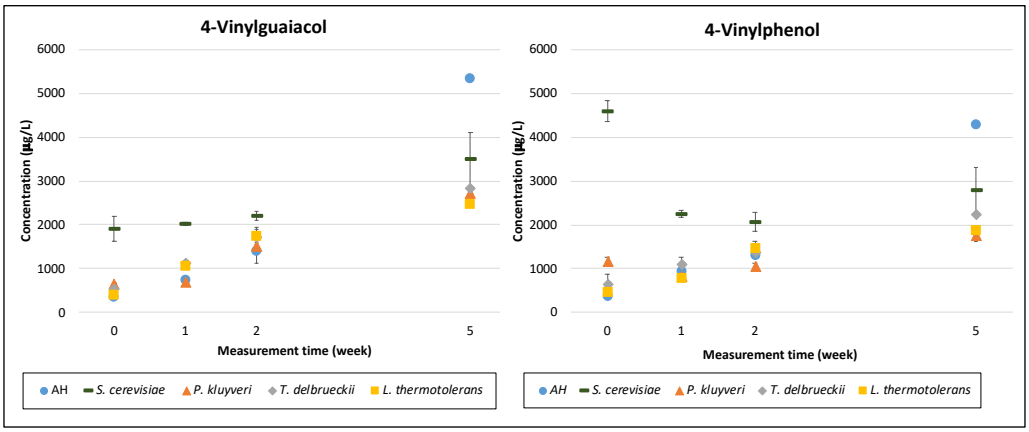

Figure 17 Evolution with aging time of the measured levels of 4-vinylguaiacol and 4-vinylphenol in fermented and unfermented samples containing aroma precursors extracted from Riesling grapes. Samples were taken at the end of fermentation (0) and after 1, 2 and 5 weeks of accelerated aging. Wines fermented exclusively with S. cerevisiae were used as controls and fermentations with non-Saccharomyces strains of P. kluyveri. T. delbrueckii and L. thermotolerans were sequentially inoculated with S. cerevisiae. AH (acid hydrolysis) was used as unfermented control of synthetic wine spiked with glycosidic precursor fraction

The unfermented controls show a continuously increasing trend, following a nearly quadratic law. Sequential-inoculation cultures are quite similar between them, having the same starting point and showing continuously increasing trends, but following straight lines. The most dissimilar is the trend followed by pure *S. cerevisiae* culture, for which initial levels are 4-8 times higher than those of the other samples. In the case of 4-vinylguaiacol there is a continuous but mild increase with time, while in the case of 4-vinylphenol, initial levels were amazingly high, there is a quick decrease in the first week of aging followed by a mild increase.

Monoterpenes are important varietal compounds with positive descriptors of citrus and floral aromas that have shown different formation and evolution

patterns. Linalool and Geraniol represent those monoterpenes quickly released or formed from precursors but not stable at wine pH, suffering further acid-catalysed reactions. Chemical rearrangements of linalool and geraniol into other monoterpenes such as α-terpineol, have been described in literature and can explain the opposite evolution of linalool and geraniol versus α-terpineol, i.e. the fast decline of linalool and geraniol could likely be due to their conversion into α-terpineol and other monoterpenes (Mateo and Jiménez, 2000; Williams et al., 1980; Wilson et al., 1984).

As aforementioned, the large levels of free linalool found in the unfermented control suggests that the precursor fraction contained fairly large amounts of some polyols, which, by a relatively fast hydrolysis, rendered the aroma (Mateo and Jiménez, 2000; Williams et al., 1980). The smaller levels found in the fermented samples may be then related to several different causes which cannot be completely discerned with the present data. Evaporation is one of them, a second one could be that the secondary enzymatic actions would induce the transformation of linalool and geraniol into other compounds. The current data, certainly do not full support this possibility, as α-terpineol levels are smaller in the fermented samples, but the transformation could have promoted the conversion into other not measured compounds such as nerol or 1,8-cineole already during fermentation (Carrau et al., 2005; Mateo and Jiménez, 2000; Wilson et al., 1984; Zoecklein et al., 1999). Clarification of this aspect would require further experimental work.

Thus, it seems that the different monoterpenes aroma volatiles contribute to wine varietal character in different stages of wines shelf-life, i.e., linalool and geraniol will probably characterize young wines while α-terpineol and several other monoterpenes are likely to be perceived in further aged wines.

The evolution pattern followed by β-damascenone, with maximum levels for the unfermented controls was expected, since in this case enzymatic action is far less efficient at producing the aroma than acid hydrolysis. This has been known for a long time, and it is one of the reasons why the analysis of grape precursors using enzymatic hydrolysis does not produce β-damascenone, implying that it does not exist as aglycone, but is related with different structures which, by rearrangement, render the molecule.

This observation draws attention to the not yet fully known mechanism that allows the release of β-damascenone from carotenoids, described in literature to require an initial oxidative cleavage followed by enzymatic transformations and finally acid-catalysed conversions (Winterhalter and Rouseff, 2001).

Regarding TDN, Riesling acetal and vitispirane, their initial contents are null or very low, and only accumulate after aging in an strain-dependent way. TDN has long been associated with aged Riesling character (Fischer, 2007; Simpson, 1978, 1978; Simpson and Miller, 1983; Zoecklein et al., 1999). As to their formation, also TDN, vitispirane and Riesling acetal have been linked to carotenoid breakdown and, similarly to β-damascenone, several intermediate steps are required in order to obtain the free odorant. Although formed from different and multiple precursors resulting from carotenoid degradation, their formation mechanisms are rather different. While one possible mechanism for β-damascenone formation has been associated with neoxanthin (Winterhalter and Rouseff, 2001) the formation of TDN is far from fully understood and linked with multiple precursors and series of hydrolysis and rearrangements. Furthermore, the chemical mechanisms of TDN, vitispirane and Riesling acetal formation are intrinsically related either by sharing or being each other's intermediate compounds (Daniel et al., 2009; Gök, 2015; Marais, 1992; Mendes-

Pinto, 2009; Waldmann and Winterhalter, 1992; Winterhalter and Rouseff, 2001).

This work has clearly demonstrated that yeast genera have a key role on TDN, vitispirane and Riesling acetal formation even though the effect is often only visible years after fermentation. Two possible explanations to this observation could be that even though the wines were centrifuged once fermentation was ceased, yeast enzymes could remain in the must after cell autolysis having extracellular activity (Loscos et al., 2009; Zoecklein et al., 1998). Nonetheless, since the accelerated aging took place at 50ºC a more plausible hypothesis is that during fermentation, enzymes segregated by yeast strains could have hydrolysed an intermediary aglycone which can undergo further slow rearrangements to form TDN, vitispirane and Riesling acetal. This enzymatic cleavage is likely strain dependent and seems to be crucial to achieve higher levels of these compounds. The most different effects of *P. kluyveri* are intriguing, since either this yeast metabolized or transformed part of the precursors into different molecules, or somehow inhibited the ability of *S. cerevisiae* to segregate glycosidases.

4-vinylguaiacol and 4-vinylphenol were the only volatile phenols formed at sensory relevant levels. Vinyl phenols can arise from grape hydroxycinnamic acids, from their corresponding ethyl and tartrate esters and, of course, from the corresponding glycosides, which would explain why these molecules appearance at significant levels, both in unfermented controls and in fermented samples (Hixson et al., 2016; Waterhouse et al., 2016).

The formation of volatile phenols from esters requires a two-step reaction catalysed first by hydroxycinnamoyl esterase, whose activity is not too abundant in yeast and second, by hydroxycinnamate decarboxylase, which can be present at different levels of activity in some strains of *S. cerevisiae*, such as the one used in the present experiment. The fact that wines fermented with non-

Saccharomyces strains have similar levels between them and to those found in the unfermented control, may indicate low or even absent hydroxycinnamate decarboxylase activity in these yeast strains, suggesting that a large fraction is produced by simple acid hydrolysis of the glycoside.

Nonetheless, normally and especially in red wines, vinyl phenols have decreasing tendencies with time due to their reactivity with anthocyanins. The formation of adducts with ethanol has also been described as potential cause for volatile phenols decreases in wine (Kennison et al., 2008; Waterhouse et al., 2016).

2. Conclusions

The sequential inoculation fermentation approach used in this study introduces a large variability in the pattern of fermentative compounds and in the wine aroma profile. Wines obtained by fermentation with *P. kluyveri* were the most distinct, containing highest levels of fusel alcohol acetates, of fatty acids and of their ethyl esters. On the other hand, samples fermented with *L. thermotolerans* contained minima levels of those compounds. Remarkably, all wines obtained by sequential inoculation fermentation contained smaller levels of isoamyl alcohol, which is a strong odour suppressor.

Levels of some relevant varietal aroma compounds were found at higher levels in unfermented controls, suggesting the existence of a pool of easily hydrolysable precursors, such as polyols, among Riesling precursors. Among fermented samples, those made with *L. thermotolerans* contained the highest levels of monoterpenes while those fermented with *P. kluyveri* contined minima levels of monoterpenes and norisoprenoids. The formation of TDN is time-dependent but it is also strongly enhanced by fermentation, suggesting that hydrolytic activities of yeasts are essential to produce acid-hydrolysable precursors of this molecule. In comparison with *S. cerevisiae*, wines made with *P. kluyveri* have a lower ability to accumulate TDN. Most remarkably, samples fermented with the three non-*Saccharomyces* yeasts had much smaller levels of vinylphenols.

In summary, results presented here further support that a sequential inoculation fermentation approach can be successfully used not only to modulate wine aroma but to control its evolution with time.

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Chapter 2

Effects of sequential inoculation with different non-Saccharomyces on the formation and further evolution of Garnacha wine aroma

Chapter 2 - The roles of yeasts on the formation and evolution of Garnacha wine aroma

1. Results and discussion

Results presented in this chapter refer to fermentations carried out with synthetic must containing or not glycosidic precursors extracted from Garnacha grapes. The global goal of the chapter is to assess the roles of yeasts on the formation and evolution with time of aroma compounds in Spanish Garnacha wines. Specific goals are to better define what is varietal aroma of Garnacha wine and to assess the influence of yeast on its development during wine aging.

A sequential inoculation protocol was followed, meaning that the sterile synthetic musts were inoculated first with a non-*Saccharomyces* strain and, after 4 days, with *S. cerevisiae* to complete the fermentation, except for one trial exclusively fermented with *S. cerevisiae,* which was kept as control. Half of the samples contained only synthetic must with all the necessary nutrients and elements to normal yeast metabolism and the second half were additionally spiked with the fraction of glycosidic precursors. Besides, unfermented control samples of synthetic wine spiked with precursors fraction were included in order to assess the role of acid hydrolysis and, particularly, enable a quantitative comparison between the efficiencies of acid versus enzymatic hydrolysis on the varietal aroma formation. A time variable, in which wine was aged in a complete anoxic environment for up to five weeks at 50ºC was also included. This set-up aims to further understand the origin and fate of the volatile compounds quantified in the wines, the role of different yeast genera and of slow hydrolytic processes, right after fermentation and during aging time.

The wines were firstly characterized according to their classical oenological parameters and then a comparative analysis between the different controls and wines spiked with glycosidic precursors was made. Different aging patterns were also identified and analysed in this section.

1.1 Classical oenological parameters of final synthetic wines

The recently fermented wines were characterized according to classical oenological parameters: pH, volatile and total acidity and residual sugars (Table 6).

Table 6 Classical oenological parameters of recently fermented wines obtained by fermentation of synthetic musts spiked (PG samples) or not (CTL samples) with Garnacha precursors. Data are means of two biological replicates.

	pH	Total acidity	Volatile acidity	Residual sugars
CTL *S. cerevisiae*	3.43 ± 0.01	6.5 ± 0.05	0.6 ± 0.03	0.75 ± 0.07
PG *S. cerevisiae*	3.43 ± 0.01	6.46 ± 0.11	0.6 ± 0.08	1.75 ± 0.78
CTL *P. kluyveri*	3.5 ± 0.08	6.27 ± 0.27	0.9 ± 0.03	1.5 ± 0.71
PG *P. kluyveri*	3.46 ± 0.02	6.16 ± 0	0.93 ± 0.03	1 ± 0.14
CTL *L. thermotolerans*	3.26 ± 0.01	8.89 ± 0.11	0.79 ± 0	5.85 ± 1.41
PG *L. thermotolerans*	3.44 ± 0.16	7.98 ± 0.32	0.71 ± 0.03	11 ± 0.85
CTL *T. delbrueckii*	3.41 ± 0.08	6.92 ± 0	0.75 ± 0.03	4.5 ± 0.14
PG *T. delbrueckii*	3.46 ± 0.01	6.57 ± 0.05	0.75 ± 0.03	8 ± 0.71

As seen in Table 6, the pH of all samples, except CTL-*L. thermotolerans*, *are* very similar. Regarding total acidity, samples fermented with *L. thermotolerans* have significantly higher acidity compared with the remaining wines, which do not differ greatly. This difference should be attributed to the production of lactic acid by this yeast. Wines fermented with different strains show large variability in their volatile acidities: samples fermented with *P. kluyveri* show the highest

levels, while those fermented with *S. cerevisiae* show the smallest. Regarding residual sugars, the wines spiked with precursors and fermented with *L. thermotolerans* and *T. delbrueckii* have not reached complete dryness, containing residual sugars of 11 and 8 g/L, respectively.

1.2 Overview of the aroma composition of final wines

Thirty-nine aroma compounds out of the 69 aroma volatiles targeted in the GC-MS system, were found above the limit of quantification. In the cases of Riesling acetal and vitispirane, a reference standard was not available, so their data were given just as relative area to the internal standard 3-octanone. The 39 volatiles could then be grouped according to whether their levels were related or not to the presence of glycosidic precursors.

Aroma compounds found at similar levels in samples fermented with or without glycosidic precursors are purely fermentative compounds. Twenty-three aroma compounds belong to this category:

- 6 alcohols (isobutanol, 1-butanol, isoamyl alcohol, 1-hexanol, methionol and β-phenylethanol);
- 7 ethyl esters (ethyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl lactate, ethyl isobutyrate, ethyl 2-methylbutyrate), 3 acetate esters (isoamyl acetate, isobutyl acetate, phenylethyl acetate);
- lactones (γ-nonalactone, γ-decalactone and γ-butyrolactone);
- 7 acids (acetic acid, butyric acid, isobutyric acid, isovaleric acid, hexanoic acid, octanoic acid and decanoic acid).

The remaining 15 compounds were found exclusively, or at much higher levels, in samples fermented with precursors. This group of compounds are genuine varietal aroma compounds and includes:

- 4 norisoprenoids (1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN), β-damascenone, vitispirane and Riesling acetal);
- 4 terpenoids (linalool, geraniol, α-terpineol and β-citronellol);
- 5 volatile phenols (guaiacol, 4-vinylguaiacol, 4-vinylphenol, 2-6-dimethoxyphenol and E-isoeugenol);
- 2 vanillin derivates (vanillin and acetovanillone).

Fermentative aroma compounds were not found in any of the unfermented samples while varietal aroma compounds, were also found in the unfermented controls containing glycosidic precursors.

The experimental setup makes it also possible to assess differences introduced in fermentative and varietal aroma fractions by non-*Saccharomyces* yeasts by comparing with the control samples fermented entirely by *S. cerevisiae*. Furthermore, samples underwent also an anoxic storage in order to study how differences evolved during aging. In order to assess the hierarchy of the effects of these three different factors (presence of precursors, yeast strain and aging) on wine aroma composition, a Principal Component Analysis (PCA) was carried out on the dataset containing all aroma compounds from all the fermented samples. Unfermented controls containing precursors were excluded. Results are shown in Figure 18.

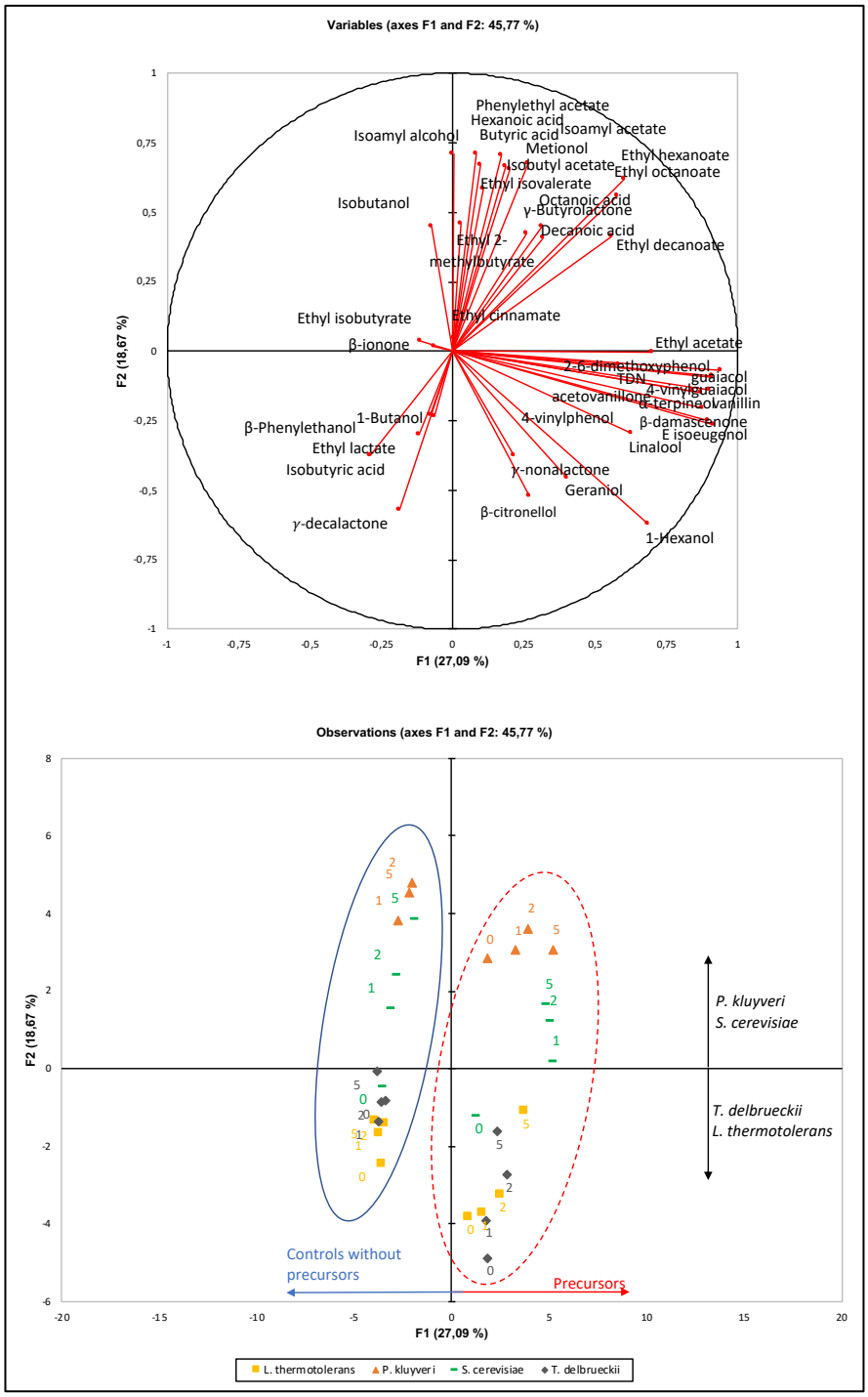

Figure 18 ***Principal component analysis carried out on the complete aroma volatiles data set*** *quantified in Garnacha from all fermented samples. The plot shows the projection of variables (top plot) or samples (bottom) -average of two biological replicates- in the plane formed by the first two components, which retained 45.77 % of the original variance. Numbers in the samples refer to the weeks of anoxic storage at 50°C.*

The plots shown in the figure summarize the results of the Principal Component Analysis, representing the projection of variables (variable loadings, top plot) and of samples (sample scores, bottom plot) in the plane formed by the two first principal components, which retained 45.77% of the original variance. The sample scores plot clearly show that the most influential factor in the whole data set is the presence or absence of glycosidic precursors in the fermenting must. The group of samples on the left side of F1 corresponds to all controls fermented without glycosidic precursors and the group of samples on the right, to the wines fermented with precursors. A look at the variable loadings plot on the upper part of the figure confirms that variables with most influence on the first component are varietal aroma compounds. It is noteworthy, however, that some fermentative compounds, such as ethyl esters of fatty acids, have relatively high loadings in this first component, suggesting that their levels are also affected by the presence of precursors in the fermenting must.

The second main source of wine differentiation is the yeast strain that carried out the first part of fermentation, which, basically, determines the position of the samples on the second axis. The influence of aging time can also be clearly seen in this second component. With the exception of *P. kluyveri*, samples with longer aging times have higher scores in this second component. Not surprisingly, variables with higher weight on the second component are higher alcohols, some of their acetates, fatty acids and their ethyl esters; i.e., fermentative aroma compounds. This indicates that these compounds are the most relevant for defining the role of yeast variety and also of aging time. For samples fermented with precursors, it can also be observed a slight but evident influence of aging time in the scores of the first component, indicating that varietal aroma compounds content changes with aging time.

In order to obtain a better understanding of the effects played by the three different factors on wine aroma composition, in the following sections, results and the corresponding discussion will be segmented into fermentative and varietal compounds.

1.3 Fermentative compounds in Garnacha wines

The distributions of variables and samples in the two first Principal Components of the PCA study carried out with the data set including only fermentative compounds can be seen in the plots given in Figure 19 (see also Supplementary data). The first two components explain 48.67% of the original variance.

By excluding the compounds derived from the precursor fraction, the factor dominating the distribution of samples within the plane is the yeast strain. It is particularly remarkable that the evident influence of aging time identified in the plots shown in Figure 18, is not clearly evident here.

Nonetheless, samples are evenly distributed into four distinctive groups containing wines fermented by the same strain, regardless of the presence or absence of precursors or aging time. Only one of the samples from *S. cerevisiae*, shares space with a sample from *T. delbrueckii*. Most remarkably, only the samples fermented with *S. cerevisiae* are represented in the middle of the plane, consistently with the fact that the *S. cerevisiae* carried out a relevant part of the alcoholic fermentation in all the cases. Accordingly, samples which were first inoculated with the three non-*Saccharomyces* yeast strains, tend to occupy extreme areas in the plot reflecting major and specifically distinctive changes in fermentative metabolism introduced by each one of the strains. This is in agreement with the fact that all non-*Saccharomyces* yeast strain selected have different metabolic description between themselves and compared to *S. cerevisiae* (Jolly et al., 2014).

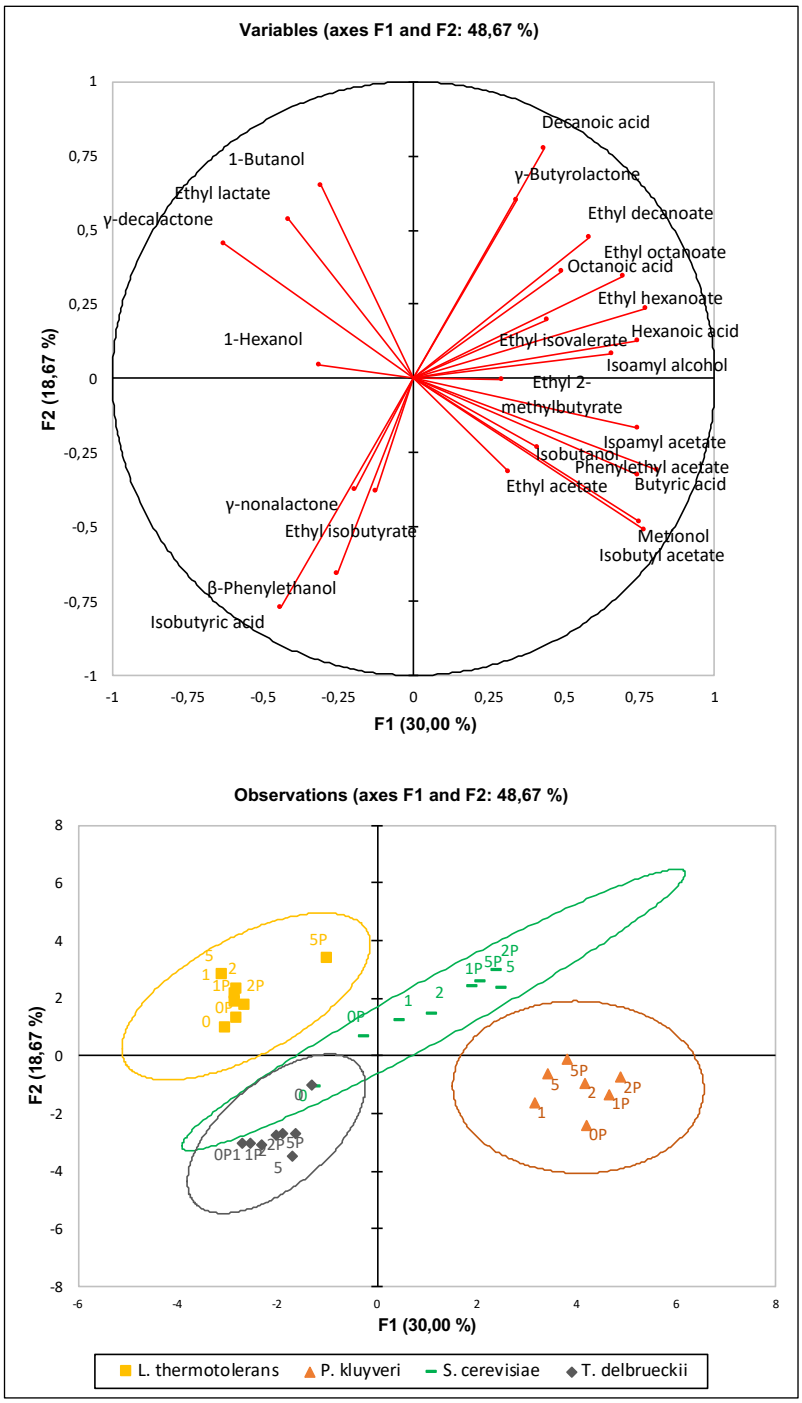

Figure 19 ***Principal component analysis on fermentative compounds*** *quantified in fermented samples with or without the precursor fraction. The plot shows the projection of variables (top plot) or samples (bottom) -average of two biological replicates- in the plane formed by the first two components, which retained 48.67 % of the original variance. Numbers in the samples refer to the weeks of anoxic storage at 50°C. P, indicates samples fermented with precursors.*

Among the wines fermented with non-*Saccharomyces*, those fermented with *P. kluyveri* seem to be the most different. This yeast is the only one from this study described as aerobic and having biofilm formation activity during fermentation (Barata et al., 2012;Escribano et al., 2017;Jolly et al., 2014). Although showing large differences in fatty acid metabolism to form the corresponding ethyl esters, *T. delbrueckii and S. cerevisiae* seem to have common features (Figure 19). *T. delbrueckii* has been described with mellow fermentative potential (Jolly et al., 2014) and thus, its metabolic activity could be closely related to *S. cerevisiae* rather than to strains like *P. kluyveri or L. thermotolerans*, explaining why the samples fermented with *T. delbrueckii and S. cerevisiae* are so close in the sample score plot.

Attending to the variable loading plot, it can be observed that wines fermented with *P. kluyveri* and *S. cerevisiae* have higher levels of acetates, fatty acids and ethyl esters, which have higher positive loadings on the first component. It should be also observed that in samples from these two groups, the presence of precursors exerts a slight but notable impact. Samples fermented with the precursor fraction are displaced to the right, suggesting that this fraction has affected to the metabolism of fermentative compounds. This could be attributed to the likely presence of other compounds in the precursor fraction, such as grape sterols indirectly affecting yeast fatty acid metabolisms, or even to the direct presence of non-volatile precursors able to yield fatty acids, which could be further metabolized into volatile esters (Liu et al., 2017; Saerens et al., 2008). Small amounts of fatty acids are often described in the aglycones of grape aroma precursors.

It is also remarkable that while fatty acids and their ethyl esters and acetate esters are fairly correlated with the first component, compounds related to yeast amino acid metabolism, are scattered throughout the plot. As can be seen in the plot, isoamyl alcohol isobutanol and methionol are positively correlated with the first

component, while isobutyric acid, β-phenylethanol and ethyl isobutyrate are slightly negatively correlated. This suggests that fatty acid metabolism is genetically determined by the yeast strain, hence, differences are mainly of quantitative nature, while, on the contrary, nitrogen metabolism would be definitively quite strain specific. For fatty acids and their derivatives, *P. kluyveri* and *S. cerevisiae* have the highest levels and *T. delbrueckii* and *L. thermotolerans* minima levels; for acetates *P. kluyveri* shows maxima levels and *L. thermotolerans* minima, while for compounds related to amino acid metabolism, the pattern is far more complicated.

Methionol is present at highest levels in samples fermented with *P. kluyveri,* while *S. cerevisiae* produced highest levels of isoamyl alcohol. *T. delbrueckii* contains much higher levels of β-phenyethanol and of isobutyric acid and its ethyl ester, while *L. thermotolerans,* in general, has minima contents of all these compounds. Regarding ethyl 2-methylbutyrate and ethyl isovalerate they were maxima in *S. cerevisiae*, following a completely different trend to that observed for isobutyric and ethyl isobutyrate.

L. thermotolerans seems to produce remarkable levels of γ-decalactone and ethyl lactate and minima levels of methionol, butyric acid and acetate esters. Finally, *T. delbrueckii* has maxima levels of isobutyric acid, ethyl isobutyrate, β-phenylethanol and γ-nonalactone and minima of decanoic and octanoic acids and their ethyl esters, as well as of γ-butyrolactone. The differences between yeast strains are also patent in the graphics shown in Figures 20-22.

Figure 20 shows that samples fermented with precursors have more than twice the amount of 1-hexanol found in fermented controls, clearly suggesting that this compound can have both origins, fermentative and varietal. In fact, 1-hexanol, is an usual component of the aglycones of glycosidic precursors of neutral grapes, from where it can be produced at small levels (less than 1 mg/L). As 1-

hexanol in fermentation is also produced at small levels (also less than 1 mg/L), both origins have relevance. This represents a clear difference between this compound and β-phenylethanol and the other higher alcohols. Some of these compounds can also be found at sub-mg/L levels in the precursor fraction; however, as they are formed during fermentation at much higher levels (>10 mg/L), the effect of the presence of precursors can be considered negligible and, thus, these compounds can be considered to have a genuine fermentative origin.

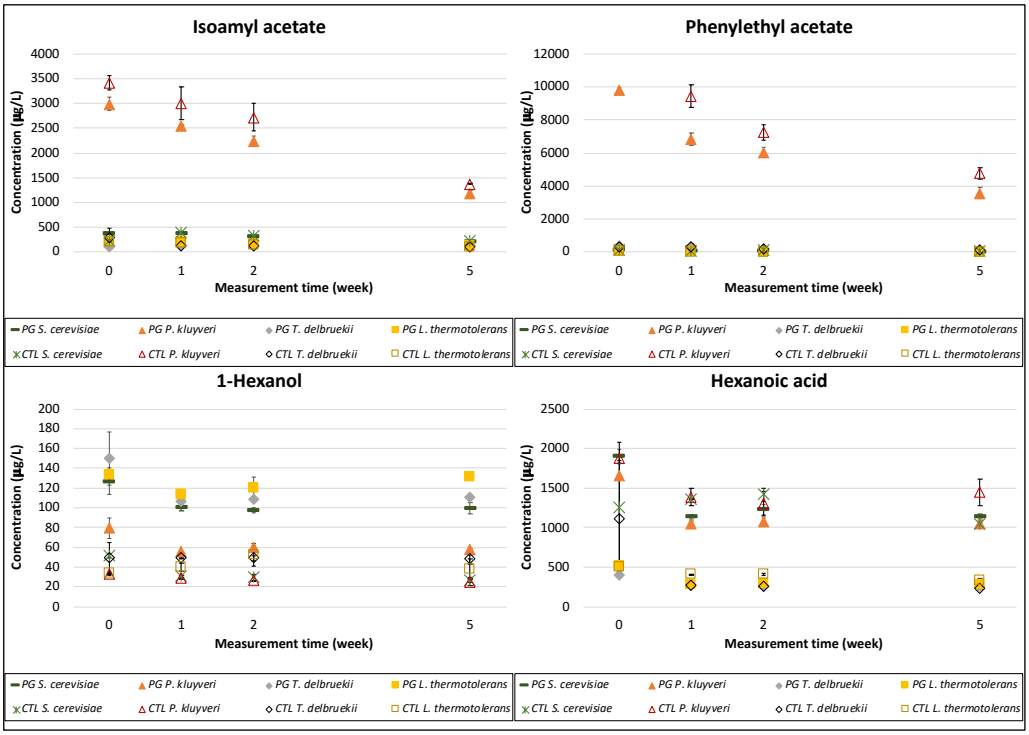

Figure 20 ***Volatile compounds showing decreasing tendencies with aging time*** *– evolution with time of two acetate esters, one alcohol and one acid (µg/L). Influence of the presence (PG) or absence (CTL) of precursors and of the yeast genera that carried fermentation.*

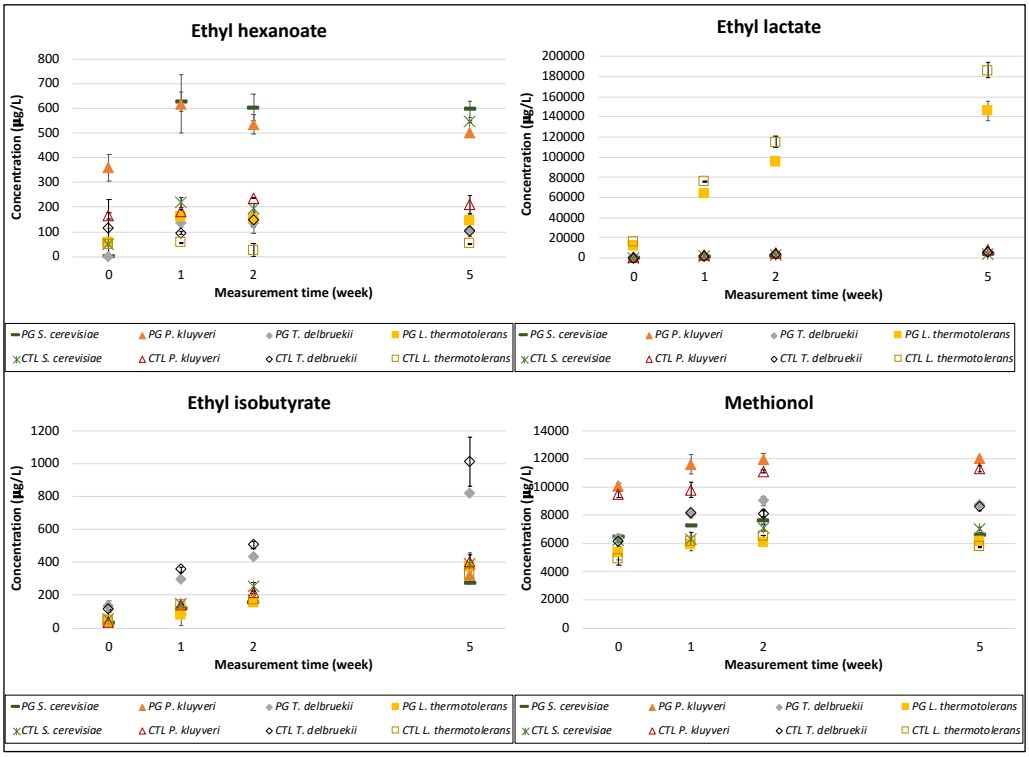

Figure 21 ***Volatile compounds showing increasing tendencies with time*** *– evolutions with time of three ethyl esters and one alcohol (µg/L). Influence of the presence (PG) or absence (CTL) of precursors and of the yeast genera that carried fermentation. The content of these volatiles increases until the first week of aging (ethyl hexanoate and methionol) or continuously increases (ethyl lactate and ethyl isobutyrate).*

The evolution pattern with time of some compounds, even if not evident in the PCA plot, can be easily identified in Figures 20-22, which give some examples of different evolutions. The case of acetates is evident with the two examples shown in Figure 20. These compounds are formed at huge levels, specifically in the fermentations with *P. kluyveri*, and their levels steadily decrease to become less than half the original after 5 weeks of accelerated aging. Levels of hexanoic acid (and the other fatty acids), also decrease slightly during the first week of aging, but afterwards, levels remain constant. This evolution is complementary to ethyl hexanoate shown in Figure 21, which increases during the first week and then remains constant. In the case of ethyl lactate and ethyl isobutyrate, also

shown in Figure 21, the increment is stronger and is observed during the whole aging time. Obviously, this is the simple consequence of esterification equilibria (Waterhouse et al., 2016). In the case of acetates, these compounds are produced by yeast in large excess in comparison to the levels corresponding to their esterification equilibrium. As wine contains relatively low levels of acetic acid and higher alcohols, the products of the hydrolysis of the acetate esters, the equilibrium is displaced towards very low levels of acetates. Hence, these compounds produced during fermentation by acetyl-transferase activities are slowly, but continuously, hydrolysed. On the contrary, in the case of ethyl esters, one of the products of hydrolysis is ethanol, which is already present at huge levels in the fermented samples. Then, depending on how much ethyl ester was produced during the fermentation, different aging trends are observed such as those of ethyl hexanoate, which is produced at relatively high levels during fermentation and, then, remains fairly stable, or of ethyl isobutyrate and ethyl lactate, produced at very low or even null levels during fermentation, and, afterwards, increasing with accelerated aging.

γ–butyrolactone (not shown) and methionol (shown in Figure 21) also showed increasing trends with time. While the increase of the former is the likely result of the internal esterification of γ-hydroxybutyric acid produced during fermentation, the case of methionol is less clear. It could be due to the release from a small fraction of yeast lees not completely eliminated by centrifugation. Levels of major alcohols, such as isoamyl alcohol and β-phenylethanol remain stable during aging (Figure 22).

The effect of the presence of the precursor fraction is most evident in the case of ethyl hexanoate for *P. kluyveri*. A look at the Figure 21 reveals that the sample spiked with precursors contained similar levels to those observed for *S. cerevisiae*, while the sample without precursors contained much smaller levels.

However, the level of hexanoic acid formed was significantly higher for this last sample, as can be seen in Figure 20. This implies that the effect of precursors on the levels of esters should not be attributed to the additional production of hexanoic acid from this fraction, but to an indirect effect of some components of the precursor fraction, such as grape esterols, on the esterification ratios.

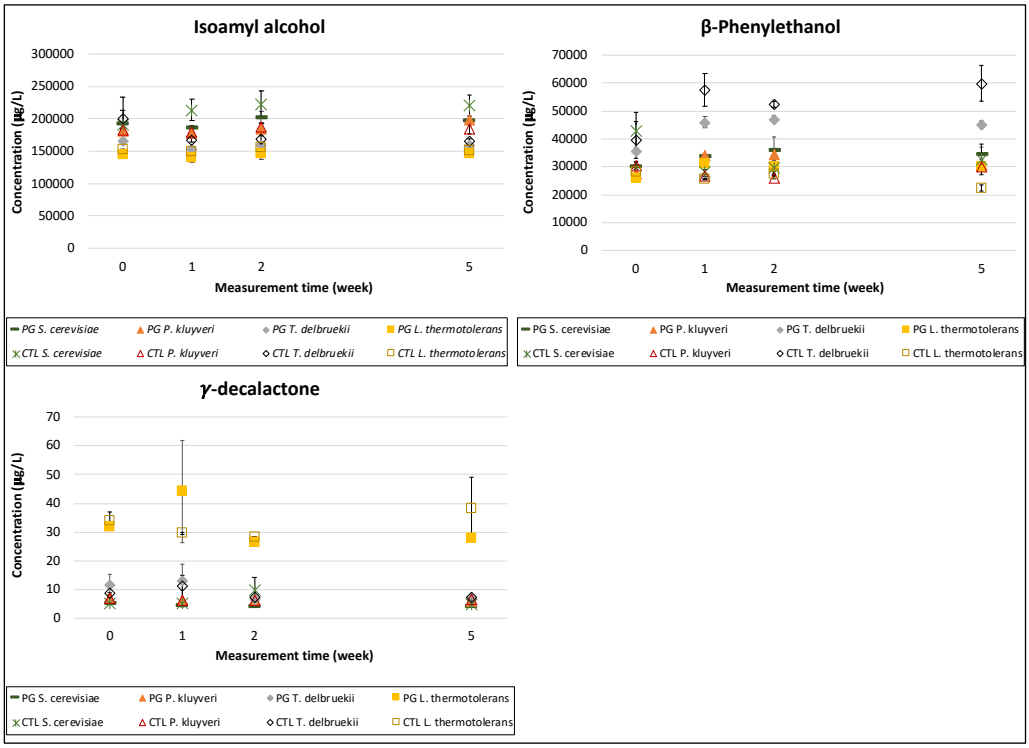

Figure 22 ***Volatile compounds that remain stable over time*** *– contents of two alcohols and one lactone (µg/L) during the aging. Effects of the presence (PG) or absence (CTL) of precursors and of the yeast genera that carried fermentation.*

1.4 Considerations about potential sensory effects of different yeast strains

Some of the compositional changes caused by the different yeast strains are going to have a strong effect on the sensory properties of the young or aged wines. In the case of *L. thermotolerans*, a major effect is due to the accumulation of lactic acid. This compound was not directly measured, but results of total acidity in Table 6 and of ethyl lactate, shown in Figure 21, clearly suggest that

the 1.5 increment in units of acidity compared to the control fermented just with *S. cerevisiae*, is due to the production of this acid. The repercussion in the pH was only observed in the case of the samples fermented without precursors, not being clear why the pH was abnormally high in one of the replicates containing precursors. Moreover, samples fermented with *L. thermotolerans* also stand out by two changes with potentially large sensory effect: the lowest levels of isoamyl alcohol and the huge production of γ-decalactone. Levels of isoamyl alcohol are 60 mg/L smaller than those of the *S. cerevisiae* controls (nearly a 30% reduction) which is going to have a strong sensory impact. Isoamyl alcohol is a strong suppressor of odour nuances, as it has been recently demonstrated (de-la-Fuente-Blanco et al., 2016) and 60 mg/L are easily detectable (de-la-Fuente-Blanco et al., 2017). Regarding γ-decalactone, this aroma compound is most usually present at levels below 10 μg/L (San Juan et al., 2012) and has a quite low sensory threshold. Levels of γ-decalactone in samples fermented by *L. thermotolerans* exceed 30 μg/L, as can be seen in Figure 22, more than 3 times higher than those measured in *S. cerevisiae*, and in large excess to those usually found in red wines (San Juan et al., 2012).

Samples fermented with *P. kluyveri* are also going to be completely different from the sensory point of view due to their huge levels of acetates, as it was shown in Figure 20. Both isoamyl and phenylethyl acetates largely exceed their odour thresholds and are, in fact, at levels in which they become impact compounds, providing a characteristic pear-like fruity-floral aroma. Finally, samples fermented with *T. delbrueckii* do contain high levels of isobutyric acid which forms large levels of ethyl isobutyrate by esterification, as seen in Figure 21. Levels of these compounds after 5 weeks of aging exceed in a factor 3 those measured in the other samples, and are much above those normally found in red wines (San Juan et al., 2012). Levels of β-phenylethanol are also much higher

(30-50%) as seen in Figure 22. Even though its sensory relevance is not as important as usually believed (de-la-Fuente-Blanco et al., 2016), the changes observed are large enough to have sensory consequences.

Fermentative compounds, some of them with a large sensory role, are affected by the distinctive yeast metabolic activities introduced by non-*Saccharomyces* yeasts in a sequential inoculation protocol. It can be said, that all non-*Saccharomyces* were able to contribute, individually, to wine volatile profile, which supports the effective contribution of this methodology to the production of more complex wines, favouring wine distinctiveness and ensuring controlled fermentations (Padilla et al., 2016).

1.5 Varietal compounds in Garnacha wines

Varietal aroma compounds were found just in the samples containing precursors. Fermented controls without precursors had null or negligible levels of these 15 aroma compounds. Figure 23 summarizes the Principal Component Analysis carried out on varietal aroma compounds quantified in the pool of samples containing precursors, fermented or not. The sample scores plot (bottom of the figure) reveals that the primary factor driving variability in such data set is the existence of fermentation. Unfermented controls lie in the left-bottom part of the plane formed by the two first components (retaining 66% of the original variance), while all fermented samples are upper and to the right, having therefore, higher scores of both F1 and F2. Attending to the variable loading plot in the upper part of the figure, this means that fermentation exerts a positive effect on the levels of most aroma compounds, particularly on volatile phenols.

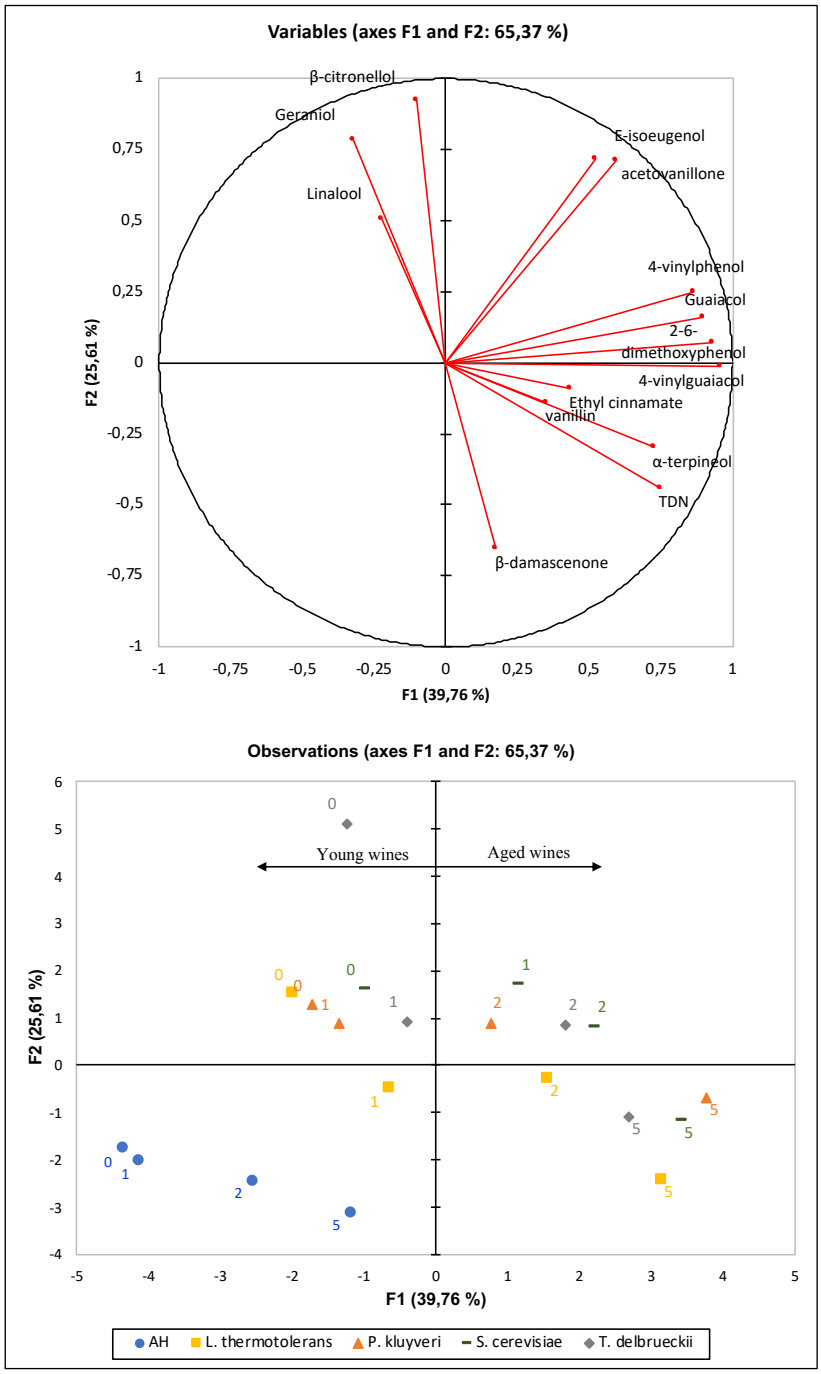

Figure 23 ***Principal Component Analysis on varietal compounds*** *quantified in fermented samples with Garnacha precursor fraction, as well as unfermented controls spiked with precursors. The plot shows the projection of variables (top plot) or samples (bottom) showing the two biological replicates- in the plane formed by the first two components, which retained 48.67 % of the original variance. Numbers in the samples refer to the weeks of anoxic storage at 50°C.*

As it will be later discussed, fermented samples contain always higher levels of varietal volatile compounds, except for some notable exceptions such as β-damascenone.

In this case, the second source of variability is time. Freshly fermented samples are positioned left and up and, during aging, samples are displaced to the right and down. A look at the variable loading plot reveals that aging implies a strongly decrease of the monoterpenes linalool, geraniol and β-citronellol and increases of α-terpineol, which is a decomposition product of monoterpenes and increases also of some norisoprenoids, such as TDN and β-damascenone.

The effect of yeast is not much evident in this representation, due to the strong influence of fermentation and aging, and also due to the fact that the effect of yeast is aging-dependent. This can be observed in the nearly outlier position taken by *T. delbrueckii* at time 0, which is not further seen at longer aging times or, in the fact that samples fermented only with *S. cerevisiae* had, at times 0, 1 and 2, highest scores in F1, moving to the centre after 5 weeks of aging. On the contrary, samples fermented by *P. kluyveri* are displaced from left positions to the right. In spite of this, it can be observed that samples fermented with *L. thermotolerans* have (except at time 0), consistently the smallest scores of the second component. These effects will be analysed in more detail with the help of Figures 24-26 which show the contents of individual compounds and their evolutions with time.

Plots in Figure 24 summarize the evolution patterns observed for monoterpenes. Linalool and geraniol, which are quite unstable compounds at wine pH and tend to decompose (Rapp and Mandery, 1986; Waterhouse et al., 2016), reach maxima levels at short aging times and then, decrease. On the contrary, α-terpineol, which is one of the products of the rearrangement of unstable monoterpenes, increases continuously or remains stable. Most likely, there are

no glycosides having α-terpineol as aglycone and this molecule is produced just by rearrangement of the other free monoterpenes, once they have been released or produced. This is consistent with the null or very low levels of α-terpineol found in the unfermented controls or in the fermented samples at time 0, respectively.

Regarding linalool and geraniol, the evolution observed in the unfermented controls is quite different to that observed in fermented samples. In unfermented controls, very low levels of aroma compounds are observed at time 0, then there is an approximately linear increase during 2 weeks and then a decrease, so that there is a maximum after two weeks of anoxic storage. By contrast, the maximum in fermented samples is found at times 0 or 1, implying that the release of aroma compounds is much faster in comparison to the unfermented controls. Practically, this implies that fermented samples contain initially 3-8 times higher levels of these two odorants, however, after 1 week of aging levels in fermented samples are just 1.5-2 times higher than those found in the unfermented controls and become equal at 2 weeks. After 5 weeks of aging the unfermented control contained maxima levels. These results can be interpreted by assuming that the release of linalool and geraniol from glycosidic precursors by acid hydrolysis is slow, implying that the pool of precursors diminishes slowly, hence, that linalool and geraniol production is active during the whole aging period. By contrast, fermented samples contain initially large levels of the odorants, implying an efficient hydrolysis of precursors during fermentation, but their levels fade quickly since the reservoir of precursors has been depleted. This is consistent with a production of the odorants by direct cleavage from the glycosides.

T. delbrueckii was particularly efficient at releasing linalool and geraniol very fast from the glycosidic precursors, so that samples fermented with this strain contain maxima levels immediately after fermentation and after one week of

aging. Differences are notable and large enough to have sensory consequences, since linalool and geraniol exert a concerted action on wine floral notes (Loscos et al., 2007; Mateo and Jiménez, 2000). As precursors are depleted very soon in these samples, levels of both odorants drop quickly from the maximum and become similar or smaller to those of other samples after 2 weeks of aging. *P. kluyveri* wines contained low levels of both monoterpenes during fermentation, but the increase during the first week of aging was very intense, thus, after one week, levels were close to the maxima of *T. delbrueckii*. In the case of linalool, increases in the first week are even higher than those observed for the unfermented control. This could be explained either by assuming that, in this case, hydrolytic enzymes remained active during the first week of aging (at 50ºC) or by assuming that enzymes transformed the initial precursors into more easily hydrolysable species. On the other hand, *L. thermotolerans* produced relatively large levels of both odorants during fermentation, but ulterior increases are smaller and so, after two weeks of fermentation, samples contained minima levels. This is consistent with the maxima levels of α-terpineol observed for this strain, which suggests that its enzymes induced the conversion of precursors into other molecules.

Comparing to the remaining monoterpenes, β-citronellol is the one produced at smaller levels. In fact, it was not even detected in unfermented controls. This data suggests that the β-citronellol glycosidic precursor exclusively releases the odorant by enzymatic hydrolysis and that by acid hydrolysis it is surely directly transformed into a different molecule. This compound seems to have great instability at wine pH, since its levels quickly decrease with aging.

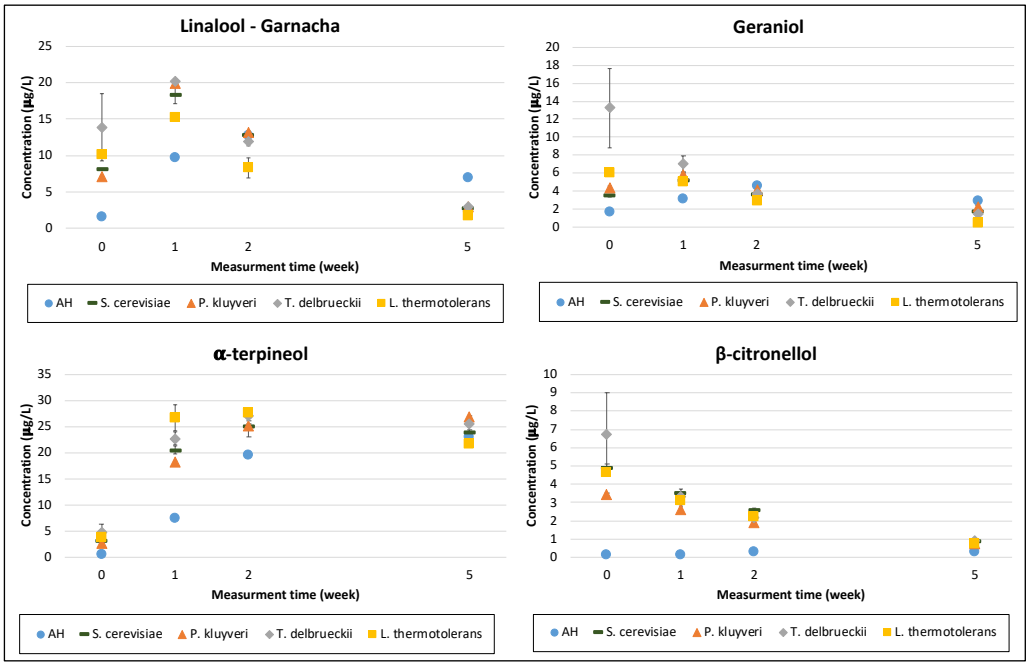

Figure 24 ***Monoterpenes in Garnacha wine*** *Concentration evolution over time of linalool, geraniol, α-terpineol and β-citronellol (µg/L) in wines spiked with Garnacha glycosidic precursors and fermented with different yeast genera. The unfermented controls are also illustrated (AH).*

The patterns followed by two sensory relevant norisoprenoids, TDN and β-damascenone, are given in Figure 25. TDN follows a quite particular pattern, with null levels in recently fermented samples and then a steady and linear increase with time in all cases, being the increase strongly linked to the yeast strain which carried fermentation. *L. thermotolerans* produced much higher levels of this compound, and the unfermented controls contained, in all cases, minima levels. The fact that TDN is not released during fermentation suggests that the precursor fraction does not contain glycosides with TDN as aglycone, which in fact is not possible in chemical terms. As described in literature, TDN can arise from multiple precursors, some of which are glycoconjugates, and some of which are not (Winterhalter, 1991; Winterhalter et al., 1990). The aforementioned works from Winterhalter support in any case that TDN

formation occurs, most likely, from the aglycones after cleavage of the glycoside or from other non-glycosidic precursors. This is consistent with our data, which suggest that TDN is formed from glycosidic precursors which only after have been separated from the sugar molecule, undergo a slow spontaneous series of chemical rearrangement at acid pH to produce the odorant. This would explain why fermentation is essential for TDN production: TDN glycosidic precursors need enzymatic action to be cleaved, and *L. thermotolerans* would be particularly efficient at that. It should be observed that in this case, the effect of yeast becomes evident only after long aging periods.

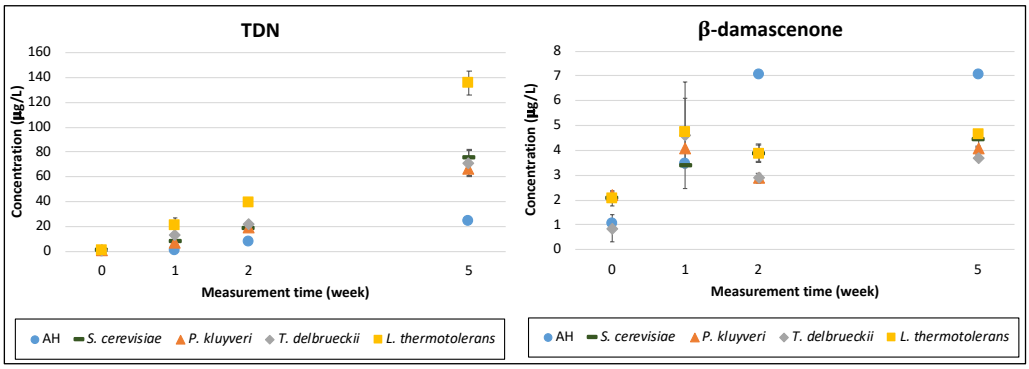

Figure 25 ***Norisoprenoids in Garnacha wine*** *Concentration evolution over time of TDN and β-damascenone (µg/L) in wines spiked with Garnacha glycosidic precursors and fermented with different yeast genera. The unfermented controls are also illustrated (AH).*

A quite different pattern is observed for β-damascenone. Levels of this compound in unfermented controls increase up to 2 weeks of aging and then remain constant. In contrast, fermented samples had, in general, higher levels of this odorant in freshly fermented samples as well as after 1 week of accelerated aging, but then, levels slightly drop or remain constant becoming, after 2 weeks, smaller than those observed in unfermented samples. Among fermented samples, even though differences between strains are small, *L. thermotolerans* had, at all sampling points, higher levels of this compound. This pattern suggests that yeasts are able to hydrolyse part of the precursor fraction during

fermentation, explaining why freshly fermented samples, with exception of *T. delbrueckii*, contain twice the level of β-damascenone compared to unfermented controls, but that a second fraction of precursor is transformed into some component which is no longer able to release an odorant.

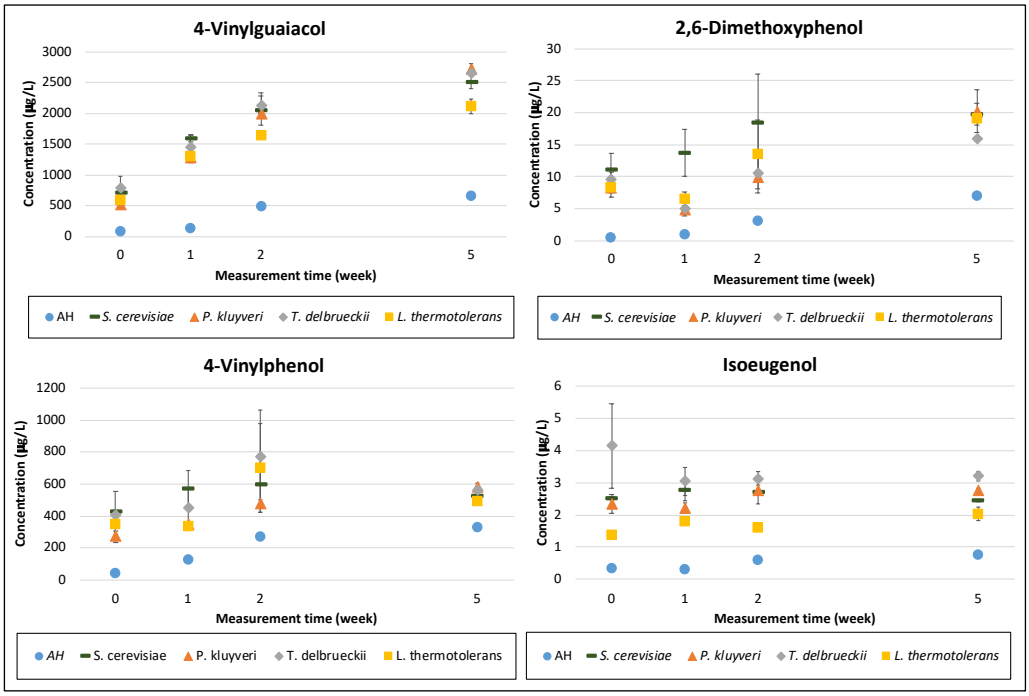

Figure 26 ***Volatile phenols in Garnacha wine*** *Evolution with time of levels of 4-vinylguaiacol, 4-vinylphenol, 2,6-dimethoxyphenol and isoeugenol (µg/L) in wines spiked with Garnacha glycosidic precursors and fermented with different yeast genera. The unfermented controls are also shown (AH).*

The evolution of volatile phenols is shown in Figure 26. As can be seen, fermented samples contain systematically higher levels of volatile phenols, implying that fermentation is essential for the release/formation of all these odorants. Regarding aging, except for 4-vinylguaiacol, the levels of odorants in fermented samples evolve in parallel to those of unfermented controls, implying that fermented samples contain a pool of precursors quantitatively equivalent to those of the unfermented controls. This further indicates that during fermentation aroma compounds from precursors different to those that rend the odorant by

simple acid hydrolysis are also produced. In the cases of 4-vinylphenol and 4-vinylguaiacol these precursors are likely coumaric and ferulic acids, or their glycosides, but in the other cases the nature of the precursor is not obvious. Additionally, in the case of 4-vinylguaiacol, the rates of accumulation of the odorant during aging are much higher in fermented samples than those observed in the unfermented controls. This suggests that in this case, yeast not only decarboxylates free ferulic acid, but also the glycoside, so that fermented samples contain higher levels of the glycoside which by acid hydrolysis forms the odorant. Therefore, fermented samples not only have higher levels of the odorant, produced either by direct decarboxylation of ferulic acid, or by hydrolysis of the glycoside, but have also higher levels of its precursors, likely produced by decarboxylation of the glycosides of ferulic acid. The specific role of distinct yeast strains does not seem to be highly relevant for volatile phenols formation. Nevertheless, *S. cerevisiae* is the most efficient at producing 2,6-dimethoxyphenol and guaiacol, *T. delbrueckii* at producing isoeugenol, and *L. thermotolerans* seems to be the least efficient at producing 4-vinylguaiacol and isoeugenol.

These observations support the hypothesis that the formation of volatile norisoprenoids and volatile phenols rely on yeast activity for a primary transformation of the precursors and that acid hydrolysis is further required to obtain a final volatile compound (Mendes-Pinto, 2009; Waterhouse et al., 2016). Regarding the potential sensory effect of these changes, levels of linalool and geraniol in recently fermented wines made with *T. delbrueckii*, are high enough to cause a sensory impact, since even if individual thresholds are not reached, these compounds act additively (Loscos et al., 2007). The highest levels of TDN produced in samples fermented with *L. thermotolerans* are also remarkable and may have a sensory impact.

2. Conclusions

Sequential inoculation of non-*Saccharomyces* and *S. cerevisiae* introduces an intense modulation of the aroma profile of wines, producing wines clearly different to those obtained in single fermentations with *S. cerevisiae*. Each yeast genera have a deep impact on the final levels of the different fermentative aroma compounds formed, causing fermentative aroma profiles to be strongly strain-specific, and specifically different to those obtained exclusively with *S. cerevisiae*. *L. thermotolerans* produced lactic acid and smaller pH, minimized the production of isoamyl alcohol and produced very high levels of γ-decalactone. *P. kluyveri* induced the production of huge levels of acetates, particularly of phenylethyl acetate, while *T. delbrueckii* produced huge levels of isobutyric acid and, hence, its aged samples were very rich in ethyl isobutyrate. Regarding varietal aroma compounds, three different yeast action patterns on the formation of these odorants have been identified. First, yeast strains strongly accelerate the hydrolysis of glycosidic precursors of linalool, geraniol and β-damascenone, making that recently fermented samples contain levels of these odorants in large excess compared to those in unfermented controls. *T. delbrueckii* was particularly efficient at releasing linalool and geraniol. The unwanted corollary is that fermented samples have a more limited aging potential since the pool of precursors is more rapidly depleted. The second pattern is seen in the case of some volatile phenols, which are produced/released during fermentation by transformation of precursors different to those able to yield the odorant by simple acid hydrolysis. In these cases, fermented samples contain higher levels of the odorant and the aging potential is preserved. Finally, in the cases of TDN and 4-vinylguaiacol, fermentation increases the pool of acid-hydrolysable precursors, further promoting the release of odorants during aging,

in fermented samples. *L. thermotolerans* was particularly effective at producing TDN precursors.

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Chapter 3

Observations, questions and conclusions derived from the comparison between varieties

Chapter 3 - Observations, questions and conclusions derived from the comparison between varieties

1. Objective

The main goal of this chapter is to compare the formation and fate of fermentative and varietal aroma compounds in a sequential fermentation approach carried out in model musts containing aroma precursors extracted from Riesling grapes with those obtained from Garnacha, in order to identify differences linked to the grape and to identify differential effects linked to the yeast strain.

2. Results and discussion

The complete data set from all the samples in the experiments carried out with Riesling and Garnacha precursors was processed firstly by PCA in order to assess the relative weights exerted by the different factors with effect in the volatile composition of the samples. Results are summarized in Figure 27 which shows the projections of samples in the plane formed by the two first components (51.3% of the variance). Five fully segregated groups can be identified in sample scores plot. Unfermented controls are represented on the left part of the plot with high negative scores in the first Component, fermented Riesling samples occupy also negative positions in the same axis, while samples from Garnacha have positive scores on the first Component. The second component basically segregates fermented samples with precursors in the upper position from the corresponding fermentative controls represented at the bottom. The unfermented controls are also segregated according to F2 where Garnacha samples are at the bottom from those of Riesling, in upper position.

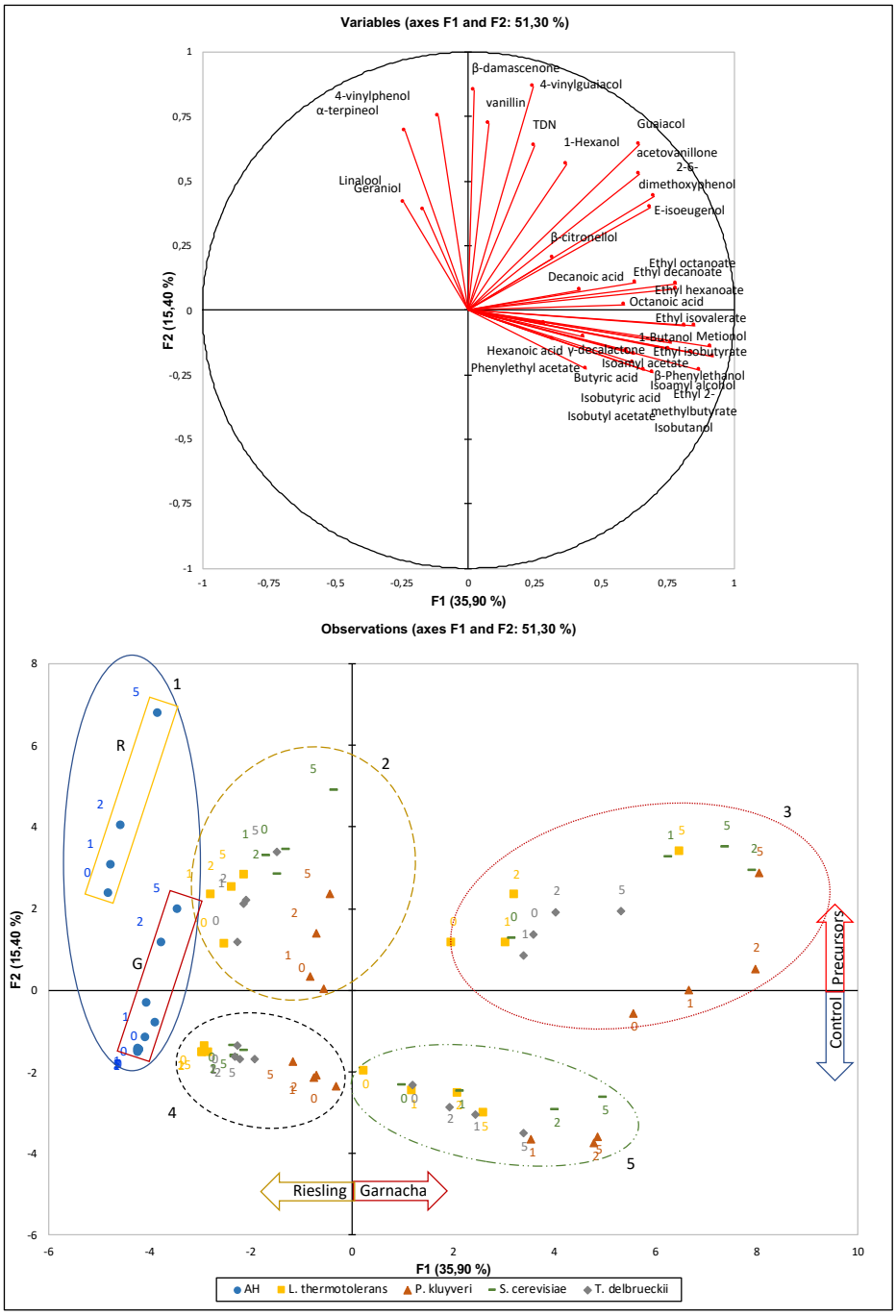

Figure 27 Principal Component Analysis carried out on the data from the two experiments carried out with precursors from Riesling and Garnacha grape varieties. Five groups are identified (from left to right): unfermented controls (group 1), samples from Riesling with (up, group 2) or without (down, group 4) precursors, samples from Garnacha with (group 3) or without (group 5) precursors. Within unfermented controls, Riesling controls are within square R and Garnacha controls within square G.

It should be noticed that samples without precursors from both varieties were produced from musts with exactly the same chemical composition. This implies that a large variability was in fact a batch effect. This could be attributed to differences in the way in which cells were activated. In Garnacha, the inoculum was prepared by rehydration of the dry yeast, following the recommended procedure, while in Riesling, the synthetic musts were inoculated with a pellet of cells previously cultured and preserved at -80ºC with glycerol. This could be the cause explaining why the fermentation rates in Garnacha were substantially slower than those observed in Riesling.

In any case, the PCA study shows that, leaving aside the non-existence of fermentation, the major source of variability is the yeast metabolic ability, which was different in both experiments, followed by the presence of precursors. The third source of variability is the yeast strain, since samples fermented with the same strain tend to occupy a delimited space within each one of the four groups of fermented samples: *L. thermotolerans* at the left and *P. kluyveri* at the centre, with the two others in between. Time also exerts remarkable effects. For Garnacha, samples aged longer have higher scores on F1 and, additionally, samples fermented with precursors have also higher scores of F2. A look at the variable loadings plot confirms that the general metabolic activity, measured as the amount of secondary metabolites produced during fermentation is correlated with the first component, while varietal compounds are correlated with the second component. Regarding varietal compounds, Figure 27 also shows that norisoprenoids and vanillin derivatives seem to have a more equal distribution while monoterpenes and phenols have opposite distributions according to the grape variety.

2.1 Norisoprenoids formation and evolution in Riesling and Garnacha

One of the most unexpected but consistent observation in the previous chapters was the role played by yeast on the formation of the norisoprenoids, specially TDN, Vitispirane and Riesling acetal and also, the surprisingly similar levels of these compounds found in both varieties, while expectations were that Riesling samples should be richer.

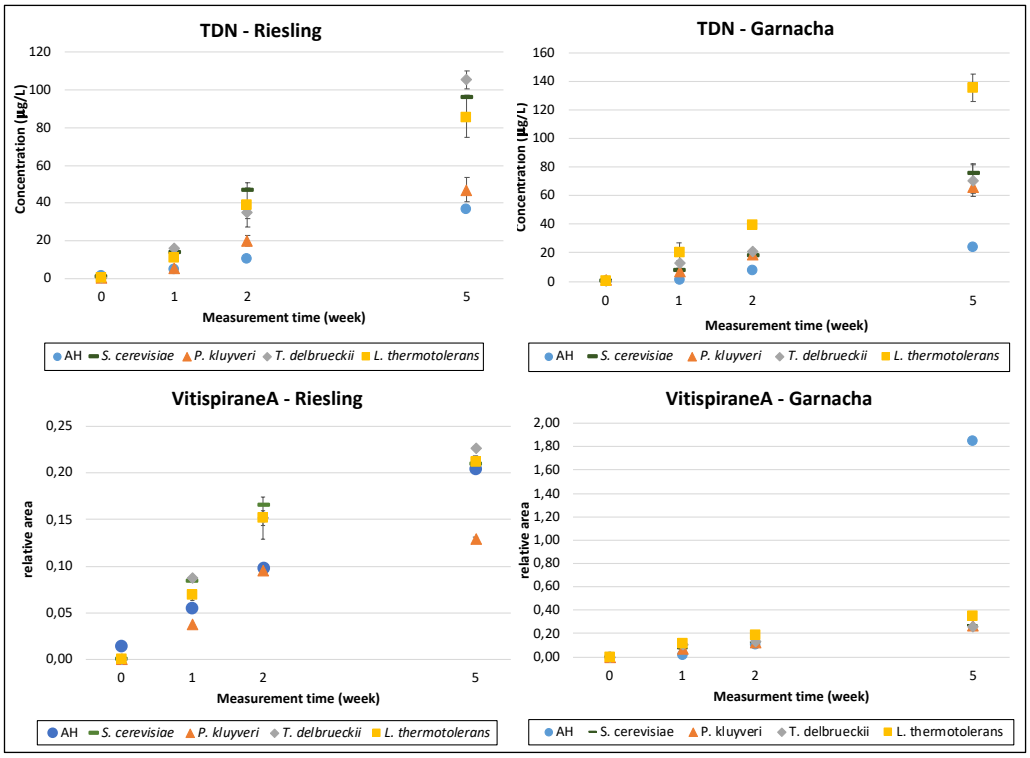

Figure 28 ***TDN and vitispirane formation and evolution in Riesling and Garnacha*** *– average concentration of TDN and vitispirane in wines fermented with different yeast strains and in unfermented controls spiked with glycosidic precursors from Riesling and Garnacha grape and its evolution during accelerated aging.*

The plot in Figure 28 reveals that levels of TDN increase with time following approximately quadratic functions, while levels of vitispirane A (also of vitispirane B) follow linear trends. Consequently, it can be deduced that the

relationships between the levels of vitispiranes and TDN follow square root functions. In fact, the plot relating levels of vitispirane A to those of TDN for each fermentation series looks as follows:

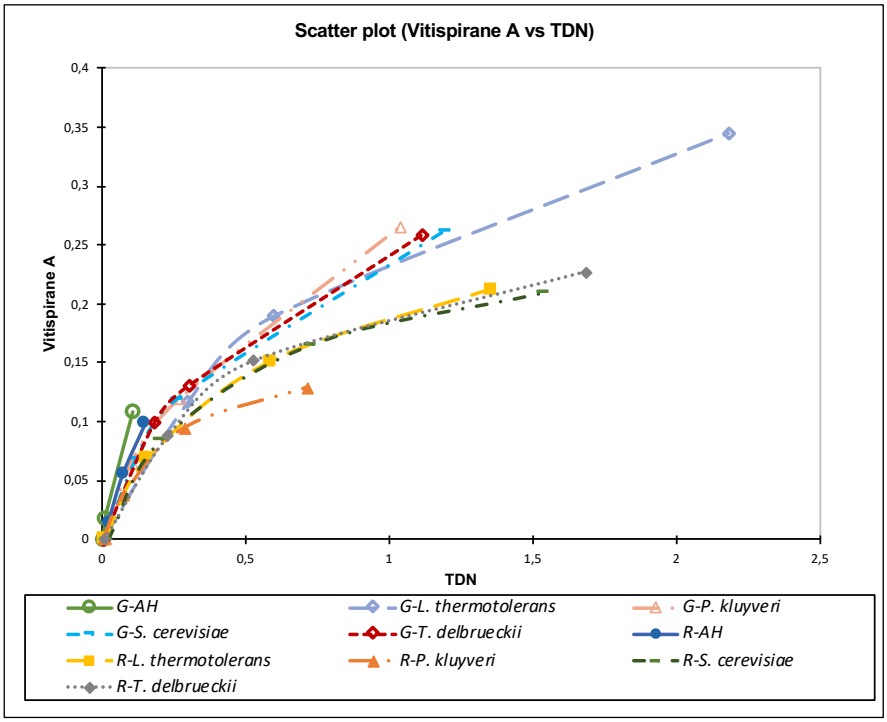

Figure 29 TDN relative areas versus those of vitispirane A for all fermented samples and unfermented controls (AH) for Garnacha (G) and Riesling (R) samples. Samples from the same condition (variety x yeast) aged different times are represented with the same code and joined by a line. In all cases, aged samples had increased levels of both compounds.

As can be seen in Figure 29, there are clear differences between the two grape varieties, and between unfermented controls and the corresponding fermented samples. It can be also observed that unfermented controls in both varieties and fermented samples with Garnacha precursors contain comparatively higher levels of vitispirane A. In the following discussion, we will divide the data set into four groups: 2 with unfermented controls of each variety and 2 others with all fermented samples from each variety.

By observing the graphs presented in Figures 28 and 29 it is possible to introduce a mathematical transformation as follows;

$$|TDN| = k_i t^2$$

$$|Vit| = k_j t$$

Where t is the aging time and k_i and k_j are constants, whose values are approximately constant for each one of the four groups.

Then it follows that:

$$|Vit| = \frac{k_j}{\sqrt{k_i}}\sqrt{|TDN|}$$

which establish that relationships between levels of vitispirane and the square root of TDN levels should follow a linear trend, whose slope is the ratio between the corresponding coefficients of the corresponding $C = f(t)$ functions. The plot in Figure 30 demonstrates the existence of such mathematical relationship between levels of TDN and those of vitispirane.

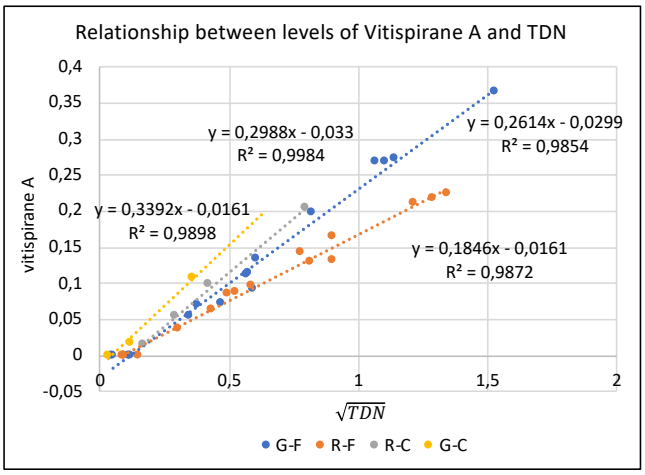

Figure 30 Levels of vitispirane A versus the square root of TDN levels. Samples were classified into four categories: unfermented controls with Garnacha precursors (G-C); unfermented controls with Riesling precursors (R-C); fermented samples spiked with Garnacha precursors (G-F); fermented samples with Riesling precursors (R-F). The plot demonstrates the existence of a linear relationship within each category

A simple *t* statistics can then be easily applied to demonstrate that the corresponding slopes of the fermented samples of each variety are different.

Effectively, simple regression analysis states that the slope for the function

$$[VitispiraneA]_{Riesling} = k_{1R}\sqrt{TDN}_R + k_{2R}$$

is $k_{1R} = 0{,}1846 \pm 0{,}0056$

While that for the function

$$[VitispiraneA]_{Garnacha} = k_{1G}\sqrt{TDN}_G + k_{2G}$$

is $k_{1G} = 0{,}2614 \pm 0{,}0085$

The difference between both slopes, divided by the combined total uncertainty is the corresponding t coefficient:

$$t = \frac{0{,}2614 - 0{,}1846}{\sqrt{0{,}0085^2 + 0{,}0056^2}} = 7{,}54$$

The probability in the two tailed t with 14 degrees of freedom is 0,0000027, implying that both slopes are significantly different at $P<10^{-5}$. Analogously, it can be also demonstrated that the intercepts are significantly different to 0, but that they do not significantly differ between them.

By contrast, the relationship between vitispirane B and $\sqrt{TDN}$ is the same for both varieties and it is only different for the unfermented controls of Riesling, as can be seen Figure 31.

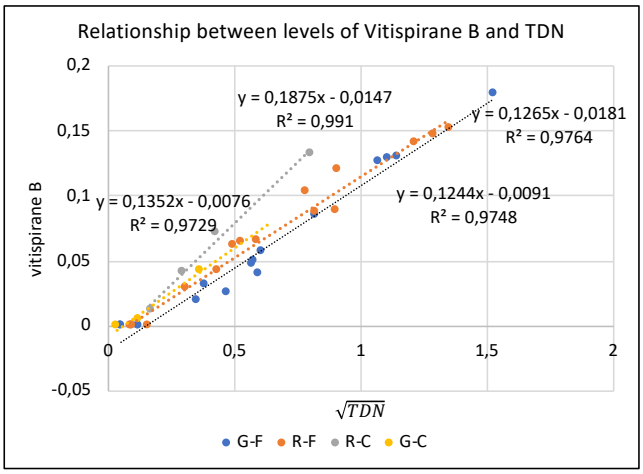

Figure 31 Mathematical relation between levels of vitispirane B and TDN in unfermented controls spiked with Garnacha (G-C) and Riesling (R-C) precursors as well as fermented samples spiked with Garnacha (G-F) and Riesling (R-F).

Hence, based on the previous plots and calculations it is possible to state that levels of vitispirane A are linearly correlated with $\sqrt{TDN}$ and that slopes follow the order:

$$k_{1G-C} > k_{1R-C} > k_{1G-F} > k_{1R-F}$$

while in the case of vitispirane B,

$$k_{1R-C} > k_{1G-C} \approx k_{1G-F} \approx k_{1R-F}$$

It should be also remembered that levels of TDN found in the samples follow approximately quadratic functions of the form: $|TDN| = k_i t^2$, which can be experimentally approximated by functions of the form:

$$\sqrt{|TDN|} = b_{1i} t + b_{oi}$$

The coefficients for each series have been estimated by linear regression and are given in Table 7:

Table 7 Experimental estimation of the coefficients which give TDN levels for each sample series

Sample series	b_{1i}	b_{0i}
Garnacha unfermented control	0.124	0.040
Riesling unfermented control	0.126	0.168
Riesling *P. kluyveri*	0.158	0.153
Garnacha *P. kluyveri*	0.188	0.152
Garnacha *T. delbrueckii*	0.196	0.164
Garnacha *S. cerevisiae*	0.201	0.151
Riesling *L. thermotolerans*	0.216	0.208
Riesling *S. cerevisiae*	0.219	0.273
Riesling *T. delbrueckii*	0.238	0.211
Garnacha *L. thermotolerans*	0.278	0.195

One possible hypothesis explaining part of these results is that original TDN and vitispirane precursors are glycosides of some unknown norisoprenoid precursor (noted as NISP) and that the set of reactions required to yield the odorants involve first the hydrolysis of glycoside. Then, the aglycone would undergo acid catalysed rearrangement reactions yielding the odorants and following a second order kinetic law for the case of TDN and a first order kinetic law for the case of both vitispiranes. The scheme shown in Figure 32 summarizes the reactions:

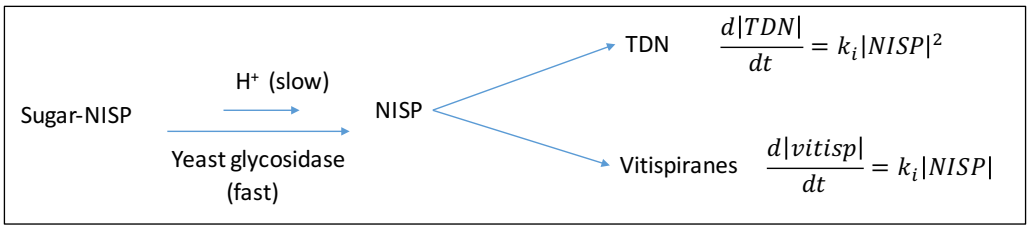

Figure 32 Scheme of the hypothesis describing the formation of TDN and vitispirane from an unknown glycosidic precursor (Sugar-NISP), whose aglycone renders both TDN and vitispiranes following 2nd and 1st order kinetics, respectively.

Fermentation would therefore, be a major effect on the increase of NISP, accelerating the production of the odorants. The different effects exerted by different yeasts may be related either to the efficiency of their glycosidases to release the NISP or, to their ability to transform the original precursor into different substances, no longer able to form TDN or vitispirane. In this last respect, P. kluyveri seems to be particularly active.

Obviously, further experimental work is required to validate this preliminary hypothesis.

It is clear, nevertheless, that precursors from both Riesling and Garnacha have potential to form TDN and vistispirane at very similar levels; that formation of TDN and vitispirane are closely correlated and that levels formed depend on the yeast carrying out fermentation.

These observations open many questions about the nature of precursors and about the formation mechanisms. While formation due to carotenoid breakdown has been demonstrated (Winterhalter et al., 1990), its linkage with environmental conditions and *Botrytis cineria* infection have been proposed, but not totally demonstrated, there is no yet clear knowledge to understand what triggers the expression of these compounds in wine (Fischer, 2007).

The case is that TDN is normally identified in the GC-trace of many old wines (Fischer, 2007; Oliveira et al., 2008; Simpson, 1978), but usually it is thought that levels are too low to cause any sensory problem. Our results show, however, that levels of TDN in Garnacha wines fermented with *L. thermotolerans* can largely exceed those obtained in Riesling. Moreover, TDN levels obtained in Garnacha fermented with *S. cerevisiae* are not much smaller than those obtained in Riesling. These observations lead to two suggestions: either TDN formation in real winemaking is affected by the presence of other specific constituents from red grape varieties, such as polyphenols and anthocyanins, or its formation

occurs normally, but those levels of TDN do not contribute to the appearance of kerosene-like descriptors in red wine-like aroma contexts. In fact, some studies have hypothesized that TDN may be integrating the aroma vector responsible for honey descriptors (Lopez et al., 2004).

β-damascenone, as discussed in previous chapters, has a different evolution pattern compared with TDN and vitispirane. Fermented samples, show very similar maximum contents and evolution patterns, which seems to be dependent on the yeast strain rather than on grape variety. *P. kluyveri* in both varieties was the yeast with least potential to form these compounds, as it was also observed for the other norisoprenoids. Differences are observed on the formation of β-damascenone in the unfermented controls as the formation of this compound from Garnacha precursors via simple acid hydrolysis is much more difficult than in Riesling. In fact, in Riesling, more than 85% of β-damascenone is in free form at the very beginning of the process. This implies, that precursors from Riesling yielded a volatile molecule in just few weeks at room temperature. By contrast, in Garnacha, less than 15% is in free form at that time and 2 weeks at 50ºC are required for the precursor to be hydrolysed completely. This certainly suggests that β-damascenone precursors in Riesling have to be completely different to those found in Garnacha.

β-damascenone formation has been connected with carotenoid degradation. Such degradation takes most likely place during grape maturation, so that each grape will contain a different pool of carotenoid degradation products, many of which will be under the form of glycosides. Our data certainly supports that the pool of β-damascenone precursors depends on grape variety (Figure 33).

β-damascenone is a compound with very low odour threshold that seems to be important for varietal character of both white and red wines, however it can integrate several aroma vectors contributing to flowery, fruity or boiled fruits

descriptors depending on the interaction with the remaining compounds in the wine (Ferreira et al., 2019).

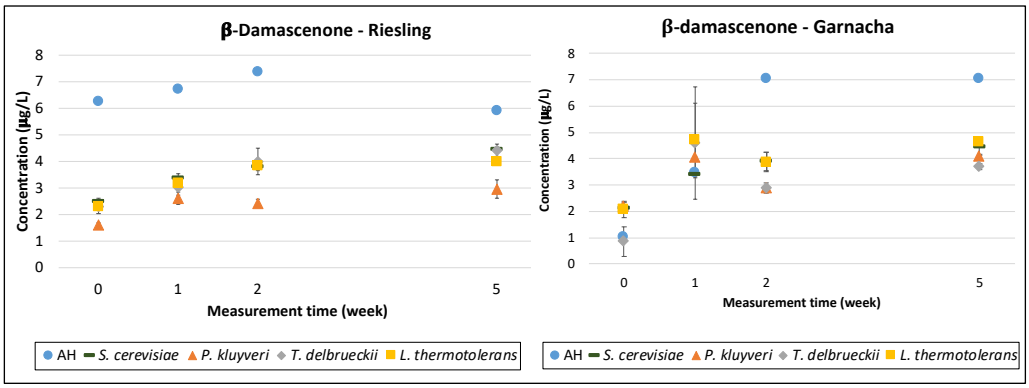

Figure 33 ***β-damascenone formation and evolution in Riesling and Garnacha*** *– average concentration of β-damascenone in wines fermented with different yeast strains and in unfermented controls spiked with glycosidic precursors from Riesling and Garnacha grape and its evolution during accelerated aging.*

2.2 Monoterpenes in Riesling and Garnacha

Monoterpenes are naturally present at much higher levels in Riesling, where they can be highly relevant in the delicate floral and citrus aromas observed in the young wines (Strauss et al., 1986). Levels in Garnacha are comparatively much lower. However, as it was already mentioned in the previous chapter, levels of linalool and geraniol together can play outstanding sensory roles, since they exert a concerted action on floral and citrus aromas. As was also observed in the case of β-damascenone, the major difference between Garnacha and Riesling, leaving aside quantitative differences, are the evolution patterns observed in the unfermented controls. In the case of Riesling, nearly all linalool is under free form initially, while in the unfermented control of Garnacha, levels of free linalool are just marginal (Figure 34). This is also in agreement with the hypothesis that in Riesling grape variety a large majority of linalool precursors may not be glycosides but polyols, which can be very easily converted into free volatiles. Furthermore, in Riesling, there is a continuous decrease because there

are very few glycosidic precursors remaining after fermentation, thus the number of linalool molecules that disappear by rearrangement cannot be replaced by new aglycones hydrolysed from the precursors. The smaller levels observed in the fermented samples are probably due to co-evaporation. On the contrary, in fermented samples from Garnacha, linalool and geraniol reached maximum levels at times 0-1 while in unfermented controls the maximum was observed after 2 weeks of aging. Besides, the levels obtained exclusively by acid hydrolysis (unfermented controls) did not outperform those found in fermented wines. This suggests that linalool production by acid hydrolysis of Garnacha glycosidic precursors is much slower compared to the production of this molecule from yeast enzymatic hydrolysis. Hence, by comparing both varieties, it seems likely that polyols are not present as linalool (and geraniol) precursors in Garnacha grapes, explaining the different rates at which linalool and geraniol can be formed due to acid or enzymatic hydrolysis in wines fermented with precursors fraction from Riesling or Garnacha.

As described in the previous chapters, α-terpineol shows a quite different evolution pattern compared to linalool and geraniol. Overall, the patterns observed suggest that there are no glycosidic precursors with an α-terpineol aglycone in none of the varieties. In both cases, this molecule is likely formed by rearrangements suffered by other monoterpenes including linalool and geraniol, at the wine pH. In fact, levels of α-terpineol at a given time are proportional to pre-existent levels of linalool and geraniol and to their decay. In other words, the faster the release/formation of linalool and geraniol the faster the appearance of α-terpineol.

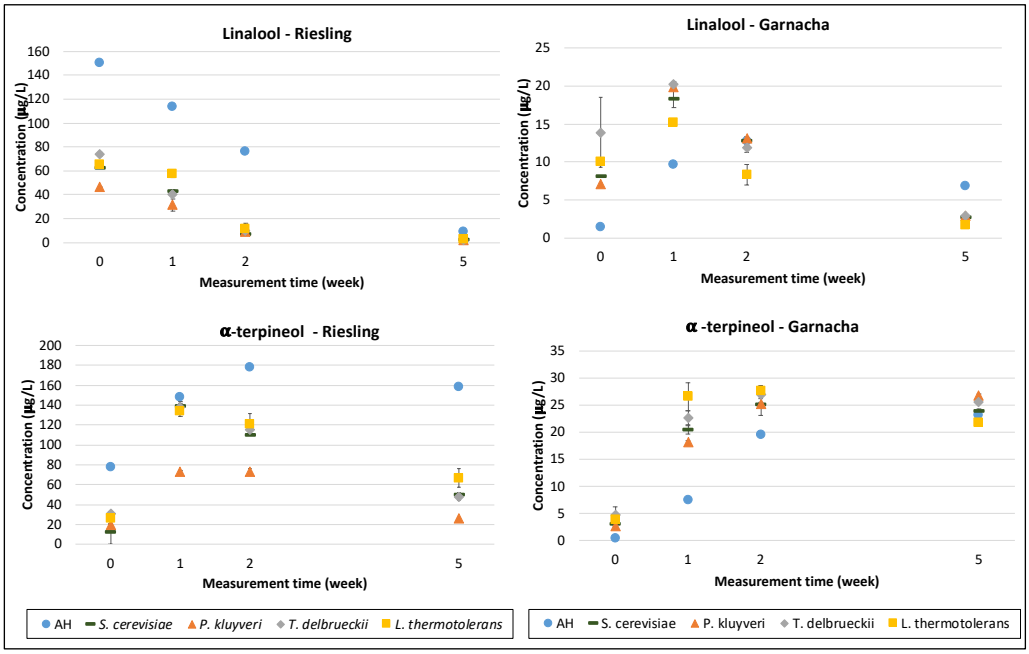

Figure 34 ***Linalool and α-terpineol formation in Riesling and Garnacha*** *– average concentration of linalool and α-terpineol in wines fermented with different yeast strains or unfermented controls spiked with glycosidic precursors from Riesling and Garnacha and its evolution during accelerated aging.*

Previous studies performed with Muscat grapes have suggested that monoterpenes could also have multiple precursors which are not necessarily glycosides, such as polyols (Williams et al., 1980). Our results suggest that these type of precursors could be particularly relevant in Riesling, but not in Garnacha. As these observations are also extended to β-damascenone, it can be hypothesized that glycosylation is in general more active in Garnacha than in Riesling. The much higher levels of free monoterpenes observed in samples from Riesling in comparison with those of Garnacha, are also in agreement with the important sensory role attributed to monoterpenes on Riesling varietal character (Schreier et al., 1976; Strauss et al., 1986).

2.3 Volatile phenols in Riesling and Garnacha

Volatile phenols are often observed in both white and red wines but the distribution of these compounds is different attending to wine type. In white wines vinyl phenols can be often observed whereas in red wines vinylphenols are present at very low levels (Chatonnet et al., 1993). These differences are due to the fact that red wine catechins inhibit the cinnamate carboxy-lyase (cinnamyl decarboxylase, CDC) activity of yeasts. Nevertheless, a look at the results of this study, summarized in the plots shown in Figure 35, reveals that the grape potential to form vinyl phenols is much higher in Riesling than in Garnacha. This is can be easily seen by comparing the levels of both phenols found in the unfermented controls after 5 weeks of accelerated aging. Both phenols were above 4 mg/L in Riesling while, in Garnacha levels did not surpass 700 µg/L.

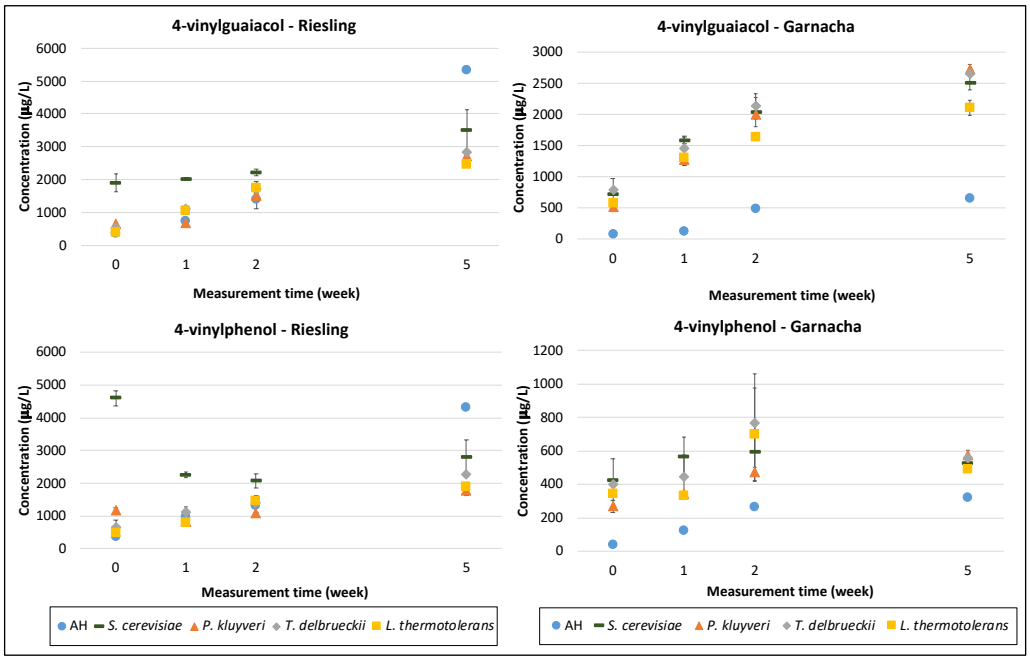

Figure 35 ***Vinyl phenols in Riesling and Garnacha*** *– average concentration of 4-vinylguaiacol and 4-vinylphenol in wines fermented by different yeast genera and unfermented controls spiked with glycosidic precursors from Riesling and Garnacha grapes.*

The presence of ethyl phenols was not observed in any of the experiments, which is consistent with their origin. This is not connected to alcoholic fermentation but rather to the action of *Brettanomyces* spp., most frequent during wood aging (Chatonnet et al., 1993).

The different patterns of evolution observed between samples fermented with Saccharomyces and the rest of yeasts in Riesling, and those observed between fermented samples and unfermented controls, support the existence of multiple precursors for vinyl phenols in both varieties. This can be seen in a very simple scheme in Figure 36.

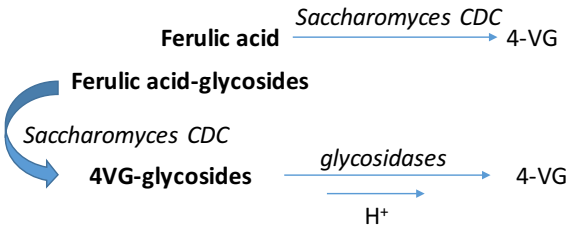

Figure 36 Scheme showing the main different processes leading to the production of 4-vinylguaiacol during fermentation and aging.

As depicted in the scheme, 4-vinylguiacol can be directly formed by *S. cerevisiae* from ferulic acid. A second possibility is that this enzyme transforms ferulic acid glycosides into 4-vinylguiacol-glycosides, which will therefore, increase the pool of 4-vinylguiacol glycosidic precursors. These precursors can release 4-vinylguiacol by fast action of glycosidases segregated during fermentation, or by slow action of acid hydrolysis. A look to the plots in Figure 35 suggests that the precursor extract from Riesling should be rich in ferulic and coumaric acids, which were massively transformed by *S. cerevisiae* into 4-vinylguiacol and 4-vinylphenol, respectively. This would explain the huge amounts of these compounds found in the samples fermented by *S. cerevisiae*. Comparatively, the Garnacha precursor extract should not contain the acids. The

fact that samples fermented with the other yeasts did not show high levels of the phenols in the recently fermented wines, would suggest that CDC activities should be active just when *S. cerevisiae* is the single fermenting species. The Riesling precursor extract should also be extremely rich in 4-vinylguiacol-glycosides, which would explain the high levels of the phenols found in aged unfermented samples. In comparison, Garnacha should contain just a limited amount of 4-vinylguiacol-glycosides and a relatively large pool of ferulic acid glycosides.

2.4 Medium chain fatty acids ethyl esters in Riesling and Garnacha

Medium chain fatty acids and their ethyl esters, as shown in the PCA from Figure 27, are present at higher levels in Garnacha wines. Figure 37 highlights the differences between varieties for ethyl hexanoate and hexanoic acid. As described in the previous sections, wines fermented with *P. kluyveri* in both varieties have the highest content of these two compounds. In both experiments, samples fermented with *L. thermotolerans* and *T. delbrueckii* produced minima levels of these compounds. The effects of the presence or not of precursors are small and inconsistent, although it is clear that in Garnacha, samples containing precursors had significantly higher levels of the ester (but not of the acid). This implies that specifically the esterification ratio is higher in these samples.

In both cases, the high levels of the fatty acid provide great stability to the esters in wine, which will have a notable sensory role in both varieties.

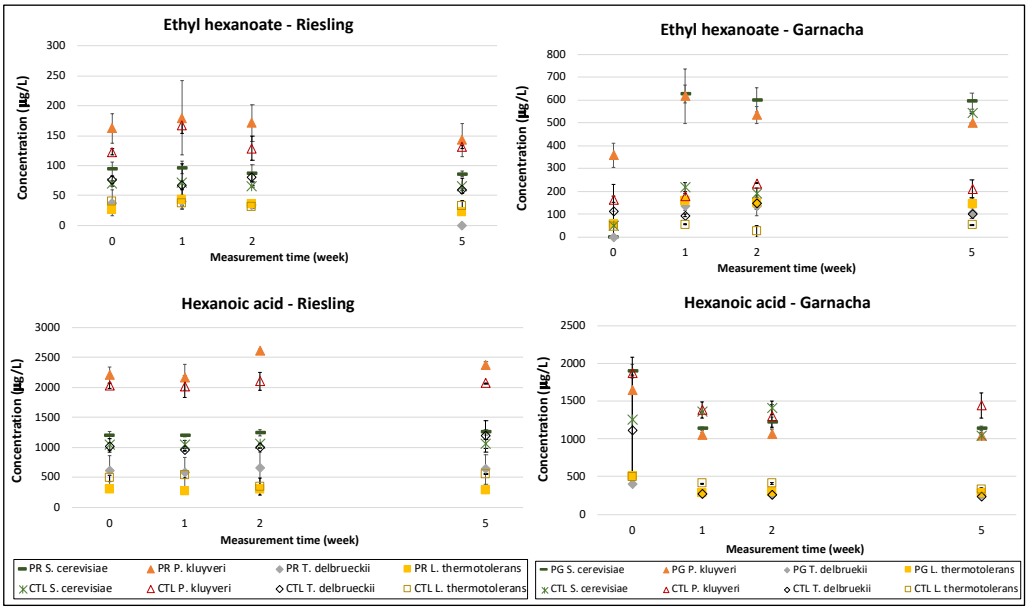

Figure 37 Hexanoic acid and ethyl hexanoate content evolution in Riesling and Garnacha wine in fermented samples with different yeast strains

2.5 Fusel alcohols in Riesling and Garnacha

In terms of sensory relevance, isoamyl alcohol and to a much lesser extent, β-phenylethanol, are highly important odorants. The plots comparing the evolution of these compounds in the 2 different experiments can be seen in Figure 38.

In spite of the large differences between the experiments, it can be seen that there are some similarities. In both cases, maxima levels of isoamyl alcohol were produced by *S. cerevisiae*, while *L. thermotolerans* and *T. delbrueckii* produced consistently minima levels. This suggests that these two yeasts can be used to produce wines with reduced levels of this strong odour suppressor (de-la-Fuente-Blanco et al., 2016; Ferreira et al., 2019).

In the case of β-phenylethanol, it is *T. delbrueckii* the one promoting high levels of this odorant, while *L. thermotolerans* is again the one consistently providing minima levels of this odorant. Although the sensory importance of β-

phenylethanol has been previously overestimated, the huge levels found in the experiment of Garnacha, suggest that it can be present at levels at which it can have sensory relevance.(de-la-Fuente-Blanco et al., 2016; Ferreira et al., 2019).

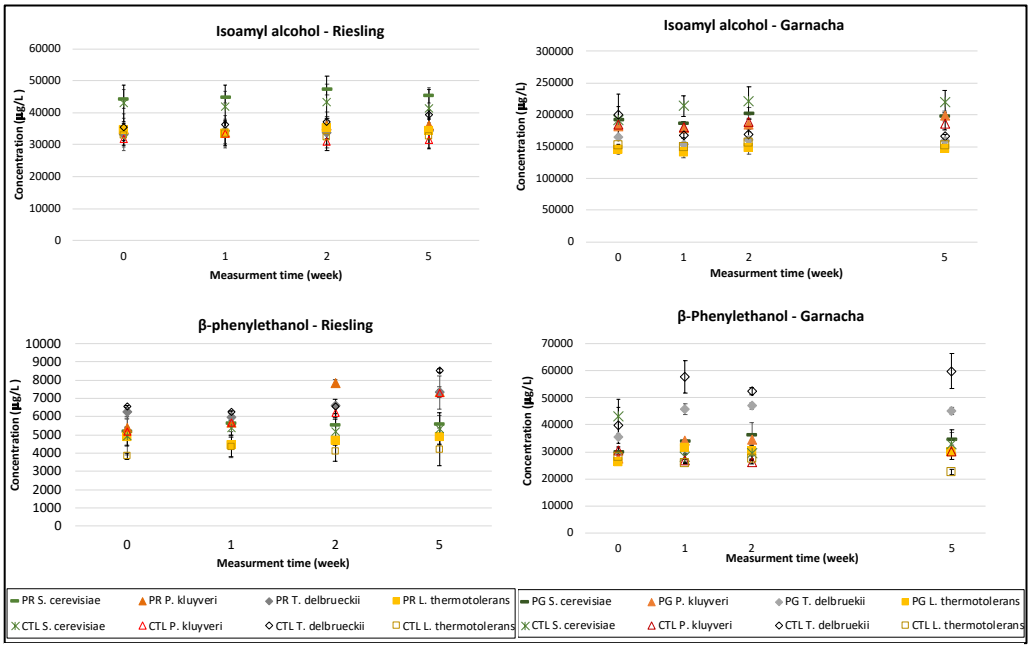

Figure 38 ***Fusel alcohols in Riesling and Garnacha*** *– average content isoamyl alcohol and β-phenylethanol in wines fermented with different yeast genera in the presence of glycosidic precursors from Riesling and Garnacha grapes and their fermentative controls without precursors fraction. No volatiles were detected in unfermented samples, thus they are not shown.*

3. Conclusions

1.- TDN and vitispirane levels are closely related, so that their ever-increasing levels during aging are perfectly connected via the mathematical law Vistispirane = $k_{ij}\sqrt{TDN}$, which suggests that both compounds derive from the same precursor.

2.- In both cases, levels are always higher in fermented samples, which suggests that such common precursor is a glycoside which requires a previous hydrolysis to form the aglycone precursor which by chemical rearrangement yields both odorants. TDN would be formed by second order kinetics, while vitispiranes by first order kinetics.

3.- The TDN potential in wine can be largely controlled by using a sequential inoculation approach. Samples fermented with *P. kluyveri* have consistent smallest TDN potential, while those fermented with *L. thermotolerans* have consistently highest TDN potentials.

4.- The TDN potential of Garnacha wines can be as high as that of Riesling.

5.- Riesling contains fast hydrolysable aroma precursors, such as polyols, for relevant varietal aroma compounds, such as linalool, geraniol and β-damascenone, so that Riesling wines contain maxima levels of these compounds immediately after fermentation, and yeast strain contributes poorly to their levels. By contrast, Garnacha does not contain those types of precursors, so that yeast strain is relevant for their release, and maxima levels are observed after some aging.

6.- In the case of vinyl phenols the patterns of formation and evolution with time of these aroma compounds are also strongly linked to grape variety. It can be hypothesized that Riesling precursor fraction is rich in cinnamic acids, which are specifically transformed by *S. cerevisiae* into the volatile phenols, and it is also

very rich in glycosides of vinyl phenols. Garnacha precursors on the other hand, should contain nearly no free cinnamic acids, slight amounts of their glycosides and just minimal amounts of the glycosides of vinyl phenols. In any case, results strongly suggest that levels of vinylphenols in white wines can be much modulated by using a sequential inoculation approach.

7.- In spite of large batch differences, which have affected to the levels of most fermentative volatiles, there are strong and consistent effects on the wine aroma profiles linked to specific sequential inoculation approaches. Levels of isoamyl alcohols were significantly reduced by all non-*Saccharomyces* strains, and levels of hexanoic acid and its ethyl esters were significantly enhanced by *P. kluyveri*, to name just a few of the most relevant.

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Section III

Aroma of German Riesling wines and influence of the *terroir*

Introduction and Methodology

Chapter 4 Sensory and chemosensory characterization of commercial young Riesling wines from different German appellations

Chapter 5 Impact of vineyard versus cellar microbiota from different harvests on the distinction of different Riesling vineyards

Introduction and Methodology

1. Section III - Introduction

Attempting to characterize a grape variety typical character is not a straightforward task due to the large variability due to sources other than grape variety. The concept *terroir* was introduced in France to define the specific wine characteristics linked to the set of regional parameters including climate, soil type, grape varieties, vine management or winemaking procedures (Fischer et al., 1999; van Leeuwen, 2010). Obviously, there is hardly two similar consecutive harvest years, nonetheless each wine region has a more or less defined climate and geomorphologic characteristics which, together with the specific cultural and technological practice of the area, are well reflected in the wines. The *Terroir* concept has been used to explain why wines made with the same grape variety, produced in different appellations, can be well distinguished, but also dealing with the fact that the same wine produced in different harvests can be really different (van Leeuwen, 2010). In fact, climate could be considered the factor with major influence on the variability of wine characteristics, since is related to location and harvest year.

Soil, considered as a more or less unchanging variable influencing wine properties, has a central role on wine quality. Soil type influences grape development and is an important provider of inorganic compounds, so important to wine aroma formation (Hopfer et al., 2015; van Leeuwen, 2010)

More recently, vineyard microbiota has raised more and more attention to the scientific community. Several studies have been conducted to try to understand the role of endogenous microorganism on the definition of *terroir*, but no definitive conclusions have been drawn (Barata et al., 2012; Varela and Borneman, 2017). Microorganism diversity has been attributed to vintage year, vineyard age and development state and studies are not very conclusive

regarding the development of specific microbiota according to vineyard location (Barata et al., 2012; Varela and Borneman, 2017).

Despite immense product diversity obtained from one single grape variety, the varietal concept can also still be well recognized by wine experts. However, describing the aroma vectors specific to a given grape variety is not an easy task, precisely because of all the variables involved in the construction of wine character. This is also translated into the specific profile of volatile components responsible for such varietal character. Riesling wines are one of the best know and consumed products world-wide (Fischer, 2007; Sacks et al., 2012), being Germany the top producer of wines from this variety followed by Australia. Not surprisingly, the wines are completely different. However, Riesling wines are readily recognized by experts even though the aroma compounds responsible for the formation of their typical nuances are not fully understood (Fischer, 2007; Sacks et al., 2012). Varietal compounds such as monoterpenes and 3-mercaptohexanol have been associated with Riesling typicity, although more commonly formed volatiles such as esters and alcohols are potentially involved in the development of these nuances (Bauer et al., 2011; Sacks et al., 2012; Schüttler et al., 2015). Aged Riesling has been extensively studied due to its almost unique formation of kerosene notes attributed to TDN formation (Fischer, 2007; Parker et al., 2017; Sacks et al., 2012; Schüttler et al., 2015; Simpson and Miller, 1983).

Difficulties arise concerning study methodologies. Often variations in chemical composition of wines are observed in quantification studies, however these changes are not necessarily reflected in sensory perception of wine. Wine aroma is built from aroma vectors formed by one or more volatile odorants in sufficient concentration to break the wine buffer. Due to the wine buffer properties, often significant wine aroma composition changes are not reflected in wine sensory

perception. Moreover, a wine aroma is a result of several volatiles physiochemical interactions in addition to perception interaction with olfactive receptors (Ferreira et al., 2019; Zucco et al., 2014).

2. Section III - Methodology Chapter 4

2.1 Project collaboration

This project had the collaboration of Kimmo Sirén regarding the selection and purchase of Riesling from different wineries in Germany.

The sensory analysis was performed during the secondment in DLR-Rheinpfalz Germany in 2018 with supervision of Professor Dr. Ulrich Fischer.

2.2 Riesling commercial wines

Six commercial German Riesling wines categorized as *Großes Gewächs* Riesling, all from 2015 harvest, were selected from different wine regions: Bürgerspital Würzburger Stein Hagemann and Fürst Centgrafenberg from Franken, Guderloch Rothenberg from Rheinhessen, Knipser Mandelpfad and Rebholz Kastanienbusch from Pfalz and Clemens-Busch Marienburg Rothenpfad from Mosel.

2.3 Sensory analysis:

The study of the aroma typicity of Riesling wines was done by carrying a sensory analysis by a panel with 15 judges (9 males and 6 females) with ages from 23 to 55 from DLR-Rheinpfalz staff.

The wines were primarily assessed through a non-verbal classification technique (napping analysis) in which the judges were asked to sort the wines on a A3 size paper according to their similitude/ dissimilitude, so that similar wines were positioned together. The judges are further asked to write the wine codes on the paper, as well as the discriminant attributes identified for each group.

Following the napping session, twelve wine aroma references were presented to judges and they were asked to discuss to relevance of those references according

to the wine attributes they have identified. Prior to the discussion session, ten aroma references were selected for the descriptive analysis. The references were prepared using food products following the principles described by (Sela Bowen et al., 2018) (Table 8): cooked apple, floral, peach/ apricot, smoky, citrus/ grape fruit, volatile acidity (VA), petrol, caramel/ butterscotch, honey, maracuja. Three tasting attributes were also included in the analysis: sweet, sour, mouthfeel; two tactile sensations: body and bitter.

Table 8 Recipe for the preparation of aroma standard for Riesling descriptive analysis

Aroma Standard	Recipe prepared in 100 ml of base wine	
1 Cooked apple	20	g quince jam
	25	ml unfiltered apple juice
2 Floral	2	drops linalool and geraniol each
3 Peach/apricot	5	ml peach juice (Granini)
	17	g apricot jam
4 Smokey	60	µl 4-vinylguiacol
	4	drops whiskey
5 Citrus/ grape fruit	1	ml citrus (orange) concentrate
	4	ml grape juice
6 Volatile acidity	18	µl ethyl acetate pure
7 Petrol (TDN)	35	µl 1mg/10ml TDN
8 Caramel/butterscotch	1.5	ml caramel syrup
	5	µl diacetyl
9 Honey	3	g honey (Blutenhonig)
10 Maracuja	0.325	ml maracuja syrup (Monin)
	3,5	ml concentrate juice

The judges participated in a training session with one wine and afterwards, the six wines were evaluated by a descriptive analysis in two replicate sessions (one session per day). Thirty ml of wine were served in random order and different for each judge using tulip-shaped tasting transparent glasses preserved at 12ºC. Intensity rating was done using 10 cm scales from 0 to 10 using Fizz Software (Biosystemes, France). Before rating the six wines for each aroma descriptor, the judges were asked to train during a few seconds with the aroma reference provided.

2.4 Chromatography-olfactometry analysis

The same Riesling wines were analysed by gas chromatography-olfactometry (GC-O) analysis.

The volatiles were capture by a purge-and -trap system using Lichrolut-EN resin cartridges and N_2 flow as described by (Escudero et al., 2014). The sniffing of the extracts was performed by a panel of 6 people (4 females and 2 males, with ages between 20-33 years old). The panellists were all trained members of the Laboratory of Analysis of Aroma and Enology (LAAE) staff.

The sniffing analysis were made using a Thermo 8000 series GC equipped with a FID detector and sniffing port (ODO-1, SGE). The column used as a DB-WAX (J&W, Folsom, CA) with 30 m x 0.32 mm x 0.5 μm. H_2 was used as carrier gas with a flow of 3 mL/min and 1 μL of sample was injected in splitless mode kept for 1 minute. Injector and detector were kept at 250ºC and sniffing port temperature was heated sequentially using a rheostat to avoid condensation of high-boiling point compounds. The column was initially at 40ºC for 5 minutes, then raised to 100ºC at 4ºC/min and afterwards temperature was raised at 6ºC/min until 200ºC, as described by (Campo et al., 2005). The judges were requested to write the retention time, descriptor and intensity (0-3).

The identification of the odorants was made by comparison of odour descriptors, chromatographic retention indexes in both DB-WAX and DB-5 chromatography column systems using data bases as references (Pherobase and Flavornet) and previous works (Ferreira and San Juan, 2012).

2.5 Volatile compounds quantification

Major and minor compounds were quantified for the six wines as described in section 1 and 2, respectively.

2.6 Data analysis

Sensory analysis was performed using Fizz Software (Biosystemes, France). The scores for each descriptor evaluated by descriptive analysis was processed by analyses of variance (ANOVA). The significance of each attribute to distinguish the six wines was evaluated by a 3-way ANOVA having as fixed factors wine, judge and replication session, as well as first-order interaction. Moreover, a pair-test analysis was performed using Fischer test (LSD).

The average score for each attribute, per session was further calculated and analysed by Principal Component Analysis (PCA).

The data obtained by GC-O analysis was processed by calculating the Modified Frequency (MF) using the following formula (Dravnieks, 1985):

$$\mathrm{MF}(\%) = \sqrt{\mathrm{F}(\%)\mathrm{I}(\%)}$$

in which MF(%) is the detection frequency of an aromatic attribute in percentage and I(%) is the average intensity expressed as percentage of the maximum intensity. Besides the minimum and maximum MF(%), as well as the ratio between them was calculated for each volatile identified. The MF(%) were then assessed by PCA.

Microsoft Excel 2016 version and XLSTAT (Addinsoft, 2018 version) were used to make data analysis, in particular, ANOVA and PCA analysis.

3. Section III - Methodology from Chapter 5

3.1 Project collaboration

This project was carried in the scope of Kimmo Sirén PhD thesis. The experimental design and full extent of results are published in Kimmo Sirén's thesis. Due to its magnitude, the project was performed in collaboration with this thesis and with Sara Mak PhD thesis.

The first secondment of this PhD thesis project took place in DLR-Rheinpfalz during harvest 2016 under supervision of Professor Dr. Ulrich Fischer and collaborating with Kimmo Sirén and Sara Mak on grape harvest, wine production and sampling.

The results herby presented refer solely to the aroma analysis carried on the wines from harvest 2015 and 2016 analysed in Zaragoza, Spain, approximately five months after wine fermentation.

3.2 Harvest and wine fermentation

Riesling grapes were harvested aseptically: use of sterile nitrile gloves, clean and sterile harvest scissors and the grapes were collected inside sterile plastic bags. Only sound grape bunches were selected and contact with soil was avoided.

Grapes were harvested in 2015 from five different vineyards from Pfalz, Germany and in 2016 from seven different vineyards from the same region.

The grapes were divided into two lots: one lot was processed aseptically in DLR-Rheinpflaz institute and the other was sent to the corresponding vineyard winery and processed according to the winery normal procedures.

Grapes processed in DLR-Rheinpflaz were intended to be processed aseptically to avoid microbial combination from the pilot cellar, thus all the winery material was sterilized with ozonated water prior to usage. The grapes were pressed using an Europress (Scharfenberger, Germany) and the grape juice was let to decant inside a stainless-steel reservoir overnight. Afterwards, the grape juice was divided into three biological replicate balloon flasks, caped with air-lock valves and kept inside a temperature-controlled sterile container were fermentation took place.

Fermentation was monitored by daily analysis on FTIR (Fourier Transform Infrared) (WineScan^{TM}, Foss).

3.3 Chemical analysis

The wines used in the studies of Chapter 3 and 4 of this section were both analysed regarding their content of important major compounds using the same methodology described in Section I.

The content of minor and trace compounds was also analysed in wines from both chapters following a Solid Phase Extraction (SPE) with Lichrolut EN resin of 15 ml of wine as described in Section II.

Wines from Chapter 4 were also characterized according to their aldehydes and polyfunctional mercaptans.

Total aldehyde content was analysed by performing a Head Space Solid Phase Microextraction followed by GC-MS analysis (HS-SPME-GC-MS) following the methodology described in Section I.

Polyfunctional mercaptans were analysed based on the method described by (Mateo-Vivaracho et al., 2010) with the usage of deuterated polyfunctional

mercaptans and by derivatizing wine samples with PFBBr from Cymit Quimica (Supelco).

The following reagents were used: n-Hexane for organic trace analysis and dichloromethane, methanol and ethanol, gradient grade for liquid chromatography (LiChrosolv), were from Merck (Darmstadt, Germany); diethyl ether for instrumental analysis and mercaptoglycerol were from Fluka (Buchs, Switzerland); anhydrous sodium sulfate was of analysis ACS-ISO quality from Panreac (Barcelona, Spain); ethylenediaminetetraacetic acid disodium salt 2-hydrate (EDTA), L-cysteine hydrochloride hydrate 99% and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were from Sigma Aldrich (Steinheim, Germany); O-Methylhydroxylamine hydrochloride purum >98% and 2,3,4,5,6-pentafluorobenzylbromide (PFBBr) (Supelco).

The compounds used to calibrate were 4-Mercapto-4-methyl-2-pentanone (MP) 1% PG and 3-mercaptohexyl acetate (MHA) were from Oxford Chemicals (Hartlepool, U.K.); 2-Furfurylthiol (FFT) and 3-mercaptohexanol (MH) were from Lancaster (Strasbourg, France); Benzyl mercaptan (BM), 2-phenylethanethiol, 4-methoxy-R-toluenethiol and 1,4-dithioerythritol, octafluoronaphthalene 96% (OFN) (Sigma Aldrich) were used as internal standards.

Bond Elut-ENV resins, prepacked in a 50 mg cartridge (1 mL total volume) were used to perform a Solid Phase Extraction (SPE).

The compounds were analysed by Gas-Chromatography-Mass Spectrometry using a Shimadzu QP-2010 Plus.

A Shimadzu PTV injector was used with initial temperature of 65ºC and kept for 25 s, afterwards temperature was raised to 260ºC at 6ºC/s and was maintained during the remaining analysis. Four microliters of sample were injected in a splitless mode kept during 4.15 minutes with a H_2 flow of 2.69 mL/min. When the split valve opened, the flow decreased to 1.44 mL/min and was maintained

during the remaining analysis. A Factor Four capillary DB-5 column from J&W Scientific with 20 m x 0.18 mm x 0.18 μm was used. The initial temperature was 40ºC and maintained for 4.15 minutes, then heated to 140ºC at 25ºC/min, afterwards temperature increased to 180ºC at 15ºC/min, then to 210 at 30ºC/min and finally at 250ºC/min to 280ºC and kept for 10 minutes.

The ion source was operated in NCI (Negative Chemical Ionization) mode and SIM mode as described in the same work.

3.4 Data analysis

The data presented in this chapter was analysed by two 1-way ANOVA assessing effects of harvest year (2015 and 2016) and the type of fermentation (aseptic and cellar) on the volatile composition of the finished wines. The significance is expressed by *: <0.0001-0.001***; 0.001-0.01**; 0.01-0.05*. Post-hoc Duncan test was also used to identify the compounds with significant differences according to the factors presented above and were expressed by the letters a-b, being a the highest average value.

The compounds above limit of quantification and/or significant on the ANOVA results were further analysed by Principal Component Analysis (PCA) and illustrated in box plots to investigate on the differences between wines attending to harvest year, vineyard location and the fermentation methodology (aseptic or in cellars).

The box plots presented in Figures 2-4 show the differences between harvest years for compounds selected by applying a TSNE algorithm (t-Distributed Stochastic Neighbour Embedding) and this work was produced by Kimmo Sirén in scope of his PhD thesis.

The ANOVA and PCA analysis as well as simple box plots were performed using Microsoft Excel 2016 version and XLSTAT (Addinsoft, 2018 version).

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Chapter 4

Sensory and chemosensory characterization of commercial young Riesling wines from different German appellation

Chapter 4 - Sensory and chemosensory characterization of commercial young Riesling wines from different German appellation

1. Results and discussion

Six high quality Riesling categorized as Großes Gewächs wines from several German wine appellations were analysed according to their sensory properties as well as aroma chemical composition. The aim of these work was to compare sensory data with semi-quantitative and chemical quantifications, linking attributes with volatile compounds analysed, attempting to identify aroma vectors that originate perceptual differences between wines.

1.1 Sensory Descriptive Analysis

The six wines were scored according to ten aroma descriptors and five tasting attributes in two replicate Descriptive Analysis (DA) sessions and the results are given in Table 9.

Table 9 Descriptive Analysis results of six German wines from different appellations carried out in two duplicate sessions and using a 10 cm scale rating to score 10 aroma attributes and 5 tactile descriptors.

Wine	Session	Petrol	VA	citrus	peach/ apricot	cooked fruit	passion fruit	floral	honey	caramel	smoky	sweet	sour	body	mouthfeel	bitter
BÜRGERSPITAL	1	4.6	2.5	3.1	2.5	3.4	2.0	2.4	2.3	2.4	3.4	3.5	5.9	6.3	4.5	3.7
	2	3.6	2.2	3.3	2.7	3.2	2.5	2.3	2.2	2.5	4.0	3.5	6.6	4.7	4.5	3.8
CLEMENS-BUSCH	1	2.1	1.2	2.4	3.4	3.5	1.5	2.7	8.1	5.6	2.2	2.6	6.2	5.7	5.0	3.9
	2	2.1	1.8	2.1	3.5	4.1	1.5	3.3	7.7	5.6	2.4	3.0	5.9	4.8	4.6	3.8
FÜRST	1	3.0	4.1	3.9	1.9	2.0	2.4	2.7	2.8	2.7	1.8	1.7	7.6	4.2	5.3	4.0
	2	2.7	4.3	3.2	1.7	1.5	2.3	2.1	2.2	1.9	2.0	2.2	6.6	4.4	5.1	3.9
GUNDERLOCH	1	6.4	1.9	3.6	2.9	3.7	2.1	2.8	2.6	2.7	5.5	2.5	5.5	4.8	5.0	4.3
	2	5.4	2.8	3.4	3.0	3.3	2.3	2.3	2.7	3.1	5.9	2.2	6.9	5.4	5.6	3.9
KNIPSER	1	4.2	2.5	4.3	3.5	2.7	2.5	3.3	2.6	2.1	2.8	3.6	5.8	5.8	3.7	3.6
	2	3.5	2.5	4.0	2.7	3.2	2.6	2.8	2.6	1.7	3.2	3.2	5.6	5.5	4.6	4.1
REBHOLZ	1	2.3	1.2	4.0	5.9	4.1	5.3	4.9	3.1	2.8	1.8	2.9	6.1	5.4	5.0	3.4
	2	1.8	1.0	3.9	5.6	2.9	4.7	5.2	2.6	2.7	1.3	2.5	6.4	5.3	5.2	3.1

The average values obtain for each descriptor were analysed by a three-way ANOVA, as shown in Table 10.

These results show that all aroma attributes and sweetness perception were highly significant to differentiate the wines. Wine effects were significant for most part of the attributes, which confirms that they are useful for characterising the observed sensory differences among this set of samples and confirmed the discrimination ability of the panel. The description of the wines is highly dependent on the judges, but they were consistent between the two replication sessions, hence the interactions between wine and replication are not highly significant for any of the attributes, reflecting the reproductivity of the panel.

Table 10 3-way ANOVA results of the sensory Descriptive Analysis (DA) of 10 aroma descriptors and 5 tasting descriptors. Significance degree is expressed using*: <0.0001-0.001***; 0.001-0.01**; 0.01-0.05*.

		wine		judge		replication		wine*judge		wine*replication		judge*replication	
	degree of freedom	5		14		1		70		5		14	
		F	Pr > F	F	Pr > F	F	Pr > F	F	Pr > F	F	Pr > F	F	Pr > F
Aroma descriptors	TDN	25.05	***	11.30	***	5.696	*	1.32	n.s.	0.46	n.s.	1.32	n.s.
	VA	10.89	***	4.54	***	0.387	n.s.	3.18	***	1.16	n.s.	2.19	**
	citrus	3.95	**	2.73	**	0.534	n.s.	2.33	***	0.32	n.s.	0.52	n.s.
	peach/apricot	15.11	***	2.85	**	0.228	n.s.	4.04	***	0.68	n.s.	2.17	**
	cooked fruit	4.44	**	3.36	***	0.398	n.s.	2.19	***	1.51	n.s.	1.13	n.s.
	passion fruit	13.37	***	1.88	*	0.017	n.s.	3.52	***	0.73	n.s.	1.76	n.s.
	floral	6.90	***	1.58	n.s.	0.122	n.s.	3.71	***	0.91	n.s.	2.38	***
	honey	46.54	***	2.83	**	0.938	n.s.	3.19	***	0.38	n.s.	0.95	n.s.
	caramel	16.60	***	4.79	***	0.265	n.s.	2.54	***	0.66	n.s.	1.46	n.s.
	smoky	22.12	***	4.14	***	0.617	n.s.	3.18	***	0.70	n.s.	0.64	n.s.
Tasting descriptors	sweet	6.06	***	12.34	***	0.003	n.s.	1.73	**	0.98	n.s.	3.44	***
	sour	2.46	*	9.49	***	0.352	n.s.	1.21	n.s.	2.37	*	0.50	n.s.
	body	3.05	*	14.34	***	2.218	n.s.	0.94	n.s.	1.92	n.s.	1.01	n.s.
	mouthfeel	1.57	n.s.[a]	2.08	*	0.309	n.s.	1.69	n.s.	0.66	n.s.	1.11	n.s.
	bitter	0.87	n.s.	17.91	***	0.049	n.s.	0.78	n.s.	0.25	n.s.	0.72	n.s.

Sensory scores, ANOVA significance scores and post-hoc results are summarized in the spider chart of Figure 39 and the wine segregation is further explained by PCA in Figure 40, where 59,51% of variance is shown. These figures show that two wines clearly stand out as the most different: Rebholz, described as the most floral, with peach/apricot and tropical fruits attributes and

Clemens-busch described with caramel, honey and cooked fruit attributes. The remaining wines were clustered together, however there were still little differences in their descriptions: Gunderloch had highest TDN and smoky notes, also observed in Bürgerspital and Knipser, but with lower intensity. Fürst was standing out for the presence of VA (volatile acidity) notes, high sourness and weak body.

Several tasting attributes, given to each wine by sensory analysis, seem to correlate with the corresponding vineyard soil type: Fürst was described as significantly sourer than the remaining wines and the grapes were grown in low pH sandstone soils. The remaining vineyards have different soil types, but with medium-high pH and thus, not contributing to sourness differences. Differences in sweetness perceptions can also be attributed to the vineyard soil type (all wines were dry): low pH sandstone soils are correlated with low sweetness (Fürst), reddish and grey slate with medium pH related to intermedium sweetness (Gunderloch, Rebholz and Clemens-bush) and limestone and shell limestone with high pH seem to be related to sweeter wines (Knipser and Bürgerspital). Soil type has not been directly linked with wine overall organoleptic properties, but has been related to the wine perception of freshness, acidity and to the wine structure (van Leeuwen, 2010).

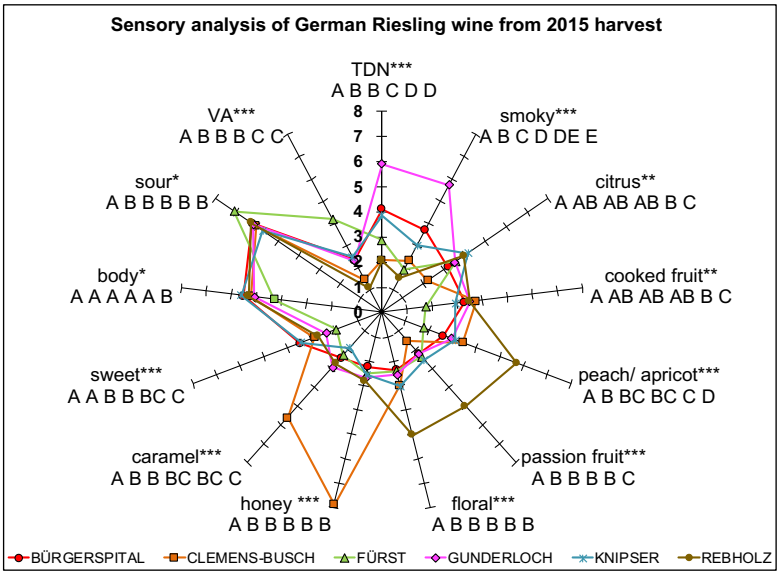

Figure 39 ***QDA results of six Riesling wines from 2015 harvest*** *- Each descriptor significance obtain with a 3-way ANOVA is given by *: <0.0001-0.001***; 0.001-0.01**; 0.01-0.05*, and the letters A-E are the results obtain by Fischer (LSD) pair-test.*

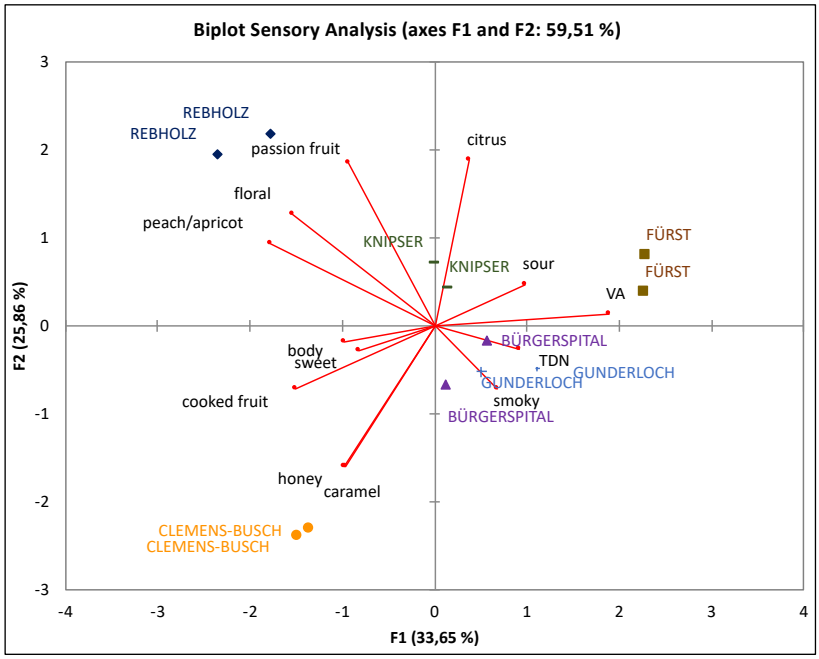

Figure 40 ***PCA obtained with sensory descriptive analysis*** *of six Riesling wines in two replicate sessions. The panel was comprised of 15 judges and before scoring each aroma attribute, a reference standard was provided.*

1.2 Semiquantitative Gas Chromatography-Olfactometry and GC quantitative analysis

To better understand the aroma compounds putatively responsible for the sensory attributes, which better describe each wine, two different approaches were followed. Firstly, a semi-quantitative odour-screening study and secondly, a GC quantitative analysis were performed.

The semiquantitative odour screening analysis was performed by means of a Gas-Chromatography Olfactometry analysis (GC-O) on extracts obtained by dynamic head space. The extracts obtained with this methodology are very clean and resemble, as much as possible, the volatile fractions reaching olfactory receptors during wine consumption. The aroma compounds potentially more relevant for explaining the sensory differences observed between the wines, were selected considering the scoring differences given by the MF%. As can be seen in the Table 11, 22 compounds with a MF% higher than 30 were identified. Only MF% above 30 are selected to ensure that at least more than three sniffers detect the aroma, discarding noise results.

Out of the 22 compounds, three could not be identified using this methodology. None of them, however, reached GC-O scores above 50%, and in fact only 1 of them reaches scores above 40% in some samples. The olfactometric list let us to state that all potentially relevant aroma compounds, except maybe one (n.i.1013) of this group of wines, are well-known odorants. It is most remarkable the absence of some aroma volatiles such as polyfunctional mercaptans, which indicates that these Riesling wines are “neutral” from the point of view of their content in these powerful aroma compounds.

Table 11 Odorants detected in Großes Gewächs Riesling wines (2015) from several German wine appellations by GC-O: gas chromatography retention index (LRI – linear retention index), olfactometry descriptors, chemical identity and modified frequency (MF%). Each compound was characterized by the average value found in all the wines, maximum (max) and minimum (min) values and slope between max and min. The table is sorted according to the slope between max and min MF% value observed.

LRI DB-5	LRI DB-WAX	Odour descriptor	Compound Identification	MF%						Max	Min	max-min
				Bürgerspital	Fürst	Gunderloch	Knipser	Rebholz	Clemens-Busch			
1344	1826	chlorine. humidity	geosmine	0	68	0	0	0	0	68	0	**68**
1122	1931	floral. red fruits	β -phenylethanol	17	30	39	38	41	76	76	17	**59**
1156	1515	paper. cucumber. oil	Z-2-nonenal	45	55	52	48	47	0	55	0	**55**
849	1056	ripe fruits. sweet. fresh	ethyl 2-methylbutyrate	57	63	33	43	43	81	81	33	**48**
825	1644	vomit. sweat. rancid	butyric acid	19	37	0	0	48	22	48	0	**48**
999	1243	melon. sweet. medicinal	ethyl hexanoate	85	83	79	83	79	41	85	41	**44**
879	1128	banana. candy	isoamyl acetate	71	51	53	55	87	68	87	51	**36**
	1745	plastic. spicy. acetone	n.i 1745[a]	0	35	36	36	0	26	36	0	**36**
833	1687	cheese. unpleasant	3-methylbutyric acid	47	54	33	53	65	63	65	33	**32**
1096	2100	leather. animal. toasted	m-cresol	36	50	18	31	18	22	50	18	**32**
1137	2086	toasted bread. curdled milk	homofuraneol	0	31	0	24	0	30	31	0	**31**
1391	1838	cooked fruit. sweet	β-damascenone	48	53	54	38	65	68	68	38	**30**
	2050	spicy. floral. honey	n.i 2050 [a]	0	0	0	30	19	0	30	0	**30**
	1013	solvent. green. medicinal	n.i 1013 [a]	36	36	17	41	46	26	46	17	**29**
<740	975	strawberry. cream. candy	isopropyl acetate	85	87	61	87	83	85	87	61	**26**
803	1038	strawberry. cream. red fruits	ethyl butyrate	75	76	57	71	83	76	83	57	**26**
1103	1562	floral. citric. fresh	linalool	31	33	29	45	39	20	45	20	**25**
854	1071	black fruits. medicinal. citric	ethyl 3-methylbutyrate	81	85	72	78	82	62	85	62	**23**
<700	1105	toasted. bitter. cheese	isobutanol	31	45	36	22	25	24	45	22	**23**
1198	1445	vegetables. wet soil. grape	ethyl octanoate	49	54	40	63	42	43	63	40	**23**
<740	1465	Vinegar	acetic acid	64	56	61	49	45	49	64	45	**19**
<740	1220	bitter. cheese. rancid	isoamyl alcohol	87	98	92	96	90	94	98	87	**11**

[a] *n.i – compound not identified*

Previous works have described polyfunctional mercaptans, specially 3-mercaptohexanol as relevant in the description of Riesling varietal character (Schüttler et al., 2015; Tominaga et al., 2000). However, these data suggest that these are not impact compounds and that further investigation may be required to confirm the real role of these powerful odorants.

The 7 most intense odorants found in the experiment, all of them at levels close to saturation ($MF\%_{max}$ >80) are all fermentative compounds: isoamyl alcohol, β-phenylethanol and several esters and acetates such as isopropyl acetate, isoamyl acetate, ethyl hexanoate, ethyl butyrate and ethyl 2-methylbutyrate. Out of these, the potentially most discriminant (based on ration max-min) compounds seem to be ethyl 2-methylbutyrate, ethyl hexanoate and isoamyl acetate. β-phenylethanol, described with pleasant flowery descriptors, seems to be the only higher alcohol with important discriminant potential.

Concerning grape derived aroma, only β-damascenone and linalool seem to be relevant, attending to the olfactometric scores.

Geosmine is the compound potentially most discriminant in the list. Its presence is limited to one sample and could be linked to fungal contamination in the grapes, even in the cellar or a bottle derived effect.

Similarly, to the sensory data, also the FM% obtained by GC-O were plotted by PCA and are shown in Figure 41, explaining 56.70% of variance. On the left side of F1 are the wines with higher level of acetate esters and other compounds described as candy and sweet aromas as well as the varietal compound β-damascenone. On the right side of F1 are the wines with higher content of fusel alcohols, fresh fruity esters and the varietal compound linalool. Overall, the same figure shows that the wines are somewhat different, even though not too many varietal compounds were identified.

The volatile composition of the wines was also quantified and normalized by means of their OAV (Odour Active Value). Only those compounds with OAV above 1 were selected and plotted in the PCA given in Figure 42. This figure explains 67.67 % of variance and illustrates that attending to their chemical composition, the wines were segregated into three groups.

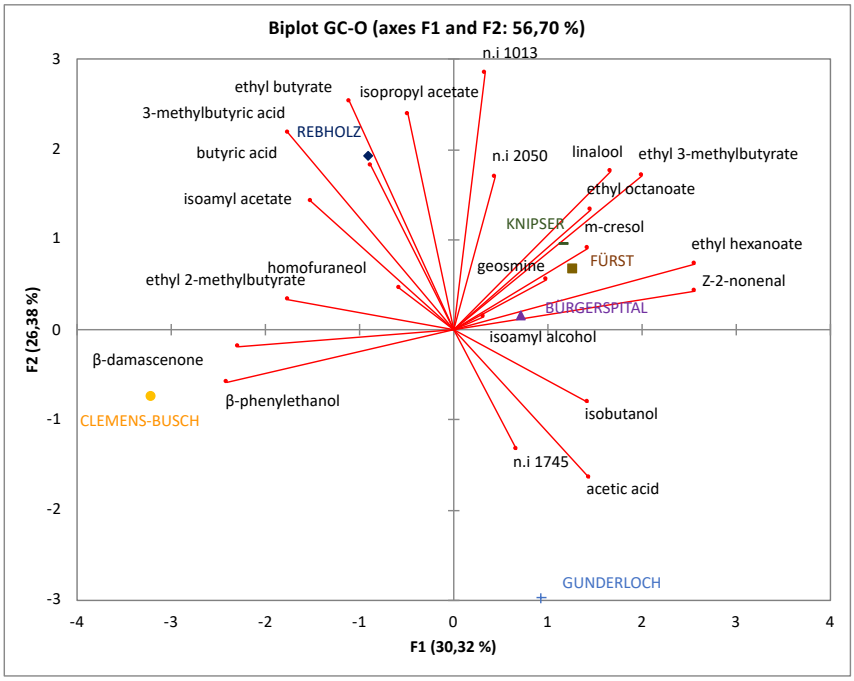

Figure 41 **PCA of *GC-O scoring results*** obtained with MF% of six Riesling sniffed by a trained panel comprised by 6 judges.

Clemens-busch stands out individually on the bottom-right side, having high level of ethyl esters (linear and branched esters), acids as well as ethyl dihydrocinnamate, γ-decalactone and β-phenylethanol. Rebholz and Bürgerspital stand on the upper-right side having also high level of linear ethyl esters, acetate esters, acids and E-isoeugenol.

On the left side of F1 are clustered together Knipser, Fürst and Gunderloch showing high content of ethyl acetate, isoamyl alcohol, ethyl isovalerate and ethyl cinnamate. Besides, these wines have the highest content of varietal compounds linalool, 4-vinylguaiacol, guaiacol and β-damascenone.

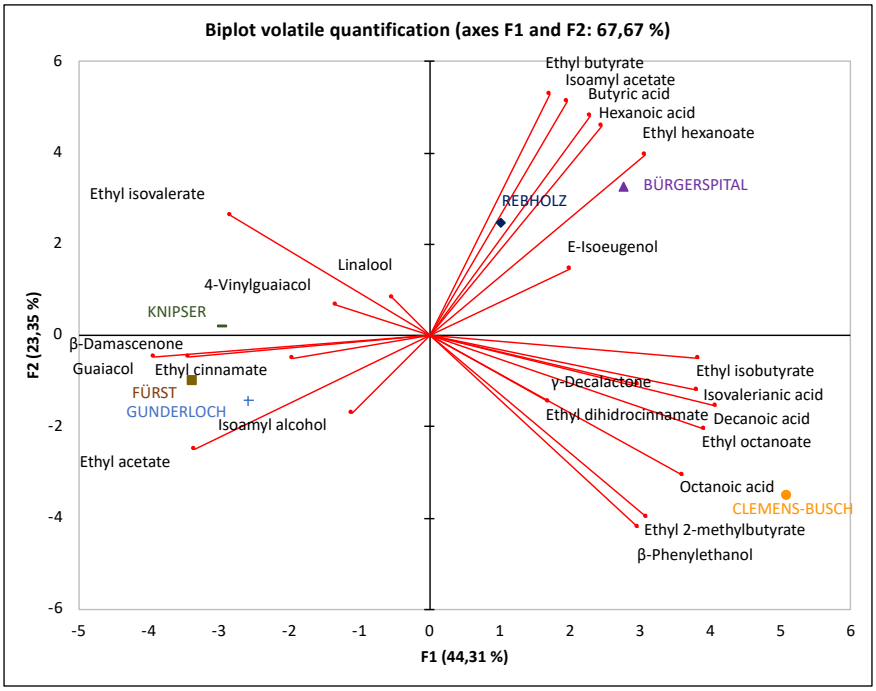

Figure 42 ***Volatile quantification*** *results obtained by GC-MS and GC-FID and normalized by OAV (Odour Active Value). Only compounds with OAV > 1 were selected.*

1.3 Integration of sensory, semiquantitative and quantitative data

Most remarkably, there is a high coherence between sensory and olfactometric data, as can be seen in Figures 40 and 41. Wine distribution attending to these two data sets are highly coincident and thus, a comparative analysis can be done with the aim to suggest which odorants detected by GC-O are potentially linked to the different sensory attributes. The direct comparison of both plots, suggests that the most different sensory character of Clemens-busch and Rebholz wines, which in Figure 40, are the most dissimilar samples, occupying empty spaces in first and third quadrants of the plane, is preserved in the GC-O study, in where

they also occupy quite specific positions in the first and third quadrants of the plane.

Both plots suggest that the odorants β-damascenone, ethyl 2-methylbutyrate and β-phenylethanol found by GC-O at highest levels in Clemens-busch, would be responsible for the sensory attributes cooked fruit, honey and caramel and may also have some relationship to the overall sweetness. All these observations are consistent with their individual descriptors and with the demonstrated implication of β-damascenone in the dry fruit descriptor (Ferreira and San Juan, 2012).

Similarly, the comparison between the GC-O and sensory PCA planes suggests that the specific floral, peach apricot and passion fruit notes observed in Rebholz wine are related to its major contents in isoamyl and isopropyl acetate, in ethylbutyrate and in 3-methylbutyric and butyric acids. The spatial relationship between Fürst and Rebholz wines with citrus notes on both planes, would likewise suggest that the citrus note is related mainly to linalool, one of whose descriptors is, in fact, orange blossom.

Finally, Figures 40 and 41 further show that the most obvious disagreement between sensory and GC-O is the most southern position of Gunderloch wines in the GC-O space. However, this is just a limitation of both plots, since in the following dimension, the Gunderloch wine is acknowledged as the most different because of its TDN and smoky character. Attending to GC-O data, the unknown compound n.i 1745 could be involved in those notes.

On the contrary, the spatial relationships shown in Figure 42, derived from quantitative data, reveal a completely different hierarchy to those observed in Figures 40 and 41. This suggests that the relationships between quantitative GC data, GC-O data and sensory data are very weak.

There are several reasons behind this apparently disappointing result, and present data do not allow to assess which one is going to have a higher weight.

A first reason is that the numbers and identities of compounds in the GC-O and GC-quantitative plots are different. For instance, only three acids were quantified by GC-O (isovaleric, butyric and acetic), while in Figure 42 there are data for octanoic, decanoic and hexanoic acids and there is missing data for acetic acid. Similarly, ethyl octanoate, decanoate, cinnamate and dihydrocinnamate, present in Figure 42, were detected by GC-O and thus, not included in Figure 41. Volatile phenols such as E-isoeugenol, guaiacol and 4-vinylguaiacol, which in fact could be related to the smoky descriptor of Bürgerspital and Gunderloch wines, were not identified by GC-O and so, are also present in Figure 42, but not in Figure 41.

A second reason is that there is evidence that quantitative data of some compounds suffering relatively specific interactions with the matrix, may not be present at enough concentration to explain their sensory notes, since volatility may depend on matrix composition (Ferreira and Cacho, 2009). However, these matrix factors should not affect the GC-O data, since the extract was obtained using a dynamic headspace sampling strategy in which, the most retained compounds are released to the headspace with more difficulty.

Finally, a third reason is that the relationships between sensory (and olfactometric) data and quantitative data are not linear, but sigmoid, and hence, depending on the concentration range, a relevant content change can have a quite limited effect on sensory properties, while in some other cases, the relationship can be far more steeped.

Anyhow, our results suggest that relating GC-quantitative data to sensory data is more difficult than relating GC-O data to sensory, even if GC-O data has a higher degree of imprecision.

Furthermore, these results clearly indicate that the sensory properties of these high-quality young Riesling wines are related to the existence of a delicate and complex balance between a pool of fermentative compounds and a limited group of compounds with varietal origin, such as β-damascenone or linalool. It seems to be also evident, that the powerful polyfunctional mercaptans 3-mercaptohexanol 4-methyl-4-mercaptopentan-2-one or to 3-mercaptohexyl acetate are no key aroma compounds in Riesling wines. Nevertheless, it can be hypothesized that at the very small concentrations at which they are usually found, they may be related to wine freshness, as it was demonstrated for Maccabeo wines (Escudero et al., 2004).

Considering the data overview, it seems that the different wine appellations highly contribute to wine character. In fact, wines from Pfalz (Rebholz and Knipser) seem to have significant different properties, which could be expected due to the different vineyard properties. Nonetheless, Rebholdz from Pfalz and Clemens-busch from Mosel stand greatly from the remaining sites. The wines from the same Franken wine region (different vineyard properties), Fürst and Bürgerspital, are closely related on sensory, but data is not that consistent attending to their chemical composition. Franken and Rheinhessen (Gunderloch) regions have closely related wines compared to Pfalz and Mosel.

2. Conclusions

The analysis of 6 wine from different German appellations according to their sensory and chemosensory properties has suggested that the wine region highly contributes to the character of the wines.

Establish a relationship between the different data is not straightforward but, nonetheless, GC-O data and sensory seem to be easier to correlate than GC-O with quantitative data, even though GC-O data has a higher degree of imprecision.

Vineyard characteristics, specially soil type, seem to be reflected on the sensory properties of the wines, with special regard to tactile sensations.

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Chapter 5

Impact of vineyard versus cellar microbiota from different harvests on the distinction of different Riesling vineyard

Chapter 5 - Impact of vineyard versus cellar microbiota from different harvests on the distinction of different Riesling vineyards

1. Results and discussion

1.1 Importance of harvest year effect and fermentation location

Compounds with significance determined by 1-way ANOVAs assessing the factors harvest year or fermentation location were further analysed by PCA and results are shown in Figure 43. The sample scores plot of this figure confirms that harvest year is the main source of variance among the wines, since all wines from harvest 2016 are represented on the left side of F1 and the wines from harvest 2015 are represented on the right side of this axis. The variables plot of Figure 43 suggests that the year factor contributes for large changes in chemical composition of wine and it seems that esters, aldehydes, volatile phenols and thiols are examples of aroma families highly affected for this vintage effect.

The differences linked to the vintage effect can be clearly seen in the box plots given in Figures 44 to 46.

Both the variables plot in Figure 43 and the box plots if Figure 44 show that wines from 2015 harvest have higher overall levels of ethyl esters as well of as β-damascenone and of 2-methyl-3-furanthiol. On the contrary, wines from 2016 harvest show higher levels of fusel alcohols, acetate esters, volatile phenols, cysteinylated derivate thiols and aldehydes, as shown in Figures 43, 45 and 46.

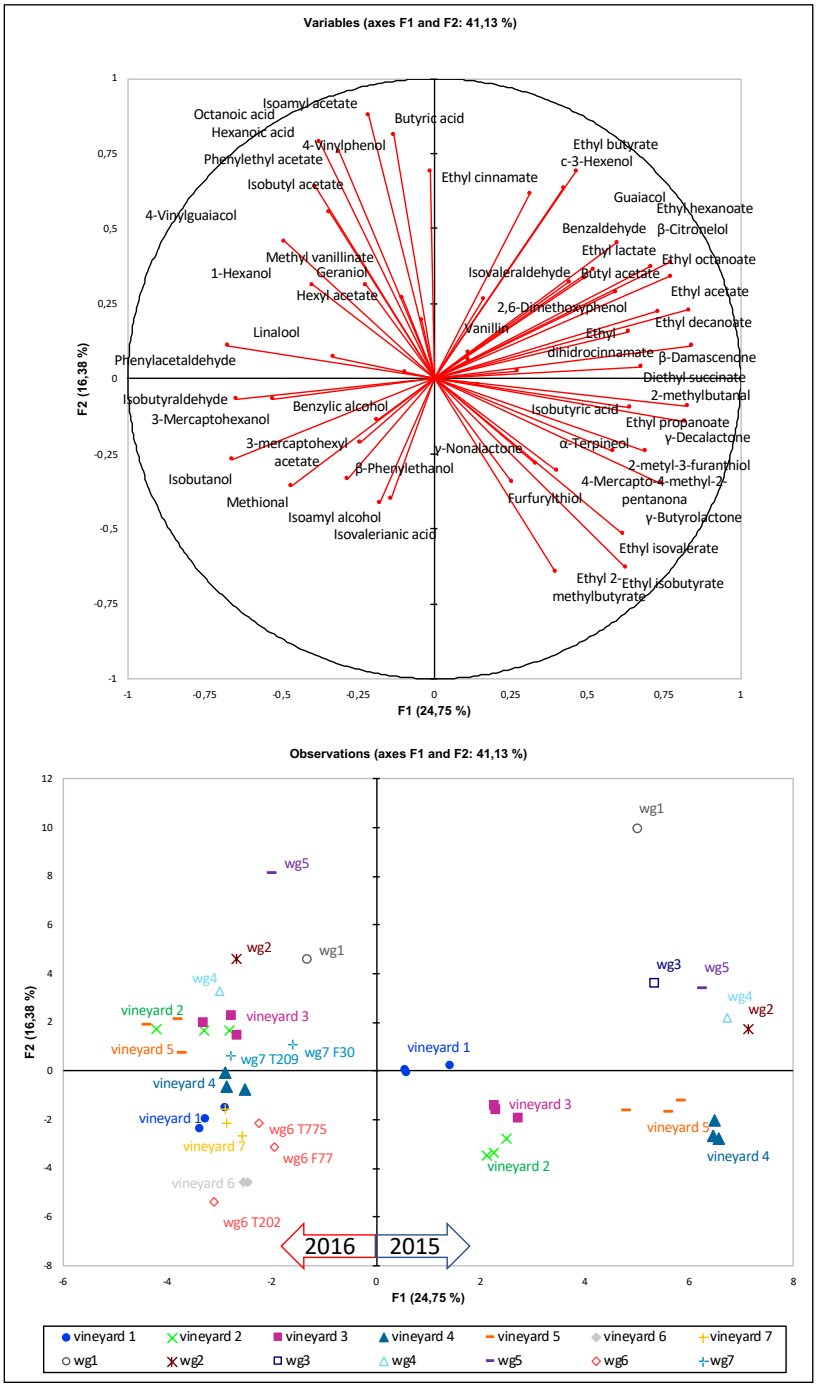

Figure 43 ***PCA analysis of Riesling wines fermented aseptically (retaining only microbiota from the vineyard) or in the cellar (microbiota from the cellar) from 7 different vineyards in harvests 2015 and 2016*** *–41.13% of initial variance. Wines fermented aseptically are expressed by vineyard name and wines fermented in cellar are expressed as wg and the number of the vineyard they were harvested from. The analysis was performed with the compounds which varied significantly between vintages (2015 and 2016).*

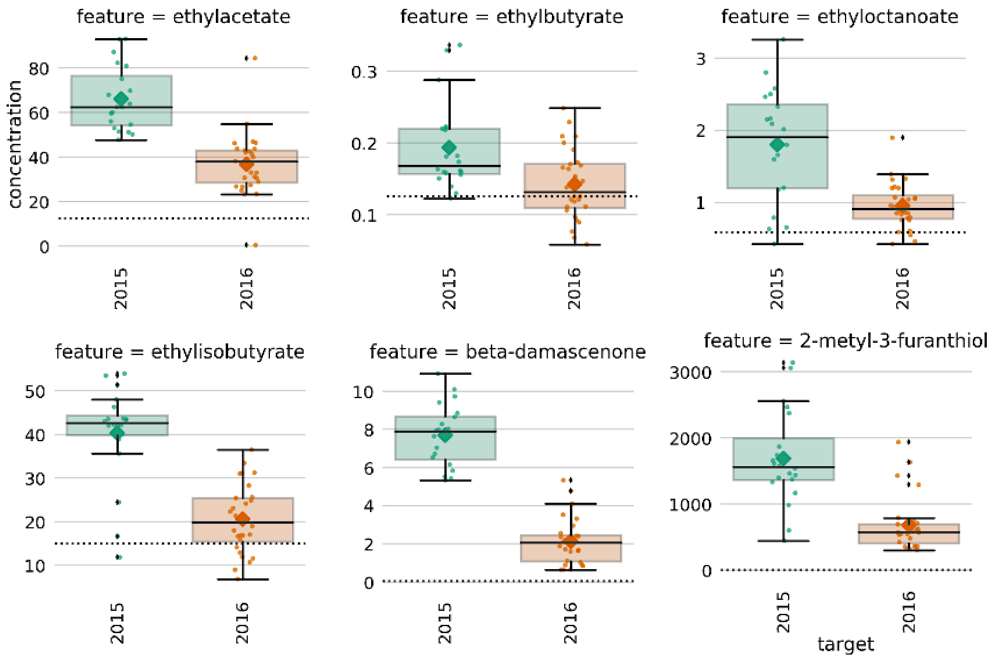

Figure 44 ***Box plots of compounds found at significantly higher levels in 2015 harvest.*** *The dot line indicates the odour thresholds of these compounds. (Figures produced by Kimmo Sirén)*

The fact that the contents of medium chain fatty acid ethyl esters are significantly higher in 2015 wines, while wines from 2016 have higher levels of the corresponding acids, reveals that the esterification rates have changed completely between both years, being much higher in 2015 than in 2016.

On the contrary, wines from 2016 were richer in fusel alcohols.

The formation of branched and fatty acids as well as their esters depends on the grape amino acid pool, which could explain the differences of these odorants between two consecutive harvests. Furthermore, and based on results from Section II, the formation of these compounds is highly strain dependent, which could give some indication that the microflora from each vineyard could also have changed between years.

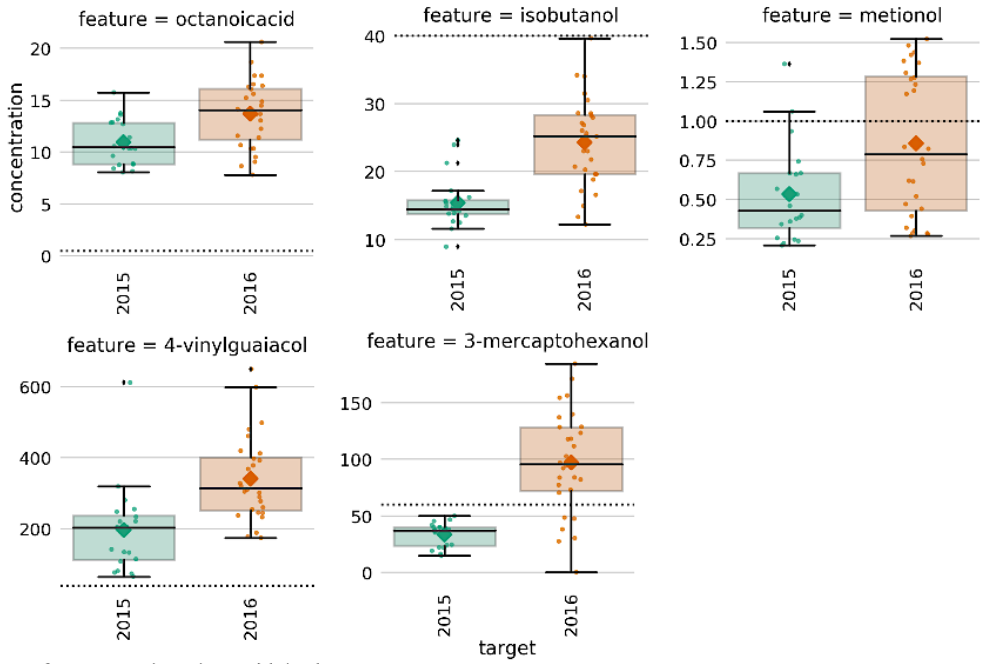

Figure 45 ***Box plots of compounds found at significantly lower levels in 2015 harvest****. The dot line indicates de odour threshold of these compounds. (Figures produced by Kimmo Sirén)*

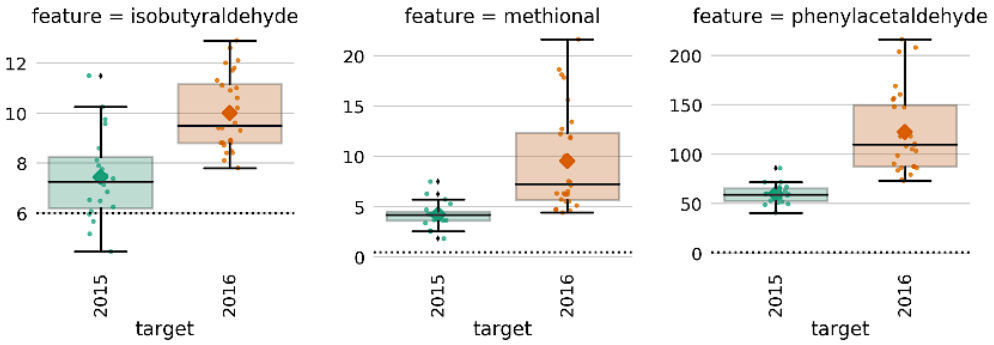

Figure 46 ***Box plots of some Strecker aldehydes in wines from 2015 and 2016 harvest.*** *The dot line indicates de odour threshold of these compounds. (Figures produced by Kimmo Sirén)*

The levels of some Strecker aldehydes shown in Figure 46 are also higher in wines from harvest 2016. As described in Section I, aldehydes were demonstrated to be formed during alcoholic fermentation by yeast. Such

formation was found to be related to the final levels of total SO_2 found in the fermenting media, which includes the SO_2 directly added to the must before fermentation, and the SO_2 formed by yeast during fermentation from different sources of sulphur, such as elemental sulphur or sulphates. The vintage factor could therefore, be related to a specifically different sulphur composition of the musts or also, to the different microbiota.

Regarding varietal aroma compounds, the plots in Figure 43 show that linalool and geraniol are present at higher levels in wines from 2016 harvest, while wines from 2015 were richer in α-terpineol. 4-vinylguaicol and 4-vinylphenol are present at higher levels in wines from harvest 2016. All these groups of varietal aroma compounds are derived from grape glycosidic precursors ant thus, their levels in finished wine are related to grape content in precursors, but as demonstrated in previous sections, the mechanisms leading to the release/formation of the odorants are also highly influenced by wine pH (acid hydrolysis processes) and by yeast enzymatic activity.

It can also be appreciated in Figures 43, 45 and 47 that 3-mercaptohexyl acetate and 3-mercaptohexanol were formed in much higher extent in wines from harvest 2016. The content of these powerful odorants is related to the grape composition in cysteinylated and glutathionylated precursors, whose contents depend on several factors related to climate, vine management and winemaking practices (Capone and Jeffery, 2011; Parker et al., 2017; Peyrot des Gachons et al., 2000, 2002). Additionally, several studies have suggested that yeast β-lyase activity can be further determinant for thiol release from their precursors (Tominaga et al., 1998). From results of Figure 47, either by grape composition changes or by different yeast activity, 2016 harvest had significantly higher potential for the formation the varietal thiols 3-mercaptohexanol and 3-mercatohexyl acetate. In fact and surprisingly, the levels of 3-mercapthexyl

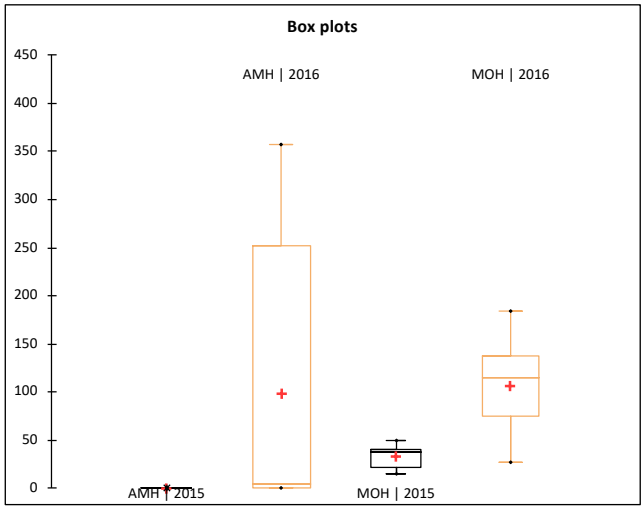

Figure 47 ***Varietal thiols in Riesling wine from 2015 and 2016 harvests*** *- content of varietal thiols 3-mercaptohexyl acetate (AMH) and 3-mercaptohexanol (MOH) in Riesling wines from two consecutive harvest years. Results are given in ng/L.*

acetate reached in a particular samples values significantly higher than many of those previously described in literature (Ribéreau-Gayon et al., 2006; Tominaga et al., 2000).

Overall, 2015 harvest seems to have higher volatile diversity however, harvest 2016 has higher levels of important and highly odoriferous volatiles such as monoterpenes, volatile phenols and thiols.

1.2 Vineyard versus winery

Results from Figure 43 also represent differences caused in wines fermented in cellar environment. The wines fermented in cellar were harvested at the same time and conditions as those fermented in aseptic environments, trying to preserve the microbial diversity of the vineyard. In 2015 harvest the segregation between aseptic and commercial cellar fermentations is higher than in 2016. In fact, in 2015 wines made in commercial wineries are completely separated than those made in the pilot cellar under aseptic conditions. It should be also observed

that both commercial and pilot fermentations from the same vineyard follow the same order, which also demonstrates that there is a clear vineyard effect. In 2016, there is a similar segregation between commercial and pilot samples from those vineyards when compared to those sampled in 2015, but commercial samples from vineyards 6 and 7, which were not included in 2015 trial, are very close to pilot wines. Moreover, as indicated by the wine name, grapes from vineyards 6 and 7 were fermented in three and two different reservoirs, respectively, which are closely related.

Overall, volatile content was significantly higher in wines fermented in commercial cellar, suggesting that the standard winemaking practices used in commercial cellars, and most importantly, cellar microbiota, have a major role on wine aroma formation and modulation (Supplementary data). Volatiles significantly different between commercial and pilot cellar are isoamyl acetate, c-3-hexanol, benzyl alcohol, ethyl lactate, butyric acid, isobutyric acid and isovaleraldehyde from the group of fermentative compounds and linalool, geraniol, guaiacol, 4-vinylguaiacol and 4-vinylphenol from the group of varietal compounds, as can be seen in Figures 48 and 49 for harvest 2016 and Figures 50 and 51 for 2015 harvest. It should be observed that in some cases there is not a clear correlation between the contents of compounds in wines, made using aseptic conditions or in the commercial cellar, from the same vineyard.

The pattern of differences between aseptic and cellar fermentation in 2016 harvest was completely different, reiterating the significance of between-vintage factors. Furthermore, regarding fermentative compounds, differences among vineyards (reflected in aseptic conditions) are, likewise, not comparable with 2016 wines supporting the hypothesis of microflora changes from harvest to harvest in vineyards. Nonetheless, comparing Figures 48 to 51, some similar patterns between harvests are observed for the varietal compounds represented and thus, further highlighting the importance of grape precursors (specially

glycosidic precursors) not only in the formation of varietal character, but also and most importantly in the construction of specific *terroir* nuances in wine.

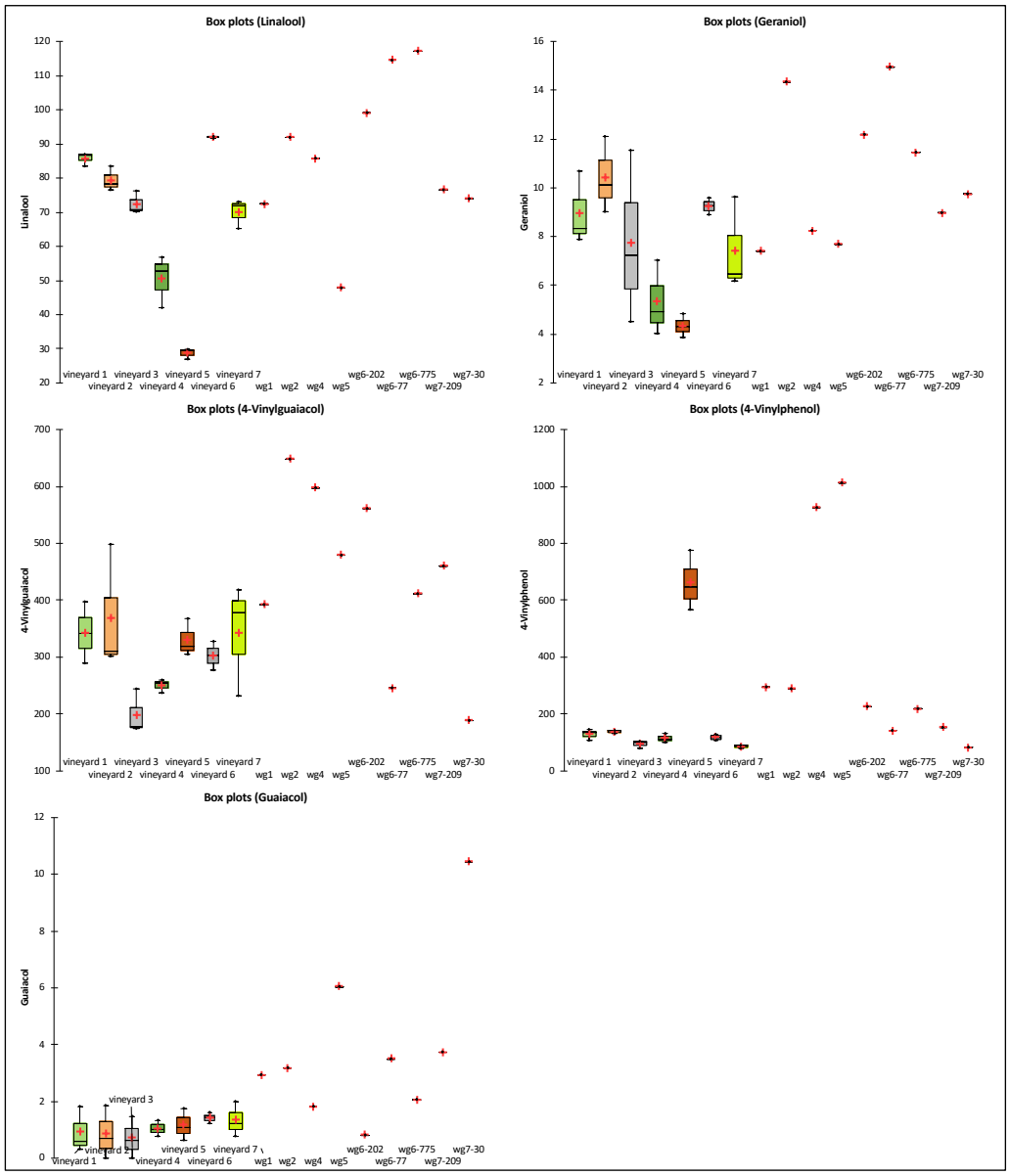

Figure 48 ***Varietal aroma compounds in wines fermented aseptically and in the cellar from grapes from 5 different vineyards in 2016 harvest*** *– Wines fermented from grapes handpicked aseptically from 7 different Pfalz regions and fermented in aseptic condition (vineyards 1-7) or in cellar (wg 1-7). Results are expressed in µg/L.*

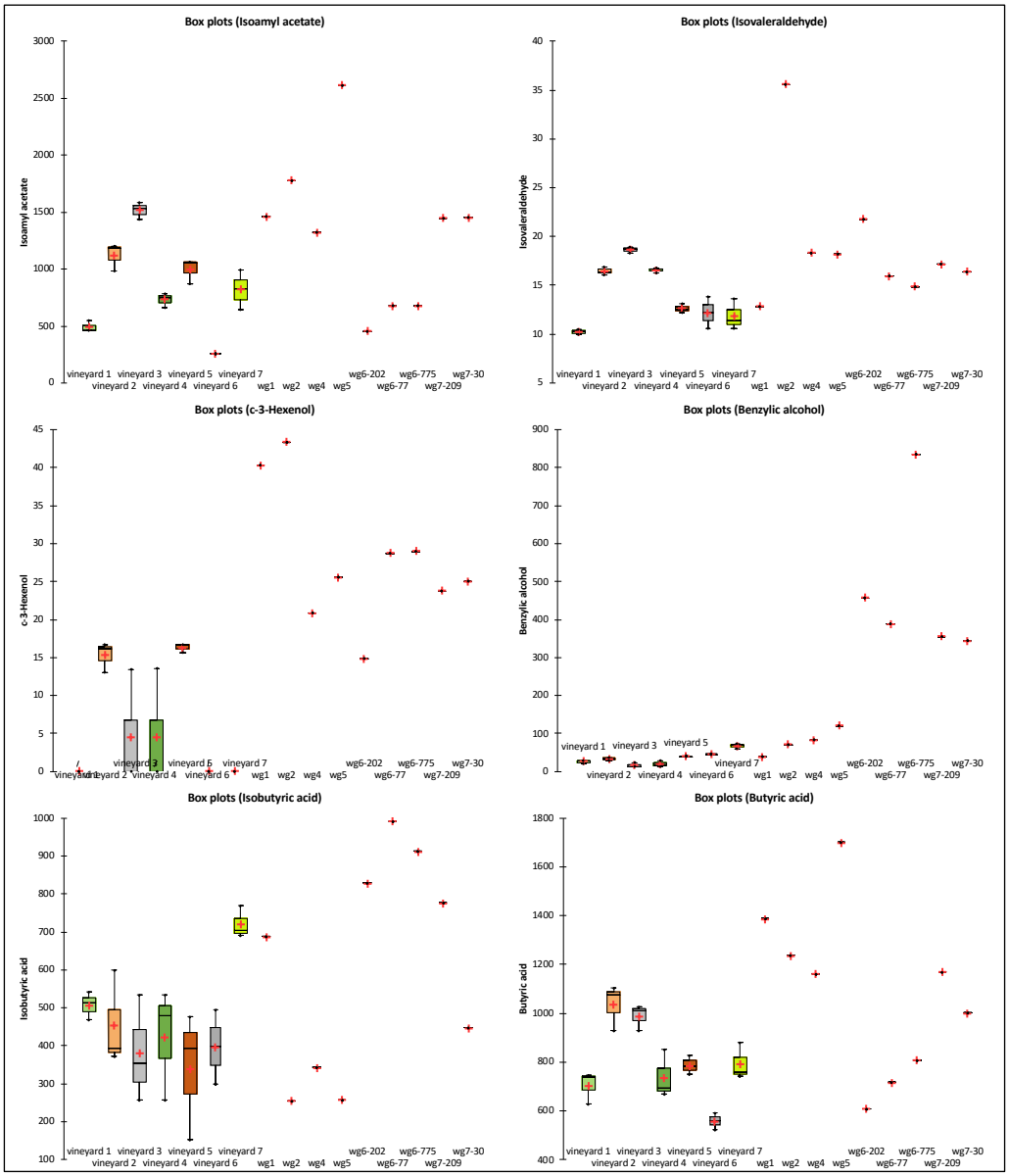

Figure 49 ***Fermentative aroma compounds in wines fermented aseptically and in the cellar from grapes from 5 different vineyards in 2016 harvest*** *– Wines fermented from grapes handpicked aseptically from 5 different Pfalz regions and fermented in aseptic condition (vineyards 1-7) or in cellar (wg 1-7). Results are expressed in µg/L.*

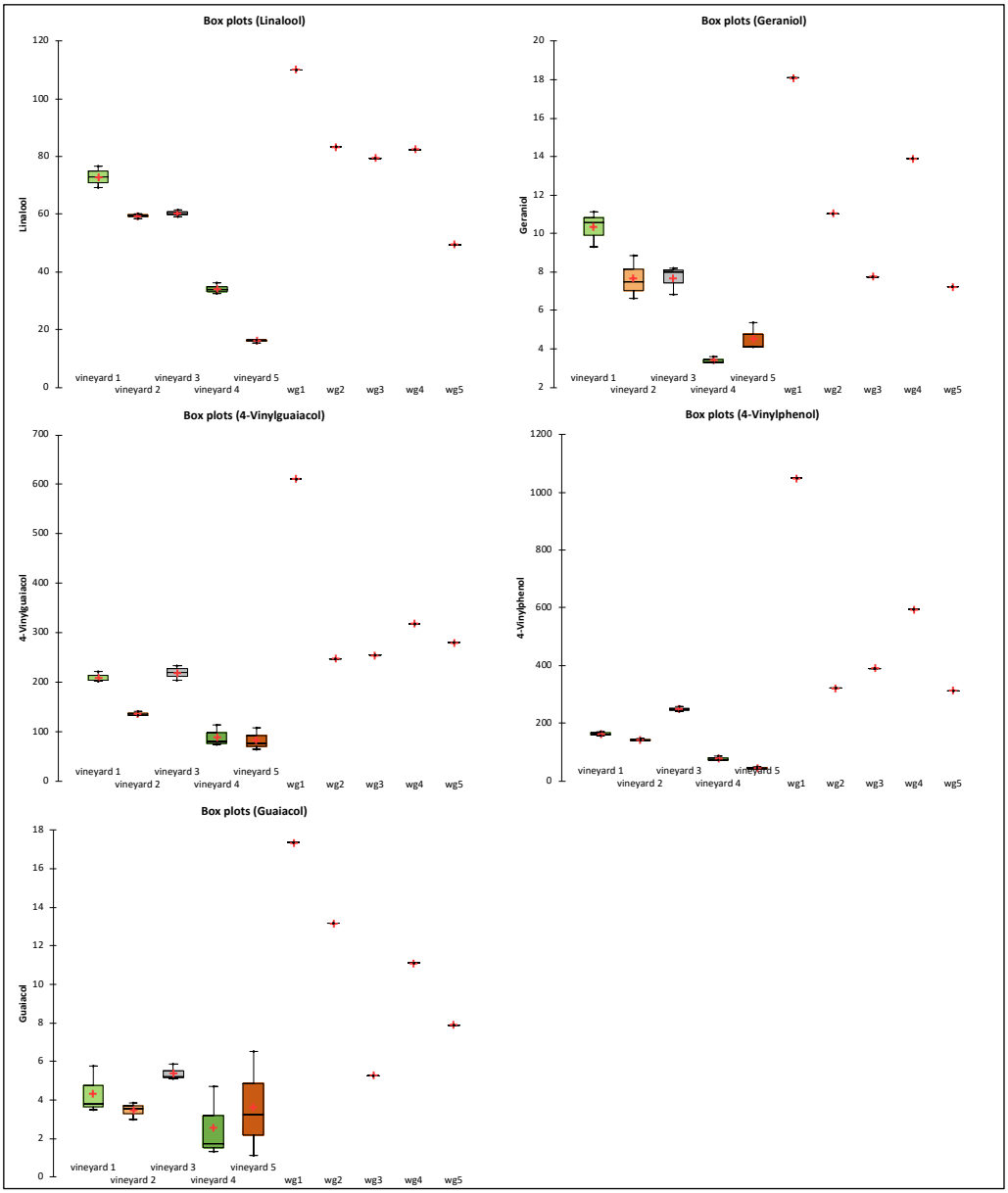

Figure 50 ***Varietal compounds in wines fermented aseptically and in the cellar from grapes from 5 different vineyards in 2015 harvest*** *– Wines fermented from grapes handpicked aseptically from 5 different Pfalz regions and fermented in aseptic condition (vineyard 1-5) or in cellar (wg 1-5). Results are expressed in µg/L.*

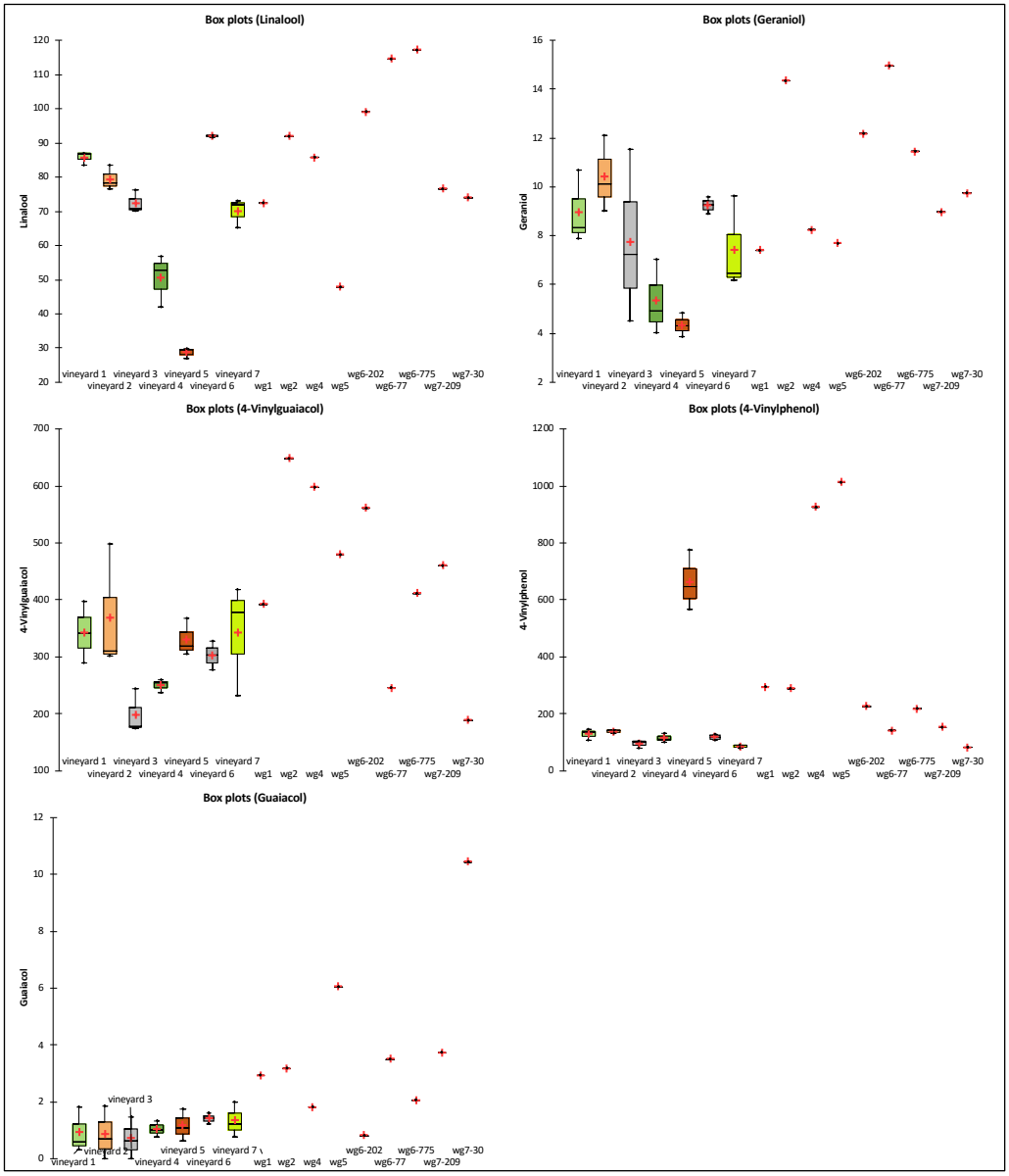

Figure 51 ***Varietal compounds in wines fermented aseptically and in the cellar from grapes from 5 different vineyards in 2016 harvest*** *– Wines fermented from grapes handpicked aseptically from 5 different Pfalz regions and fermented in aseptic condition (vineyard 1-5) or in cellar (wg 1-5). Results are expressed in µg/L.*

2. Conclusions

Wines made from the same grape variety and vineyard in two consecutive harvests show a completely different aroma composition. These differences are the likely result of specific climate conditions of each year, which closely relate to the vine management, including the degree of maturity of the grapes achieved in each year and also, the little changes in the winemaking practices introduce as a response to the specific conditions at which the grapes arrive to the cellar each harvest. These differences should translate into both changes in vineyard microflora and changes in grape berry composition, both of which can be potentially important aroma modulators.

Although in this specific work, specifically related to the preliminary study of volatile compounds, it is not possible to make a definitive assessment of whether the observed changes are due to chemical differences or to differences in the microbiota, the study shows a relevant influence of the *terroir* within each vintage.

Fermentative compounds such as esters highly reflect the harvest effect, whereas varietal compounds formed from grape glycosidic precursors show similar formation patterns in wines from 2015 and 216 harvest. On the contrary, polyfunctional mercaptans seem to be highly affected and their formation changes significantly from harvest to harvest.

Grapes picked from the same vineyards and fermented under aseptic conditions or in commercial cellar environment originate different wines in which cellar microflora should have a major effect.

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Supplementary data

1. Odour thresholds

S.d. Table 1 Odour threshold of all the compounds quantified organized by aroma family

Aroma family	Compounds	Odor Threshold (µg/L)	Odor Threshold (mg/L)	Reference
Aldehydes	Isobutyraldehyde	6		Culleré et al. 2007
	2-methylbutanal	16		Culleré et al. 2007
	Isovaleraldehyde	4.6		Culleré et al. 2007
	methional	0.5		Escudero et al. 2000
	Phenylacetaldehyde	1		Culleré et al. 2007
	Diacetyl	100		Peinado et al. 2004
CARBONYL COMPOUNDS	Acetaldehyde		0.5	Guth 1997
	Acetoine		150	Ferreira et al. 2000
ACETATES	Ethyl acetate		7.5	Maarse and Visscher 1989
	Isoamyl acetate		0.03	Guth 1997
	Hexyl acetate		1.5	Etievant, 1991
ACETATES	Isobutyl acetate		1.6	Ferreira et al. 2002
	Butyl acetate		1.8	Etievant, 1991
	Phenylethyl acetate	250		Ferreira et al. 2000
LINEAL ETHYL ESTERS	Ethyl propanoate		5.5	San Juan et al. 2012
	Ethyl butyrate		0.125	Ferreira et al. 1995; Guth 1997; Ferreira et al. 2000
	Ethyl hexanoate		0.062	Baumes et al. 1986; Ferreira et al. 2000
	Ethyl octanoate		0.58	Ferreira et al. 1995; Guth 1997; Ferreira et al. 2000
	Ethyl decanoate		0.2	Ferreira et al. 2000
BRANCHED ETHYL ESTERS	Ethyl isobutyrate	15		Guth 1997; Ferreira et al. 2000
	Ethyl 2-methylbutyrate	18		Guth 1997; Ferreira et al. 2000
	Ethyl isovalerate	3		Guth 1997; Ferreira et al. 2000
ALCOHOLS	Isobutanol		40	Guth 1997; Ferreira et al. 2000
	1-Butanol		150	Etievant, 1991
	Isoamyl alcohol		30	Baumes et al. 1986; Ferreira et al. 2000
	1-Hexanol		8	Baumes et al. 1986; Ferreira et al. 2000
	c-3-Hexenol		0.4	Guth 1997
	Metionol		0.5	Baumes et al. 1986; Ferreira et al. 2000
	Benzylic alcohol		200	Escudero et al. 2007
	β-Phenylethanol		14	Guth 1997; Ferreira et al. 2000
MISCELANEOUS ESTERS	Ethyl lactate		154	Simpson and Miller 1984
	Diethyl succinate		200	Etievant, 1991
ACIDS	Acetic acid		300	Ferreira et al. 2002
	Butyric acid	1,73		Ferreira et al. 2000
	Isobutyric acid		2.3	Guth 1997; Ferreira et al. 2000
	Isovalerianic acid	0.33		Ferreira et al. 2000
	Hexanoic acid		0.42	Marais and Pool 1980; Shinohara 1985; Ferreira et al. 2000
	Octanoic acid		0.5	Marais and Pool 1980; Shinohara 1985
	Decanoic acid		1	Shinohara 1985; Ferreira et al. 2000
CARBONYL COMPOUND	Benzaldehyde	2000		Guth 1997
MONOTERPENOLS	Linalool	25		Ferreira et al. 2000
	α-Terpineol	250		Ribéreau-Gayon et al. 1975
	β-Citronelol	100		Etievant, 1991
	Geraniol	20		Ribéreau-Gayon et al. 1975
NORISOPRENOIDS	TDN	20		Simpson and Miller 1983
	β-Damascenone	0.05		Guth 1997
	α-Ionone	2.6		Kotseridis et al. 1998, 1999; Ferreira et al. 2000
	β-Ionone	0.09		Kotseridis et al. 1998, 1999; Ferreira et al. 2000

S.d. Table 1 (cont) Odour threshold of all the compounds quantified organized by aroma family

		Odor Threshold (µg/L)	Odor Threshold (ng/L)	
PHENOLS	Guaiacol	9.5		Chatonnet and Boidron 1988; Ferreira et al. 2000
	o-Cresol	31		Etievant, 1991
	4-Ethylguaiacol	33		Schreier et al. 1980; Ferreira et al. 2000
	m-Cresol	68		Ferreira et al. 2009
	4-Propylguaiacol	10		López et al. 2002
	Eugenol	6		Chatonnet and Boidron 1988; Ferreira et al. 2000
	4-Ethylphenol	35		Chatonnet and Boidron 1988
	4-Vinylguaiacol	40		Versini and Tomasi 1983; Ferreira et al. 2000
	E-Isoeugenol	6		Escudero et al. 2007
	2,6-Dimethoxyphenol	570		López et al. 2002
	4-Vinylphenol	180		Chatonnet and Boidron 1988
	4-Alyl-2,6-dimethoxyphenol	1200		Gamert and Nettenbreijer 1977
CINAMATES	Ethyl dihidrocinnamate	1.6		Aubry et al. 1997; Ferreira et al. 2000
	Ethyl cinnamate	5.1		Aubry et al. 1997; Ferreira et al. 2000
LACTONES	γ-Butyrolactone	35000		Escudero et al. 2007
	t-Whiskylactone	790		Etievant, 1991
	c-Whiskylactone	67		Etievant, 1991
	γ-Nonalactone	25		Nakamura et al. 1988
	γ-Decalactone	3,86		Ferreira et al. 2001
VANILLIN DERIVATIVES	Vanillin	995		Escudero et al. 2007
	Methyl vanillinate	3000		López et al. 2002
	Ethyl vanillate	990		López et al. 2002
	Acetovanillone	1000		López et al. 2002
	Siringaldehyde	50000		Gamert and Nettenbreijer 1977
POLYFUNCTIONAL MERCAPTANS	2-Methyl-3-Furanthiol		4	Tominaga and Dubourdieu 2006
	2-Furfurylthiol		0.4	Tominaga et al. 2000
	4-Mercapto-4-methyl-2-pentanone		0.8	Tominaga et al. 1998
	3-Mercaptohexyl acetate		4	Tominaga et al. 1998
	3-Mercaptohexanol		60	Tominaga et al. 1998
	Benzylmercaptane		0.3	Tominaga et al. 2003

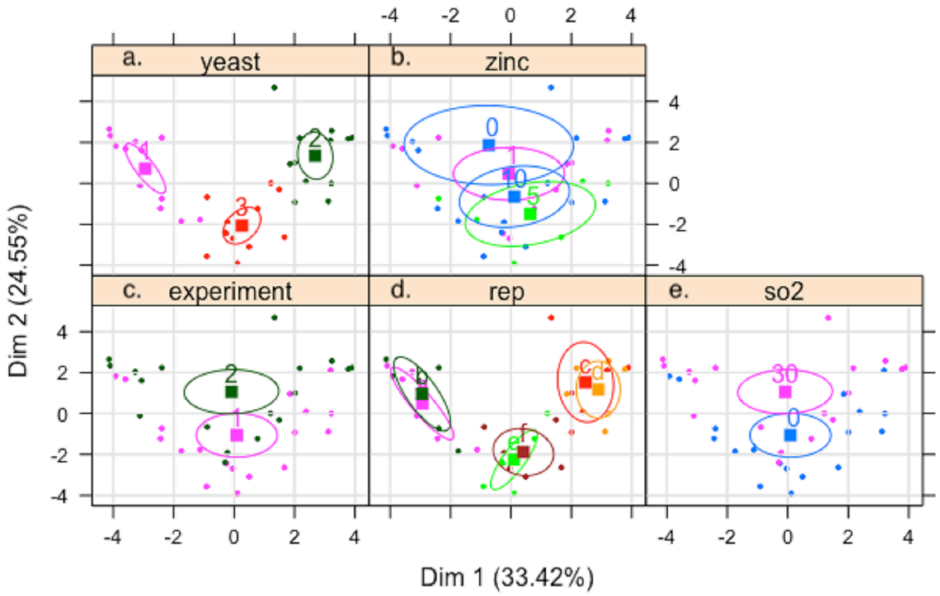
2. Supplementary data from Section I

S.d. Table 2 Average levels of aroma compounds and of some relevant ratios, segregated attending to levels of zinc and strain of yeast. Concentration data are in mg/L except aldehydes which are in µg/L. Results are shown for the experiment without external SO_2 and with 30 mg/L of external SO_2

		Yeast zinc	L1			L2			L3		
			10 mg/L	5 mg/L	1 mg/L	10 mg/L	5 mg/L	1 mg/L	10 mg/L	5 mg/L	1 mg/L
Without SO2	2-methylbutanal/ isovaleraldehyde		0.10	0.09	0.11	0.13	0.14	0.18	0.11	0.10	0.08
	medium chain fatty acids										
	hexanoic acid		1.75	1.67	1.91	1.17	1.06	1.23	1.09	0.98	0.71
	decanoic acid		0.65	0.75	1.25	0.45	0.65	0.56	0.55	0.33	0.31
	esteres										
	ethyl octanoate		0.18	0.19	0.78	0.13	0.12	0.38	0.16	0.09	0.39
	ethyl decanoate		0.11	0.12	0.54	0.10	0.08	0.31	0.09	0.04	0.17
		Yeast zinc	L1			L2			L3		
			10 mg/L	1 mg/L	0 mg/L	10 mg/L	1 mg/L	0 mg/L	10 mg/L	1 mg/L	0 mg/L
With SO_2	**Aldehydes**										
	isovaleraldehyde		34.14	28.74	27.22	28.44	22.53	28.55	36.43	22.30	42.72
	phenylacetaldehyde		24.60	23.30	23.12	13.16	13.86	11.62	38.66	13.86	37.57
	fusel Alcohols										
	isobutanol		16.39	15.29	12.10	55.28	42.50	36.11	18.24	22.12	16.56
	phenylethanol		34.77	32.09	25.35	26.35	25.56	21.60	20.47	25.91	24.47
	aldehyde/ alcohol ratio										
	isovaleraldehyde/ isoamyl alcohol		0.18	0.16	0.19	0.10	0.09	0.14	0.27	0.13	0.33
	phenylacetaldehyde/phenylethanol		0.71	0.73	0.91	0.51	0.55	0.55	1.89	0.54	1.54
	aldehyde/ acid Ratio										
	isovaleraldehyde/ isovaleric acid		29.66	25.11	21.62	14.50	11.27	13.35	36.51	15.64	35.97

S.d. Table 3 Results of three-way ANOVA on the subset data with equivalent Zn levels showing the significant results for the three factors: yeast. zinc and SO_2, as well as their interactions.

	yeast	zinc	SO_2	zinc*yeast	SO_2*zinc	SO_2*yeast
total SO2	< 0.0001	0.007	< 0.0001	0.001	-	0.011
aldehydes						
isobutyraldehyde	0.027	-	-	-	-	-
isovaleraldehyde	-	0.006	0.018	-	-	-
2-methylbutanal	0.045	-	-	-	-	-
methional	0.001	-	0.01	-	-	-
phenylacetaldehyde	0.009	-	< 0.0001	0.044	0.030	-
fusel Alcohols						
isobutanol	< 0.0001	-	0.006	-	-	-
isoamyl alcohol	< 0.0001	-	0.001	-	-	-
methionol	< 0.0001	-	-	0.006	0.012	-
phenylethanol	< 0.0001	0.002	< 0.0001	0.026	0.011	-
iso-acids						
isobutyric acid	< 0.0001	-	-	-	-	-
isovaleric acid	< 0.0001	0.003	-	-	-	-
aldehyde/ alcohol ratio						
isobutyraldehyde/ isobutanol	0.036	-	-	-	-	-
isovaleraldehyde/ isoamyl alcohol	< 0.0001	0.001	-	0.005		
methional/ methionol	0	-	0.002	-	-	-
phenylacetaldehyde/ phenylethanol	0.002	0.018	< 0.0001	0.010	-	-
aldehyde/ acid ratio						
isobutyraldehyde/ isobutyric acid	-	-	-	-	-	-
isovaleraldehyde/ isovaleric acid	0.006	0.004	-	-	-	-
isobutanol/ isobutyric Acid	-	-	0.006	-	-	-
isoamyl Alcohol/ isovaleric acid	0.043	-	-	-	-	-
2-methylbutanal/ isovaleraldehyde	0.003	-	0.004	-	-	-
medium chain fatty acids						
hexanoic acid	< 0.0001	-	0.019	-	-	-
octanoic acid	< 0.0001	-	-	0.036	-	-
decanoic acid	0.002	-	-	-	-	-
esteres						
isoamyl acetate	< 0.0001	-	< 0.0001	-	-	-
ethyl hexanoate	0.018	< 0.0001	0.004	-	< 0.0001	-
ethyl octanoate	0	0.001	-	-	0.000	-
ethyl decanoate	0.001	0.000	0.001	-	0.000	-
ratios						
ethyl hexanoate/ hexanoic acid	0.018	< 0.0001	0.000	-	< 0.0001	-
ethyl octanoate/ octanoic acid	-	< 0.0001		-	0.000	-
ethyl decanoic/ decanoic acid	-	0.000	< 0.0001	-	0	-



S.d. Figure 1 ***Summary of the results obtained by Principal Component Analysis****: distribution of the two data sets having into consideration the three main factors, the biological replicates and set-up. The plots show the first two dimensions explain 58,7% of the variance. The 90% confidence ellipses are also shown. a. distribution of yeast strains; b. distribution of samples according to Zn level; c. segregation of the two set-ups; d. distribution of the biological replicates; e. distribution of the samples according to* SO_2 *supplementation.*

3. Supplementary data from Section II: Chapter 1

*S.d. Table 4 3-way ANOVA assessing the effect of the factors: presence or absence of precursors, yeast strain, aging and their interaction on the volatile composition of Riesling synthetic wine. F and significance are indicated to each factor. Significance is expressed as ***:** <0.0001-0.001***; 0.001-0.01**; 0.01-0.05*.*

	Precursors		Yeast		Aging		Precursors*yeast		Yeast*Aging	
Ethyl acetate	18.0	***	12.6	***	1.3	n.s.	3.6	*	0.7	n.s.
Isoamyl acetate	6.8	*	401.1	***	24.2	***	8.5	***	21.6	***
Ethyl hexanoate	0.0	n.s.	96.7	***	1.7	n.s.	6.8	***	0.5	n.s.
Ethyl octanoate	0.0	n.s.	81.9	***	1.8	n.s.	4.8	**	1.2	n.s.
Ethyl decanoate	2.4	n.s.	28.8	***	2.2	n.s.	2.6	*	1.1	n.s.
Isobutanol	0.1	n.s.	128.7	***	0.5	n.s.	2.1	n.s.	0.4	n.s.
Isoamyl alcohol	0.7	n.s.	136.2	***	0.1	n.s.	1.4	n.s.	0.1	n.s.
Metionol	12.6	***	221.4	***	5.4	**	1.5	n.s.	2.7	n.s.
β-Phenylethanol	0.9	n.s.	111.8	***	3.7	*	1.0	n.s.	1.5	n.s.
Ethyl lactate	0.5	n.s.	16.1	***	5.6	**	1.0	n.s.	1.0	n.s.
γ-Butyrolactone	1.7	n.s.	34.9	***	45.2	***	0.4	n.s.	2.6	n.s.
Butyric acid	0.6	n.s.	2.1	n.s.	1.1	n.s.	0.8	n.s.	0.7	n.s.
Isobutyric acid	28.5	***	141.5	***	1.2	n.s.	6.2	***	1.7	n.s.
Hexanoic acid	0.6	n.s.	367.6	***	0.7	n.s.	12.5	***	0.6	n.s.
Octanoic acid	0.8	n.s.	340.0	***	1.1	n.s.	9.6	***	0.9	n.s.
Decanoic acid	1.0	n.s.	18.9	***	1.1	n.s.	0.1	n.s.	0.9	n.s.
Ethyl isobutyrate	4.4	*	48.4	***	76.7	***	2.1	n.s.	9.8	***
Isobutyl acetate	19.6	***	916.5	***	57.6	***	23.4	***	61.2	***
Ethyl 2-methylbutyrate	0.2	n.s.	43.4	***	91.1	***	1.8	n.s.	10.2	***
Phenylethyl acetate	27.9	***	1197.0	***	61.7	***	33.2	***	68.9	***
γ-nonalactone	10.9	**	145.0	***	3.6	*	2.1	n.s.	0.8	n.s.
γ-decalactone	1.1	n.s.	328.5	***	7.6	***	2.1	n.s.	1.9	n.s.
TDN	34.2	***	1.2	n.s.	11.1	***	1.2	n.s.	0.4	n.s.
β-damascenone	1100.1	***	24.4	***	8.2	***	34.6	***	0.8	n.s.
Linalool	78.0	***	4.8	**	15.3	***	5.0	**	0.5	n.s.
α-terpineol	186.8	***	4.4	**	12.0	***	4.4	**	0.4	n.s.
β-citronellol	26.4	***	3.8	**	23.8	***	1.0	n.s.	1.6	n.s.
Geraniol	46.6	***	3.7	*	1.4	n.s.	4.1	**	2.1	n.s.
4-vinylguaiacol	122.7	***	1.8	n.s.	14.3	***	1.9	n.s.	0.7	n.s.
4-vinylphenol	166.9	***	8.3	***	6.4	***	8.3	***	2.7	**
vanillin	52.4	***	7.3	***	6.3	***	8.0	***	2.3	*
acetovanillone	1102.0	***	50.3	***	1.5	n.s.	47.3	***	1.5	n.s.

S.d. Table 5 Average concentration of volatiles measured above the limit of quantification in control wines and those spiked with glycosidic precursors from Riesling grapes in unfermented controls (Acid Hydrolysis) and in wines fermented with S. cerevisiae. P. kluyveri. T. delbrueckii and L. thermotolerans.

Mosto	Control				PR			CTL	CTL	CTL	CTL	PR			CTL				PR			
Levadura				AH				*S. cerevisiae*							*P. kluyveri*							
Aging	0	1	2	5	0	1	2	0	1	2	5	0	1	2	5	0	1	2	5	0	1	2	5
Ethyl acetate	0	0	0	0	0	0	0	5625±103	9936±1171	13483±1107	16193±1708	30264±262	29553±1016	13523±13523	23211±778	28147±996	29245±131	27402±1792	20699±950	23011±22656	22606±21452	32724±2401	24590±592
Isoamyl acetate	0	0	0	0	0	0	0	30.6±2.9	19.0±3.8	14.8±0.7	14.9±4.5	32.4±1.2	24.1±0.0	17.1±5.9	15.3±1.0	492±35	385±38	416±69	120±19	395±27	295±65	283±1	95.7±13.9
Isobutyl acetate	1.75	1.50	1.18	1.26	2.39	1.60	1.79	1.39	17.2±1.2	16.3±1.1	16.9±1.5	18.1±1.2	20.5±1.6	20.9±0.3	20.7±0.1	21.1±1.0	263±1	211±3	165±6	59.0±5.0	194±4	148±12	128±6	51.5±2.4
Phenylethyl acetate	0.00	0.75	0.00	0.00	4.84	4.54	4.92	3.75	22.7±1.1	17.1±0.6	13.1±0.6	9.4±0.5	23.6±0.6	18.7±0.2	15.0±0.2	11.4±0.1	3727±55	3078±53	2477±119	864±55	2713±62	2112±215	1780±16	671±12
Ethyl hexanoate	0	0	0	0	0	0	0	0	70.1±4.3	71.4±31.0	65.1±1.5	66.2±12.0	94.7±11.9	96.7±10.1	86.8±14.7	86.3±3.9	123±5	167±14	129±20	131±3	162±25	180±62	171±30	143±28
Ethyl octanoate	0	0	0	0	0	0	0	0	104±5	73.1±30.8	99.3±28.9	0.0±0.0	138±20	103±4	111±8	118±10	168±13	160±17	189±30	150±1	146±28	144±13	148±10	172±33
Ethyl decanoate	0	0	0	0	0	0	0	0	34.2±1.2	66.1±11.4	54.3±5.8	46.2±27.5	47.9±6.4	62.3±13.6	48.0±9.7	51.3±3.4	87.9±6.4	144±47	92.1±10.1	52.8±4.7	141±25	130±83	73.8±1.1	72.1±17.7
Ethyl isobutyrate	0	0	0	0	0	0	0	0	1.1±1.1	10.0±0.5	21.9±1.9	38.4±6.1	1.4±0.0	6.2±0.4	13.6±0.6	25.1±2.7	4.2±0.7	26.9±1.6	48.8±2.3	57.1±1.2	2.7±0.0	16.5±0.8	33.3±0.7	51.2±17.0
Ethyl 2-methylbutyrate	0	0	0	0	0	0	0	0	0.0±0.0	1.0±0.1	2.3±0.0	3.6±0.5	0.6±0.1	0.8±0.0	2.0±0.0	3.0±0.5	0.0±0.0	1.2±0.1	2.4±0.1	5.5±0.1	0.6±0.1	1.0±0.1	2.5±1.0	4.4±0.9
Ethyl isovalerate	0	0	0	0	0	0	0	0	0.0±0.0	0.9±0.1	1.8±0.2	3.4±0.3	0.1±0.0	0.7±0.0	1.4±0.1	2.8±0.1	0.1±0.0	0.9±0.0	1.4±0.1	3.9±0.2	0.1±0.0	0.6±0.0	1.3±0.1	3.1±0.1
Ethyl lactate	0	0	0	0	0	0	0	0	100±9	437±9	668±27	867±68	110±3	474±18	841±29	1158±14	105±24	413±110	618±136	1032±311	117±11	424±19	785±146	1226±105
Isobutanol	0	0	0	0	0	0	0	0	8115±946	7823±1038	8226±1900	7602±966	8645±441	8753±201	9917±197	9198±159	8251±510	9662±714	8694±186	7136±552	9407±3	9009±1814	8744±372	8409±30
1-Butanol	0	0	0	0	0	0	0	0	60.0±0.5	55.5±4.0	55.2±3.8	61.4±2.4	63.8±0.1	57.3±6.6	58.4±7.8	57.4±8.8	47.1±1.6	58.8±13.3	63.6±8.5	55.7±0.7	54.0±2.4	52.2±0.7	53.8±0.9	73.0±18.4
Isoamyl alcohol	0	0	0	0	0	0	0	0	42922±5794	41979±6773	43341±8181	41487±5854	44279±2897	44837±1801	47300±1647	45515±2382	31823±1978	33373±3472	31011±2881	31604±2823	34994±266	33848±3496	35941±1470	36035±874
1-Hexanol	0	0	0	0	0	0	0	0	11.7±0.1	7.5±7.5	12.3±0.1	9.9±9.9	128±4	137±1	132±3	141±1	0.0±0.0	0.0±0.0	0.0±0.0	5.4±5.4	27.8±0.4	31.3±2.0	35.6±0.5	39.1±0.7
Methionol	0	0	0	0	0	0	0	0	2960±112	3319±360	3110±439	3192±220	3390±166	3555±64	3604±38	3630±107	3490±75	4190±251	4436±217	4855±23	3940±29	4165±860	5577±733	5002±90
β-Phenylethanol	0	0	0	0	0	0	0	0	4913±521	5379±717	5172±788	5336±643	5206±139	5633±257	5533±40	5601±215	5157±53	5652±391	6235±352	7316±42	5364±188	5663±580	7835±983	7353±46
γ-Butyrolactone	0	0	0	0	0	0	0	0	215±41	932±148	959±98	965±248	192±8	870±68	1025±108	1050±58	128±86	875±16	1021±157	979±50	240±22	850±99	1288±233	1169±124
γ-nonalactone	0.65	0	0	0.47	0	0	0	0	2.6±1.6	4.7±0.1	4.6±0.0	4.9±0.3	4.9±0.1	4.9±0.2	5.0±0.1	5.3±0.2	4.4±0.1	5.0±0.0	5.2±0.1	5.2±0.1	5.0±0.3	5.0±0.1	5.4±0.4	5.5±0.5
γ-decalactone	0	0	0	0	0	0	0	0	2.8±0.4	2.9±0.2	3.1±0.1	3.0±0.1	2.3±0.1	2.8±0.0	2.7±0.2	2.9±0.1	3.3±0.3	3.9±0.1	3.7±0.2	4.2±0.2	3.2±0.1	3.7±0.2	3.9±0.3	4.3±0.3
Butyric acid	0	0	0	0	0	0	0	0	285±39	161±27	104±30	133±42	360±150	185±15	210±74	248±3	2395±2151	217±6	442±73	230±82	278±114	348±186	262±38	239±12
Isobutyric acid	0	0	0	0	0	0	0	0	198±16	219±19	184±25	233±20	111±2	98.7±20.7	133±5	141±13	493±24	500±6	638±54	332±111	309±9	272±7	339±1	294±20
Hexanoic acid	0.28	0.56	0.57	0.08	0.31	0.33	0.71	0.10	1037±113	1049±63	1055±15	1060±78	1203±61	1193±18	1245±58	1255±43	2030±48	2017±179	2100±146	2069±9	2208±134	2169±222	2609±37	2380±51
Octanoic acid	0	0	0	0	0	0	0	0	2321±122	2223±200	2077±97	2574±69	2614±95	2728±33	2605±155	2936±113	3664±374	3559±229	3948±460	4032±134	3960±214	3754±358	4185±171	4602±213
Decanoic acid	0	0	0	0	0	0	0	0	960±126	606±63	610±18	1067±234	852±149	741±114	893±105	1033±17	1035±131	796±45	1271±308	1417±18	982±58	1177±414	1327±5	1736±226
TDN	0.84	0.27	0.27	0.20	1.61	5.06	10.4	37	1.5±0.2	0.2±0.0	0.3±0.0	0.5±0.5	1.4±0.0	14.3±0.7	47.4±0.3	96.6±0.4	0.5±0.0	0.7±0.0	0.0±0.0	0.4±0.0	0.5±0.0	5.3±0.8	20.1±2.9	46.9±6.4
β-damascenone	0	0	0	0	6.28	6.72	7.37	5.92	0.0±0.0	0.1±0.0	0.3±0.0	0.3±0.1	2.5±0.0	3.4±0.2	3.8±0.0	4.5±0.1	0.4±0.0	0.5±0.0	0.4±0.0	0.3±0.0	1.6±0.0	2.6±0.2	2.4±0.2	3.0±0.3
β-ionone	0.28	0.56	0.57	0.08	0.31	0.33	0.71	0.10	0.5±0.0	0.2±0.0	0.6±0.1	0.5±0.0	0.4±0.0	0.2±0.0	0.5±0.0	0.4±0.0	0.8±0.0	0.3±0.0	0.4±0.0	0.1±0.0	0.8±0.1	0.3±0.0	0.6±0.2	0.3±0.4
Ethyl cinnamate	0	0.13	0.00	0.02	0.05	0.09	0.10	0.15	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Linalool	0.76	2.10	1.98	0.29	150	114	76.7	9.37	4.2±0.3	1.2±0.1	2.8±0.1	2.1±0.1	62.9±0.7	42.8±1.3	7.1±0.1	2.5±0.0	2.2±0.1	1.4±0.1	1.9±0.0	0.5±0.0	46.3±0.7	31.5±4.9	9.4±1.7	1.9±1.4
α-terpineol	0.65	0.92	1.07	0.46	78.5	148	178	158	1.1±0.1	1.5±0.0	0.9±0.5	1.0±0.2	12.5±12.0	139±4	111±1	50.1±1.2	1.0±0.0	0.9±0.0	0.9±0.1	0.3±0.0	20.2±0.4	73.1±1.1	73.1±2.7	26.1±0.7
β-citronellol	0.23	0.71	0.63	0	0.43	0.60	0.86	0	2.3±0.2	0.5±0.0	0.8±0.1	0.8±0.2	6.9±0.1	2.1±0.0	1.1±0.1	0.7±0.1	1.3±0.0	0.4±0.1	0.0±0.0	0.5±0.0	0.6±0.0	0.0±0.0	4.5±0.3	1.6±0.1	1.1±0.2	0.3±0.5
Geraniol	0	1.75	0	0	38.7	36.2	26.6	1.88	2.6±1.4	0.0±0.0	0.4±0.0	0.3±0.3	9.6±0.1	12.1±0.0	1.7±0.0	0.3±0.3	0.4±0.1	0.0±0.0	0.5±0.0	0.0±0.0	5.5±0.0	8.9±1.6	51.2	38.5±9.5
Guaiacol	0.06	0.04	0.06	0.00	0.41	0.38	0.58	1.86	0.3±0.0	0.1±0.0	0.1±0.0	0.3±0.1	2.2±0.1	2.1±0.1	0.9±0.3	5.3±3.8	0.2±0.0	0.1±0.0	0.1±0.0	0.4±0.1	0.9±0.1	0.7±0.1	1.0±0.5	2.4±0.6
4-vinylguaiacol	27.3	44.5	28.6	55.5	348	746	1404	5335	16.0±9.9	27.1±7.3	11.3±4.4	67.0±44.9	1902±283	2016±38	2207±100	3496±618	61.4±2.1	24.0±0.3	34.4±8.1	106±28	656±11	682±7	1506±382	2705±16
2-6-dimethoxyphenol	0.16	0	0	0.09	0	0	0	0	0.6±0.2	0.1±0.1	0.0±0.0	0.5±0.5	1.5±1.5	0.0±0.0	0.0±0.0	4.1±0.7	0.2±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.3±0.0	0.0±0.0	0.0±0.0	1.4±0.1
E-isoeugenol	0	0	0	0	0	0.19	0	0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.4±0.0	0.0±0.0	0.2±0.2	0.5±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.1
4-vinylphenol	14.6	15.9	14	36.1	364	944	1318	4299	149±23	26.0±1.5	16.7±3.6	54.0±36.8	4599±237	2255±78	2079±214	2791±523	70.7±0.5	18.4±1.3	35.2±13.6	124±26	1165±85	825±9	1065±51	1774±153
vanillin	1.58	0.67	0	0.78	13.16	14.3	41.9	91	1.8±0.7	2.5±1.2	1.0±0.0	3.7±2.5	9.8±1.6	9.5±0.0	10.0±0.8	23.5±9.5	1.6±0.2	0.9±0.1	1.1±0.1	1.8±0.4	7.2±0.4	7.4±1.4	6.6±0.3	8.7±1.4
acetovanillone	0.76	0	0	0	5.02	5.75	8.30	13.9	0.0±0.0	1.0±0.7	0.0±0.0	2.3±1.8	58.4±0.8	60.7±0.1	58.8±4.7	58.2±2.2	1.5±1.5	0.3±0.3	0.2±0.2	0.0±0.0	329±0.9	29.4±1.4	30.8±1.4	32.1±1.7

S.d. Table 5 (cont) Average concentration of volatiles measured above the limit of quantification in control wines and those spiked with glycosidic precursors from Riesling grapes in unfermented controls (Acid Hydrolysis) and in wines fermented with S. cerevisiae. P. kluyveri. T. delbrueckii and L. thermotolerans.

Mosto	CTL				PR				CTL				PR			
Levadura	*T. delbrueckii*								*L. thermotolerans*							
Aging	0	1	2	5	0	1	2	5	0	1	2	5	0	1	2	5
Ethyl acetate	15005±370	14768±1645	13958±244	6059±5986	43321±1201	38412±772	28832±84	17938±1208	13398±2392	12579±2117	11574±1377	10963±479	26460±12517	33153±3367	26992±3771	17095±3153
Isoamyl acetate	17.2±2.2	16.9±1.5	11.1±1.4	0.0±0.0	10.8±1.9	11.0±3.5	11.5±1.9	0.0±0.0	16.9±2.4	12.3±1.1	13.0±3.3	0.0±0.0	11.0±1.4	16.3±3.6	13.8±2.0	12.5±0.1
Isobutyl acetate	22.4±0.2	21.2±1.0	18.1±0.9	15.6±0.6	20.2±5.4	18.0±4.8	10.0±1.6	13.8±3.3	9.2±0.4	8.0±0.8	7.5±0.3	7.5±0.3	5.9±1.8	6.4±1.3	11.9±5.5	7.5±1.3
Phenylethyl acetate	48.9±3.1	39.4±3.0	32.7±2.1	20.6±1.1	48.0±5.2	36.1±4.0	16.6±10.7	17.3±1.2	3.6±0.0	2.9±0.1	2.6±0.0	2.5±0.1	5.3±1.3	4.4±0.9	18.7±14.4	5.0±0.5
Ethyl hexanoate	76.3±6.1	67.1±7.9	80.2±7.6	59.6±1.9	37.8±21.0	39.7±11.5	34.7±5.9	0.0±0.0	39.9±1.5	37.9±5.2	31.5±2.4	32.3±9.8	26.0±5.6	43.7±17.8	35.1±6.0	21.5±1.1
Ethyl octanoate	77.5±10.3	66.7±12.1	26.5±26.5	0.0±0.0	0.0±0.0	27.5±27.5	49.7±19.8	0.0±0.0	37.3±6.1	27.0±27.0	40.9±4.7	34.7±5.4	0.0±0.0	20.9±0.7	26.5±9.4	25.1±3.3
Ethyl decanoate	62.1±0.2	53.8±11.0	60.4±1.5	74.8±15.2	0.0±0.0	48.0±1.1	26.6±1.7	0.0±0.0	19.0±19.0	43.2±10.4	0.0±0.0	27.5±1.1	0.0±0.0	0.0±0.0	16.5±1.1	0.0±0.0
Ethyl isobutyrate	4.0±0.3	25.7±3.3	44.2±2.0	93.2±4.3	4.9±1.2	27.0±6.3	25.8±9.8	77.9±9.3	2.2±0.4	5.2±5.2	7.9±7.9	31.2±1.6	1.1±1.1	6.4±1.5	28.2±17.9	26.6±5.0
Ethyl 2-methylbutyrate	0.6±0.0	1.7±0.0	2.7±0.2	5.9±0.5	0.6±0.0	1.8±0.2	1.4±0.5	5.2±0.7	0.3±0.3	0.3±0.3	1.0±0.2	1.1±0.1	0.5±0.0	0.4±0.0	1.5±0.8	2.3±0.4
Ethyl isovalerate	0.0±0.0	0.6±0.0	1.1±0.1	2.4±0.2	0.0±0.0	0.6±0.1	0.6±0.1	2.1±0.5	0.0±0.0	0.1±0.1	0.2±0.2	0.6±0.1	0.0±0.0	0.1±0.1	0.7±0.4	0.4±0.4
Ethyl lactate	93.9±10.8	335±5	602±49	1067±184	119±34	363±93	588±169	944±176	364±179	2313±1097	3475±1413	5141±2459	1852±1664	1060±156	1839±399	2910±606
Isobutanol	7668±225	7857±293	8389±482	8074±654	7332±1567	7865±1237	7408±1272	7344±1344	5254±632	5136±244	5104±197	5394±277	4451±452	4663±962	4894±917	4227±675
1-Butanol	93.2±1.8	105±10	113±2	110±0	104±6	102±4	92.1±13.5	93.5±14.9	110±2	115±3	102±16	96.9±10.8	109±1	110±0	106±4	91.3±10.1
Isoamyl alcohol	35329±1164	36262±1071	37246±957	39467±1418	33266±5029	34338±4879	33867±4951	34361±4040	33012±1798	33355±102	32447±744	32648±730	34520±5166	33537±4542	35077±5092	34306±5380
1-Hexanol	12.1±0.3	13.7±0.8	14.0±0.8	19.4±2.0	130±4	129±1	137±5	138±3	12.0±0.4	17.6±2.1	14.4±2.5	16.1±0.5	159±10	156±2	156±3	164±5
Metionol	4114±1	3871±346	4315±146	5400±930	4355±71	4220±38	4446±79	5587±952	1483±54	1616±90	1307±18	1616±12	2404±114	2188±84	2291±185	2389±129
β-Phenylethanol	6538±155	6240±257	6571±210	8521±2431	6276±304	5947±347	6607±431	7334±677	3834±148	4351±22	4111±133	4192±320	4861±911	4465±567	4680±938	4894±879
γ-Butyrolactone	173±6	657±56	823±31	1089±204	237±23	716±38	811±54	1076±141	95.2±49.5	959±20	1022±138	1115±9	682±503	819±41	977±65	1149±36
γ-nonalactone	4.2±0.0	4.2±0.0	4.6±0.0	4.5±0.1	4.8±0.1	4.8±0.1	4.7±0.5	5.3±0.2	3.2±0.1	3.1±0.2	3.2±0.2	3.6±0.0	3.6±0.3	3.7±0.0	4.6±0.8	4.2±0.2
γ-decalactone	2.9±0.0	2.9±0.0	3.1±0.1	3.2±0.2	2.6±0.1	2.8±0.1	2.6±0.2	3.0±0.1	1.7±0.2	2.1±0.0	2.2±0.0	1.9±0.2	1.9±0.1	2.1±0.2	2.5±0.6	2.1±0.2
Butyric acid	102±39	172±3	89.0±27.3	113±63	116±64	78.2±23.3	168±38	0.0±0.0	494±59	150±21	356±226	160±59	229±114	317±225	242±102	240±104
Isobutyric acid	479±13	479±13	489±11	413±82	387±59	410±49	409±26	446±13	98.5±44.8	166±2	203±37	227±47	155±6	144±11	140±22	143±18
Hexanoic acid	1007±59	948±4	996±8	1187±263	603±265	578±256	650±279	637±249	488±51	540±54	342±142	556±12	301±8	264±1	291±18	279±14
Octanoic acid	1824±31	1884±207	1766±172	1680±386	1073±513	1072±535	1183±481	1024±502	984±144	885±55	895±151	1002±95	549±116	505±85	549±60	602±64
Decanoic acid	531±36	500±63	508±98	559±211	364±178	1099±929	509±211	433±197	628±56	434±32	762±361	689±73	814±596	530±318	559±264	954±457
TDN	0.5±0.0	0.2±0.0	0.7±0.0	0.8±0.3	0.5±0.0	15.9±1.2	35.2±3.3	105±5	1.3±0.0	0.1±0.1	0.1±0.1	0.1±0.1	0.5±0.0	10.9±0.7	38.8±11.7	85.4±10.5
β-damascenone	0.2±0.1	0.3±0.0	0.3±0.0	0.1±0.1	2.3±0.3	3.1±0.3	4.0±0.5	4.4±0.2	0.3±0.0	0.1±0.1	0.1±0.1	0.2±0.0	2.3±0.3	3.2±0.1	3.9±0.2	4.0±0.1
β-ionone	0.6±0.0	0.5±0.0	0.3±0.0	0.6±0.3	0.7±0.0	0.5±0.0	0.3±0.0	0.4±0.0	0.4±0.0	0.3±0.1	0.5±0.0	0.4±0.0	0.6±0.0	0.2±0.0	0.2±0.0	0.3±0.0
Ethyl cinnamate	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.0	0.2±0.0	0.2±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.0	0.2±0.0
Linalool	2.4±0.1	3.0±0.0	1.5±0.0	2.5±1.2	73.8±1.7	40.6±1.1	10.2±0.7	2.1±0.0	3.5±0.0	1.2±0.1	1.7±0.6	1.8±0.1	65.3±1.6	57.3±2.5	11.2±4.5	2.8±0.5
α-terpineol	1.0±0.0	1.6±0.0	1.2±0.0	1.0±0.3	30.8±1.9	138±4	116±3	48.4±3.8	1.1±0.0	1.2±0.2	0.9±0.3	0.7±0.1	26.6±1.6	134±6	121±11	66.8±9.0
β-citronellol	1.2±0.0	0.8±0.0	0.5±0.0	0.7±0.3	5.6±0.7	2.2±0.2	0.7±0.0	0.6±0.1	1.4±0.1	0.4±0.1	0.3±0.3	0.5±0.1	4.4±0.1	1.7±0.2	0.8±0.1	0.7±0.1
Geraniol	1.1±0.1	0.0±0.0	0.3±0.1	0.5±0.6	13.3±0.9	12.5±1.4	2.9±0.1	0.0±0.0	1.1±0.0	1.3±1.0	1.4±1.0	0.4±0.1	12.4±0.0	17.8±1.1	3.5±1.2	0.0±0.0
Guaiacol	0.1±0.1	0.1±0.0	0.1±0.0	0.2±0.2	0.9±0.3	0.4±0.0	0.8±0.0	1.1±0.0	0.3±0.1	0.1±0.0	0.1±0.0	0.1±0.0	0.7±0.1	0.7±0.0	0.9±0.3	0.9±0.0
4-vinylguaiacol	17.6±5.1	13.0±6.0	23.0±3.0	60.3±35.7	525±156	1114±65	1719±117	2827±25	45.9±15.0	13.9±10.9	28.8±24.8	22.2±1.0	399±58	1055±29	1748±186	2458±56
2-6-dimethoxyphenol	0.9±0.9	0.0±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	1.9±1.9	0.0±0.0	0.0±0.0	0.3±0.1	0.2±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
E-isoeugenol	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.5±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
4-vinylphenol	22.1±2.8	21.4±6.5	15.8±1.6	30.4±23.8	643±230	1094±169	1383±96	2243±41	134±71	75.7±58.4	80.9±57.4	10.2±10.2	469±126	793±82	1463±164	1874±21
vanillin	8.1±7.1	1.0±0.1	0.9±0.0	1.6±0.5	5.5±0.6	7.4±0.5	7.4±0.1	16.5±10.0	1.1±0.3	0.7±0.4	0.4±0.4	1.3±0.1	5.4±0.4	7.9±0.5	8.2±0.3	5.2±0.2
acetovanillone	5.8±5.8	0.4±0.4	0.2±0.2	0.6±0.1	49.6±4.5	43.6±3.9	41.5±2.3	36.6±9.7	0.5±0.5	0.0±0.0	0.4±0.4	0.0±0.0	48.8±3.5	47.2±7.1	42.8±3.2	27.8±2.3

S.d. Table 6 Average values of compounds above the limit of quantification according the yeast strain that carried fermentation and acidic hydrolysis (AH) of wines with or without Riesling precursors. The letters a-d express the ducan post-hoc test being a the highest value.

	AH	*P. kluyveri*	*S. cerevisiae*	*T. delbrueckii*	*L. thermotolerans*
ethyl acetate	0 c	26053 a	17723 b	22287 ab	19027 b
isoamyl acetate	0 b	310 a	21 b	9.8 b	12 b
isobutyl acetate	1.6 c	152 a	19 b	17.4 b	8 c
phenylethyl acetate	2.3 b	2177 a	16.4 b	32.4 b	5.6 b
ethyl hexanoate	0 e	151 a	79.7 b	49.4 c	33.5 d
ethyl octanoate	0 d	160 a	93.3 b	31 c	26.5 c
ethyl decanoate	0 c	99 a	51.3 b	40.7 b	13.2 c
ethyl isobutyrate	0 d	29.7 b	14.5 c	37.8 a	13.6 c
ethyl 2-methylbutyrate	0 d	2.2 a	1.7 b	2.5 a	0.9 c
ethyl lactate	0 b	590 b	582 b	514 b	2369 a
isobutanol	0 d	8664 a	8534 a	7742 b	4890 c
isoamyl alcohol	0 c	33579 b	43957 a	35517 b	33612 b
metionol	0 d	4457 a	3344 b	4538 a	1912 c
β-phenylethanol	0 d	6322 a	5346 b	6754 a	4424 c
ethyl lactate	0 b	590 b	582 b	514 b	2369 a
γ-butyrolactone	0 c	819 ab	776 ab	698 b	852 a
γ-nonalactone	0 d	5.1 a	4.6 b	4.6 b	3.7 c
γ-decalactone	0 d	3.8 a	2.8 b	2.9 b	2.1 c
butyric acid	0 b	551 a	210 ab	105 b	274 ab
isobutyric acid	0 d	397 b	165 c	439 a	159 c
hexanoic acid	0 e	2198 a	1137 b	826 c	383 d
octanoic acid	0 e	3963 a	2510 b	1438 c	746 d
decanoic acid	0 d	1218 a	845 b	563 c	671 bc
TDN	7 a	9.2 a	20.3 a	19.9 a	17.1 a
β-damascenone	3.3 a	1.4 c	1.9 b	1.8 b	1.8 b
linalool	44.5 a	11.8 b	15.7 b	17 b	18.1 b
α-terpineol	70.9 a	24.5 b	39.7 b	42.2 b	44 b
β-citronellol	0.4 b	1.2 a	1.9 a	1.5 a	1.3 a
Geraniol	13.1 a	10.003 ab	3.381 c	3.840 c	4.733 bc
4-vinylguaiacol	999 ab	722 b	1218 a	787.5 ab	721.2 b
4-vinylphenol	876 b	636 b	1496 a	681.8 b	612.3 b
vanillin	20.4 a	4.4 b	7.7 b	6.1 b	3.8 b
acetovanillone	4.2 d	15.9 c	29.9 a	22.3 b	20.9 b

4. Supplementary data from Section II: Chapter 2

S.d. Table 7 3-way ANOVA assessing the effect of the factors: presence or absence of precursors. yeast strain. aging and their interaction on the volatile composition of Garnacha synthetic wine.

Pr > F	Precursors	Yeast	Aging	Precursors* Yeast	Precursors* Aging	Yeast* Aging
Ethyl acetate	< 0.0001	0.00	n.s	n.s	0.02	n.s
Isoamyl acetate	n.s[a]	< 0.0001	n.s	n.s	n.s	0.01
Ethyl hexanoate	0.00	< 0.0001	0.00	0.01	0.03	0.02
Ethyl octanoate	0.00	< 0.0001	0.01	0.01	n.s	n.s
Ethyl decanoate	0.01	0.00	n.s	n.s	n.s	n.s
Isobutanol	n.s	0.01	0.01	n.s	n.s	n.s
1-Butanol	n.s	0.00	0.05	n.s	n.s	n.s
Isoamyl alcohol	0.01	< 0.0001	n.s	n.s	n.s	n.s
1-Hexanol	< 0.0001	< 0.0001	0.00	0.00	0.01	n.s
Metionol	0.01	< 0.0001	< 0.0001	n.s	n.s	n.s
β-Phenylethanol	n.s	< 0.0001	n.s	0.04	n.s	n.s
Ethyl lactate	0.04	< 0.0001	< 0.0001	0.02	n.s	< 0.0001
γ-Butyrolactone	0.00	< 0.0001	< 0.0001	0.01	n.s	< 0.0001
Butyric acid	n.s	< 0.0001	0.00	n.s	n.s	0.00
Isobutyric acid	0.05	< 0.0001	n.s	n.s	n.s	n.s
Hexanoic acid	n.s	< 0.0001	0.01	n.s	n.s	n.s
Octanoic acid	n.s	n.s	n.s	n.s	n.s	n.s
Decanoic acid	0.01	< 0.0001	0.01	0.00	n.s	0.02
Ethyl isobutyrate	0.01	< 0.0001	< 0.0001	n.s	n.s	0.00
Isobutyl acetate	0.05	< 0.0001	0.00	0.04	n.s	0.01
Ethyl 2-methylbutyrate	0.00	< 0.0001	< 0.0001	0.03	0.05	0.01
Ethyl isovalerate	0.00	< 0.0001	< 0.0001	0.01	0.05	< 0.0001
Phenylethyl acetate	0.01	< 0.0001	< 0.0001	0.00	n.s	< 0.0001
γ-nonalactone	0.01	0.01	n.s	n.s	n.s	0.05
γ-decalactone	n.s	< 0.0001	n.s	n.s	n.s	n.s
TDN	< 0.0001	n.s	< 0.0001	n.s	< 0.0001	n.s
β-damascenone	< 0.0001	n.s	0.00	n.s	0.00	n.s
Linalool	< 0.0001	n.s	< 0.0001	n.s	< 0.0001	n.s
α-terpineol	< 0.0001	n.s	< 0.0001	n.s	< 0.0001	n.s

Pr > F	Precursors	Yeast	Aging	Precursors* Yeast	Precursors* Aging	Yeast* Aging
β-citronellol	< 0.0001	n.s	< 0.0001	n.s	0.03	n.s
Geraniol	< 0.0001	0.01	0.00	n.s	n.s	0.03
Guaiacol	< 0.0001	0.01	0.00	0.01	0.00	n.s
4-vinylguaiacol	< 0.0001	0.04	< 0.0001	0.03	< 0.0001	n.s
2-6-dimethoxyphenol	< 0.0001	0.02	0.00	0.03	0.00	n.s
E-isoeugenol	< 0.0001	0.00	n.s	0.00	n.s	n.s
4-vinylphenol	< 0.0001	n.s	0.00	n.s	0.01	n.s
vanillin	< 0.0001	n.s	n.s	n.s	n.s	n.s
acetovanillone	< 0.0001	n.s	n.s	n.s	n.s	n.s

[a] n.s – not significant

S.d. Table 8 Average concentration of volatiles measured above the limit of quantification in control wines and those spiked with glycosidic precursors from Garnacha grapes in unfermented controls (Acid Hydrolysis) and in wines fermented with S. cerevisiae. P. kluyveri. T. delbrueckii and *L. thermotolerans.*

Precursors	Control				PG			Control				PG				Control				PG			
Yeast				AH				*S. cerevisiae*								*P. kluyveri*							
Aging	0	1	2	5	0	1	2	5	0	1	2	5	0	1	2	5	0	1	2	5	0	1	2	5
Ethyl acetate	0	0	0	159	81.4	113	89.8	47916 ± 25983	40643 ± 400	51373 ± 815	79038 ± 3552	98265 ± 815	103863 ± 1277	105766 ± 8826	111643 ± 2094	115396 ± 3465	53785 ± 53547	134323 ± 6716	126562 ± 6215	188802 ± 1965	181048 ± 3838	171344 ± 4821	154900 ± 482
Isoamyl acetate	0	0	0	0	0	0	0	226 ± 107	407 ± 18	325 ± 8	233 ± 5	377 ± 24	319 ± 27	224 ± 2	3415 ± 147	2998 ± 332	2727 ± 279	1378 ± 17	2997 ± 128	2557 ± 10	2235 ± 100	1191 ± 60
Isobutyl acetate	0	0	0.66	0.63	7.3	4.2	6.1	5.3	94.6	88.6 ± 5.4	96.2 ± 7.0	87.6 ± 4.6	88.2 ± 0.3	83.1 ± 2.9	82.3 ± 0.7	80.6 ± 1.8	219 ± 12	195 ± 10	186 ± 5	171 ± 13	252 ± 5	205 ± 0	197 ± 3	176 ± 7
Phenylethyl acetate	0	0	0	0	0	0	0	0	133	96.9 ± 5.2	150 ± 67	54.5 ± 1.2	150 ± 3	103 ± 0	92.6 ± 4.3	59.9 ± 0.9		9454 ± 676	7253 ± 446	4765 ± 365	9850 ± 94	6837 ± 363	6056 ± 318	3570 ± 354
Ethyl hexanoate	0	0	0	0	49.5 ± 0.5	218 ± 20	192 ± 22	544 ± 4	0.0 ± 0.0	628 ± 40	602 ± 54	595 ± 35	165 ± 13	179 ± 12	236 ± 1	210 ± 39	359 ± 54	618 ± 120	535 ± 39	501 ± 2
Ethyl octanoate	0	0	0	0	123 ± 123	294 ± 124	369 ± 27	929 ± 13	382 ± 82	1038 ± 83	1046 ± 66	1028 ± 17	235 ± 38	198 ± 75	408 ± 44	308 ± 86	199 ± 37	694 ± 42	827 ± 47	793 ± 40
Ethyl decanoate	0	0	0	0	75.7 ± 75.7	104 ± 51	128 ± 11	234 ± 1	109 ± 9	493 ± 37	424 ± 40	128 ± 128	89.2 ± 11.4	50.6 ± 5.5	57.9 ± 57.9	77.5 ± 32.7	109 ± 13	265 ± 96	317 ± 15	155 ± 17
Ethyl isobutyrate	0	0	0	0	55.5	146 ± 9	250 ± 29	386 ± 6	30.8 ± 0.5	117 ± 10	157 ± 3	277 ± 0	35.0 ± 5.2	141 ± 3	216 ± 15	400 ± 44	40.2 ± 5.1	112 ± 11	177 ± 21	320 ± 7
Ethyl isovalerate	0	0	0	0	6.6	21.1 ± 1.5	34.5 ± 2.4	58.8 ± 4.1	4.7 ± 0.1	16.3 ± 1.1	23.8 ± 0.1	51.6 ± 3.4	4.4 ± 0.1	14.8 ± 0.3	26.0 ± 0.1	54.8 ± 4.5	3.6 ± 0.0	13.1 ± 0.8	20.3 ± 0.3	45.6 ± 1.1
Ethyl 2-methylbutyrate	0	0	0	0	5.0	14.9 ± 1.2	24.3 ± 0.1	43.4 ± 2.3	3.6 ± 0.0	11.8 ± 0.8	17.5 ± 0.4	37.5 ± 1.8	4.7 ± 0.3	15.2 ± 0.2	26.4 ± 0.7	53.3 ± 4.2	4.5 ± 0.9	11.1 ± 0.6	17.3 ± 0.6	40.2 ± 0.8
Ethyl lactate	0	0	0	0	597 ± 0	1973 ± 3	2929 ± 12	4638 ± 64	524 ± 20	2027 ± 58	2914 ± 42	5187 ± 84	724 ± 26	2895 ± 38	4954 ± 3	7909 ± 163	771 ± 12	3111 ± 55	4739 ± 220	8145 ± 156
Isobutanol	0	0	0	0	45235 ± 1531	43427 ± 6037	74678 ± 12438	41391 ± 4333	32287 ± 2788	33469 ± 1743	80738 ± 43781	34710 ± 62	50869 ± 6169	46562 ± 4653	72764 ± 21711	46414 ± 1564	45094 ± 1215	60932 ± 20067	86547 ± 11814	43866 ± 750
1-Butanol	0	0	0	0	282 ± 84	319 ± 28	568 ± 91	307 ± 36	361 ± 43	340 ± 8	811 ± 480	354 ± 34	205 ± 20	184 ± 1	290 ± 95	187 ± 14	186 ± 20	290 ± 102	401 ± 45	220 ± 3
Isoamyl alcohol	0	0	0	0	191149 ± 2160	213686 ± 16738	221999 ± 21432	220444 ± 17295	192907 ± 1604	187282 ± 1231	202726 ± 8881	197827 ± 7893	183988 ± 396	180723 ± 8648	188716 ± 4510	185446 ± 91.39	182229 ± 2320	178591 ± 5591	184375 ± 10388	194878 ± 892
1-Hexanol	0	0	0	0	51.1 ± 2.2	31.0 ± 1.0	29.2 ± 3.6	27.0 ± 2.4	127 ± 14	101 ± 4	97.2 ± 2.4	99.6 ± 5.5	32.5 ± 0.6	28.5 ± 0.3	26.5 ± 0.3	24.9 ± 3.5	79.0 ± 10.3	55.0 ± 0.4	59.3 ± 2.0	57.4 ± 1.5
Methionol	0	0	0	0	6215 ± 63	6312 ± 500	7113 ± 223	7042 ± 105	6453 ± 209	7297 ± 18	7650 ± 703	6612 ± 209	9467 ± 187	9795 ± 574	11083 ± 103	11266 ± 208	10042 ± 207	11590 ± 696	11968 ± 402	12035 ± 82
β-Phenylethanol	0	0	0	0	43093 ± 6427	28107 ± 972	29620 ± 2488	32684 ± 5416	30153 ± 1324	33933 ± 112	36173 ± 4712	34833 ± 2229	30452 ± 1547	26911 ± 655	26205 ± 643	30113 ± 165	27534 ± 803	34197 ± 423	34468 ± 2013	30689 ± 422
γ-Butyrolactone	0.79	0	0.67	0.56	0.97	0.95	1.43	1.54	7.6	8.1 ± 0.2	14.5 ± 6.9	7.6 ± 0.1	10.0 ± 0.3	9.1 ± 0.4	9.4 ± 0.3	9.0 ± 0.1	8.6 ± 0.1	7.7 ± 0.2	8.2 ± 0.1	8.3 ± 0.5	10.4 ± 0.1	10.0 ± 0.1	10.1 ± 0.1	9.9 ± 0.4
γ-Nonalactone	0	0	0	0	1128 ± 192	6520 ± 8	8341 ± 622	9626 ± 48	1384 ± 121	6994 ± 90	8923 ± 562	10098 ± 234	870 ± 38	4026 ± 76	5497 ± 197	6007 ± 123	972 ± 21	4820 ± 357	6143 ± 371	7403 ± 443
γ-decalactone	0	0	0	0	4.9	5.1 ± 0.0	9.5 ± 4.9	4.6 ± 0.1	5.1 ± 0.4	4.3 ± 0.1	4.1 ± 0.1	4.3 ± 0.0	6.7 ± 0.1	6.2 ± 0.2	6.2 ± 0.0	6.3 ± 0.4	6.8 ± 0.1	6.2 ± 0.0	6.0 ± 0.4	5.8 ± 0.0
Butyric acid	0	0	0	0	652 ± 107	520 ± 18	543 ± 70	504 ± 10	660 ± 26	541 ± 2	646 ± 52	675 ± 32	4020 ± 197	2777 ± 215	2718 ± 174	1887 ± 335	3421 ± 103	3180 ± 213	2767 ± 246	1476 ± 109
Isobutyric acid	0	0	0	0	4618 ± 2256	2467 ± 80	2467 ± 200	2548 ± 117	1831 ± 162	1796 ± 11	1902 ± 102	1905 ± 48	2732 ± 127	2480 ± 154	2588 ± 128	2701 ± 194	2113 ± 135	2145 ± 139	2232 ± 53	2032 ± 72
Hexanoic acid	0	0	0	0	1257 ± 826	1339 ± 5	1413 ± 86	1044 ± 40	1905 ± 83	1139 ± 26	1227 ± 65	1140 ± 28	1874 ± 31	1382 ± 109	1304 ± 155	1443 ± 170	1652 ± 6	1048 ± 39	1068 ± 51	1042 ± 12
Octanoic acid	0	0	0	0	3121 ± 2071	3658 ± 68	4202 ± 174	3590 ± 26	5384 ± 158	3851 ± 198	4451 ± 643	4107 ± 18	4965 ± 71	4112 ± 152	3942 ± 438	4264 ± 470	4853 ± 176	3847 ± 46	3946 ± 13	3235 ± 16
Decanoic acid	0	0	0	0	333 ± 333	752 ± 304	961 ± 82	1191 ± 12	727 ± 188	961 ± 237	991 ± 13	1349 ± 157	676 ± 12	479 ± 1	1146 ± 135	1087 ± 216	655 ± 8	632 ± 130	825 ± 10	908 ± 76
TDN	0	0	0	0	0.5	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.0	8.4 ± 0.5	18.8 ± 1.6	75.9 ± 6.5	0.7 ± 0.1	0.3 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.1	7.1 ± 1.6	19.1 ± 2.1	66.3 ± 4.9
β-damascenone	0	0	0	0	0.4	0.2 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	2.1 ± 0.1	3.4 ± 0.1	3.9 ± 0.4	4.5 ± 0.3		0.2 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	2.2 ± 0.1	4.1 ± 0.0	1.4 ± 1.4	4.1 ± 0.1
β-ionone	0	0	0	0	0.6	0.6 ± 0.1	0.3 ± 0.3	0.2 ± 0.0	0.5 ± 0.0	0.7 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.8 ± 0.0	0.3 ± 0.0	0.5 ± 0.3	0.4 ± 0.3	0.5 ± 0.0	0.6 ± 0.2	0.5 ± 0.2	0.1 ± 0.0
Ethyl cinnamate	0	0	0	0	0.0	0.0 ± 0.0	8.6 ± 8.6	1.1 ± 0.1	0.0 ± 0.0	0.2 ± 0.0	0.6 ± 0.1	1.6 ± 0.2	0.0 ± 0.0	0.1 ± 0.0	0.5 ± 0.2	0.0 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.7 ± 0.4	1.0 ± 0.4
Linalool	0	0	0	1.5	9.6	12.7	6.9	3.3	2.3 ± 0.1	1.0 ± 0.2	0.8 ± 0.0	8.2 ± 0.0	18.4 ± 1.2	12.8 ± 0.1	2.8 ± 0.1	2.4 ± 0.1	2.0 ± 0.1	1.7 ± 0.3	0.8 ± 0.5	7.1 ± 0.1	19.9 ± 0.2	13.1 ± 0.3	2.9 ± 0.1
α-terpineol	0	0	0	0	7.4	19.5	23.0	1.1	2.4 ± 0.1	1.6 ± 0.2	3.0 ± 0.0	20.5 ± 0.8	25.1 ± 2.0	23.8 ± 0.5	0.7 ± 0.1	1.5 ± 0.0	2.0 ± 0.1	1.8 ± 0.1	2.7 ± 0.1	18.1 ± 0.3	25.2 ± 0.3	26.7 ± 0.3
β-citronellol	0	0	0	0	0	0	0	2.6	1.8 ± 0.1	1.1 ± 0.1	0.3 ± 0.0	4.9 ± 0.2	3.5 ± 0.2	2.6 ± 0.1	0.9 ± 0.1	2.2 ± 0.2	1.4 ± 0.1	1.1 ± 0.1	0.4 ± 0.2	3.4 ± 0.1	2.6 ± 0.1	1.9 ± 0.2	0.8 ± 0.0
Geraniol	1	0	1	1	2	3	5	3	1.8	0.9 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.5 ± 0.2	5.2 ± 0.3	3.6 ± 0.2	1.7 ± 0.0	2.5 ± 0.0	1.3 ± 0.1	1.1 ± 0.0	0.8 ± 0.8	4.4 ± 0.2	5.8 ± 0.2	4.2 ± 0.4	2.3 ± 0.2
Guaiacol	0	0	0	0	0	1	1	2	0.2	0.2 ± 0.0	0.1 ± 0.0	4.3 ± 0.3	4.2 ± 0.1	5.1 ± 0.9	5.8 ± 0.6	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	3.2 ± 0.5	2.4 ± 0.2	4.0 ± 0.3	6.3 ± 0.5
4-vinylguaiacol	12.7	5.6	5.8	16.5	77.6	127	481	649	20.2	16.3 ± 3.1	18.8 ± 0.6	6.2 ± 1.3	720 ± 5	1588 ± 49	2045 ± 236	2516 ± 123	5.7 ± 1.3	13.7 ± 1.8	12.7 ± 4.3	21.9 ± 0.7	512 ± 59	1277 ± 87	1999 ± 5	2731 ± 75
2,4-dimethoxyphenol	2	0	0	0	0	0.9	3.1	7.0	0.2	0.3 ± 0.1	0.5 ± 0.3	1.0 ± 0.8	11.2 ± 2.6	13.8 ± 3.7	18.4 ± 7.7	19.8 ± 1.7	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.8 ± 0.3	8.3 ± 0.9	4.9 ± 1.0	9.9 ± 0.7	20.2 ± 3.4
E-isoeugenol	0	0	0	0	0	0	0.6	0.7	0.0	0.0 ± 0.0	0.0 ± 0.0	2.5 ± 0.0	2.8 ± 0.3	2.7 ± 0.4	2.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.3 ± 0.3	2.2 ± 0.2	2.8 ± 0.0	2.8 ± 0.1
4-vinylphenol	9.7	4.1	5.3	8.9	38.6	123	264	324	21.5	9.7 ± 9.7	19.9 ± 0.7	9.3 ± 1.8	424 ± 5	569 ± 116	598 ± 174	525 ± 5	11.6 ± 2.7	17.8 ± 1.3	18.3 ± 4.6	22.0 ± 0.1	270 ± 36	346 ± 10	477 ± 24	578 ± 26
Vanillin	10.2	0.6	2.0	1.7	6.9	8.9	21.2	36.8	1.5	0.7 ± 0.1	2.3 ± 1.0	5.7 ± 3.7	10.8 ± 0.7	36.6 ± 23.8	21.5 ± 2.4	20.5 ± 1.2	1.0 ± 0.2	0.8 ± 0.1	1.3 ± 0.4	2.6 ± 1.5	9.4 ± 0.5	9.6 ± 0.2	13.9 ± 4.2	21.5 ± 0.6
Acetovanillone	7.5	1.2	1.1	0.9	53.2	4.2	6.4	12.9	2.5	1.2 ± 0.3	1.5 ± 0.8	3.3 ± 3.3	126 ± 45	194 ± 17	186 ± 6	166 ± 5	0.7 ± 0.1	1.4 ± 0.7	1.0 ± 0.3	2.6 ± 0.5	170 ± 10	131 ± 16	145 ± 34	185 ± 4

S.d. Table 8 (cont) Average concentration of volatiles measured above the limit of quantification in control wines and those spiked with glycosidic precursors from Garnacha grapes in unfermented controls (Acid Hydrolysis) and in wines fermented with S. cerevisiae. P. kluyveri. T. delbrueckii and L. thermotolerans.

Precursors	Control				PG				Control				PG			
Yeast	*T. delbrueckii*								*L. thermotolerans*							
Aging	0	1	2	5	0	1	2	5	0	1	2	5	0	1	2	5
Ethyl acetate	47032 ± 24509	75440 ± 1213	91377 ± 61	92847 ± 577	166213 ± 2999	155637 ± 7184	148887 ± 15598	127574 ± 458	59220 ± 56	66765 ± 299	67184 ± 1116	75513 ± 265	140554 ± 504	135488 ± 307	131983 ± 1955	100561 ± 177
Isoamyl acetate	295 ± 182	132 ± 1	122 ± 1	109 ± 8	111 ± 18	144 ± 0	137 ± 3	133 ± 2	217 ± 13	181 ± 7	150 ± 0	113 ± 4	233 ± 33	207 ± 0	167 ± 9	1901 ± 1784
Isobutyl acetate	88.6 ± 1.6	90.5 ± 1.4	87.6 ± 0.3	111 ± 6	140 ± 45	92.5 ± 0.2	98.2 ± 7.8	118 ± 4	66.4 ± 0.6	61.5 ± 2.6	62.2 ± 3.2	57.9 ± 3.2	67.4 ± 0.4	59.9 ± 3.7	60.4 ± 6.9	68.4 ± 5.6
Phenylethyl acetate	320 ± 9	327 ± 83	188 ± 1	142 ± 3	398 ± 139	351 ± 158	168 ± 5	116 ± 5	57.0 ± 7.3	41.2 ± 2.2	37.3 ± 2.5	36.0 ± 11.9	48.2 ± 0.3	57.0 ± 17.7	34.8 ± 1.7	32.4 ± 0.7
Ethyl hexanoate	115 ± 115	95.5 ± 6.5	148 ± 25	103 ± 2	0.0 ± 0.0	135 ± 19	137 ± 41	107 ± 24	48.2 ± 4.7	55.6 ± 1.1	25.2 ± 25.2	54.2 ± 0.2	57.2 ± 57.2	159 ± 2	152 ± 33	144 ± 30
Ethyl octanoate	268 ± 210	167 ± 7	167 ± 19	177 ± 21	63.8 ± 16.8	154 ± 4	157 ± 33	135 ± 1	93.1 ± 53.2	51.2 ± 8.0	51.4 ± 5.4	63.6 ± 8.1	180 ± 132	181 ± 18	134 ± 6	193 ± 45
Ethyl decanoate	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	49.5 ± 4.9	30.8 ± 3.4	34.2 ± 0.6	61.9 ± 38.1	59.3 ± 14.5	137 ± 40	54.8 ± 54.8	118 ± 27
Ethyl isobutyrate	114 ± 4	357 ± 15	505 ± 24	1011 ± 149	132 ± 36	296 ± 0	435 ± 4	816 ± 4	47.9 ± 1.6	139 ± 11	175 ± 36	327 ± 21	38.9 ± 2.1	78.6 ± 64.1	154 ± 19	371 ± 88
Ethyl isovalerate	2.2 ± 2.2	10.7 ± 0.4	16.8 ± 0.9	34.8 ± 2.6	5.2 ± 1.6	9.9 ± 0.1	15.6 ± 0.2	32.9 ± 1.4	2.3 ± 0.2	8.1 ± 0.0	13.2 ± 0.0	26.7 ± 1.8	1.7 ± 0.0	7.2 ± 0.2	11.8 ± 0.7	27.4 ± 0.2
Ethyl 2-methylbutyrate	4.6 ± 0.0	14.1 ± 0.8	21.8 ± 1.1	45.6 ± 2.9	5.7 ± 1.6	12.3 ± 0.4	20.7 ± 0.1	41.6 ± 0.9	2.9 ± 0.2	8.5 ± 0.4	14.2 ± 0.0	28.8 ± 0.7	2.1 ± 0.0	9.2 ± 0.1	12.7 ± 0.2	28.6 ± 0.8
Ethyl lactate	545 ± 54	2442 ± 21	3745 ± 28	6199 ± 11	662 ± 74	2531 ± 94	3882 ± 141	6348 ± 83	16111 ± 817	75862 ± 685	115387 ± 5327	186688 ± 7348	11845 ± 769	63983 ± 1630	95974 ± 2571	146020 ± 9999
Isobutanol	46525 ± 2868	75502 ± 20250	42812 ± 1791	45437 ± 1610	47816 ± 456	45798 ± 1892	44777 ± 335	50035 ± 4681	28429 ± 918	46129 ± 19150	59208 ± 32857	45903 ± 20089	24362 ± 3697	28357 ± 3159	24301 ± 1321	28164 ± 1491
1-Butanol	314 ± 61	391 ± 108	200 ± 5	219 ± 16	300 ± 5	270 ± 8	275 ± 26	319 ± 27	400 ± 9	643 ± 245	862 ± 474	700 ± 299	436 ± 1	448 ± 90	389 ± 3	474 ± 41
Isoamyl alcohol	199904 ± 33337	167528 ± 3780	169586 ± 8688	165996 ± 3231	165229 ± 782	153452 ± 2168	160373 ± 1153	159103 ± 1830	152611 ± 307	149919 ± 4033	156226 ± 7191	152385 ± 2096	145238 ± 6798	140945 ± 8614	147328 ± 9539	146940 ± 3959
1-Hexanol	49.7 ± 15.2	49.0 ± 0.8	49.1 ± 2.6	47.8 ± 4.4	149 ± 27	107 ± 10	108 ± 0	110 ± 2	33.3 ± 0.4	40.0 ± 3.8	52.8 ± 11.5	38.0 ± 10.7	133 ± 1	113 ± 1	120 ± 11	131 ± 3
Metionol	6110 ± 9	8165 ± 167	8098 ± 203	8572 ± 224	6363 ± 4	8104 ± 292	9006 ± 325	8776 ± 48	4911 ± 411	6167 ± 11	6519 ± 67	5753 ± 56	5324 ± 485	5890 ± 394	6072 ± 49	6112 ± 39
β-Phenylethanol	39683 ± 6635	57655 ± 5942	52489 ± 1223	59920 ± 6367	35655 ± 1161	45883 ± 2002	47021 ± 1223	45122 ± 1393	28106 ± 1776	25719 ± 68	27636 ± 789	22502 ± 1023	26220 ± 566	31365 ± 1782	30121 ± 2571	30003 ± 393
γ-Butyrolactone	1061 ± 110	3325 ± 133	3987 ± 169	4650 ± 311	919 ± 37	3213 ± 79	4194 ± 144	4543 ± 66	1548 ± 14	5418 ± 513	6681 ± 371	6665 ± 12	1626 ± 40	5445 ± 307	6666 ± 25	7160 ± 127
γ-nonalactone	9.8 ± 0.2	13.1 ± 4.9	8.6 ± 0.3	8.3 ± 0.3	17.7 ± 6.0	20.9 ± 10.8	10.2 ± 0.3	10.4 ± 0.5	8.1 ± 0.1	7.1 ± 0.1	6.7 ± 0.2	9.9 ± 3.1	9.1 ± 0.0	13.5 ± 5.2	7.7 ± 0.2	8.2 ± 0.1
γ-decalactone	8.4 ± 0.3	10.9 ± 3.9	7.0 ± 0.1	7.1 ± 0.3	11.5 ± 3.8	12.7 ± 6.2	6.2 ± 0.1	6.7 ± 0.3	33.9 ± 3.0	29.4 ± 0.4	28.2 ± 0.3	38.1 ± 11.2	31.7 ± 0.2	44.1 ± 17.7	26.4 ± 0.3	27.8 ± 0.1
Butyric acid	639 ± 73	397 ± 32	362 ± 35	494 ± 95	540 ± 29	426 ± 68	499 ± 52	445 ± 54	373 ± 12	329 ± 13	381 ± 54	269 ± 15	423 ± 100	275 ± 22	339 ± 24	289 ± 2
Isobutyric acid	4645 ± 1870	7041 ± 179	6593 ± 288	7413 ± 767	6339 ± 283	5603 ± 340	6046 ± 374	5682 ± 189	2012 ± 55	2195 ± 92	2466 ± 35	2765 ± 193	2098 ± 174	2048 ± 137	2137 ± 221	2298 ± 225
Hexanoic acid	1110 ± 698	263 ± 2	255 ± 6	231 ± 0	395 ± 26	262 ± 7	258 ± 13	253 ± 8	498 ± 43	409 ± 2	407 ± 12	330 ± 26	509 ± 57	274 ± 30	295 ± 31	279 ± 8
Octanoic acid	2696 ± 1634	922 ± 37	732 ± 7	871 ± 4	888 ± 120	728 ± 123	731 ± 10	672 ± 26	1263 ± 134	1138 ± 35	1093 ± 64	909 ± 112	1227 ± 80	886 ± 56	827 ± 29	12884 ± 12019
Decanoic acid	419 ± 371	96.2 ± 1.5	98.3 ± 2.3	151 ± 11	19.9 ± 19.9	105 ± 0	110 ± 0	161 ± 16	505 ± 31	585 ± 99	563 ± 26	736 ± 120	1096 ± 115	1316 ± 393	824 ± 39	1254 ± 318
TDN	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	12.8 ± 5.3	21.4 ± 0.9	70.7 ± 10.9	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	20.7 ± 6.2	39.3 ± 0.4	136 ± 9
β-damascenone	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.6	4.6 ± 2.1	2.9 ± 0.2	3.7 ± 0.1	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.1 ± 0.3	4.7 ± 1.4	3.9 ± 0.4	4.6 ± 0.1
β-ionone	0.6 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	0.8 ± 0.2	0.3 ± 0.3	0.2 ± 0.0	0.1 ± 0.0	0.8 ± 0.1	0.5 ± 0.3	0.1 ± 0.1	0.2 ± 0.0	0.4 ± 0.0	0.1 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
Ethyl cinnamate	0.0 ± 0.0	1.2 ± 0.7	0.6 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	3.0 ± 2.7	0.7 ± 0.2	1.2 ± 0.4	0.0 ± 0.0	0.1 ± 0.1	1.6 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.2 ± 2.0	1.0 ± 0.4	1.0 ± 0.3
Linalool	3.3 ± 0.0	3.6 ± 0.4	2.3 ± 0.1	0.8 ± 0.1	13.9 ± 4.6	20.2 ± 0.4	11.9 ± 0.5	2.9 ± 0.1	3.4 ± 0.1	2.6 ± 0.2	1.3 ± 0.1	0.5 ± 0.1	10.1 ± 0.3	15.3 ± 0.1	8.3 ± 1.3	1.7 ± 0.0
α-terpineol	1.2 ± 0.0	3.0 ± 0.2	3.5 ± 0.1	3.4 ± 0.0	4.7 ± 1.6	22.7 ± 1.3	26.9 ± 0.8	25.5 ± 0.1	1.2 ± 0.1	2.6 ± 0.5	3.0 ± 0.1	1.8 ± 0.1	3.8 ± 0.0	26.6 ± 2.6	27.6 ± 1.0	21.7 ± 0.2
β-citronellol	2.7 ± 0.1	2.0 ± 0.4	1.2 ± 0.1	0.3 ± 0.3	6.7 ± 2.3	3.4 ± 0.0	2.2 ± 0.0	0.9 ± 0.1	3.3 ± 0.3	1.9 ± 0.0	1.2 ± 0.0	0.2 ± 0.2	4.6 ± 0.1	3.1 ± 0.3	2.2 ± 0.3	0.7 ± 0.0
Geraniol	5.9 ± 0.5	2.2 ± 0.7	1.3 ± 0.4	0.6 ± 0.6	13.3 ± 4.4	7.0 ± 0.9	3.7 ± 0.7	1.4 ± 0.4	3.9 ± 0.0	1.2 ± 0.1	0.4 ± 0.4	0.0 ± 0.0	6.0 ± 0.4	5.0 ± 0.1	2.9 ± 0.3	0.5 ± 0.5
Guaiacol	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	3.2 ± 0.4	1.9 ± 0.0	3.0 ± 0.5	4.4 ± 0.2	0.2 ± 0.0	0.2 ± 0.1	0.7 ± 0.1	0.2 ± 0.0	2.2 ± 0.7	2.8 ± 0.1	3.6 ± 0.9	5.6 ± 0.2
4-vinylguaiacol	19.2 ± 11.8	13.0 ± 2.4	10.0 ± 2.1	14.9 ± 5.3	787 ± 188	1453 ± 208	2133 ± 204	2657 ± 13	17.5 ± 0.3	19.9 ± 3.8	48.4 ± 25.2	10.7 ± 2.7	582 ± 5	1305 ± 32	1644 ± 54	2113 ± 120
2-6-dimethoxyphenol	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	9.6 ± 1.1	5.0 ± 0.8	10.6 ± 3.1	15.9 ± 0.1	0.8 ± 0.4	0.4 ± 0.4	2.4 ± 0.0	0.0 ± 0.0	8.3 ± 1.6	6.6 ± 1.0	13.5 ± 5.5	19.0 ± 0.3
E-isoeugenol	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.1 ± 1.3	3.1 ± 0.4	3.1 ± 0.2	3.2 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 0.0	1.8 ± 0.1	1.6 ± 0.1	2.0 ± 0.2
4-vinylphenol	23.0 ± 3.4	18.6 ± 2.9	11.5 ± 1.0	0.0 ± 0.0	403 ± 149	447 ± 128	770 ± 290	560 ± 17	20.5 ± 4.3	25.5 ± 7.9	206 ± 119	0.0 ± 0.0	343 ± 7	331 ± 5	698 ± 277	491 ± 12
Vanillin	1.0 ± 0.3	0.5 ± 0.3	3.2 ± 1.6	2.0 ± 0.0	13.0 ± 4.1	9.3 ± 0.8	25.8 ± 3.2	11.5 ± 11.5	2.7 ± 1.7	1.2 ± 0.3	1.1 ± 1.1	1.2 ± 0.6	22.4 ± 2.1	8.1 ± 0.3	15.4 ± 2.3	20.1 ± 3.2
Acetovanillone	1.5 ± 0.9	0.0 ± 0.0	2.0 ± 0.8	1.2 ± 1.2	212 ± 78	71.7 ± 40.1	172 ± 3	102 ± 60	2.9 ± 2.0	1.0 ± 0.1	0.9 ± 0.9	0.8 ± 0.8	163 ± 1	32.6 ± 2.5	165 ± 3	92.6 ± 60.1

S.d. Table 9 Average concentration of volatile compounds according to the yeast strain or acidic hydrolysis controls of wines with and without Garnacha precursors. Letters a-d are the results of Duncan post-hoc test; compounds with different letters indicate significant differences

	AH	*P. kluyveri*	*S. cerevisiae*	*T. delbrueckii*	*L. thermotolerans*
Ethyl acetate	55.4 d	144395 a	79313 c	113126 b	97159 bc
Isoamyl acetate	0 c	2298 a	311 bc	148 bc	396 b
Ethyl hexanoate	0 c	377 a	354 a	105 b	86.9 bc
Ethyl octanoate	0 d	490 b	651 a	161 c	118 c
Ethyl decanoate	0 c	147 ab	212 a	0 c	68.1 bc
Ethyl isobutyrate	0.2 c	201 b	178 b	458 a	166 b
Ethyl 2-methylbutyrate	0 d	24 a	19.8 b	20.8 b	13.4 c
Ethyl isovalerate	0 d	25.5 a	27.2 a	16 b	12.3 c
Isobutyl acetate	3 e	197 a	87.6 c	103 b	63 d
Phenylethyl acetate	0.1 b	6826 a	105 b	251 b	42.8 b
Isobutanol	0 d	57426 a	48253 b	49838 ab	35606 c
1-Butanol	0 d	251 c	418 b	286 c	544 a
Isoamyl alcohol	0 e	185508 b	203502 a	167646 c	148949 d
1-Hexanol	0.01 d	47.3 c	70.3 b	83.8 a	82.7 a
Metionol	0 e	11111 a	6837 c	7899 b	5844 d
β-Phenylethanol	0 d	30017 c	33575 b	47929 a	27709 c
Ethyl lactate	0 b	4646 b	2599 b	3294 b	88984 a
Butyric acid	0 d	2604 a	593 b	475 b	335 c
Isobutyric acid	0 c	2327 b	2442 b	6170 a	2252 b
Hexanoic acid	0 c	1277 a	1311 a	378 b	375 b
Octanoic acid	0c	4028 a	4046 a	1030 bc	2529 ab
Decanoic acid	0 c	819 a	908 a	145 b	860 a
γ-nonalactone	0.9 c	9.2 b	9.4 b	12.4 a	8.8 b
γ-decalactone	0.1 c	6.2 b	5.2 b	8.8 b	32.4 a
γ-Butyrolactone	0 e	4981 c	6627 a	3236 d	5151 b
TDN	4 a	13.4 a	13.1 a	13.1 a	24.5 a
β-damascenone	2.3 a	1.7 a	1.8 a	1.5 a	2 a
β-ionone	0.2 b	0.4 a	0.4 a	0.3 ab	0.3 ab
Ethyl cinnamate	0.1 a	0.4 a	1.5 a	0.8 a	0.7 a
Linalool	3.9 a	6.8 a	6.2 a	7.4 a	5.4 a
α-terpineol	6.3 a	11.1 a	9.9 a	11.4 a	11 a
β-citronellol	0.1 c	1.7 b	2.2 ab	2.4 a	2.2 ab

Geraniol	1.877 b	2.832 b	2.090 b	4.423 a	2.486 b
Guaiacol	0.511 b	2.352 a	2.508 a	1.631 a	1.943 a
4-vinylguaiacol	171.828 b	938.134 a	866.362 a	885.829 a	717.636 a
2-6-dimethoxyphenol	1.841 b	6.373 a	8.140 a	5.182 ab	6.373 a
E-isoeugenol	0.247 d	1.440 b	1.306 b	1.711 a	0.864 c
4-vinylphenol	97.249 b	247.035 a	272.127 a	279.034 a	264.342 a
vanillin	11.057 a	8.445 a	12.465 a	8.276 a	9.035 a
acetovanillone	10.937 b	90.920 a	85.067 a	70.348 a	57.372 a

5. Supplementary data from Section III: Chapter 2

S.d. Table 10 Average content of volatile odorants quantified in Riesling wine from harvest 2015, from five different vineyards handpicked aseptically. Wines referred as vineyards were fermented under aseptic conditions to assess the effect of vineyard on the aroma composition and wines referred as wineries were harvest at the same sites but fermented in the respective wineries to evaluate the effect of setting during fermentation.

	Vineyards				Wineries				
Compound	WB1A	WB2A	WB3A	WB5A	Wgt1	Wgt2	Wgt3	Wgt4	Wgt5
Acetate Esters									
Ethyl acetate	55497 ± 4868	57968 ± 2488	49576 ± 1435	67079 ± 2464	55883,2	87078,3	92977,3	74982,7	62243,4
Isoamyl acetate	961 ± 92	641 ± 41	512 ± 17	223 ± 34	2378,6	861,1	1829,1	978,4	938,4
Hexyl acetate	138 ± 31	50.1 ± 4.8	48.0 ± 4.9	274 ± 24	12,1	0,0	0,0	14,8	31,8
Isobutyl acetate	13.4 ± 1.6	12.0 ± 1.1	12.1 ± 0.6	11.8 ± 1.0	11,2	10,2	11,6	8,1	10,7
Butyl acetate	8.7 ± 0.9	8.7 ± 1.7	1.4 ± 0.3	3.6 ± 0.7	8,4	5,5	10,5	6,7	4,6
Phenylethyl acetate	75.2 ± 1.1	48.3 ± 3.0	28.4 ± 1.4	14.5 ± 2.7	74,7	135,7	66,3	44,9	55,7
Ethyl esters									
Ethyl propanoate	49.5 ± 35.0	0.0 ± 0.0	0.0 ± 0.0	44.2 ± 31.3	0,0	65,3	77,8	0,0	0,0
Ethyl butyrate	220 ± 2	134 ± 12	152 ± 9	170 ± 11	336,1	180,6	328,6	220,2	287,2
Ethyl hexanoate	633 ± 125	572 ± 94	1369 ± 60	2058 ± 252	2491,1	1799,7	3196,2	2127,3	3071,8
Ethyl octanoate	740 ± 317	878 ± 235	1715 ± 87	2374 ± 209	2497,1	2145,7	2324,6	3255,1	2796,2
Ethyl decanoate	68.0 ± 36.4	96.3 ± 23.9	246 ± 40	267 ± 24	297,0	269,0	135,2	295,1	215,9
Ethyl isobutyrate	42.3 ± 1.5	41.3 ± 4.4	46.8 ± 5.0	42.7 ± 0.7	11,8	38,8	16,6	53,5	24,4
Ethyl 2-methylbutyrate	1.1 ± 0.1	1.7 ± 0.1	1.6 ± 0.2	2.4 ± 0.2	0,0	2,2	1,2	1,9	1,5
Ethyl isovalerate	3.0 ± 0.3	6.4 ± 0.3	4.5 ± 0.3	7.2 ± 0.9	1,3	8,2	3,7	6,9	5,4
Miscelaneous esters									
Ethyl lactate	19517 ± 411	8142 ± 192	6236 ± 193	5274 ± 297	67817,9	124695,3	121062,4	114919,0	123751,4
Diethyl succinate	1077 ± 52	1183 ± 95	799 ± 39	1129 ± 58	3227,6	1751,2	510,0	757,2	625,8
Fusel alcohols									
Isobutanol	23235 ± 1444	13818 ± 997	13859 ± 327	15144 ± 500	14424,3	11549,5	12633,4	13870,8	8916,6
1-Butanol	512 ± 19	512 ± 38	586 ± 45	808 ± 108	1291,3	742,1	1895,6	1037,1	677,4
Isoamyl alcohol	138317 ± 2376	131111 ± 6206	104765 ± 2671	100462 ± 7387	120011,7	111426,4	136635,7	118682,9	121572,8
1-Hexanol	1015 ± 7	1001 ± 58	815 ± 23	1037 ± 20	2208,1	1277,3	780,5	1243,4	883,0
c-3-Hexenol	19.5 ± 0.2	0.0 ± 0.0	14.9 ± 1.1	28.7 ± 0.9	76,0	37,4	28,5	28,9	30,0
Metionol	689 ± 37	1118 ± 180	368 ± 19	220 ± 11	253,6	458,3	243,2	358,6	398,7
Benzylic alcohol	143 ± 3	20.2 ± 2.0	13.2 ± 1.3	8.0 ± 0.8	31,5	121,1	17,7	52,9	82,8
β-Phenylethanol	15081 ± 782	19455 ± 1356	15767 ± 526	12566 ± 2313	12175,5	47968,2	13709,4	12368,7	15801,3
Acids									
Butyric acid	692 ± 14	479 ± 9	530 ± 26	779 ± 18	1069,4	740,7	1222,0	921,0	1364,4
Isobutyric acid	472 ± 8	399 ± 16	400 ± 20	341 ± 33	652,5	930,7	727,7	1169,8	788,3
Isovalerianic acid	226 ± 16	364 ± 30	275 ± 18	300 ± 19	257,9	525,8	377,3	558,5	369,3
Hexanoic acid	5434 ± 211	3229 ± 338	3060 ± 117	3352 ± 227	5419,9	3579,5	3806,0	3622,3	4450,3
Octanoic acid	13240 ± 370	8561 ± 304	10354 ± 23	10282 ± 468	15737,4	13579,1	11400,0	12692,0	12819,9
Decanoic acid	2500 ± 707	2360 ± 149	2851 ± 133	2009 ± 304	3321,7	2271,5	2146,7	2909,2	2151,7
Monoterpenes									
Linalool	72.9 ± 3.1	59.4 ± 0.7	60.3 ± 1.0	16.1 ± 0.6	110,1	83,2	79,5	82,4	49,4
Linalool acetate	0.8 ± 0.1	1.0 ± 0.2	0.2 ± 0.0	0.8 ± 0.2	0,3	0,3	1,0	0,5	0,7
α-Terpineol	53.6 ± 3.0	49.7 ± 0.9	54.2 ± 0.8	32.2 ± 0.4	45,7	69,9	55,5	54,2	48,2
β-Citronelol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.8 ± 0.1	3,8	2,1	4,5	4,2	6,7
Geraniol	10.3 ± 0.8	7.6 ± 0.9	7.7 ± 0.6	4.5 ± 0.6	18,1	11,0	7,8	13,9	7,2
Norisoprenoids									
β-Damascenone	8.2 ± 1.1	6.1 ± 0.4	8.4 ± 0.4	7.7 ± 1.9	9,4	6,7	10,9	7,8	8,6
α-Ionone	0.7 ± 0.0	0.5 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1,5	0,8	0,7	0,0	0,0
β-Ionone	0.3 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0,0	0,0	0,3	0,3	0,3

S.d. Table 10 (cont) Average content of volatile odorants quantified in Riesling wine from harvest 2015, from five different vineyards handpicked aseptically. Wines referred as vineyards were fermented under aseptic conditions to assess the effect of vineyard on the aroma composition and wines referred as wineries were harvest at the same sites but fermented in the respective wineries to evaluate the effect of setting during fermentation.

	Vineyards				Wineries				
Compound	WB1A	WB2A	WB3A	WB5A	Wgt1	Wgt2	Wgt3	Wgt4	Wgt5
Phenols									
Guaiacol	4.3 ± 1.0	3.5 ± 0.4	5.4 ± 0.3	3.6 ± 2.2	17,3	13,2	5,3	11,1	7,9
o-Cresol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,0	0,0	0,0	0,5	0,7
4-Ethylguaiacol	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.2 ± 0.1	0,6	29,0	0,6	0,7	0,4
m-Cresol	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0,3	0,3	0,0	0,0	0,0
Eugenol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,7	6,8	0,0	0,5	0,7
4-Ethylphenol	0.5 ± 0.0	0.7 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0,6	3,6	0,6	0,4	0,4
4-Vinylguaiacol	209 ± 8	136 ± 4	219 ± 12	83.3 ± 17.9	610,6	247,5	255,0	318,9	280,2
E-Isoeugenol	1.8 ± 0.4	2.0 ± 0.0	2.7 ± 0.1	1.9 ± 0.3	3,8	4,4	1,9	3,0	2,4
2,6-Dimethoxyphenol	2.1 ± 0.8	1.6 ± 0.2	3.0 ± 0.3	2.6 ± 1.8	11,8	18,8	3,1	6,9	4,1
4-Vinylphenol	163 ± 6	142 ± 3	248 ± 7	44.4 ± 3.0	1048,4	321,2	390,2	594,5	312,3
4-Alyl-2,6-dimethoxyphenol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,0	1,3	0,0	0,0	0,0
Cinamates									
Ethyl dihidrocinnamate	0.0 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	1.0 ± 0.1	0,5	1,1	0,5	1,2	1,1
Ethyl cinnamate	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3,6	2,1	1,1	1,1	0,0
Lactones									
t-Whiskylactone	2.0 ± 0.1	1.2 ± 0.0	0.2 ± 0.3	0.7 ± 0.5	0,6	29,4	0,0	0,0	0,0
c-Whiskylactone	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,0	84,0	0,0	0,0	0,0
γ-Butyrolactone	4852 ± 126	6474 ± 284	7303 ± 333	7646 ± 50	3285,2	6227,7	4664,5	9548,9	11561,6
γ-Nonalactone	4.5 ± 0.3	2.2 ± 0.0	2.7 ± 0.0	3.1 ± 0.0	4,0	2,7	1,1	4,1	3,6
γ-Decalactone	12.7 ± 1.6	21.8 ± 1.8	0.0 ± 0.0	41.7 ± 4.1	20,3	21,1	40,4	17,4	21,4
Vanillin derivates									
Vanillin	5.6 ± 0.5	4.5 ± 0.0	4.6 ± 0.3	5.6 ± 0.8	5,1	6,8	4,8	8,7	6,7
Methyl vanillinate	56.1 ± 4.3	39.5 ± 1.4	50.9 ± 1.8	48.5 ± 2.4	84,6	65,2	44,7	86,7	89,7
Ethyl vanillate	1.0 ± 0.7	0.7 ± 0.1	1.6 ± 0.2	0.5 ± 0.4	18,5	3,4	1,1	4,5	11,6
Acetovanillone	34.7 ± 1.8	26.9 ± 0.1	36.1 ± 1.2	34.0 ± 0.6	84,0	56,5	36,1	78,2	103,5
Aldehydes									
Benzaldehyde	0.6 ± 0.4	0.7 ± 0.1	0.6 ± 0.0	1.3 ± 1.0	5,6	1,6	1,2	3,8	3,7
Isobutyraldehyde	7.7 ± 0.2	6.7 ± 0.5	5.9 ± 0.2	8.8 ± 0.7	4,5	7,1	6,5	5,2	6,8
Isovaleraldehyde	8.2 ± 0.4	15.1 ± 1.4	11.4 ± 0.6	15.1 ± 1.9	17,0	15,2	12,0	12,4	40,8
2-methylbutanal	2.6 ± 0.1	3.0 ± 0.1	2.6 ± 0.1	3.1 ± 0.4	2,9	2,7	3,1	2,5	3,7
Methional	3.7 ± 0.3	4.9 ± 0.6	4.2 ± 0.0	2.3 ± 0.4	3,6	5,3	4,1	7,5	6,3
Phenylacetaldehyde	61.4 ± 1.7	61.3 ± 2.6	65.5 ± 5.5	52.1 ± 2.4	52,7	66,7	40,6	71,4	85,7
Polyfunctional mercaptans									
2-metyl-3-furanthiol	1.6 ± 0.2	1.5 ± 0.1	1.3 ± 0.1	1.9 ± 0.9	1,655	1,611	0,444	2,375	1,739
Furfurylthiol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,0	0,011	0,005	0,001	0,003
4-Mercapto-4-methyl-2-pentanona	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,0	0,0	0,0	0,015	0,0
3-mercaptohexyl acetate	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,0	0,0	0,0	0,0	0,0
3-Mercaptohexanol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,037	0,040	0,024	0,040	0,035
Benzylmercaptan	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,001	0,008	0,001	0,000	0,007

S.d. Table 11 Average content of volatile odorants quantified in Riesling wine from harvest 2016, from five different vineyards handpicked aseptically. Wines referred as vineyards were fermented under aseptic conditions to assess the effect of vineyard on the aroma composition and wines referred as wineries were harvest at the same sites but fermented in the respective wineries to evaluate the effect of setting during fermentation.

	Vineyards				Wineries								
Compound	wb1a-16	wb2a-16	wb3a-16	wb5A-16	wgt1-16	wgt2-16	wgt4-16	wgt5-16	wgt6 f77-16	wgt6 t775-16	wgt6 t202	wgt7 t209-16	wgt7 f30-16
Acetate Esters													
Ethyl acetate	27171 ± 2311	31174 ± 607	43823 ± 2122	37677 ± 3913	84319,2	46065,6	46231,6	54812,3	23257,1	23100,7	24926,9	43530,9	38480,5
Isoamyl acetate	503 ± 45	1121 ± 96	1516 ± 63	995 ± 90	1458,2	1775,9	1321,0	2611,8	676,9	679,0	456,3	1448,3	1451,3
Hexyl acetate	123 ± 2	223 ± 24	350 ± 28	308 ± 56	352,3	335,1	273,8	570,1	110,0	80,2	112,5	162,8	173,9
Isobutyl acetate	9.3 ± 0.6	16.4 ± 2.0	24.7 ± 1.0	26.6 ± 2.7	28,4	24,4	21,5	24,0	9,4	10,3	6,8	18,0	19,1
Butyl acetate	1.5 ± 0.5	3.1 ± 0.5	5.3 ± 0.5	0.7 ± 1.0	7,9	5,6	0,0	4,3	0,0	1,3	2,5	0,2	11,9
Phenylethyl acetate	59.7 ± 0.4	86.8 ± 8.3	81.4 ± 1.8	147 ± 14	136,7	96,0	57,8	155,7	50,4	56,0	35,6	64,0	84,3
Ethyl esters													
Ethyl propanoate	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Ethyl butyrate	92.5 ± 3.6	175 ± 41	181 ± 43	114 ± 5	163,3	208,6	172,3	247,9	105,7	146,2	91,4	169,9	168,1
Ethyl hexanoate	671 ± 19	592 ± 421	1012 ± 74	524 ± 348	1181,2	896,3	922,6	1567,7	780,4	693,2	368,2	936,1	936,5
Ethyl octanoate	950 ± 97	1149 ± 64	1094 ± 83	1163 ± 517	1066,0	1386,6	877,8	1324,5	938,8	456,1	380,1	951,9	543,0
Ethyl decanoate	110 ± 26	102 ± 1	131 ± 6	140 ± 71	94,7	169,5	72,4	109,0	91,8	28,7	0,0	81,7	39,0
Ethyl isobutyrate	23.9 ± 0.9	15.6 ± 2.9	15.1 ± 5.5	19.7 ± 5.2	17,0	10,7	11,5	6,8	30,9	31,1	43,4	21,3	17,9
Ethyl 2-methylbutyrate	1.6 ± 0.3	0.8 ± 0.3	1.3 ± 0.1	1.0 ± 0.3	0,4	1,5	0,5	0,5	1,6	2,7	2,9	1,7	0,8
Ethyl isovalerate	2.8 ± 1.5	0.8 ± 1.1	1.5 ± 1.1	0.9 ± 1.0	0,0	2,7	0,4	1,0	4,9	8,1	9,1	3,8	4,6
Miscelaneous esters													
Ethyl lactate	4469 ± 240	11981 ± 2370	8132 ± 2339	4242 ± 562	20528,6	13930,3	4159,9	1260,0	57884,5	22239,6	11194,6	25721,4	5871,6
Diethyl succinate	496 ± 2	407 ± 34	211 ± 2	292 ± 51	2140,6	556,9	305,9	95,8	1065,5	475,1	557,2	407,1	414,3
Fusel alcohols													
Isobutanol	25131 ± 3395	23333 ± 11598	19949 ± 518	27524 ± 2792	22976,1	16539,0	27911,4	12196,6	34154,7	25178,3	37894,1	27104,1	25604,2
1-Butanol	621 ± 104	776 ± 401	871 ± 41	367 ± 18	1445,4	584,9	1437,6	458,4	417,3	348,3	361,8	410,2	643,8
Isoamyl alcohol	120420 ± 832	126929 ± 3443	107351 ± 3216	98344 ± 1624	88936,5	118196,4	131106,0	100278,0	184730,7	173601,8	250179,9	168212,4	159184,1
1-Hexanol	1206 ± 33	1296 ± 47	1152 ± 36	1329 ± 12	1197,0	1471,0	1275,8	1145,5	1284,3	1438,0	1690,8	1228,4	1082,2
c-3-Hexenol	0.0 ± 0.0	15.3 ± 1.6	4.5 ± 6.4	16.3 ± 0.5	40,3	43,4	20,8	25,5	28,7	29,0	14,8	23,7	25,0
Metionol	1347 ± 72	795 ± 47	287 ± 11	663 ± 66	284,1	266,9	318,1	393,0	1369,5	1479,8	1740,7	1170,8	1192,3
Benzylic alcohol	28.1 ± 0.2	32.9 ± 2.8	15.4 ± 4.8	39.2 ± 1.2	38,1	70,8	81,7	120,0	388,1	834,3	457,5	354,6	343,6
β-Phenylethanol	23728 ± 1849	22835 ± 1627	12652 ± 1020	24555 ± 1765	8441,3	14471,3	11944,9	15318,2	28819,1	32557,9	42459,9	21442,8	23044,2
Acids													
Butyric acid	743 ± 5	1036 ± 77	989 ± 43	787 ± 33	1387,5	1237,5	1161,8	1701,3	717,3	808,5	609,0	1169,2	1001,9
Isobutyric acid	491 ± 21	455 ± 102	382 ± 115	340 ± 137	688,2	255,5	342,7	257,8	991,6	913,1	828,6	776,3	446,8
Isovalerianic acid	370 ± 11	320 ± 18	313 ± 23	242 ± 26	213,4	353,5	244,9	353,9	765,9	927,5	1101,6	580,8	511,8
Hexanoic acid	3889 ± 172	5719 ± 351	5430 ± 328	4535 ± 232	6109,8	6733,8	5311,3	8113,0	4252,2	4856,8	2923,3	5342,4	5035,9
Octanoic acid	11897 ± 338	17185 ± 1044	15846 ± 1321	14715 ± 643	14854,8	17355,0	13634,0	20598,0	13034,9	13671,6	7335,4	16278,5	15215,4
Decanoic acid	2521 ± 289	2494 ± 954	2336 ± 308	2282 ± 266	2048,8	1940,9	2242,3	2716,9	2441,7	1591,0	1127,0	2149,6	2019,7
Monoterpenes													
Linalool	85.2 ± 1.6	79.4 ± 2.9	72.4 ± 2.8	28.7 ± 1.3	72,4	92,0	85,7	47,8	114,6	117,2	99,2	76,6	74,1
Linalool acetate	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0,0	0,2	0,0	0,0	0,0	0,2	0,0	0,0	0,0
α-Terpineol	45.4 ± 0.2	34.6 ± 1.5	33.4 ± 1.6	15.8 ± 2.5	33,8	43,2	35,7	20,8	46,4	47,7	41,4	27,3	29,3
β-Citronelol	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.9	0.0 ± 0.0	2,5	0,0	0,0	3,7	0,0	0,0	0,0	0,0	0,9
Geraniol	9.3 ± 1.4	10.4 ± 1.3	7.8 ± 2.9	4.3 ± 0.4	7,4	14,4	8,2	7,7	15,0	11,4	12,2	9,0	9,8
Norisoprenoids													
β-Damascenone	2.2 ± 1.3	1.1 ± 0.7	1.4 ± 0.8	1.6 ± 0.7	5,3	1,7	3,3	4,1	2,3	1,6	2,8	2,0	4,8
α-Ionone	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.4	0.0 ± 0.0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
β-Ionone	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0

S.d. Table 9 (cont) Average content of volatile odorants quantified in Riesling wine from harvest 2016, from five different vineyards handpicked aseptically. Wines referred as vineyards were fermented under aseptic conditions to assess the effect of vineyard on the aroma composition and wines referred as wineries were harvest at the same sites but fermented in the respective wineries to evaluate the effect of setting during fermentation.

	Vineyards				Wineries								
Compound	wb1a-16	wb2a-16	wb3a-16	wb5A-16	wgt1-16	wgt2-16	wgt4-16	wgt5-16	wgt6 f77-16	wgt6 f775-16	wgt6 t202	wgt7 t209-16	wgt7 f30-16
Phenols													
Guaiacol	1.1 ± 0.7	0.9 ± 0.8	0.7 ± 0.6	1.2 ± 0.5	2,9	3,2	1,8	6,0	3,5	2,1	0,8	3,7	10,4
o-Cresol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,0	0,0	0,4	0,0	0,6	0,0	0,6	0,4	0,4
4-Ethylguaiacol	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0,2	2,9	0,4	0,3	0,3	0,1	0,7	1,1	0,2
m-Cresol	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Eugenol	0.2 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0,6	0,6	1,0	1,1	1,2	0,7	1,1	0,8	2,0
4-Ethylphenol	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.7 ± 0.2	0,4	0,4	0,9	1,0	0,5	0,2	0,6	0,4	0,2
4-Vinylguaiacol	343 ± 54	369 ± 91	199 ± 32	331 ± 27	392,2	648,8	597,9	479,3	245,2	411,5	561,0	460,6	188,4
E-Isoeugenol	1.2 ± 0.1	2.6 ± 1.3	1.4 ± 0.1	4.1 ± 0.9	3,8	2,0	5,3	7,8	5,9	1,4	6,0	4,5	3,2
2,6-Dimethoxyphenol	0.4 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,9	0,0	0,0	0,0	0,0	0,0	0,0	0,0	9,8
4-Vinylphenol	139 ± 6	138 ± 5	94.2 ± 10.4	663 ± 87	293,5	288,3	926,0	1013,5	141,3	216,8	225,6	152,5	80,7
4-Alyl-2,6-dimethoxyphenol	0.0 ± 0.0	0.3 ± 0.2	0.1 ± 0.2	0.5 ± 0.1	0,8	0,7	0,6	1,2	1,0	0,7	0,9	1,1	1,1
Cinamates													
Ethyl dihidrocinnamate	0.4 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.3 ± 0.2	0,5	0,3	0,3	1,0	0,6	0,4	0,0	0,0	0,5
Ethyl cinnamate	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.1	0,0	0,0	0,8	1,0	0,0	0,0	0,0	0,0	0,0
Lactones													
t-Whiskylactone	0.0 ± 0.0	0.2 ± 0.2	0.3 ± 0.2	0.0 ± 0.0	0,0	0,6	0,0	0,0	0,0	0,0	0,0	0,0	0,0
c-Whiskylactone	4.6 ± 4.6	2.5 ± 3.6	0.0 ± 0.0	4.2 ± 3.0	5,0	0,0	0,0	9,4	9,2	0,0	2,8	4,8	4,0
γ-Butyrolactone	4289 ± 7	3279 ± 305	3179 ± 222	4921 ± 307	3440,9	3077,3	5355,5	4549,2	6028,3	6069,9	7233,0	4249,7	4180,7
γ-Nonalactone	0.9 ± 0.5	0.7 ± 0.5	0.4 ± 0.6	0.0 ± 0.0	0,6	1,4	0,0	0,0	6,7	10,3	9,2	2,0	3,0
γ-Decalactone	1.9 ± 1.9	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.4	3,1	0,0	0,0	0,0	0,0	0,0	1,4	7,0	0,0
Vanillin derivates													
Vanillin	2.6 ± 1.3	1.8 ± 0.8	2.7 ± 1.8	5.0 ± 2.3	4,9	3,6	10,2	6,4	7,3	4,1	9,9	6,2	8,8
Methyl vanillinate	67.3 ± 2.2	70.7 ± 6.3	68.2 ± 2.2	53.7 ± 1.6	52,8	85,5	87,3	75,7	0,0	107,9	88,5	85,8	88,8
Ethyl vanillate	0.5 ± 0.0	0.7 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	1,3	2,9	1,7	5,4	4,1	4,9	5,5	3,2	2,0
Acetovanillone	26.4 ± 1.3	29.4 ± 2.0	24.2 ± 0.5	24.2 ± 0.6	27,9	44,5	61,8	70,3	54,8	53,3	55,5	48,2	50,3
Aldehydes													
Benzaldehyde	0.5 ± 0.0	1.0 ± 0.7	0.1 ± 0.1	0.0 ± 0.0	0,0	0,6	0,0	2,4	1,5	4,2	0,0	0,6	0,0
Isobutyraldehyde	7.9 ± 0.1	8.9 ± 0.5	12.0 ± 0.1	11.4 ± 1.1	8,8	11,3	10,2	9,4	10,9	8,9	12,1	8,5	11,1
Isovaleraldehyde	10.1 ± 0.1	16.4 ± 0.3	18.6 ± 0.3	12.6 ± 0.4	12,8	35,6	18,3	18,2	15,9	14,9	21,7	17,1	16,4
2-methylbutanal	1.2 ± 0.0	1.6 ± 0.1	1.8 ± 0.0	1.4 ± 0.0	1,6	2,1	1,9	1,9	1,1	1,2	1,6	1,3	1,4
Methional	9.7 ± 2.1	5.2 ± 0.4	5.4 ± 0.6	7.6 ± 1.2	4,4	6,5	4,7	5,7	21,6	18,1	24,1	17,8	18,6
Phenylacetaldehyde	210 ± 6	93.5 ± 6.8	109 ± 1	152 ± 6	156,2	86,0	155,0	168,7	104,9	117,8	142,4	87,2	83,4
Polyfunctional mercaptans													
2-metyl-3-furanthiol	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0,479	0,543	0,625	0,364	1,630	1,428	1,544	1,936	1,290
Furfurylthiol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,002	0,001	0,002	0,002	0,003	0,002	0,002	0,002	0,005
4-Mercapto-4-methyl-2-pentanona	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
3-mercaptohexyl acetate	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,117	0,000	0,000	0,032	1,330	0,000	0,000	0,117	0,127
3-Mercaptohexanol	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0,097	0,129	0,000	0,084	0,048	0,092	0,056	0,073	0,084
Benzylmercaptan	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,003	0,006	0,002	0,020	0,006	0,005	0,003	0,002	0,005

S.d. Table 6 Average content of odorants fermented in wines from harvest 2015 and 2016with different additives

	2015					2016		
Compound	21 A	22 A	30 A	33	34	so2 med A-16	element a-16	7f9a-16
Acetate Esters								
Ethyl acetate	58606 ± 9924	39129 ± 459	78116 ± 8044	52660,7	56444,1	26193 ± 3530	28778 ± 909	23785 ± 171
Isoamyl acetate	505 ± 12	617 ± 1	623 ± 170	414,1	502,3	414 ± 56	582 ± 19	223 ± 8
Hexyl acetate	105 ± 1	128 ± 28	109 ± 9	130,6	137,3	105 ± 27	69.4 ± 5.9	29.0 ± 3.8
Isobutyl acetate	11.3 ± 0.9	10.3 ± 0.6	10.3 ± 0.3	12,3	11,1	11.1 ± 5.6	8.2 ± 0.0	3.8 ± 0.2
Butyl acetate	3.4 ± 1.3	3.2 ± 0.4	4.7 ± 0.1	3,5	4,4	1.0 ± 1.5	2.1 ± 0.8	1.7 ± 0.1
Phenylethyl acetate	33.0 ± 1.9	39.7 ± 0.9	39.5 ± 8.2	33,1	38,9	35.9 ± 4.8	90.9 ± 2.5	34.5 ± 0.2
Ethyl esters								
Ethyl propanoate	69.4 ± 10.8	0.0 ± 0.0	26.0 ± 26.0	0,0	78,2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Ethyl butyrate	177 ± 16	143 ± 3	226 ± 18	152,0	156,8	81.4 ± 9.8	60.3 ± 16.3	82.4 ± 16.2
Ethyl hexanoate	1481 ± 626	565 ± 18	640 ± 59	668,3	759,4	571 ± 105	428 ± 26	570 ± 18
Ethyl octanoate	1493 ± 274	749 ± 27	615 ± 135	883,6	884,4	729 ± 167	671 ± 1	529 ± 142
Ethyl decanoate	165 ± 29	98.4 ± 1.9	51.0 ± 16.6	119,8	114,5	75.5 ± 16.0	75.0 ± 1.5	67.3 ± 11.1
Ethyl isobutyrate	57.1 ± 4.8	121 ± 4	38.6 ± 2.5	23,6	24,8	25.0 ± 19.6	46.3 ± 1.9	44.5 ± 2.5
Ethyl 2-methylbutyrate	1.6 ± 0.2	2.7 ± 0.1	1.4 ± 0.1	0,9	0,9	1.4 ± 0.6	2.7 ± 1.2	4.6 ± 0.4
Ethyl isovalerate	5.4 ± 0.6	7.1 ± 0.1	4.8 ± 0.2	2,2	2,6	3.2 ± 1.7	11.0 ± 1.2	9.6 ± 1.5
Miscelaneous esters								
Ethyl lactate	6197 ± 66	6297 ± 149	3229 ± 447	13302,8	12613,4	4579 ± 819	4584 ± 137	4980 ± 238
Diethyl succinate	910 ± 37	834 ± 49	364 ± 14	329,8	385,2	466 ± 147	539 ± 38	501 ± 45
Fusel alcohols								
Isobutanol	14485 ± 47	25041 ± 186	20180 ± 280	9344,0	9874,8	28096 ± 12995	27662 ± 1455	21434 ± 2448
1-Butanol	410 ± 1	227 ± 11	586 ± 83	597,8	674,6	519 ± 111	366 ± 32	412 ± 58
Isoamyl alcohol	76299 ± 2091	128039 ± 4359	118899 ± 4508	79937,8	85136,5	124684 ± 20513	212095 ± 8332	170652 ± 2664
1-Hexanol	1254 ± 13	1274 ± 64	1367 ± 96	1014,2	1094,3	1032 ± 15	1222 ± 27	1385 ± 15
c-3-Hexenol	74.9 ± 2.1	75.1 ± 1.5	74.6 ± 1.1	65,3	71,0	7.9 ± 11.2	0.0 ± 0.0	0.0 ± 0.0
Metionol	455 ± 17	736 ± 28	200 ± 15	154,8	164,5	1293 ± 475	1622 ± 51	1109 ± 36
Benzylic alcohol	92.8 ± 19.2	569 ± 29	44.1 ± 1.6	27,4	37,0	28.9 ± 4.2	19.7 ± 3.5	27.1 ± 0.4
β-Phenylethanol	10059 ± 1719	13831 ± 92	8343 ± 103	7792,8	8373,5	19778 ± 2028	46768 ± 699	37981 ± 60
Acids								
Butyric acid	723 ± 6	545 ± 15	1041 ± 42	651,9	709,9	637 ± 65	575 ± 17	659 ± 3
Isobutyric acid	608 ± 19	1126 ± 25	550 ± 15	336,4	332,9	513 ± 327	860 ± 72	912 ± 65
Isovalerianic acid	421 ± 23	639 ± 25	440 ± 4	266,4	256,4	395 ± 83	1025 ± 11	1225 ± 1
Hexanoic acid	4515 ± 1024	4640 ± 197	4886 ± 117	5313,6	5712,8	3742 ± 138	2235 ± 60	2686 ± 36
Octanoic acid	12274 ± 193	11147 ± 22	8163 ± 366	13132,4	12382,0	11386 ± 810	7258 ± 174	8808 ± 181
Decanoic acid	2833 ± 370	2668 ± 225	1369 ± 258	2871,4	3068,8	1846 ± 516	1083 ± 81	1445 ± 53
Monoterpenes								
Linalool	84.5 ± 4.1	74.2 ± 3.5	67.4 ± 1.2	71,5	76,9	74.4 ± 10.1	79.6 ± 0.4	81.0 ± 1.1
Linalool acetate	0.7 ± 0.5	0.8 ± 0.2	0.9 ± 0.2	0,5	0,8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
α-Terpineol	53.3 ± 2.7	50.6 ± 2.0	70.6 ± 1.9	53,6	60,2	63.5 ± 9.3	40.4 ± 0.4	40.0 ± 2.0
β-Citronelol	3.3 ± 1.1	1.4 ± 1.4	6.4 ± 1.1	4,8	0,0	0.4 ± 0.6	0.6 ± 0.6	0.0 ± 0.0
Geraniol	11.1 ± 0.1	9.9 ± 0.0	6.7 ± 0.9	10,0	9,4	5.6 ± 2.4	8.9 ± 0.6	10.3 ± 1.0
Norisoprenoids								
β-Damascenone	12.7 ± 1.5	13.6 ± 1.0	9.9 ± 0.6	15,6	13,2	1.2 ± 0.4	2.0 ± 1.0	2.5 ± 1.1
α-Ionone	0.4 ± 0.3	0.7 ± 0.0	0.5 ± 0.0	0,6	0,6	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3
β-Ionone	0.2 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0,3	0,3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Phenols								
Guaiacol	5.6 ± 1.0	4.9 ± 1.2	5.3 ± 1.3	5,0	5,0	1.0 ± 0.3	1.0 ± 0.1	1.1 ± 0.6
o-Cresol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,0	0,0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
4-Ethylguaiacol	0.3 ± 0.0	0.6 ± 0.2	0.5 ± 0.1	0,2	0,4	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1
m-Cresol	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0,2	0,2	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
Eugenol	0.2 ± 0.2	0.0 ± 0.0	0.6 ± 0.0	0,4	0,5	0.5 ± 0.1	0.4 ± 0.2	0.6 ± 0.3
4-Ethylphenol	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0,3	0,5	0.1 ± 0.2	0.3 ± 0.2	0.3 ± 0.3
4-Vinylguaiacol	102 ± 6	251 ± 3	225 ± 24	130,4	119,3	414 ± 246	278 ± 21	271 ± 46
E-Isoeugenol	2.4 ± 0.2	2.1 ± 0.3	2.0 ± 0.2	2,3	2,3	2.3 ± 1.2	2.6 ± 1.6	3.7 ± 2.1
2,6-Dimethoxyphenol	4.6 ± 1.4	3.1 ± 1.3	3.1 ± 1.2	2,1	2,5	0.3 ± 0.4	0.0 ± 0.0	0.0 ± 0.0
4-Vinylphenol	72.8 ± 1.4	103 ± 4	208 ± 21	69,6	80,8	187 ± 41	123 ± 5	119 ± 1
4-Alyl-2,6-dimethoxyphenol	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0,0	0,0	0.1 ± 0.2	0.2 ± 0.2	0.7 ± 0.3
Cinamates								
Ethyl dihidrocinnamate	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 0.0	0,5	0,8	0.1 ± 0.2	0.8 ± 0.2	1.8 ± 0.0
Ethyl cinnamate	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,0	0,0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Lactones								
t-Whiskylactone	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,0	0,0	0.0 ± 0.0	0.4 ± 0.4	0.0 ± 0.0
c-Whiskylactone	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,0	0,0	2.2 ± 3.1	2.6 ± 2.6	0.0 ± 0.0
γ-Butyrolactone	6337 ± 222	6976 ± 156	3672 ± 143	5552,6	5938,0	3913 ± 712	5854 ± 90	4678 ± 20
γ-Nonalactone	1.4 ± 0.1	1.3 ± 0.1	2.6 ± 0.1	1,1	1,4	0.4 ± 0.6	0.7 ± 0.7	0.5 ± 0.5
γ-Decalactone	17.8 ± 17.8	12.7 ± 1.3	31.0 ± 0.8	19,6	22,4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Vanillin derivates								
Vanillin	5.3 ± 1.3	6.5 ± 0.2	7.1 ± 1.4	5,3	6,1	4.7 ± 3.3	5.0 ± 2.9	3.7 ± 2.4
Methyl vanillinate	59.2 ± 4.5	49.1 ± 3.0	59.0 ± 2.8	41,3	47,8	62.5 ± 6.3	59.0 ± 0.2	60.2 ± 0.3
Ethyl vanillate	2.0 ± 2.0	1.7 ± 0.2	2.4 ± 0.2	1,7	2,1	0.4 ± 0.1	0.6 ± 0.0	0.5 ± 0.0
Acetovanillone	50.0 ± 6.5	44.8 ± 2.0	54.2 ± 3.3	40,0	44,7	25.0 ± 2.1	24.5 ± 0.9	21.3 ± 2.0
Aldehydes								
Benzaldehyde	1.3 ± 0.2	1.7 ± 0.2	2.2 ± 0.2	2,0	1,9	0.2 ± 0.3	0.0 ± 0.0	0.4 ± 0.4
Isobutyraldehyde						10.1 ± 2.1	12.9 ± 0.0	13.5 ± 0.5
Isovaleraldehyde						15.9 ± 2.7	20.1 ± 0.5	18.4 ± 2.6
2-methylbutanal						1.5 ± 0.2	2.6 ± 0.1	2.3 ± 0.0
Methional						11.4 ± 2.5	14.5 ± 0.2	12.5 ± 0.2
Phenylacetaldehyde						207 ± 11	240 ± 21	248 ± 18
Polyfunctional mercaptans								
2-metyl-3-furanthiol	1.2 ± 0.4	1.4 ± 0.7	0.6 ± 0.2	1,362	0,466	0.4 ± 0.3	0.7 ± 0.2	0.6 ± 0.2
Furfurylthiol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,005	0,003	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
4-Mercapto-4-methyl-2-pentanona	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0,000	0,000	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3-mercaptohexyl acetate	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,000	0,000	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0
3-Mercaptohexanol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,031	0,029	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
Benzylmercaptan	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0,002	0,002	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0