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Yohanna Alegre Martínez

Influence of different oenological processes in the generation of aromas from their precursors
Influencia de diferentes procesos enológicos en la generación de aromas a partir de sus precursores

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INFLUENCE OF DIFFERENT OENOLOGICAL PROCESSES IN THE GENERATION OF AROMAS FROM THEIR PRECURSORS
INFLUENCIA DE DIFERENTES PROCESOS ENOLÓGICOS EN LA GENERACIÓN DE AROMAS A PARTIR DE SUS PRECURSORES

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TESIS DOCTORAL

Influence of different oenological processes in the generation of aromas from their precursors

Influencia de diferentes procesos enológicos en la generación de aromas a partir de sus precursores

Memoria presentada por

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UNIVERSIDAD DE ZARAGOZA

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Química Analítica

Dña PURIFICACIÓN HERNÁNDEZ ORTE, profesora Titular del Departamento de Química Analítica, y Don VICENTE FERREIRA GONZALEZ, Catedrático del Departamento de Química Analítica de la Universidad de Zaragoza,

CERTIFICAN

Que la presente memoria, titulada "*Influencia de diferentes procesos enológicos en la generación de aromas a partir de sus precursores*", presentada para optar al grado de Doctor por la Universidad de Zaragoza, ha sido realizada bajo nuestra dirección por Dña. Yohanna Alegre Martínez, autorizando su presentación para proseguir los trámites oportunos y proceder a su calificación por el tribunal correspondiente.

Y para que conste, expedimos la siguiente autorización

En Zaragoza, a 30 de enero de 2020

Fdo. Vicente Ferreira González

Fdo. Purificación Hernández-Orte

Este trabajo ha sido realizado gracias a

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concedida a Dña Yohanna Alegre Martínez en 2015.

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The noblest art is that of making others happy

P. N. Barnum

Por ese punto de locura que nos hace ser nosotros mismos

Sombrerero loco (Alicia en el país de las maravillas)

On ne voit bien qu'avec le coeur.

L'essentiel est invisible pour les yeux

Antoine de Saint-Exupéry (Le Petit Prince)

Apendix

The thesis presented has resulted in the following publications:

Chapter I resulted in three publications:

- María-Pilar Sáenz-Navajas, Yohanna Alegre, Arancha De-La-Fuente, Vicente Ferreira, David García, Sara Eizaguirre, Iosu Razquin, Purificación Hernández-Orte. *Rapid sensory-directed methodology for the selection of high-quality aroma wines*. (2016). Journal of the science of food and agriculture, 96-12, pp. 4250-62.
- Yohanna Alegre, María-Pilar Sáenz-Navajas, Vicente Ferreira, David García, Iosu Razquin, Purificación Hernández-Orte. (2017). *Rapid strategies for the determination of sensory and chemical differences between a wealth of similar wines*. European food Research and technology, pp. 1 – 15.
- Yohanna Alegre, Arancha de la Fuente, Purificación Hernández-Orte, Vicente Ferreira, María-Pilar Sáenz-Navajas. *Aplicació de noves tècniques ràpides d'anàlisi sensorial a la recerca enològica: prova de categorització de la qualitat seguida d'un perfilat ràpid*. (2015). ACE: revista de enología, 32-102, pp. 12 – 17.

Chapter II resulted in two publications:

- Yohanna Alegre, Laura Culleré, Vicente Ferreira, Purificación Hernández-Orte. *Study of the influence of varietal amino acid profiles on the polyfunctional mercaptans released from their precursors*. (2017). Food research international, 100, pp. 740 -747.
- Yohanna Alegre, Vicente Ferreira, Purificación Hernández-Orte. *How does the addition of antioxidants and other sulfur compounds affect the metabolism of polyfunctional mercaptan precursors in model fermentations?* (2019). Food research international, 122, pp. 1 - 9.

Apendix

Chapter III resulted in two publications:

- Yohanna Alegre, Ignacio Arias-Pérez, Purificación Hernández-Orte, Vicente Ferreira. *Development of a new strategy for studying the aroma potential of winemaking grapes through the accelerated hydrolysis of phenolic and aromatic fractions (PAFs)* (2020). Food research international, 127, 108728.
- Yohanna Alegre, María-Pilar Sáenz-Navajas, Purificación Hernández-Orte, Vicente Ferreira. *Sensory, olfactometric and chemical characterization of the aroma potential of Grenache and Tempranillo winemaking grapes*. (2020). Food chemistry (Submitted).

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You will never be completely at home again,

because part of your heart will always be elsewhere.

That is the price you pay for the richness of loving

and knowing people in more than one place.

Miriam Adeney

A papa y mama, y aunque ya no esté,

al pequeño de la casa.

Por su apoyo incondicional durante todos estos años,

por creer en mí y por darme ánimos cuando lo necesitaba.

Dreams don't work unless you do.

John C. Maxwell

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PRESENTATION

This doctoral thesis was hosted at the Laboratorio de Análisis del Aroma y Enología (LAAE) of the Department of Analytical Chemistry of the University of Zaragoza. This work is included within one of the main lines of research of the group, focused on the study of the formation of the different aromatic compounds present in the wine from the different precursors present in the grape.

The work is composed of a general introduction in Spanish, a summary in both English and Spanish, 3 chapters that will cover each type of precursor (amino acids, cysteinylated and glutathionylated precursors and glycosidic precursors), and the final conclusions in both English and Spanish.

The work has obtained financing from the different projects of the group:

1. Project: Nuevos suplementos para la generación de aromas diferenciadores (AROMAS). RTC-2014-2002-2 (2014-2016). Financed by Ministerio de Economía y Competitividad (MINECO).
2. Project: GRUPO CONSOLIDADO T53 LABORATORIO DE ANÁLISIS DEL AROMA Y ENOLOGÍA T53 (2015 y 2016). Financed by Diputación General de Aragón (DGA).
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6. Project: REALIZACIÓN DE ANÁLISIS RELACIONADOS CON LA QUÍMICA ANALÍTICA (2018 y 2019). Financed by several enterprises.
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PRESENTACIÓN

La presente tesis doctoral se ha realizado en el grupo de investigación Laboratorio de Análisis del Aroma y Enología (LAAE) del departamento de Química Analítica de la Universidad de Zaragoza. Se trata de un trabajo que se engloba dentro de unas de las líneas principales de investigación del grupo, centrada en el estudio de la formación de los diferentes compuestos aromáticos presentes en el vino a partir de los diferentes precursores presentes en la uva.

La memoria se compone de una introducción general en español, un resumen tanto en inglés como en español, 3 capítulos que versarán cada uno de un tipo de precursores (aminoácidos, precursores cisteínicos y de glutatión, y precursores glicosídicos), y unas conclusiones generales tanto en inglés como en español.

El trabajo se inserta y ha obtenido financiación de los proyectos del grupo:

1. Nombre del proyecto: Nuevos suplementos para la generación de aromas diferenciadores (AROMAS). RTC-2014-2002-2 (2014-2016). Financiado por Ministerio de Economía y Competitividad (MINECO).
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7. Nombre del proyecto: ANÁLISIS DE VOLÁTILES (2019). Financiado por varias empresas.

ABBREVIATION LIST

Abbreviation	Definition	Definición
3MH	3-mercaptohexanol	3-mercaptohexanol
3MHA	3-mercaptohexyl acetate	Acetato de 3 mercaptohexilo
4MMP	4-mercapto-4-methyl-2-pentanone	4-mercapto-4-metil-2-pentanona
AAT	Alcohol acetyltransferase	Alcohol acetiltransferasa
Acetyl-CoA	Acetyl coenzime A	Acetil coenzima A
AF	Alcoholic fermentation	Fermentación alcohólica
Ala	Alanine	Alanina
ANOVA	Analysis of variance	Análisis de la varianza
AQC	6-aminoquinolyl-N-Hydroxysuccinimidyl carbamate	6-aminoquinolil-N-hidroxisucinimidil carbamato
Arg	Arginine	Arginina
arPAF	Acelerated hydrolyzed rPAF	PAF tras la hidrólisis acelerada
Asn	Asparagine	Asparragina
Asp	Ascaptic acid	Ácido aspártico
ATT	Alcohol acetyltransferase	Alcohol acetiltransferasa
CA	Correspondence analysis	Análisis de correspondencia
cDNA	Complementary DNA	ADN complementario
cRNA	Complementary RNA	ARN complementario
CT	Categorization task	Tarea de catogorización
Cys	Cysteine	Cisteína
CysGly-3MH	S-3-hexan-1-ol-cysteinyl-glycine	S-3-hexan-1-ol-cisteinil-glicina
CysGly-4MMP	S-4-mercapto-4-methyl-2-pentanone-l-cysteinyl-glycine	S-4-mercapto-4-metil-2-pentanona-l-cisteinil-glicina
CYSMH	Cysteine-3-mercaptohexanol	Cisteína-3-mercaptohexanol
CYSMP	Cysteine-4-mercapto-4-methyl-2-pentanone	Cisteína-4-mercapto-4-metil-2-pentanona
DAP	Di-ammonium phosphate	Fosfato diamónico
DBU	1,8-diazabicyclo[5,4,0]undec-7-ene	1,8-diazabiciclo[5,4,0]undec-7-eno
DCM	Dichloromethane	Diclorometano
DEPC	Diethylpyrocarbonate	Dietilpirocarbonato
DMS	Dimethyl sulfide	Dimetilsulfuro
EDTA	Ethylendiaminetetracetic acid	Ácido etilendiaminotetraacético

Abbreviation list

FID	Flame ionization detector	Detector de inoización de llama
FP	Flash profiling	Perfilado rápido
GABA	Gamma-amminobutyric acid	Ácido gamma aminobutírico
GC	Gas chromatograph	Cromatógrafo de gases
GC-GC-O-MS	Multidimensional gas chromatography- olfactometry – mass spectrometry	Cromatografía de gases multidimensional acoplada a olfatometría y espectrometría de masas
GC-O	Gas chromatography-olfactometry	Cromatografía de gases acoplada a olfatometría
GC-PFPD	Gas chromatography with pulsed flame photometric detection	Detección fotométrica de llama pulsada con cromatografía de gases
Gln	Glutamine	Glutamina
Glu	Glutamic acid	Ácido glutámico
GLUMH	Glutathione-3-mercaptohexanol	Glutatión-3-mercaptohexanol
GLUMP	Glutathione-4-mercapto-4-methyl-2-pentanone	Glutatión-4-mercapto-4-metil-2-pentanona
Gly	Glycine	Glicina
GPA	Generalized procruster analysis	Análisis procrusteano generalizado
GSH	Glutathione	glutatión
HCA	Hierarchical cluster analysis	Análisis de agrupamiento jerárquico
His	Histidine	Histidina
HPLC	High performance liquid chromatography	Cromatografía Líquida de alta eficiencia
HQ_R	High quality red wines	Vinos tintos de alta calidad
HQ_W	High quality white wines	Vinos blancos de alta calidad
HS-SPME	Solid-phase microextraction of automated head space with a carboxy-polydimethylsiloxane fiber	Microextracción en fase sólida de espacio de cabeza automatizada con una fibra de carboxen-polidimetilsiloxano
ICVV	Institute of grapevine and wine sciences	Instituto de las ciencias de la vid y el vino
ID	Identification	Identification
Ile	Isoleucine	Isoleucina
LAAE	Laboratory for Analysis of Aroma and Enology	Laboratorio del análisis del aroma y enología
LC-MS	Liquid chromatography coupled to mass spectrometry	Cromatografía líquida acoplada a espectrometría de masas
Leu	Leucine	Leucina
LM_W	Los Molinos wine	Vino Los Molinos
LQ_R	Low quality red wines	Vinos tintos de baja calidad

LQ_W	Low quality white wines	Vinos blancos de baja calidad
Lys	Lysine	Lisina
MDS	Multidimensional scaling analysis	Análisis de escalamiento multidimensional
Met	Methionine	Metionina
MF	Modified frequency	Frecuencia modificada
mRNA	Messenger RNA	ARN mensajero
N_2	Nitrogen	Nitrógeno
NAD^+	Oxidized form of nicotinamide adenine dinucleotide	Forma oxidada del nicotin adenin dinucleótido
NADH	Reduced form of nicotinamide adenine dinucleotide	Forma reducida del nicotin adenin dinucleótido
NCI	Negative chemical ionization	Ionización química negativa
OAV	Odour activity values	Valor de la actividad aromática
OD	Optic density	Densidad optica
OFN	Octafluoronaphthalene	Octafluoronaftaleno
Orn	Ornithine	Ornitina
PAF	Phenolic and aroma precursor fraction	Fracción de fenoles y precursores del aroma
PCA	Principal component analysis	Análisis de componentes principales
PFBBr	2,3,4,5,6-pentafluorobenzylbromide	Bromuro de 2,3,4,5,6-pentafluorobencilo
Phe	Phenylalanine	Fenilalanina
Pro	Proline	Prolina
PTV	Programmable temperature vaporization	Temperatura de vaporización programable
RI	Retention index	Índice de retención
rPAF	Reconstituted PAF in model wine	PAF reconstituida en vino sintético
rsPAF	Reconstituted PAF in model wine with sugar	PAF reconstituida en vino sintético con azúcar
SAM	S-adenosylmethionine	S-adenosilmetionina
SB	Sauvignon blanc	Sauvignon blanc
Ser	Serine	Serina
SIM	Single ion monitoring	Monitorización de iones individuales
SO_2	Sulfur dioxide	Dióxido de azufre
SPE	Solid phase extraction	Extracción en fase sólida
TDN	1,1,6-trimethyl-1,2-dihydronaphthalene	1,1,6-trimetil-1,2-dihidronaftaleno
Thr	Threonine	Treonina
TPI	Total polyphenol index	Indice de polifenoles totales
Tyr	Tyrosine	Tirosina

Abbreviation list

UHPLC	Ultrahigh liquid performance chromatography	Cromatografía líquida de ultraalta eficacia
Val	Valine	Valina
V_B	Breakthrough volume	Volumen de ruptura
VSCs	Volatile sulfur compounds	Compuestos azufrados volátiles
YAN	Yeast assimilable nitrogen	Nitrogeno facilmente asimilable
α-ABA	Alpha-amminobutyric acid	Ácido alfa aminobutírico
γGluCys-3MH	S-3-hexan-1-ol-γ-glutamyl-cysteine	S-3-hexan-1-ol-γ-glutamil-cisteína
γGluCys-4MMP	S-4-mercapto-4-methylpentan-2-one-N-(l-γ-glutamyl)-l-cysteine	S-4-mercapto-4-metil-2-pentanona-N-(l-γ-glutamil) -l-cisteína

SUMMARY / RESUMEN

SUMMARY

1. INTRODUCTION

During the winemaking process, aromatic compounds with great importance in the quality of the wine are released or produced. Several compounds derived from grapes play a decisive role in the quality and typicality of wines.

In general, the winemaking grape is an aromatically neutral product that contains an aromatic potential in the form of odorless precursors (Hewitt, Mackay, Konigsbacher, & Hasselstrom, 1956; Weurman, 1961). These precursors can be transformed into aromatic compounds either by enzymatic action, either by spontaneous (although slow) reactions to the wine pH, or by a combination of both.

There are two large groups of precursors in grapes: i) specific precursors, non-volatile and odorless molecules, which can produce an aromatic compound by hydrolysis of the chemical bond and/or by spontaneous chemical rearrangement (Delfini, Cocito, Bonino, Schellino, Gaia, & Baioccchi., 2001; López, Ezpeleta, Sánchez, Cacho, & Ferreira, 2004; Tominaga, Peyrot des Gachons, & Dubourdieu, 1998; Waterhouse, Sacks, & Jeffery, 2016); and ii) non-specific precursors, molecules that can be transformed into aromatic compounds as a result of the metabolic action of yeasts (Ferreira & López, 2019). An example is the amino acid leucine that is metabolized by yeast and can lead to compounds such as isoamyl alcohol or isoamyl acetate, among others (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008). However, leucine is not a specific precursor since these compounds can be formed even if there is no leucine in musts (Ferreira & López, 2019).

In this thesis, the formation of different aromatic compounds present in the wine from the different precursors present in the grape (cysteinylated and glutathionylated precursors,

glycosidic precursors, as well as amino acids) has been studied. The thesis is composed of 3 chapters, one chapter for each precursor group: amino acids (chapter I), cysteinylated and glutathionylated precursors (chapter II) and glycosidic precursors (chapter III).

2. CHAPTER I

2.1. Introduction

The main non-specific precursors of grapes are amino acids (Albers, Larsson, Lidén, Niklasson, & Gustafsson, 1996; Hernández-Orte, Cacho, & Ferreira, 2002).

Ferreira et al., found that the levels of higher alcohols, their acetates, acids and their ethyl esters were linked to the grape variety with which the wine had been made (Ferreira, López, & Cacho, 2000). These compounds are mainly produced by yeasts during alcoholic fermentation through the Ehrlich pathway (Bell & Henschke, 2005; Hazelwood et al., 2008). From the amino acids present in the juice (valine, leucine, isoleucine, methionine, threonine and phenylalanine) intermediate compounds such as aldehydes are formed, which can be reduced and form higher alcohols, or on the contrary, can be oxidized and form the corresponding volatiles acids with an atom of carbon less than the corresponding amino acid (Hazelwood et al., 2008).

Traditionally, juices fermentation has been carried out spontaneously with yeasts present in both grapes and wineries. *Non-Saccharomyces* yeasts appear in the initial stages of fermentation and are then replaced by those of the *Saccharomyces* genus (Henick-Kling, Edinger, Daniel, & Monk, 1998). The variability of yeasts generates an uncertainty in the vinification since it puts in risk the stability and reproducibility of the fermentation year after year. Currently, commercial *Saccharomyces cerevisiae* yeasts are inoculated in the

cellars, leaving *non-Saccharomyces* yeasts aside. This ensures the success of the fermentation, but generates an organoleptic homogenization of the wine.

The contribution of *non-Saccharomyces* yeasts to the aroma of wine depends on the concentration of the metabolites formed, which depends on the characteristics of the must and the concentration of precursors. The beneficial characteristics of *non-Saccharomyces* for wine quality is currently known. However, due to the high biodiversity of this group of yeasts, there are still many opportunities for exploitation in wine production, studying not only *non-Saccharomyces* yeasts but also their interaction with *Saccharomyces* yeasts.

In Chapter I, two rapid sensory analysis methodologies have been applied to select wines fermented with more than 100 *non-Saccharomyces* yeasts and identify the key compounds responsible for the generation of the different aromatic profiles among a large number of similar samples. Likewise, the capacity of selected *non-Saccharomyces* yeasts to generate similar aromatic profiles between musts of different terroirs and vintages has been studied and to evaluate if small variations in the composition of the must affect the generated aromatic profile.

2.2. Methods

2.2.1. Samples

In the first year, in the company Lev2050 (Pamplona, Spain), microfermentations with a must of the Verdejo variety and another of the Tempranillo variety with 50 *non-Saccharomyces* yeasts for each variety (W1-W50 in the case of the Verdejo variety, and R1-R50 in the case of Tempranillo) were carried out.

In the second and third year, 5 musts of Verdejo variety (MV1-MV5) and 5 of Tempranillo (MT1-MT5) from different terroirs were fermented with the 5 *non-Saccharomyces* yeasts selected during the first year.

2.2.2. Sensory analyses

Categorization task. In the categorization task the panelists grouped the wines according to their quality in "very high", "high", "medium", "low" and "very low".

Flash profiling. Flash profiling consisted of 2 sessions with an inter-session. In the first session, the panelists individually generated a list of attributes. During the inter-session, the attributes were grouped into a global list that was given to the panelists during the second session. In the second session, the panelists ordered the samples according to their intensity in each of the chosen attributes.

Sorting task. Sorting task consisted of grouping the wines into groups according to the odor similarity between them.

2.2.3. HPLC fractionation

The fractionation procedure was based on the method described by Ferreira et al., (Ferreira, Hernández-Orte, Escudero, López, & Cacho, 1999), using an high performance liquid chromatography (HPLC) with C18 column, and water and ethanol as mobile phases. Twelve fractions of 6 mL each were collected.

The extracts injected into HPLC were obtained following the method described by López et al., (López, Aznar, Cacho, & Ferreira, 2002). 750 mL of wine were passed through 1000 mg cartridges of LiChrolut EN resin. The retained compounds were eluted with 10 mL of ethanol. After a concentration stage by demixture following the method described by Ferreira et al., (Ferreira, Escudero, López, & Cacho, 1998), a 1: 4 dilution was performed for fractionation with an HPLC.

The fractions obtained by HPLC were sensory evaluated by 3 wine experts who described each of them.

2.2.4. Identification of the odorants by GC-O

Gas chromatography coupled to Olfactometry (GC-O) analyses of two types of samples were performed: i) wines selected from flash profiling (5 white and 5 red wines), and ii) selected fracciones obtained by HPLC (8 fractions for each variety).

2.2.4.1. Sample extraction for analysis by GC-O

Samples obtained from the selected wines. Total wine extracts were obtained by SPE following the method described by López et al., (López et al., 2002). 100 mL of wine was percolated on 100 mg of LiChrolut EN resin. The aromatic compounds were eluted with 1 mL of ethanol. Then, 1 mL of the extract was reconstituted in 99 mL of synthetic wine. Extracts for GC-O analysis were obtained using the dynamic head space sampling technique (San Juan, Pet'ka, Cacho, Ferreira, & Escudero, 2010).

Samples obtained from the selected fractions. The selected fractions were diluted with water at pH 3.5 to adjust the ethanol to 11% and then extracted by SPE (López et al., 2002). Between 30-55 mL of sample were passed through 50 mg cartridges of LiChrolut EN, which were then eluted with 600 µL of dichloromethane with 5% methanol.

2.2.4.2. Análisis GC-O

GC-O. The different extracts (1 µL) were analyzed with a Trace GC gas chromatograph, with a DB-WAX column. The panel consisted of six judges in the case of wines, and 3 judges in the case of fractions. The identification of the odorants was carried out by comparison of their descriptors, retention rates in DB-WAX and DB-5 with the indexes of pure references.

GC-GC-O-MS. In order to identify the compounds that could not be identified by their retention rates in both columns, 40 µL of the samples were analyzed by multidimensional

gas chromatography coupled to olfactometry and mass spectrometry (GC-GC-O-MS). The column of the first chromatograph was DB-WAX and the second was VF-5MS. With the data obtained in the first chromatograph, selective cuts were made to isolate the odorants of interest that were transferred to the second chromatograph and monitored by olfactometry with simultaneous mass detection.

GC-O data analysis was based on the calculation of the modified frequency (MF) following the formula given by Dravnieks (Dravnieks, 1985).

2.2.5. Quantitative analyses

Amino acids. The different amino acids were quantified by HPLC coupled to a fluorescence detector following the method proposed by Hernández-Orte et al., (Hernández, Ibarz, Cacho, & Ferreira, 2003). The method is based on derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC).

Cysteinylated and glutathionylated precursors. The precursors were analyzed following the method proposed by Concejero et al., (Concejero, Peña-Gallego, Fernández-Zurbano, Hernández-Orte, & Ferreira, 2014). For that end, 5 mL of the samples were centrifuged and the supernatant was filtered with 2 tandem filters of 0.45 and 0.20 µm. The samples were analyzed by ultra high resolution liquid chromatography (UHPLC) coupled to triple quadrupole mass spectrometer.

Major compounds (mg/L). The method was developed by Ortega et al., (Ortega, López, Cacho, & Ferreira, 2001) by means of a liquid-liquid extraction with dichloromethane and subsequent analysis by gas chromatography with a FID (flame ionization detector). The quantification of the samples was carried out by interpolation in the calibration curves made with model wines.

Minor compounds (µg/L). The method used consisted of a SPE extraction following the method developed by López et al., (López et al., 2002) with some modifications: 15 mL of wine was percolated through 65 mg of LiChrolut EN resin previously conditioned. Elution of the compounds was carried out with 0.6 mL of dichloromethane with 5% methanol. The extract was analyzed with a GC-ion trap mass spectrometry detection.

Polyfunctional mercaptans (ng/L). The method used was developed by Mateo-Vivaracho et al., (Mateo-Vivaracho, Zapata, Cacho, & Ferreira, 2010). The analysis was performed with a GC with mass detection with negative chemical ionization (NCI).

2.3. Results and discussion

To select the non-*Saccharomyces* yeasts that generated wines with a high aromatic quality, a sensory screening of the 50 wines obtained for each variety was carried out by means of a categorization task of the samples. Once the *non-Saccharomyces* yeasts were selected, a flash profiling of the wines obtained with these *non-Saccharomyces* yeasts was performed. Nine Verdejo and seven Tempranillo wines were classified in the highest quality category presenting different aromatic profiles: citrus, stewed fruit, vegetal/boxwood, tropical fruit and cereal in the case of Verdejo wines, and red fruit, strawberry yogurt, stewed fruit, white fruit and banana in the case of the Tempranillo wines.

Wines with distinctive aromatic profiles were analyzed by GC-O. Five white wines obtained with the yeasts W39_*sacch* (citrus), W20 (citrus), W47 (tropical fruit and vegetal/boxwood), W36 (stewed fruit and cereal) and W12 (stewed fruit) and another 5 red wines fermented with the yeasts R20_*sacch* (strawberry yogurt, red fruit and toffee), R22 (red fruit, strawberry yogurt, stewed fruit, white fruit and banana), R24, R45 and R27 (stewed fruit, white fruit and banana). The main compounds responsible for stewed fruit aromas were isoamyl acetate, β-damascenone and 2-phenylethyl acetate. On the other hand,

3-mercaptohexyl acetate and ethyl butyrate were responsible for the tropical fruit and red fruit notes, respectively.

The selected yeasts were used to ferment 5 musts of the Verdejo variety and another 5 of the Tempranillo variety obtained with grapes from different terroirs. The wines obtained from each variety were sensory analyzed by means of a sorting task. The panelists formed 4 clusters in the case of Verdejo wines (cluster 1 was described as stewed fruit, sweet, green, must/grape and unpleasant; cluster 2, tropical fruit, stewed fruit, ripe fruit and sweet; cluster 3, tropical fruit, reduction and stagnant water; and cluster 4, tropical fruit, white fruit and fruit), and 4 in the case of the Tempranillo (cluster 1 had descriptors such as reduction, rotten eggs, sewer and fruit; cluster 2, red fruit, caramel/lactic and sweet; cluster 3, stewed fruit, white fruit, green, cereal and tropical fruit; and cluster 4, tropical fruit, white fruit and stewed fruit).

With this analysis we found that, in the case of white wines, the same yeast produced wines with a similar aromatic profile regardless of the origin of the must, 3 yeasts generated a similar aromatic profile with the exception of one must for each of them, and another yeast generated wines with different aromatic profiles depending on the must. In the case of Tempranillo musts, 2 yeasts generated wines with a similar aromatic profile, 2 yeasts generated a similar aromatic profile with the exception of one must for each of them, and one produced different wines depending on the origin of the must.

To identify the compounds that could affect the generation of the different aromatic profiles, the amino acids and the cysteinylated and glutathionylated precursors present in the must were analyzed. On the other hand, the key aromas responsible for the different aromatic profiles obtained by means of the sorting task were identified.

Regarding to the precursors of the grapes, it was observed that in some cases the must composition can influence the aromatic profile, as in the case of Verdejo musts in which the presence of fruity aromas could be due to a higher concentration of amino acids, or the absence of tropical fruit notes could be due to a lower concentration in the cysteinylated and glutathionylated precursors. However, the influence of the composition of Tempranillo musts on the sensory characteristics was not so clear.

For the identification of the key aromas responsible for positive notes, 3 representative samples of each variety were selected. Selected white samples were described as fresh fruit aromas, sweet fruit aromas and green and negative notes. The selected red wines had descriptors such as red fruit, fruity aromas and green and cereal notes. The 6 samples were fractionated by HPLC. Each fraction was sensory described and the fractions whose aromas were similar to wine were characterized by GC-O.

In the case of Verdejo wines, the tropical fruit aroma could be explained by the high modified frequencies (MF) of 3-mercaptohexanol and 3-mercaptohexyl acetate. In the wines with sweet fruit aroma, higher MF of isoamyl acetate, 2-phenylethyl acetate, benzyl acetate, and β-damascenone were found. On the other hand, isobutyl acetate, ethyl hexanoate, dihydromyrcenol and linalool oxide were associated with fresh fruit notes. The green notes were related to 3-mercaptohexanol, dihydromyrcenol and linalool oxide, while the unpleasant notes with 3-methylbutyric acid. In the case of Tempranillo wines, fruity notes (stewed, tropical and white fruit) were found in the samples with high MF of isoamyl acetate, ethyl hexanoate, 3-mercaptohexyl acetate, 2-phenylethyl acetate and β-damascenone. The green notes were related to Z-3-hexenal, dihydromyrcenol and linalool oxide, while the cereal notes could be due to the presence of 2-isopropyl-3-methoxypyrazine. On the other hand, the notes of red, lactic and caramel fruit were related to the presence of isobutyl acetate, β-damascenone and furaneol.

In the third year, microfermentations with the same selected *non-Saccharomyces* yeasts and with the 5 musts of the different terroirs of each variety were performed. Sensory analyses in order to group them according to odor similarities were carried out as it was done in the previous vintage. For white wines, it was found that two of the yeasts generated similar aromatic profiles between the different musts, and in comparison with the different musts from the previous vintage. Therefore, for these two yeasts the composition of the must did not affect their metabolism, while the other yeasts generated different aromatic profiles not only among different musts, but also between different vintages. On the other hand, for red wines, it was observed that the 5 yeasts generated similar aromatic profiles between the different musts, but only two of them generated the same aromatic profile when fermenting musts from different vintages.

As with the samples from the previous vintage, in 2016 the quantitative analysis of the precursors present in the musts was also carried out. In both vintages, the amino acids and the cysteinylated and glutathionylated precursors of the 5 musts of each variety were analyzed. Important differences were found in the relationship between amino acids, although the relationship between the different terroirs was not maintained between the two years of study.

From these studies, we found that two yeasts for white and two for red musts generated a similar aromatic profile after fermentation of musts from different terroirs and vintages, so it could be said that the aromas generated by these yeasts were quite independent of the must composition, and therefore they were selected.

Finally, these 4 selected *non-Saccharomyces* yeasts were used to study whether the aromatic profile generated was kept when they were fermented in co-inoculation with *Saccharomyces cerevisiae*. Samples obtained after co-inoculation were sensory analyzed with a sorting task.

In the case of white wines, co-inoculation with *S. cerevisiae* did not generate changes in the aromatic profiles obtained with the two *non-Saccharomyces* yeasts. Thus, the wines obtained with co-inoculation with *S. cerevisiae* and the yeasts W20 and W36 had aromatic profiles similar to those observed previously when fermenting musts from different terroirs of 2015 and 2016 only with these two *non-Saccharomyces* yeasts. On the contrary, in red wines, when the 2 *non-Saccharomyces* yeasts were used in co-inoculation with *S. cerevisiae*, no differences were found between the aromatic profiles generated by the different *non-Saccharomyces* yeasts, but the aroma was determined mainly by *Saccharomyces cerevisiae*.

In order to study whether these differences observed in the sensory analysis were explained with the quantitative data, the major and minor compounds and polyfunctional mercaptans were analyzed. In the case of Verdejo wines, the concentrations of the compounds were higher in wines with co-inoculation than in wines without it. However, these quantitative differences did not explain the lack of effect of co-inoculation observed in the sensory analysis. On the contrary, in the case of Tempranillo wines, the separation observed in the sensory analysis between the fermented samples with and without co-inoculation with *S. cerevisiae*, could not be quantitatively explained by the compounds analyzed. The different aromatic profiles observed could be due to compounds that have not been analyzed or to the interactions between the different compounds present in the samples.

2.4. Conclusions

The categorization task followed by flash profiling and GC-O have proven to be a fast and effective sensory methodology for the selection of high quality wines. This methodology allowed to select different *non-Saccharomyces* yeasts (7 in the case of Verdejo and 5 for Tempranillo), capable of generating high quality wines described with notes of citrus,

stewed fruit, vegetal/boxwood, tropical fruit and cereal for Verdejo and red fruit or stewed fruit for Tempranillo, as well as identifying the compounds linked to these aromas: β-damascenone (stewed fruit), 3-mercaptohexyl acetate (tropical fruit) and ethyl butyrate (red fruit).

On the other hand, the sorting task followed by HPLC fractionation and GC-O analysis has also proven to be a rapid sensory methodology for the identification of compounds responsible for the distinctive aromas of wines. This methodology allowed the identification of isoamyl acetate, β-damascenone and 2-phenylethyl acetate as compounds linked to aromas of ripe and stewed fruit; 3-mercaptohexyl acetate and 3-mercaptohexanol as compounds linked to tropical fruit notes; isobutyl acetate, ethyl hexanoate and dihydromyrcenol with white fruit aromas; and 3-methylbutyric acid as responsible for unpleasant aromas in Verdejo wines. In the case of Tempranillo, Z-3-hexenal was identified as a compound linked to green aromas; isoamyl acetate, ethyl hexanoate, 3-mercaptohexyl acetate, 2-phenylethyl acetate and β-damascenone as compounds capable of producing fruity notes; and isobutyl acetate, furaneol and β-damascenone as compounds that can generate aromas of caramel and red fruit. The application of this methodology also allowed the identification of four *non-Saccharomyces* yeasts that could generate wines with a similar aromatic profile when fermenting musts not only from different terroirs, but also from different vintages. Therefore, these *non-Saccharomyces* yeasts did not depend on the composition of the must.

In the case of Verdejo wines, the aromatic profile generated with the selected yeasts was maintained even with co-inoculation with *S. cerevisiae*, while the aromatic profile generated by the yeasts used in Tempranillo wines after co-inoculation was determined by the *S. cerevisiae*, and there was no difference between the *non-Saccharomyces* yeasts.

Therefore, these sensory methodologies are effective and fast tools in the detection and characterization of quality aromatic profiles.

For more information, see articles:

- Sáenz-Navajas, M. P.; **Alegre, Y**.; de-la-Fuente-Blanco, A.; Ferreira, V.; García, D.; Eizaguirre, S.; Razquin, I.; Hernández-Orte, P., Rapid sensory-directed methodology for the selection of high-quality aroma wines. Journal of the Science of Food and Agriculture 2016, 96 (12), 4250-4262.
- **Alegre, Y**.; Sáenz-Navajas, M. P.; Ferreira, V.; García, D.; Razquin, I.; Hernández-Orte, P., Rapid strategies for the determination of sensory and chemical differences between a wealth of similar wines. European Food Research and Technology 2017, 243 (8), 1295-1309.

3. CHAPTER II

3.1. Introduction

The polyfunctional mercaptans 4-mercapto-4-methyl-2-pentanone (4MMP) and 3-mercatohexanol (3MH) are aromatic compounds impact on wine since they are usually found in concentrations above their olfaction threshold (Tominaga, Murat, & Dubourdieu, 1998). These compounds are responsible for fresh notes in wines and are associated with aromas of tropical fruit, grapefruit, cassis, boxwood and guava (Lund Thompson, Benkwitz, Wohler, Triggs, Gardner, Heymann, & Nicolau, 2009; Mestres, Busto, & Guasch, 2000; Swiegers & Pretorius, 2007; Tominaga, Murat, et al., 1998).

In grapes, these polyfunctional mercaptans are linked to a cysteine and/or glutathione molecule forming the odorless cysteinylated and glutathionylated precursors (cysteine-3-mercaptohexanol, CYSMH, cysteine-4-mercapto-4-methyl-2-pentanone, CYSMP,

glutathione-3-mercaptohexanol, GLUMH, and glutathione-4-mercapto-4-methyl-2-pentanone, GLUMP) (Fedrizzi, Pardon, Sefton, Elsey, & Jeffery, 2009; Peyrot des Gachons, Tominaga, & Dubourdieu, 2002; Tominaga, Peyrot des Gachons, et al., 1998).

During alcoholic fermentation (AF), the β-lyase enzymatic action of the yeast produces the cleavage of the carbon-sulfur bond resulting in the release of polyfunctional mercaptans (Tominaga, Peyrot des Gachons, et al., 1998). However, the concentration of 3MH and 4MMP in the wine is not correlated with the concentration of its precursors in the must, in fact, the conversion level is less than 5% (Bonnaffoux, Delpech, Rémond, Schneider, Roland, & Cavelier, 2018; Concejero, Hernández-Orte, Astrain, Lacau, Baron, & Ferreira, 2016; Peyrot des Gachons, Tominaga, & Dubourdieu, 2000; Roland, Schneider, Razungles, Le Guerneve, & Cavelier, 2010). These facts indicate that there are aspects related to the uptake and use of precursors that remain poorly understood.

Chapter II has 4 different aims: i) to study the effect of different amino acid profiles on the yeast metabolism of cysteinylated and glutathionylated precursors; ii) to identify the precursors preferred by yeast; iii) to determine the effect of different concentrations and sources of sulfur on the release of polyfunctional mercaptans from precursors; and iv) to study the effect of the addition of cysteine and glutathione on genes related to the metabolism of polyfunctional mercaptans precursors.

3.2. Methods

3.2.1. Synthetic juice

Synthetic juice adapted from Bely et al., (Bely, Sablayrolles, & Barre, 1990) was prepared with some modifications:

Experiment 1. Effect of the amino acid profile. Fermentations were performed with synthetic juices prepared with 150 mg N/L. The only difference between juices were their

amino acid profiles. Nine synthetic musts were prepared with the amino acid profiles of 7 different varieties (Hernández-Orte et al., 2002). The total nitrogen in the amino acid profiles was adjusted by percentage.

Experiment 2. Effect of the addition of precursors by separate. Two concentrations of nitrogen (120 and 150 mg/L) obtained by adjusting the percentages of the amino acid profile of the Chardonnay variety were studied. The low nitrogen concentration was used in order to try to force the use of these precursors by yeast.

For experiments 3 to 6, the juice was prepared with a nitrogen concentration of 200 mg/L by adjusting in percentage the amino acid profile of Chardonnay. In this synthetic juice different additions were made.

Experiment 3. Different sulfur compounds were added at different concentrations: elemental sulfur (1 mg/L), glutathione (50 and 70 mg/L), cysteine (20 mg/L), methionine (30 and 50 mg/L) and sulfur dioxide (20 mg/L).

Experiment 4. The amino acids that are part of the glutathione (GSH): cysteine (10, 20 and 30 mg/L), glutamic acid (50 mg/L) and glycine (10 mg/L) were added.

Experiment 5. Sulfur dioxide (SO_2) was added at different concentrations (20, 30, 50 and 70 mg/L).

Experiment 6. Transcriptomic analyses during fermentation. GSH and cysteine were added at different concentrations. Three sets of fermentations were performed. In the first one, the growth of *S. cerevisiae* populations was evaluated. In the second one, it was studied the moment when the polyfunctional mercaptans were released. With the data obtained, it was decided the moments during the fermentation in which samples would be collected for the transcriptomic analyses that were performed in the third set of fermentations.

In all juices, the 4 precursors of polyfunctional mercaptans were added (50 µg/L of CYSMP and GLUMP, 100 µg/L of CYSMH and 1000 µg/L of GLUMH). In addition, in experiment 2 the cysteinylated and glutathionylated precursors were added separately. In all experiments, the juices were inoculated with *Saccharomyces* yeasts (Zymaflore X5) and the fermentations were carried out in triplicate at 20 °C until reaching a constant weight.

3.2.2. Analyses

At the end of the alcoholic fermentation, the polyfunctional mercaptans and the remaining precursors were analyzed following the methods previously described in section 2.2.5

In addition, in the experiment 6, samples were collected at different times, in the first and second set in order to evaluate the release of polyfunctional mercaptans and the growth of yeast populations. In the third set, for transcriptomic analyses, samples were taken at 26 and 150 h.

Cell counting. The counting of the yeast population was performed with an electronic particle counter. The samples were diluted to be in the range of 20000 to 80000 cells/mL and then the aggregates were separated by sonication.

Transcriptomic analyses. The mRNAs were extracted from the cells and submited to retro-transcription and then transcription in the presence of nucleotides labeled with a fluorochrome. The labeled complementary RNAs (cRNAs) were purified and hybridized on the DNA chips (glass plates containing single stranded cDNA strands to which the mRNAs bind). The labeled cRNA fragments bind to DNA strands by complementarity of the bases. Differences in the genetic expression were evaluated based on fluorescence intensity (Duc, Pradal, Sanchez, Noble, Tesnière, & Blondin, 2017). The data processing was carried out with the software R version R.3.6.1.

3.3. Results and discussion

In the first experiment, the effect of the amino acid profile on the release of polyfunctional mercaptans, as well as on the consumption of their precursors, was studied. Nine different juices were prepared simulating the amino acid profiles of 7 different varieties and 2 versions of one of the varieties. In the wines obtained, significant differences were found in the consumption of precursors and in the release of polyfunctional mercaptans depending on the amino acid profile. A higher consumption of precursors and higher release of 3MH and 4MMP was observed in wines with the amino acid profile of the Chardonnay variety.

In order to determine if the type of precursor influences the yeast metabolism, synthetic juices with different amounts of total nitrogen (120 and 150 mg N/L) were prepared to which cysteinylated precursors were added and others to which glutathionylated precursors were added, as well as a control with all the precursors together. Wines whose juices were added with cysteinylated precursors released practically the same concentrations of 4MMP as the control wines that possessed both cysteinylated and glutathionylated precursors together, while the wines containing only glutathionylated precursors generated only 25% of the 4MMP concentration compared to the control. Moreover, the precursor analyses indicated that the highest consumption of the 4MMP precursors occurred when the precursors added were the cysteinylated. This seems to indicate that the main precursor of 4MMP is CYSMP. By contrast, the main precursor of 3MH was GLUMH. Both data were similar at the two nitrogen concentrations tested.

The low conversion factor between polyfunctional mercaptans and their precursors could be due to the fact that yeasts use precursors as a source of some nutrient that could be some sulfur compound. To verify this, fermentation of synthetic juice was carried out to which different sulfur compounds were added: elemental sulfur, SO_2, GSH, methionine and

cysteine. It was observed that GLUMH is the precursor most consumed and that yeast could transform GLUMP into CYSMP. However, although there was a significant effect in all cases, the effects of greater magnitude were observed in the case of GLUMH. The addition of GSH led to a decrease in the metabolism of GLUMH, so yeasts could use this precursor as a source of GSH. On the other hand, the only case in which an increase in the GLUMH metabolism in comparison with the control was observed was with the addition of SO_2. Regarding the release of polyfunctional mercaptans, the clearest effect was an increase in polyfunctional mercaptans concentrations when SO_2 and cysteine were added at 20 mg/L. The addition of GSH caused a significant increase of 4MMP and a decrease of 3MH.

To explain the reason why the addition of GSH decreased the metabolism of GLUMH, the effect of the amino acids that are part of the GSH was studied. The addition of glutamic acid and glycine did not have any relevant effect on the metabolization of these precursors, unlike the addition of cysteine. For this reason, different levels of cysteine were studied. The strongest effect was caused by the addition of high amounts of cysteine, observing a lower metabolism of GLUMH. This seems to indicate that yeasts use this precursor as a source of cysteine. In the case of the effect on polyfunctional mercaptans, a dose-dependent effect was observed. The low cysteine concentration did not generate differences, the average concentration generated an increase in the 3 aromatic compounds and the highest concentration caused a decrease in the three compounds. This lower concentration is consistent with the fact that, in this sample, the GLUMH precursor was less metabolized than in the controls.

Since SO_2 was the only one that produced a higher consumption of GLUMH, the effect of different levels of SO_2 was studied. However, although the lower concentration produced an increase in the metabolism of GLUMH, intermediate concentrations had opposite results. Surprisingly, the effects of SO_2 were much more intense in CYSMH, which was

more consumed when the must contained SO_2. Which could indicate that SO_2 prevents the formation of CYSMH from GLUMH. With respect to the effect on polyfunctional mercaptans, the addition of 20 mg/L of SO_2 caused an increase in the concentrations of 4MMP and 3MH. On the other hand, the addition of higher levels did not have an effect on the concentration of 4MMP but caused an increase in the concentration of 3MH. Therefore, in addition to preventing the oxidation of these compounds, SO_2 can produce some metabolic effect.

The results obtained previously in which it was observed that there was a lower disappearance of GLUMH when the samples contained GSH and cysteine led us to think that these differences in the metabolism of the volatile thiol precursors could be due to a regulation of the genes of the yeast involved in their metabolic pathway. Therefore, the effect of the addition of these compounds on the genes involved in the metabolism of precursors of polyfunctional mercaptans was studied. For this, fermentations containing cysteine and glutathione at different concentrations, as well as a control without additions were carried out. At 26 and 150 h, transcriptomic analysis was performed.

Previously it had been observed that the addition of cysteine at 10 mg/L did not cause changes in the concentration of polyfunctional mercaptans with respect to the control without additions. In the transcriptomic study, we also found that few changes happened; only 4 genes were differentially expressed at 26 h and 1 gene at 150 h. However, none of these genes was related to either the sulfur cycle, or the metabolic pathways of sulfur amino acids and/or GSH, or genes related to the metabolism of polyfunctional mercaptans precursors. Similar results were observed in wines obtained with the additions of GSH at 70 mg/L and cysteine at 30 mg/L despite the changes previously observed in the volatile compounds. Therefore, the reason for this lower consumption of GLUMH in the samples

with the addition of GSH at 70 mg/L and cysteine at 30 mg/L could be due to a nutritional necessity of the yeast and not to a regulation at genetic level.

On the other hand, intermediate concentrations of these compounds resulted in an increase in the number of differentially expressed genes. In both cases, genes involved in the metabolic pathways of cysteine, methionine and sulfur were observed. The most interesting case was observed with the addition of cysteine at 20 mg/L, which generated a significantly higher concentration of volatile thiols compared to the control and an overexpression of the IRC7 gene that encode for an enzyme with β-lyase activity. Thus, the addition of cysteine at 20 mg/L produces an increase in yeast β-lyase activity, which leads to a higher concentration of polyfunctional mercaptans.

3.4. Conclusions

The amino acid profile affects not only the release of polyfunctional mercaptans but also the consumption of their precursors. Being the amino acid profile the only difference between the juices, it was observed that in wines with the amino acid profile of the Chardonnay variety there was a higher concentration of 4MMP and 3MH. In addition, it was observed that yeasts consumed preferably the cysteinylated precursor of the 4MMP and the glutathionylated precursor of the 3MH.

Yeasts could use the cysteinylated and glutathionylated precursors as a source of GSH and/or cysteine since the addition of high concentrations of both GSH and cysteine resulted in significantly lower consumption of the precursors.

The addition of cysteine at 20 mg/L led to an overexpression of the IRC7 gene that encodes an enzyme with β-lyase activity, resulting in a release of higher concentrations of polyfunctional mercaptans.

For more information, see articles:

- **Alegre, Y**.; Culleré, L.; Ferreira, V.; Hernández-Orte, P., Study of the influence of varietal amino acid profiles on the polyfunctional mercaptans released from their precursors. Food Res. Int. 2017, 100, 740-747.
- **Alegre, Y**.; Ferreira, V.; Hernández-Orte, P., How does the addition of antioxidants and other sulfur compounds affect the metabolism of polyfunctional mercaptan precursors in model fermentations? Food Research International 2019, 122, 1-9.

4. CHAPTER III

4.1. Introduction

The glycosidic precursors are formed by an aglycone linked to a glycone (sugar moiety) (Gunata, Bitteur, Brillouet, Bayonove, & Cordonnier, 1988; Williams, Strauss, Wilson, & Massy-Westropp, 1982). More than 100 different aglycones classified in terpenoles, norisoprenoids, lactones, volatile phenols, vainillin derivates and miscellaneous compounds have been identified. These aglycones are released during the vinification process by exogenous or endogenous glycosidases (Gunata, Bayonove, Tapiero, & Cordonnier, 1990; Sánchez-Palomo, Díaz-Maroto Hidalgo, González-Viñas, & Pérez-Coello, 2005), or by slow acid hydrolysis (López et al., 2004; Skouroumounis & Sefton, 2000).

The study of these compounds is complicated because, in some cases, the aglycone is the aromatic compound (Strauss, Wilson, Gooley, & Williams, 1986; Wilson, Strauss, & Williams, 1984), but in other cases, the aroma is formed after spontaneous chemical rearrangements of the original aglycone.

Most research is based on the indirect study of non-volatile precursor fractions extracted from grapes. These precursor fractions are generally "revealed" by two main strategies:

enzymatic hydrolysis and/or harsh acid hydrolysis (Delfini et al., 2001; Loscos, Hernández-Orte, Cacho, & Ferreira, 2009). The problem of enzymatic hydrolysis is that some of the most relevant aromatic compounds, such as β-damascenone, TDN or β-ionone, require chemical rearrangement and therefore, these compounds are not even form (Waterhouse et al., 2016). On the other hand, harsh acid hydrolysis provides information on these molecules, but implies an intense degradation of a high proportion of relevant aromatic compounds such as terpenols (Loscos et al., 2009). The best aromatic results are obtained using mild acid hydrolysis, which takes a long time. Under the conditions commonly used, there is strong evidence to suggest that exist a noticeable oxidation and deterioration of the aroma during strong acid hydrolysis.

Therefore, the main objective of this chapter is the development of a new strategy to measure the aromatic potential of winemaking grapes. This main objective is divided into the following sub-objectives: i) to develop a method for the extraction of the aroma precursors; ii) to develop an accelerated hydrolysis method to determine the aromatic potential of the grapes; and iii) to apply the developed strategy to determine the aromatic potential of grapes from different varieties, wineries, terroirs and ripeness states.

4.2. Methods

4.2.1. Development of an extraction method

The development of the extraction method was carried out with Tempranillo grapes from Bodegas Pingus, and Grenache from Bodegas Ilurce.

Preparation of the ethanolic musts (mistelles). Ten kg of Tempranillo and Grenache grapes were destemmed and pressed in the presence of ethanol (15%) and potassium metabisulfite.

Optimization of cartridges and breakthrough volume (V_B). Three types of samples were prepared (mistelle, mistelle diluted at 50% and mistelle dealcoholized). Cartridges of 7 and

10 g of C18 were studied. After conditioning the cartridges, the 3 types of sample were passed through them and 5 mL-fractions were collected. In these fractions the total polyphenol index (TPI) was analyzed (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). To determine the breakthrough volume, a loss of TPI less than 15% was considered compared to the control.

Determination of elution volume. To determine the elution volume, the cartridges were eluted with ethanol and different fractions were collected. In order to determine the presence or absence of glycosidic precursors in the fractions, acid hydrolysis was performed at 100 °C (Ibarz, Ferreira, Hernández-Orte, Loscos, & Cacho, 2006) and the fractions were analyzed in a sensory manner. In these fractions, the tannins and anthocyanins were also analyzed (Ribéreau-Gayon et al., 2006).

Extraction of phenolic and aromatic fractions (PAFs, phenolic and aromatic fractions). 750 mL of mistelle was dealcoholized and passed through a 10 g-C18 cartridge, then, the cartridges were eluted with 100 mL of ethanol.

4.2.2. Hydrolysis conditions

Three types of samples were prepared: i) mistelles; ii) phenolic and aromatic fractions reconstituted in synthetic wine (rPAFs); and phenolic and aromatic fractions reconstituted in synthetic wine with sugar (rsPAFs). These samples were incubated at 45 °C for 2, 4 and 7 weeks both in anoxia and in the presence of oxygen. In addition, Tempranillo rPAFs were incubated at 75 °C for different times from 3 to 72 h. The compounds released were analyzed in a sensory manner, by GC-O and by GC-MS.

4.2.3. Sensory analyses

The samples were submitted to 4 different sensory analyses. The first was carried out to determine the elution volume. The second and third consisted of a descriptive task for samples obtained both with accelerated hydrolysis at 45 °C and 75 °C. The fourth sensory analysis consisted of a triangular test for rPAFs obtained at 75 ºC.

In addition, once the method of the extraction and accelerated hydrolysis was developed, the characterization of 33 Grenache and Tempranillo grapes from different origins, terroirs and ripeness states was carried out. For the characterization of these samples, 2 sensory analyses were carried out. The first was a sorting task to group the 33 different rPAFs of Tempranillo and Grenache according to odor similarities, and the second a flash profiling to obtain a deeper characterization of the selected rPAFs from the groups formed by sorting task. These last two sensory analyses were performed as described in section 2.2.2.

Determination of elution volume. This sensory analysis was performed to determine the presence/absence of the aromas released from glycosidic precursors in each of the collected fractions. Panelists indicated “yes” or “no” depending on whether there was presence or absence of aroma, respectively.

Descriptive analyses. Panelists had to smell the fractions and wrote a free description using 1 to 5 attributes. They were also asked to indicate the intensity of each sample by defining them as low, medium or high.

Triangular test. Tempranillo rPAF incubated at 75 °C for 14, 24, 38 and 48 h was submitted to triangular tests in order to identify if there were differences between the different pairs of rPAFs: i) rPAF incubated for 14 h vs rPAF incubated for 24 h ; ii) 24 h vs 38 h; and iii) 24 h vs 48 h. The panelists were presented with 3 glasses and had to indicate which was the different sample.

4.2.4. Quantitative analyses

Aromas released from glycosidic precursors. Quantification was carried out following the method developed by López et al., (López et al., 2002) for the analysis of minor compounds (section 2.2.5) but with different chromatographic method. Two µL of the sample was injected into a GC-MS QP2010 following the method described by Oliveira et al., (Oliveira & Ferreira, 2019).

Volatile sulfur compounds. They were determined following the method described by Franco-Luesma et al., (Franco-Luesma & Ferreira, 2014). A solid-phase microextraction of automated head space (HS-SPME) with a carboxy-polydimethylsiloxane fiber was used followed by a gas chromatography with pulsed flame photometric detection (GC-PFPD). The concentrations of the compounds were calculated based on the interpolation in the calibration curves.

Amino acids. Amino acids were analyzed as described in section 2.2.5.

Metals. The most abundant and oenologically relevant transition metals (Fe, Cu, Mn and Zn) were determined by measuring the most abundant isotopes (^{56}Fe, ^{63}Cu, ^{55}Mn and ^{66}Zn) by inductively coupled plasma mass spectrometry using a procedure published by Grindlay et al., (Grindlay, Mora, de Loos-Vollebregt, & Vanhaecke, 2014).

4.2.5. GC-O

The GC-O analysis was carried out following the method described in the section 2.2.4.2.

4.3. Results and discussion

The main objective was to develop a method for determining the aromatic potential of the grapes. To that end, firstly, a method for the extraction not only glycosidic precursors but

also the polyphenolic grape fraction (phenolic and aromatic fractions, PAFs) was developed.

4.3.1. Development of the method of the extraction of PAFs

For the development of the strategy, it was necessary to optimize the resin, the preparation of the mistelle from which the PAFs will be extracted, the breakthrough volume (V_B) and the elution volume. The grapes were processed immediately in the presence of ethanol to avoid fermentation and to mimic the extraction obtained in the production of red wine. As a result, ethanolic musts or "mistelles" were obtained.

Three types of samples (mistelle, dealcoholized mistelle and diluted mistelle) were prepared. Cartridges of 7 and 10 g of C18 resins were used. First, the V_B of the polyphenols was determined. To that end, the different types of mistelle were percolated through the previously conditioned cartridges and the fractions were collected every 5 mL. In each collected fraction, TPI was measured.

In the case of the untreated mistelle, a 15% loss of the TPI was obtained by passing only 7 and 10 mL through the 7 and 10 g cartridges, respectively. The dilution had a positive effect, the V_B increased until 31 and 45.5 mL, respectively. The best results were observed with the dealcoholized mistelle in which the V_B was 240 and 750 mL, respectively.

In order to determine the elution volume, the dealcoholized mistelle was percolated in a 10 g C18 cartridge and then it was eluted with ethanol. Fractions were collected every 50 mL, and in each fraction, tannins and anthocyanins were analyzed, as well as an indirect analysis by acid hydrolysis of the glycosidic precursors present in the fractions. Only in the first two fractions, the aroma released was detected. In addition, 98% of anthocyanins and more than 90% of tannins were present in the first two fractions. Thus, 100 mL were considered as the elution volume. Therefore, the final extraction method allowed to extract more than

90% of the grape polyphenols and practically all the precursors that release aromas by acid hydrolysis.

4.3.2. Hydrolysis conditions

4.3.2.1. Accelerated hydrolysis at 45 ºC

Once the method of the PAFs extraction was developed, a method for the accelerated hydrolysis of the PAFs was developed to release the aromatic compounds from glycosidic precursors. The PAFs were reconstituted in synthetic wine and aged at 45 °C under strict anoxic conditions at different times (2, 4 and 7 weeks). In order to study the effect of sugar and the losses produced during the extraction process, the mistelle, as well as the rsPAF were also aged. To assess the effect of oxygen, a sample of each type was also aged for 7 weeks without anoxia conditions. Compounds released from glycosidic precursors were sensory and quantitatively studied.

The aged mistelles developed strong aromas of caramel and raisins. The addition of sugars to the PAF induced the formation of kerosene notes attributed to TDN. The presence of oxygen caused a strong distortion of the sensory profiles. On the contrary, the reconstitution of the PAF in synthetic wine (rPAF) followed by anoxic aging caused the development of intense aromas similar to the aromas observed in red wines.

Therefore, rPAF aged at 45 °C need at least 7 weeks to release the compounds that produce intense fruity aromas similar to some of the most appreciated aroma nuances of wines. However, 7 weeks is too long, so in order to minimize the time an accelerated hydrolysis at 75 °C was performed.

4.3.2.2. Accelerated hydrolysis at 75 ºC

Reconstituted PAF (rPAF) were incubated under strict anoxia conditions for different times from 3 to 72 h. At 75 °C, in just 24 h, there was a satisfactory development of the aroma. In addition, the triangular tests performed showed that there were no significant differences between 24, 38 and 48 hours of incubation. The aroma developeded in the fractions were also studied by GC-O and GC-MS. The results confirmed that the hydrolysates obtained at 75 °C in just 24 h, were sensory, chemically and olfactometrically, very close to those obtained after 7 weeks at 45 °C.

At least 32 different odorants were identified at relevant levels from the sensory point of view in the hydrolysates. Odorants can be classified into five categories: i) lipid derivatives, including *Z*-3-hexenal, 1-octen-3-one, *Z* -1,5-octadien-3-one, *Z*-3-hexenol, *E*-2-octenal, *Z*-2-nonenal, *E*-2-nonenal, *E*,*Z*-2,6-nonadienal, *E*,*E*-2,4-nonadienal, γ-decalactone and massoia lactone; ii) volatile phenols and vanillins, including guaiacol, cresols, eugenol, 2,6-dimethoxyphenol, *E*-isoeugenol and vanillin; iii) norisoprenoids and terpenes, including linalool oxide (and/or dihydromyrcenol), linalool, TDN, β-damascenone and β-ionone; iv) amino acid derivatives, including methional and sotolon; and v) miscellaneous compounds, including β-phenylethanol, ethyl cinnamate, furaneol, 3-mercaptohexanol and three unidentified compounds.

The odorants in categories i and iv will probably be transformed by yeast during alcoholic fermentation, so they may not reach the wine. However, the odorants in categories i, iii and v will be essential for the development of wine aroma. The presence of 3-mercaptohexanol is surprising, since for the first time it is suggested that this important aromatic compound could be formed from the precursor without the action of yeast.

4.3.3. Characterzation of aromatic potential of Tempranillo and Grenache grapes

With already developed method, the characterization of 33 lots of Tempranillo and Grenache grapes from different wineries, different terroirs and with different ripeness states was carried out. Using the developed strategy, PAFs were prepared which were subsequently reconstituted in synthetic wine and submitted to accelerated hydrolysis at 75 °C for 24 h. The characterization of these samples was then carried out by sensory analysis, GC-O and GC-MS.

4.3.3.1. Sensory characterization

A sorting task was performed in which samples were grouped according odor similarities. Two large groups were observed, one mainly composed of the Grenache grapes that were subdivided into 2 clusters (clusters i and ii), and a second large group composed mainly of Tempranillo grapes and in which we found clusters iii, iv and v. Five clusters were observed: i) tropical fruit/citrus, floral; ii) floral, fruit in syrup; iii) woody/toasty, red fruit, in syrup and black fruit; iv) vegetal; and v) vegetal and in syrup. From these clusters, a representative PAF was selected and it was submitted to flash profiling for a more depth sensory characterization.

In the flash profiling, the sample from cluster i was described with notes of tropical fruit/citrus, floral and kerosene; the sample selected in cluster iii was described with notes of woody/toasty, red fruit and fruit in syrup; the sample belonging to cluster iv was described with alcoholic notes; and the selected samples of clusters ii and v were described mainly with notes of fruit in syrup and alcoholic. In addition, all rPAFs had vegetal notes. Therefore, these samples were described very similarly in both flash profiling and sorting

task. Interestingly, the sensory data did not show any relevant effect on the geographical origin of the grapes or the ripeness state.

4.3.3.2. GC-O

In order to identify the odorants responsible for the distinctive descriptions among the clusters, the 5 rPAFs selected and characterized by flash profiling were analyzed by GC-O. The GC-O revealed that 3MH, linalool oxide and dihridromyrcenol, 2 unidentified odorants, phenylacetaldehyde and *Z*-1,5-octadien-3-one were the most discriminating odorants. In addition, several lipid derivatives including 7 aldehydes, 2 ketones and 2 lactones could be responsible for the vegetal background. Other compounds were also identified as 4 volatile phenols, ethyl cinnamate, β-ionone, β-damascenone, linalool, α-terpineol and furaneol.

4.3.3.3. GC-MS

Quantitative data showed that the concentrations of some compounds were very similar among rPAFs that belonged to the same cluster in the sorting task. The odorants that generated the greatest variability at the sensory level were β-damascenone, TDN, linalool, limonene, furaneol and 4-vinylphenol. In addition, massoia lactone also showed a high discriminant potential. On the other hand, β-ionone, geraniol, 1,8-cineole, guaiacol and 4-vinylphenol could also induce sensory differences.

Grenache samples were rich in norisoprenoids (except ionones), terpenoles (except limonene) and in vanillin derivatives, while Tempranillo samples were rich in volatile phenols. Of the two Grenache clusters (clusters i and ii), the samples in cluster ii contained high levels of β-ionone, β-damascenone, linalool, limonene and massoia lactone, while those in cluster i had high concentrations of TDN. These compounds explain the differences found in the sensory analyses, as well as the kerosene notes observed in the sample

belonging to cluster i. The tropical fruit/citrus notes could not be explained by these compounds, but they can be explained by the presence of 3MH, linalool oxide and dihydromyrcenol in the GC-O analysis. On the other hand, in the case of Tempranillo (clusters iii, iv and v), the samples from cluster iv contained lower concentrations in most of the compounds, which explains their vegetal character. Samples from cluster v also had low concentrations of most of the compounds except for ionones and β-damascenone which would explain the fruit in syrup character along with the vegetal notes. Samples from cluster iii samples had higher levels of volatile phenols which would explain the woody/toasty notes.

4.4. Conclusions

Most of the glycosidic precursors and grape polyphenols present in 750 mL of mistelle could be isolated by SPE from dealcoholized mistelle using 10 g of C18. However, amino acids, metal cations and DMS precursors were not extracted in these PAFs. In addition, the reconstitution of the PAF in synthetic wine followed by aging in anoxia for 7 weeks at 45 °C or for 24 h at 75 °C led to the development of strong aromas reminiscent of some odor nuances observed in aged red wines. On the contrary, similarly aged mistelles developed strong aromas of caramel and raisins. The addition of sugars to the PAF induced the formation of kerosene notes attributed to TDN. The presence of oxygen caused a strong distortion of the aromatic profiles.

The 32 odorants identified by GC-O were similar between samples aged at 45 and 75 °C and belonged to 4 main categories (lipid derivatives; volatile phenols and vanillins; and norisoprenoids and terpenes) and a fifth miscellaneous group that surprisingly included 3MH. In addition, GC-MS and GC-O analyzes of rPAF incubated for 7 week at 45 °C were also relatively similar to those obtained after 24 h at 75 °C, suggesting that this rapid

hydrolysis of PAFs may be a promising tool for the study of the aromatic potential of winemaking grapes.

In addition, the characterization of the 33 grape samples allowed us to identify five different aromatic profiles: i) vegetal; ii) fruit in syrup and vegetal; iii) floral and fruit in syrup; iv) floral and tropical fruit/citrus; and v) woody/toasty, red fruit, black fruit and fruit in syrup. GC-O revealed that these aromatic profiles were due to 19 odorants: i) constitutive odorants: eight aldehydes that provide a common "vegetal" background and β-damascenone and massoia lactone that provide a "fruit in syrup" background, and ii) discriminant odorants: linalool, linalool oxide, 3MH, furaneol, guaiacol and methoxieugenol that seem to be responsible for the distintive aromatic nuances.

Quantitative analyses confirmed the existence of the five different profiles. Grenache was rich in norisoprenoids, terpenoles and vanillin derivatives and Tempranillo was rich in volatile phenols.

For more information, see articles:

- **Alegre, Y**.; Arias-Pérez, I.; Hernández-Orte, P.; Ferreira, V. Development of a new strategy for studying the aroma potential of winemaking grapes through the accelerated hydrolysis of phenolic and aromatic fractions (PAFs). Food Research International 2020, 108728.
- **Alegre, Y**.; Sáenz-Navajas, M. P.; Hernández-Orte, P.; Ferreira, V. Sensory, olfactometric and chemical characterization of the aroma potential of Grenache and Tempranillo winemaking grapes. (2020). Food chemistry (Submitted).

RESUMEN

1. INTRODUCCIÓN

Durante el proceso de vinificación se liberan o producen compuestos aromáticos con gran importancia en la calidad del vino. Varios compuestos derivados de las uvas juegan un papel decisivo en la calidad y tipicidad de los vinos.

En general, la uva de vinificación es un producto aromáticamente neutro que contiene un cierto potencial aromático bajo la forma de precursores inodoros (Hewitt, Mackay, Konigsbacher, & Hasselstrom, 1956; Weurman, 1961). Estos precursores pueden ser transformados en compuestos aromáticos bien por acción enzimática, bien por reacciones espontáneas (aunque lentas) al pH del vino, bien por una combinación de ambas.

Hay dos grandes grupos de precursores en las uvas: i) precursores específicos, moléculas no volátiles e inodoras, que pueden producir un compuesto aromático por la hidrólisis del enlace químico y/o por reordenamiento químico espontáneo (Delfini, Cocito, Bonino, Schellino, Gaia, & Baioccchi., 2001; López, Ezpeleta, Sánchez, Cacho, & Ferreira, 2004; Tominaga, Peyrot des Gachons, & Dubourdieu, 1998; Waterhouse, Sacks, & Jeffery, 2016); y ii) precursores inespecíficos, moléculas que pueden transformarse en compuestos aromáticos como consecuencia de la acción metabólica de levaduras (Ferreira & López, 2019). Un ejemplo es el aminoácido leucina que es metabolizado por la levadura y puede dar lugar a compuestos como el alcohol isoamílico o acetato de isoamilo, entre otros (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008). Sin embargo, la leucina no es un precursor específico ya que estos compuestos pueden formarse incluso si no hay leucina en los mostos (Ferreira & López, 2019).

En esta tesis se ha estudiado la formación de diferentes compuestos aromáticos presentes en el vino a partir de los diferentes precursores presentes en la uva (precursores cisteínicos y glutatiónicos, precursores glicosídicos, así como aminoácidos). La tesis está compuesta por 3 capítulos, uno por cada grupo de precursor: aminoácidos (capítulo I), precursores de cisteína y glutatión (capítulo II) y precursores glicosídicos (capítulo III).

2. CAPÍTULO I

2.1. Introducción

Los principales precursores no específicos de la uva son los aminoácidos (Albers, Larsson, Lidén, Niklasson, & Gustafsson, 1996; Hernández-Orte, Cacho, & Ferreira, 2002).

Ferreira et al., (Ferreira, López, & Cacho, 2000) encontraron que los niveles de alcoholes superiores, sus acetatos, los ácidos y sus ésteres etílicos estaban vinculados a la variedad de uva con la que el vino se había elaborado. Estos compuestos principalmente son producidos por las levaduras durante la fermentación alcohólica a través de la denominada ruta de Ehrlich (Bell & Henschke, 2005; Hazelwood et al., 2008). A partir de los aminoácidos presentes en el medio (valina, leucina, isoleucina, metionina, treonina y fenilalanina) se forman compuestos intermedios como aldehídos, los cuales pueden reducirse y formar alcoholes superiores, o por el contrario pueden oxidarse y dar lugar a los ácidos volátiles correspondientes con un átomo de carbono menos que el aminoácido correspondiente (Hazelwood et al., 2008).

Tradicionalmente, la fermentación de mostos se ha llevado a cabo de forma espontánea con levaduras presentes tanto en las uvas como en las bodegas. Las levaduras *no-Saccharomyces* aparecen en fases iniciales de la fermentación y después son sustituidas por las del género *Saccharomyces* (Henick-Kling, Edinger, Daniel, & Monk, 1998). La

variabilidad de levaduras genera una gran incertidumbre en la vinificación ya que pone en riesgo la estabilidad y reproducibilidad de la fermentación año tras año. Actualmente en las bodegas se inoculan levaduras *Saccharomyces cerevisiae* comerciales dejando a un lado las levaduras *no-Saccharomyces.* Esto asegura el éxito de la fermentación, pero genera una homogenización organoléptica del vino.

La contribución de las levaduras *no-Saccharomyces* al aroma del vino depende de la concentración de los metabolitos formados, lo que depende a su vez de las características del mosto y de la concentración de precursores. Actualmente se conoce el potencial beneficioso que tienen las *no-Saccharomyces* para la calidad del vino. Sin embargo, debido a la elevada biodiversidad de este grupo de levaduras, todavía quedan muchas oportunidades para su explotación en la producción de vino, estudiando no solo las levaduras *no-Saccharomyces* sino también su interacción con las levaduras *Saccharomyces*.

En el capítulo I se han aplicado dos metodologías de análisis sensorial rápido para seleccionar vinos fermentados con más de 100 levaduras *no-Saccharomyces* e identificar los compuestos clave responsables de la generación de diferentes perfiles aromáticos entre un gran número de muestras similares. Asimismo, se ha estudiado la capacidad que tienen las levaduras *no-Saccharomyces* seleccionadas para generar perfiles aromáticos similares entre mostos de diferentes terroirs y añadas y evaluar si pequeñas variaciones en la composición del mosto afectan al perfil aromático generado.

2.2. Métodos

2.2.1. Muestras

En el primer año, en la empresa Lev2050 (Pamplona, Spain), se realizaron microfermentaciones con un mosto de la variedad Verdejo y otro de la variedad

Tempranillo con 50 levaduras *no-Saccharomyces* para cada variedad (W1-W50 en el caso de la variedad Verdejo, y R1-R50 en el caso de la Tempranillo).

En el segundo y tercer año se fermentaron 5 mostos de la variedad Verdejo (MV1-MV5) y 5 de la Tempranillo (MT1-MT5) de diferentes terroirs con las 5 levaduras *no-Saccharomyces* seleccionadas el primer año.

2.2.2. Análisis sensoriales

Tarea de categorización (categorization task). En la tarea de categorización los panelistas agruparon los vinos en función de su calidad en "muy alta", "alta", "media", "baja" y "muy baja".

Perfilado rápido (flash profiling). El perfilado rápido consistió en 2 sesiones con una inter-sesión. En la primera sesión los panelistas generaron individualmente una lista de atributos. Durante la inter-sesión los atributos fueron agrupados en una lista global que fue dada a los panelistas durante la segunda sesión. En la segunda sesión los panelistas ordenaron las muestras según la intensidad de las mismas en cada uno de los atributos elegidos.

Tarea de agrupación (sorting task). La tarea de agrupación consistió en agrupar los vinos en grupos en función de la similitud aromática entre ellos.

2.2.3. Fraccionamiento mediante HPLC

El procedimiento de fraccionamiento se basó en el método descrito por Ferreira et al., (Ferreira, Hernández-Orte, Escudero, López, & Cacho, 1999), utilizando HPLC con columna C18, y agua y etanol como fases móviles. Se recogieron 12 fracciones de 6 mL cada una.

Los extractos inyectados en el HPLC se obtuvieron siguiendo el método descrito por López et al., (López, Aznar, Cacho, & Ferreira, 2002). 750 mL de vino se pasaron por cartuchos

de 1000 mg de resina LiChrolut EN. Los compuestos retenidos se eluyeron con 10 mL de etanol. Después de una etapa de concentración mediante desmezcla siguiendo el método descrito por Ferreira et al., (Ferreira, Escudero, López, & Cacho, 1998), se realizó una dilución 1:4 para su fraccionamiento con cromatografía líquida de alta resolución (HPLC).

Las fracciones obtenidas por HPLC fueron evaluadas sensorialmente por 3 expertos en vino que describieron cada una de ellas.

2.2.4. Identificación de los odorantes por GC-O

Se realizaron análisis de cromatografía de gas acoplada a olfatometría (GC-O) de dos tipos de muestras: i) vinos seleccionados a partir del perfilado rápido (5 vinos blancos y 5 tintos), y ii) fracciones de HPLC seleccionadas (8 fracciones para cada variedad).

2.2.4.1. Extracción de las muestras para su análisis por GC-O

Muestras extraídas de los vinos seleccionados. Los extractos totales de vino se obtuvieron por SPE siguiendo el método descrito por López et al., (López et al., 2002). Sobre 100 mg de resina LiChrolut EN se percolaron 100 mL de vino. Los compuestos aromáticos se eluyeron con 1 mL de etanol. Seguidamente, 1 mL del extracto se reconstituyó en 99 mL de vino sintético. Los extractos para el análisis por GC-O fueron obtenidos mediante la técnica de muestreo del espacio de cabeza dinámico (San Juan, Pet'ka, Cacho, Ferreira, & Escudero, 2010).

Muestras extraídas de las fracciones seleccionadas. Las fracciones seleccionadas se diluyeron con agua ácida para ajustar el etanol al 11% y seguidamente se extrajeron por SPE (López et al., 2002). Entre 30-55 mL de muestra se pasaron por cartuchos de 50 mg de LiChrolut EN que fueron seguidamente eluídos con 600 µL de diclorometano con 5% de metanol.

2.2.4.2. Análisis GC-O

GC-O. Los diferentes extractos (1 µL) fueron analizados con un cromatógrafo de gases Trace GC, con una columna DB-WAX. El panel estaba formado por seis jueces en el caso de los vinos, y 3 jueces en el caso de las fracciones. La identificación de los odorantes se llevó a cabo por comparación de sus descriptores, índices de retención en DB-WAX y DB-5 con los índices de las referencias puras.

GC-GC-O-MS. Con el fin de identificar los compuestos que no pudieron ser identificados por sus índices de retención en ambas columnas, 40 µL de las muestras se analizaron mediante cromatografía de gases multidimensional acoplada a olfatometría y espectrometría de masas (GC-GC-O-MS). La columna del primer cromatógrafo fue DB-WAX y del segundo fue VF-5MS. Con los datos obtenidos en el primer cromatógrafo se realizaron cortes selectivos para aislar los odorantes de interés que se transfirieron al segundo cromatógrafo y se monitorearon por olfatometría con detección simultánea de masas.

El análisis de datos de GC-O se basó en el cálculo de la frecuencia modificada (MF) siguiendo la fórmula dada por Dravnieks (Dravnieks, 1985).

2.2.5. Análisis cuantitativo

Aminoácidos. Los diferentes aminoácidos se determinaron por HPLC acoplado a un detector de fluorescencia siguiendo el método propuesto por Hernández-Orte et al., (Hernández, Ibarz, Cacho, & Ferreira, 2003). El método se basa en la derivatización con 6-aminoquinolil-N-hidroxisuccinimidil carbamato (AQC).

Precursores cisteínicos y glutatiónicos. Los precursores se analizaron siguiendo el método propuesto por Concejero et al., (Concejero, Peña-Gallego, Fernández-Zurbano, Hernández-Orte, & Ferreira, 2014). Para ello, 5 mL de las muestras se centrifugaron y el sobrenadante

se filtró con 2 filtros en tándem de 0.45 y 0.20 µm. Las muestras se analizaron mediante cromatografía líquida de ultra alta resolución (UHPLC) acoplada a espectrómetro de masas de triple cuadrupolo.

Compuestos mayoritarios (mg/L). El método utilizado fue desarrollado por Ortega et al., (Ortega, López, Cacho, & Ferreira, 2001) mediante una extracción líquido-líquido con diclorometano y posterior análisis por cromatografía de gases con un detector FID. La cuantificación de las muestras se llevó a cabo por interpolación en las rectas de calibrado realizadas en vinos modelo.

Compuestos minoritarios (µg/L). El método utilizado consistió en una extracción SPE siguiendo el método desarrollado por López et at., (López et al., 2002) con algunas modificaciones: 15 mL de vino se percolaron a través de 65 mg de resina LiChrolut EN previamente acondicionada. La elución de los compuestos se llevó a cabo con 0.6 mL de dicloromentano con 5% de metanol. El extracto se analizó con un sistema GC con detección de espectrometría de masas con trampa de iones.

Mercaptanos polifuncionales (ng/L). El método utilizado fue desarrollado y validado por Mateo-Vivaracho et al., (Mateo-Vivaracho, Zapata, Cacho, & Ferreira, 2010). El análisis se realizó con cromatógrafo de gases con detección de masas con ionización química negativa (NCI).

2.3. Resultados y discusión

Para seleccionar las levaduras *no Saccharomyces* que generaron vinos con una calidad aromática alta, se realizó un screening sensorial de los 50 vinos obtenidos para cada variedad mediante un análisis de categorización de las muestras (Categorization task). Una vez seleccionadas las levaduras *no-Saccharomyces* que generaron vinos de mayor calidad, se realizó un perfilado rápido (Flash profiling) de los mismos. Nueve vinos de Verdejo y

siete Tempranillo se clasificaron en la categoría de calidad más alta presentando diferentes perfiles aromáticos: cítrico, fruta compotada, vegetal/boj, fruta tropical y cereal en el caso de los Verdejos, y fruta roja, yogurt de fresa, fruta compotada, fruta blanca y plátano en el caso de los Tempranillos.

Los vinos con perfiles aromáticos más diferentes fueron analizados mediante GC-O. Se seleccionaron 5 vinos blancos obtenidos con las levaduras W39_sacch (cítrico), W20 (cítrico), W47 (fruta tropical y vegetal/boj), W36 (fruta compotada y cereal) y W12 (fruta compotada) y otros 5 vinos tintos fermentados con las levaduras R20_sacch (yogurt de fresa, fruta roja y tofe), R22 (fruta roja, yogurt de fresa, fruta compotada, fruta blanca y plátano), R24, R45 y R27 (fruta compotada, fruta blanca y plátano). Los principales compuestos responsables de los aromas a fruta compotada fueron acetato de isoamilo, β-damascenona y acetato de 2-feniletilo. Por otro lado, acetato de 3-mercaptohexilo y butirato de etilo fueron responsables de las notas a fruta tropical y fruta roja, respectivamente.

Las levaduras seleccionadas se usaron para fermentar 5 mostos de la variedad verdejo y otros 5 de la variedad Tempranillo obtenidos a partir de uvas de diferentes zonas. Los vinos obtenidos de cada variedad fueron analizados sensorialmente mediante un test de agrupación (Sorting task). Los panelistas formaron 4 grupos en el caso de los vinos Verdejo (el grupo 1 fue descrito como fruta compotada, dulce, verde, mosto/uva y desagradable; el grupo 2, fruta tropical, fruta compotada, fruta madura y dulce; el grupo 3, frutas tropicales, reducción y agua estancada; y el grupo 4, fruta tropical, fruta blanca y fruta), y 4 en el caso de los Tempranillo (grupo 1 tenía descriptores como reducción, huevos podridos, alcantarillado y fruta; el grupo 2, fruta roja, caramelo/láctico y dulce; el grupo 3, fruta compotada, fruta blanca, verde, cereal y fruta tropical; y el grupo 4, fruta tropical, fruta blanca y fruta compotada).

Con este análisis encontramos que, en el caso de los vinos blancos, una misma levadura producía vinos con un perfil aromático similar independientemente del origen del mosto, 3 levaduras generaron un perfil aromático similar con la excepción de un mosto para cada una de ellas, y otra levadura generó vinos con perfiles aromáticos diferentes en función del mosto de partida. En el caso de los mostos de Tempranillo, 2 levaduras generaron vinos con un perfil aromático similar, 2 levaduras generaron un perfil aromático similar con la excepción de un mosto para cada una de ellas, y una produjo vinos diferentes en función del origen del mosto.

Para identificar los compuestos que podrían afectar a la generación de los diferentes perfiles aromáticos se analizaron los precursores cisteínicos y glutatiónicos, así como los aminoácidos presentes en los mostos. Por otro lado, se identificaron los aromas claves responsables de los diferentes perfiles aromáticos obtenidos tras la tarea de agrupación.

Con respecto a los precursores de las uvas, se observó que en algunos casos la composición del mosto puede influir en el perfil aromático como en el caso de los mostos Verdejo donde la presencia de aromas afrutados podría ser debida a una mayor concentración de aminoácidos o la ausencia de notas de frutas tropicales, debida a una menor concentración en los precursores cisteínicos y glutatiónicos. Sin embargo, la influencia de la composición de los mostos Tempranillo en las características sensoriales no fue tan clara.

Para la identificación de los aromas clave responsables de notas positivas se seleccionaron 3 muestras representativas de cada variedad. Las muestras de blanco seleccionadas se describían como aromas a fruta fresca, aromas a fruta dulce, notas verdes y negativas. Los vinos tintos seleccionados tenían descriptores como fruta roja, aromas afrutados y notas a verde y cereal. Las 6 muestras fueron fraccionadas por HPLC. Cada fracción fue descrita sensorialmente y las fracciones cuyos aromas fueron similares al vino se caracterizaron mediante GC-O.

En el caso de los Verdejos, el aroma a fruta tropical se pudo explicar por las elevadas frecuencias modificadas (MF) de 3-mercaptohexanol y acetato de 3-mercaptohexilo. En los vinos con aroma a fruta dulce se encontraron MF más altas de acetato de isoamilo, acetato de 2-feniletilo, acetato de bencilo y β-damascenona. Por otro lado, acetato de isobutilo, hexanoato de etilo, dihidromircenol y óxido de linalol estuvieron asociados a las notas a fruta fresca. Las notas verdes estuvieron relacionadas con 3-mercaptohexanol, dihidromircenol y óxido de linalol, mientras que las notas desagradables con el ácido 3-metilbutírico. En el caso de los vinos de Tempranillo, las notas afrutadas (fruta compotada, tropical y blanca) se encontraron en las muestras con MF altas de acetato de isoamilo, hexanoato de etilo, acetato de 3-mercaptohexilo, acetato de 2-feniletilo y β-damascenona. Las notas verdes estuvieron relacionadas con *Z*-3-hexenal, dihidromircenol y óxido de linalol, mientras que las notas a cereal podrían deberse a la presencia de 2-isopropil-3-metoxipirazina. Por otro lado, las notas a fruta roja, láctico y caramelo fueron relacionadas con la presencia de acetato de isobutilo, β-damascenona y furaneol.

En el tercer año, se volvieron a realizar microfermentaciones con las mismas levaduras *no-Saccharomyces* seleccionadas y con los 5 mostos de los diferente terroirs de cada variedad. Se realizaron análisis sensoriales de todas las muestras para agruparlas en función de su similitud aromática como se hizo en la añada anterior. Para los vinos blancos, se encontró que dos de las levaduras generaron perfiles aromáticos similares tanto entre los diferentes mostos como con los de la añada anterior, es decir, para estas dos levaduras la composición del mosto no afectó a su metabolismo, mientras que las otras levaduras generaron diferentes perfiles aromáticos no solo entre los diferentes mostos, sino también entre las diferentes añadas. Por otro lado, para los vinos tintos, se observó que las 5 levaduras generaron perfiles aromáticos similares entre los diferentes mostos, pero solo dos de ellas generaron el mismo perfil aromático al fermentar mostos de diferentes añadas.

Al igual que con las muestras de la añada anterior, en el 2016 también se realizó el análisis cuantitativo de los precursores presentes en los mostos. En ambas añadas se analizaron los aminoácidos y los precursores cisteínicos y glutatiónicos de los 5 mostos de cada variedad. Se encontraron diferencias importantes en la relación entre los aminoácidos, aunque la relación entre los diferentes terroirs no se mantuvo entre las dos cosechas.

De estos estudios encontramos que dos levaduras para blanco y otras dos para tintos generaron un perfil aromático similar después de la fermentación de mostos de diferentes terroirs y añadas, por lo que se podría decir que los aromas generados por estas levaduras fueron bastante independientes de la composición del mosto, y por ello fueron seleccionadas.

Por último, estas 4 levaduras *no-Saccharomyces seleccionadas* se usaron para estudiar si su comportamiento se mantenía en la co-inoculación con *Saccharomyces cerevisiae*. Las muestras obtenidas tras la co-inoculación se analizaron sensorialmente con un test de agrupación (sorting task).

La co-inoculación con *S. cerevisiae* no generó cambios en los perfiles sensoriales obtenidos con las dos levaduras *no-Saccharomyces* en los vinos blancos. Es decir, los vinos obtenidoc con *S. cerevisiae* y las levaduras W20 y W36 tuvieron perfiles aromáticos similares a los observados anteriormente al fermentar mostos de diferentes terroirs de 2015 y 2016 solo con estas dos levaduras *no-Saccharomyces*. Por el contrario, en los vinos tintos, cuando las 2 levaduras *no-Saccharomyces* fueron fermentadas con *S. cerevisiae*, no se encontraron diferencias entre los perfiles aromáticos generados por las diferentes levaduras *no-Saccharomyces*, sino que el aroma estuvo determinado principalmente por *Saccharomyces cerevisiae*.

Con el fin de estudiar si estas diferencias observadas a nivel sensorial se explicaban con los datos cuantitativos, se analizaron los compuestos mayoritarios, minoritarios y los mercaptanos polifuncionales. En el caso de los vinos de Verdejo, las concentraciones de los compuestos analizados fueron más elevadas en los vinos con co-inoculación que sin ella. Estas diferencias a nivel cuantitativo no explicaron la ausencia de efecto de la co-inoculación observada sensorialmente. Por el contrario, en el caso de los vinos de Tempranillo, la separación observada a nivel sensorial entre las muestras fermentadas con y sin co-inoculación con *S. cerevisiae*, no pudo explicarse cuantitativamente con los compuestos analizados. Los diferentes perfiles observados podrían deberse a compuestos que no han sido analizados o a las interacciones entre los diferentes compuestos presentes en las muestras.

2.4. Conclusiones

La tarea de categorización seguida del perfilado rápido y GC-O han demostrado ser una metodología sensorial rápida y efectiva para la selección de vinos de alta calidad. Esta metodología permitió seleccionar diferentes levaduras *no-Saccharomyces* (7 en el caso del Verdejo y 5 para el Tempranillo), capaces de generar vinos de alta calidad descritos con aromas cítricos, fruta compotada, vegetal/boj, fruta tropical y cereal para Verdejo y frutas rojas o frutas compotadas para Tempranillo, así como identificar los compuestos vinculados a dichos aromas: β-damascenona (fruta compotada), acetato de 3-mercaptohexilo (fruta tropical) y butirato de etilo (fruta roja).

Por otro lado, la tarea de clasificación seguida de fraccionamiento y análisis GC-O también ha demostrado ser una metodología sensorial rápida para la identificación de compuestos responsables de los aromas distintivos de los vinos. Esta metodología permitió identificar el acetato de isoamilo, β-damascenona y acetato de 2-feniletilo como compuestos

vinculados a aromas a fruta madura y compotada; acetato de 3-mercaptohexilo y 3-mercaptohexanol como compuestos vinculados a notas a fruta tropical; acetato de isobutilo, hexanoato de etilo y dihidromircenol a aromas de fruta blanca; y ácido 3-metilbutírico como responsable de aromas desagradables en los vinos Verdejo. En el caso de los Tempranillo, el *Z*-3-hexenal se identificó como un compuesto vinculado a los aromas verdes; acetato de isoamilo, hexanoato de etilo, acetato de 3-mercaptohexilo, acetato de 2-feniletilo y β-damascenona como compuestos capaces de producir notas frutales; y acetato de isobutilo, furaneol y β-damascenona como compuestos que pueden generar aromas de caramelo y fruta roja. La aplicación de esta metodología permitió también identificar cuatro levaduras *no-Saccharomyces* que pudieron generar vinos con un perfil aromático similar al fermentar mostos no solo de diferentes terroirs, sino también de diferentes añadas. Por lo tanto, estas levaduras *no-Saccharomyces* no dependieron de la composición del mosto.

En el caso de los vinos de Verdejo, el perfil aromático generado con las levaduras seleccionadas se mantuvo incluso con co-inoculación con *Saccharomyces cerevisiae*, mientras que el perfil aromático generado por las levaduras empleadas en los vinos de Tempranillo tras la co-inoculacion fue determinado por *Saccharomyces cerevisiae*, y no hubo diferencia entre levaduras *no-Saccharomyces*.

Por lo tanto, estas metodologías sensoriales son herramientas efectivas y rápidas en la detección y caracterización de perfiles aromáticos de calidad.

Para más información, mirar los artículos:

- Sáenz-Navajas, M. P.; **Alegre, Y**.; de-la-Fuente-Blanco, A.; Ferreira, V.; García, D.; Eizaguirre, S.; Razquin, I.; Hernández-Orte, P., Rapid sensory-directed methodology for the selection of high-quality aroma wines. Journal of the Science of Food and Agriculture 2016, 96 (12), 4250-4262.

- **Alegre, Y**.; Sáenz-Navajas, M. P.; Ferreira, V.; García, D.; Razquin, I.; Hernández-Orte, P., Rapid strategies for the determination of sensory and chemical differences between a wealth of similar wines. European Food Research and Technology 2017, 243 (8), 1295-1309.

3. CAPÍTULO II

3.1. Introducción

Los precursores cisteínicos y glutatiónicos son precursores de los mercaptanos polifuncionales 4-mercapto-4-metil-2-pentanona (4MMP) y 3-mercatohexanol (3MH) que son compuestos aromáticos impacto en el vino dado que suelen encontrarse en concentraciones por encima de su umbral de olfacción (Tominaga, Murat, & Dubourdieu, 1998). Estos compuestos son responsables de notas frescas en los vinos y están asociados a aromas a fruta tropical, pomelo, casis, boj y guayaba (Lund Thompson, Benkwitz, Wohler, Triggs, Gardner, Heymann, & Nicolau, 2009; Mestres, Busto, & Guasch, 2000; Swiegers & Pretorius, 2007; Tominaga, Murat, et al., 1998).

En la uva, estos mercaptanos polifuncionales están unidos a una molécula de cisteína y/o de glutatión formando los precursores inodoros (cisteína-3-mercapto-hexan-1-ol, CYSMH, cisteína-4-mercapto-4-metil-2-pentanona, CYSMP, glutatión-3-mercapto-hexan-1-ol, GLUMH, y glutatión-4-mercapto-4-metil-2-pentanona, GLUMP) (Fedrizzi, Pardon, Sefton, Elsey, & Jeffery, 2009; Peyrot des Gachons, Tominaga, & Dubourdieu, 2002; Tominaga, Peyrot des Gachons, et al., 1998).

Durante la fermentación alcohólica, la acción de la enzima β-liasa producida por la levadura escinde el enlace carbono-sulfuro de los precursores dando lugar a la liberación de los mercaptanos polifuncionales (Tominaga, Peyrot des Gachons, et al., 1998). Sin embargo,

la concentración del 3MH y 4MMP en el vino no está correlacionada con la concentración de sus precursores en el mosto, de hecho, el nivel de conversión es inferior al 5% (Bonnaffoux, Delpech, Rémond, Schneider, Roland, & Cavelier, 2018; Concejero, Hernández-Orte, Astrain, Lacau, Baron, & Ferreira, 2016; Peyrot des Gachons, Tominaga, & Dubourdieu, 2000; Roland, Schneider, Razungles, Le Guerneve, & Cavelier, 2010). Estos hechos indican que hay aspectos relacionados con el metabolismo de los precursores que aún son desconocidos.

El capítulo II tiene 4 objetivos diferentes: i) estudiar el efecto de diferentes perfiles de aminoácidos en el consumo por parte de la levadura de los precursores cisteínicos y glutatiónicos; ii) identificar los precursores preferidos por la levadura; iii) determinar el efecto de diferentes concentraciones y fuentes de azufre en la liberación de los mercaptanos polifuncionales a partir de los precursores; y iv) estudiar el efecto de la adición de cisteína y glutatión en los genes relacionados con el metabolismo de los precursores de mercaptanos polifuncionales.

3.2. Métodos

3.2.1. Mosto sintético

Se preparó mosto sintético adaptado de Bely et al., (Bely, Sablayrolles, & Barre, 1990) con algunas modificaciones:

Experimento 1. Efecto del perfil de aminoácidos. Se realizaron fermentaciones con mostos sintéticos preparados con 150 mg N/L. Los mostos únicamente se diferenciaron por su perfil aminoacídico. Se prepararon 9 mostos sintéticos con los perfiles aminoacídicos de 7 variedades diferentes (Hernández-Orte et al., 2002). El nitrógeno total de los perfiles se ajustó porcentualmente.

Experimento 2. Efecto de la adición de precursores por separado. Se estudiaron 2 concentraciones de nitrógeno (120 y 150 mg/L) obtenidas mediante el ajuste porcentual del perfil aminoacídico de la variedad Chardonnay. La baja concentración de nitrógeno se utilizó con el fin de intentar forzar a la levadura a utilizar dichos precursores.

Para los experimentos del 3 al 6, el mosto se preparó con una concentración de nitrógeno de 200 mg/L (mediante el ajuste porcentual del perfil aminoacídico de Chardonnay). En este mosto sintético se realizaron diferentes adiciones.

Experimento 3. Se añadieron diferentes compuestos azufrados a diferentes concentraciones: azufre elemental (1 mg/L), glutatión (50 y 70 mg/L), cisteína (20 mg/L), metionina (30 y 50 mg/L) y dióxido de azufre (20 mg/L).

Experimento 4. Se adicionaron los aminoácidos que componen el glutatión (GSH): cisteína (10, 20 y 30 mg/L), ácido glutámico (50 mg/L) y glicina (10 mg/L).

Experimento 5. Se adicionó dióxido de azufre (SO_2) a diferentes concentraciones (20, 30, 50 y 70 mg/L).

Experimento 6. Estudio transcriptómico durante la fermentación. Se añadió GSH y cisteína a diferentes concentraciones. Se realizaron 3 sets de fermentaciones. En el primero se evaluó el crecimiento de las poblaciones de *S. cerevisiae*. En el segundo se estudió cuando se liberaban los mercaptanos polifuncionales. Con los datos obtenidos se decidieron los momentos durante la fermentación en los que se tomarían muestras para los análisis de transcriptómica que se realizaron en el tercer set.

En todos los mostos se añadieron los 4 precursores de los mercaptanos polifuncionales (50 µg/L de CYSMP y GLUMP, 100 µg/L de CYSMH y 1000 µg/L de GLUMH). Además, en el experimento 2 los precursores cisteínicos y gluatiónicos se añadieron por separado. En todos los experimentos los mostos se inocularon con levaduras *Saccharomyces* (Zymaflore

X5) y se realizaron las fermentaciones por triplicado a 20 ºC hasta obtener un peso constante.

3.2.2. Análisis

Al final de la fermentación alcohólica se analizaron los mercaptanos polifuncionales y los precursores remanentes siguiendo los métodos descritos previamente en la sección 2.2.5 del resumen.

Además, en el experimento 6, se tomaron muestras a diferentes tiempos, en el primer y segundo set con el fin de evaluar la liberación de mercaptanos polifuncionales y el crecimiento de las poblaciones de levadura, respectivamente. En el tercer set para los análisis transcriptómicos se tomaron muestras a las 26 y 150 h.

Conteo celular. El conteo de las poblaciones se realizó con un contador electrónico de partículas. Las muestras se diluyeron para estar en el rango de 20000 a 80000 células/mL y luego los agregados se separaron por sonicación.

Análisis transcriptómico. Los ARNm se extrajeron de las células y se sometieron a retro-transcripción y luego a transcripción en presencia de nucleótidos marcados con un fluorocromo. Los ARN complementarios marcados (ARNc) se purificaron e hibridaron en los chips de ADN (placas de vidrio que contienen hebras de ADNc monocatenario a las que se unen los ARNm). Los fragmentos de ARNc marcados se unen a las cadenas de ADN por complementariedad de bases. Las diferencias en la expresión genética se evaluaron en función de la intensidad de la fluorescencia (Duc, Pradal, Sanchez, Noble, Tesnière, & Blondin, 2017). El tratamiento de los datos se llevó a cabo con el software R versión R.3.6.1.

3.3. Resultados y discusión

En el primer experimento, se estudió el efecto del perfil de aminoácidos sobre la liberación de mercaptanos polifuncionales, así como sobre el consumo de sus precursores. Se prepararon 9 mostos distintos simulando los perfiles aminoacídicos de 7 variedades distintas y 2 versiones de una de las variedades. En los vinos obtenidos se encontraron diferencias significativas en el consumo de precursores y en la liberación de mercaptanos funcionales en función del perfil aminoacídico. Se observó un mayor consumo de los precursores, así como una mayor liberación de 3MH y 4MMP en los vinos con el perfil de aminoácidos de la variedad Chardonnay.

Con el fin de determinar si el tipo de precursor influye en el metabolismo de la levadura, se prepararon mostos sintéticos con diferentes cantidades de nitrógeno total (120 y 150 mg N/L) a los que se añadieron precursores cisteínicos y otros a los que se añadieron precursores glutatiónicos, así como un control con todos los precursores juntos. En los vinos cuyos mostos fueron adicionados de precursores cisteínicos se encontraron las mismas concentraciones de 4MMP que en los vinos control que contenían tanto los precursores cisteínicos como glutatiónicos juntos, mientras que en los vinos que contenían solo los precursores glutatiónicos la concentración de 4MMP fue solo un 25% de la del control. Además, el análisis de precursores indicó que el mayor consumo de los precursores de la 4MMP se produjo cuando los precursores adicionados fueron los cisteínicos. Esto parece indicar que el principal precursor de la 4MMP es la CYSMP. Por otro lado, el principal precursor del 3MH fue el GLUMH. Ambos datos fueron similares con las dos concentraciones de nitrógeno probadas.

El bajo factor de conversión entre los mercaptanos polifuncionales y sus precursores podría ser debido a que las levaduras usan los precursores como fuente de algún nutriente que

podría ser algún compuesto azufrado. Para comprobarlo se realizaron fermentaciones de mosto sintético a los que se adicionaron diferentes compuestos azufrados: azufre elemental, dióxido de azufre, glutatión, metionina y cisteína. Se observó que GLUMH es el precursor más consumido y que la levadura podría transformar GLUMP en CYSMP. Sin embargo, aunque hubo un efecto significativo en todos los casos, los efectos de mayor magnitud se observaron en el caso de GLUMH. Al agregar GSH se produjo una disminución en la metabolización de GLUMH, por lo que las levaduras podrían usar este precursor como fuente de GSH. Por otro lado, la adición de SO_2 provocó un aumento en el nivel de metabolización de GLUMH respecto al control, único caso en que se observó aumento. Respecto a la liberación de mercaptanos polifuncionales, el efecto más claro fue un aumento en los mercaptanos polifuncionales cuando se añadieron SO_2 y cisteína a 20 mg/L. La adición de GSH causó un aumento significativo de 4MMP y una disminución de 3MH.

Para explicar la razón por la cual la adición de GSH disminuyó la metabolización de GLUMH, se estudió el efecto de los aminoácidos que componen el GSH. La adición de ácido glutámico y glicina no tuvo un efecto relevante en la metabolización de estos precursores, al contrario que la adición de cisteína. Por esta razón, se estudiaron diferentes niveles de cisteína. El efecto más fuerte fue causado por la adición de altas cantidades de cisteína, observando una menor metabolización de GLUMH. Esto parece indicar que las levaduras usan este precursor como fuente de cisteína. En el caso del efecto sobre los mercaptanos polifuncionales, se observó un efecto dependiente de la dosis. La concentración de cisteína baja no generó diferencias, la concentración media generó un aumento en los 3 compuestos aromáticos y la concentración alta provocó una disminución de los tres compuestos. Esta menor concentración es consistente con el hecho de que, en esta muestra, el precursor de GLUMH fue menos metabolizado que en los controles.

Debido a que el SO_2 fue el único que produjo un consumo mayor de GLUMH, se estudió el efecto de diferentes niveles de SO_2. Sin embargo, aunque la concentración más baja produjo un aumento en la metabolización de GLUMH, las concentraciones intermedias dieron resultados opuestos. Sorprendentemente, los efectos del SO_2 fueron mucho más intensos en CYSMH, que fue más consumido cuando el mosto contenía SO_2. Lo que podría indicar que SO_2 previene la formación de CYSMH a partir de GLUMH. Con respecto al efecto sobre los mercaptanos polifuncionales, la adición de 20 mg/L de SO_2 provocó un aumento en las concentraciones de 4MMP y 3MH. Por otro lado, la adición de niveles más altos no tuvo un efecto sobre la concentración de 4MMP pero provocó un aumento en la concentración de 3MH. Por lo tanto, además de evitar la oxidación de estos compuestos, el SO_2 puede producir algún efecto metabólico.

Los resultados obtenidos previamente en los que se observó que se produce una menor desaparición de GLUMH cuando las muestras contienen GSH y cisteína nos llevaron a pensar que estas diferencias en el metabolismo de los precursores de los tioles volátiles podría deberse a una regulación de los genes de la levadura implicados en la ruta metabólica de los mismos. Por ello se estudió el efecto de la adición de estos compuestos sobre los genes implicados en el metabolismo de los precursores de los mercaptanos polifuncionales. Para ello, se realizaron fermentaciones que contenían cisteína y glutatión a diferentes concentraciones además de un control sin adiciones. A las 26 y 150 h se realizaron análisis de transcriptómica.

Previamente se había observado que la adición de cisteína a 10 mg/L no provocaba cambios en la concentración de los mercaptanos polifuncionales con respecto al control sin adicionar. En el estudio transcriptómico también encontramos que se producían pocos cambios, solo 4 genes se expresaron diferencialmente a las 26 h y 1 gen a las 150 h. Sin embargo, ninguno de estos genes tenía relación ni con ciclo del azufre, ni con las rutas de

biosíntesis de los aminoácidos azufrados y GSH, ni con genes relacionados con el metabolismo de los mercaptanos polifuncionales. Resultados similares fueron observados en los vinos obtenidos con las adiciones de GSH a 70 mg/L y cisteína a 30 mg/L a pesar de que se habían observado cambios en los compuestos volátiles. Por lo tanto, la razón de este menor consumo del GLUMH con la adición de GSH a 70 mg/L y cisteína a 30 mg/L podría ser debida a una necesidad nutricional por parte de la levadura y no a una regulación a nivel genético.

Las concentraciones intermedias de estos compuestos, por el contrario, dieron como resultado un aumento del número de genes expresados diferencialmente. En ambos casos se observaron genes implicados en las rutas metabólicas de la cisteína, metionina y azufre. El caso más llamativo se observó con la adición de cisteína a 20 mg/L que generó una concentración de tioles volatiles significativamente mayor en comparación con el control, y además, en esta muestra se observó también una sobre-expresión del gen IRC7 que codifica a un enzima con actividad β-liasa. La adición de cisteína a 20 mg/L produce un aumento en la actividad β-liasa de la levadura lo que genera mayor concentración de mercaptanos polifuncionales.

3.4. Conclusiones

El perfil de aminoácidos afecta no solo al consumo de los precursores de los mercaptanos polifuncionales sino también a la liberación de los mismos. Siendo el perfil de aminoácidos la única diferencia entre los mostos, se observó que en los vinos con el perfil de la variedad Chardonnay se producía una mayor concentración de 4MMP y 3MH. Además, se observó que las levaduras consumían preferentemente el precursor cisteínico de la 4MMP y el precursor glutatiónico del 3MH.

Las levaduras podrían usar los precursores cisteínicos y glutatiónicos como fuente de GSH y/o cisteína dado que la adición de concentraciones altas tanto de GSH como de cisteína generó un consumo significativamente menor de los precursores.

La adición de cisteína a 20 mg/L generó una sobre-expresión del gen IRC7 que codifica a una enzima Irc7p con actividad β-liasa, dando lugar a la generación de mayores concentraciones de mercaptanos polifuncionales.

Para más información, mirar los artículos:

- **Alegre, Y**.; Culleré, L.; Ferreira, V.; Hernández-Orte, P., Study of the influence of varietal amino acid profiles on the polyfunctional mercaptans released from their precursors. Food Res. Int. 2017, 100, 740-747.
- **Alegre, Y**.; Ferreira, V.; Hernández-Orte, P., How does the addition of antioxidants and other sulfur compounds affect the metabolism of polyfunctional mercaptan precursors in model fermentations? Food Research International 2019, 122, 1-9.

4. CAPÍTULO III

4.1. Introducción

Los precursores glicosídicos están formados por una aglicona unida a una o varias gliconas (moléculas de azúcar) (Gunata, Bitteur, Brillouet, Bayonove, & Cordonnier, 1988; Williams, Strauss, Wilson, & Massy-Westropp, 1982). Se han identificado más de 100 agliconas diferentes clasificadas en terpenoles, norisoprenoides, lactonas, fenoles volátiles, derivados de las vainillinas y misceláneos. Estas agliconas son liberadas durante el proceso de vinificación mediante glicosidasas exógenas o endógenas (Gunata, Bayonove, Tapiero, & Cordonnier, 1990; Sánchez-Palomo, Díaz-Maroto Hidalgo, González-Viñas, & Pérez-

Coello, 2005), o mediante hidrólisis ácida lenta (López et al., 2004; Skouroumounis & Sefton, 2000).

El estudio de estos compuestos es complicado debido a que, en algunos casos, la aglicona es el compuesto aromático como tal (Strauss, Wilson, Gooley, & Williams, 1986; Wilson, Strauss, & Williams, 1984), pero en otros casos, el aroma se forma después de reordenamientos químicos espontáneos de la aglicona original.

La mayoría de las investigaciones se basan en el estudio indirecto de las fracciones de precursores no volátiles extraídas de la uva. Estas fracciones precursoras generalmente son "reveladas" por dos estrategias principales: hidrólisis enzimática y/o hidrólisis ácida fuerte (Delfini et al., 2001; Loscos, Hernández-Orte, Cacho, & Ferreira, 2009). El problema de la hidrólisis enzimática es que algunos de los compuestos aromáticos más relevantes, como la β-damascenona, TDN o β-ionona, no son agliconas como tal y por lo tanto, no se forman con este tipo de hidrólisis (Waterhouse et al., 2016). Por otra parte, la hidrólisis ácida fuerte, proporciona información sobre estas últimas moléculas, pero destruye una elevada proporción de compuestos aromáticos relevantes como terpenoles (Loscos et al., 2009). Los mejores resultados aromáticos se obtienen usando hidrólisis ácida leve, la cual conlleva mucho tiempo. En las condiciones utilizadas habitualmente existen fuertes evidencias que sugieren que se produce una fuerte oxidación y deterioro del aroma durante la hidrólisis ácida fuerte.

Por ello, el principal objetivo de este capítulo consiste en el desarrollo de una nueva estrategia metodológica para medir el potencial aromático de las uvas de vinificación. Este objetivo principal se divide en los siguientes sub-objetivos: i) desarrollar un método para la extracción de precursores del aroma; ii) desarrollar un método de hidrólisis acelerada para determinar el potencial aromático de las uvas; y iii) aplicar la metodología desarrollada

para determinar el potencial de uvas de diferentes variedades, bodegas, terroirs y estado de madurez.

4.2. Métodos

4.2.1. Desarrollo del método de extracción

El desarrollo del método de extracción se llevó a cabo con uvas de la variedad Tempranillo de Bodegas Pingus, y uvas de la variedad Garnacha de Bodegas Ilurce.

Preparación de los mostos etanólicos (mistelas). Diez kg de uvas de Tempranillo y Garnacha se despalillaron y prensaron. A la pasta obtenida se le añadió etanol (15%) y metabisulfito potásico.

Optimización del cartucho y volumen de ruptura. Se prepararon 3 tipos de muestras (mistela, mistela diluida al 50% y mistela desalcoholizada). Se estudiaron cartuchos de 7 y 10 g de C18. Tras el acondicionamiento de los cartuchos, los 3 tipos de muestra se pasaron a través de los mismos y se recogieron fracciones de 5 mL. En estas fracciones se analizó el índice de polifenoles totales (TPI) (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). Para determinar el volumen de ruptura, se consideró una pérdida del IPT inferior al 15% en comparación al control.

Determinación del volumen de elución. Para determinar el volumen de elución los cartuchos se eluyeron con etanol y se recogieron diferentes fracciones. Con el fin de determinar la presencia o ausencia de precursores glicosídicos en las fracciones, se hizo una hidrólisis ácida a 100 ºC (Ibarz, Ferreira, Hernández-Orte, Loscos, & Cacho, 2006) y las fracciones se analizaron sensorialmente. En estas fracciones también se analizaron los taninos y antocianos (Ribéreau-Gayon et al., 2006).

Extracción de las fracciones fenólicas y aromáticas (PAFs, phenolic and aromatic fractions). 750 mL de mistela se desalcoholizó y se pasó a través de un cartucho C18 de 10 g, seguidamente se eluyó con 100 mL de etanol.

4.2.2. Condiciones de la hidrólisis

Se prepararon 3 tipos de muestras: i) mistelas; ii) fracciones fenólicas y aromáticas reconstituidas en vino sintético (rPAFs); y fracciones fenólicas y aromáticas recontituidas en vino sintético con azúcar (rsPAFs). Estas muestras se incubaron a 45 ºC durante 2, 4 y 7 semanas tanto en anoxia como en presencia de oxígeno. Además, las PAFs de Tempranillo reconstituidas en vino sintético (rPAF) se incubaron a 75 ºC durante diferentes tiempos de 3 a 72 h. Los componentes liberados se analizaron sensorialmente, por GC-O y por GC-MS.

4.2.3. Análisis sensoriales

Las muestras fueron caracterizadas mediante 4 análisis sensoriales diferentes. El primero fue llevado a cabo para determinar el volumen de elución. El segundo y el tercero consistieron en una tarea descriptiva para ambas muestras obtenidas con la hidrólisis acelerada a 45 °C y a 75 °C. El cuarto análisis sensorial consistió en un test triangular para las rPAFs obtenidas a 75 °C.

Además, una vez desarrollado el método de extracción e hidrólisis acelerada, se llevó a cabo la caracterización de 33 uvas de Garnacha y Tempranillo de diferentes orígenes, terroirs y estados de madurez. Para la caracterización de estas muestras se llevaron a cabo 2 análisis sensoriales. El primero fue un test de agrupamiento (sorting task) para agrupar según similitud aromática 33 rPAFs diferentes de Tempranillo y Garnacha, y el segundo un perfilado rápido (flash profiling) para obtener una mayor caracterización de las rPAFs

seleccionadas a partir del test de agrupamiento. Estos dos últimos se realizaron como se han descrito en la sección 2.2.2.

Determinación del volumen de elución. Este análisis sensorial se realizó para determinar la presencia/ausencia de los aromas liberados de los precursores glicosídicos en cada una de las fracciones recogidas. Los panelistas indicaron "si" o "no" según si había presencia o ausencia de aroma, respectivamente.

Análisis descriptivo. Los panelistas tuvieron que oler las fracciones y describirlas libremente usando de 1 a 5 atributos. Asimismo, se les pidió que indicaran la intensidad de cada muestra definiéndolas como baja, media o alta.

Test triangular. La rPAF de Tempranillo incubada a 75 ºC durante 14, 24, 38 y 48 h fue sometida a test triangulares con el fin de identificar si había diferencias entre los diferentes pares de rPAFs: i) rPAF incubada durante 14 h *vs* rPAF incubada durante 24 h; ii) 24 h *vs* 38 h; y iii) 24 h *vs* 48 h. A los panelistas se les presentaron 3 copas y tuvieron que indicar cuál era la copa diferente.

4.2.4. Análisis cuantitativos

Aromas liberados de los precursores glicosídicos. La cuantificación se llevó a cabo siguiendo el método desarrollado por López et al., (López et al., 2002) para el análisis de los compuestos minoritarios (sección 2.2.5) pero cambiando el método cromatográfico. Dos µL de la muestra se inyectaron en un GC-MS QP2010 siguiendo el método descrito por Oliveira et al., (Oliveira & Ferreira, 2019).

Compuestos azufrados volátiles. Se determinaron siguiendo el método de Franco-Luesma et al., (Franco-Luesma & Ferreira, 2014). Se usó una microextracción en fase sólida de espacio de cabeza automatizada (HS-SPME) con una fibra de carboxen-polidimetilsiloxano seguida de una detección fotométrica de llama pulsada con cromatografía de gases (GC-

PFPD). Las concentraciones se calcularon en función de la interpolación en las rectas de calibración.

Aminoácidos. Se analizaron los aminoácidos como se describió en la sección 2.2.5.

Metales. Los metales de transición más abundantes y enológicamente relevantes (Fe, Cu, Mn y Zn) se determinaron midiendo los isótopos más abundantes (^{56}Fe, ^{63}Cu, ^{55}Mn y ^{66}Zn) por espectrometría de masas de plasma acoplada inductivamente usando un procedimiento publicado por Grindlay et al., (Grindlay, Mora, de Loos-Vollebregt, & Vanhaecke, 2014).

4.2.5. GC-O

Los análisis GC-O se llevaron a cabo siguiendo el método descrito en la sección 2.2.4.2.

4.3. Resultados y discusión

El objetivo principal fue desarrollar un método para la determinación del potencial aromático de la uva. Para ello, en primer lugar, se desarrolló un método de extracción no solo de los precursores glicosídicos sino también de la fracción polifenólica de la uva (phenolic and aromatic fractions, PAFs).

4.3.1. Desarrollo del método extracción de las PAFs

Se optimizaron la resina a utilizar, la preparación de la mistela a partir de la cual se extraerían las PAFs, el volumen de ruptura y el de elución. Las uvas se procesaron inmediatamente en presencia de etanol para evitar la fermentación e imitar la extracción obtenida en la elaboración del vino tinto. Como resultado se obtuvieron mostos etanólicos o "mistelas".

Se prepararon 3 tipos de muestras (mistela, mistela desalcoholizada y mistela diluida), y se utilizaron resinas C18 ya preparadas en cartuchos de 7 y 10 g. En primer lugar, se determinó

el volumen de ruptura de los polifenoles. Para ello se percolaron los diferentes tipos de mistela a través del cartucho previamente acondicionado y se recogieron las fracciones cada 5 mL. En cada fracción recogida se midió el IPT (índice de Polifenoles Totales). En el caso de la mistela sin tratar, el 15% de pérdida del IPT se obtuvo al pasar únicamente 7 y 10 mL a través de los cartuchos de 7 y 10 g, respectivamente. La dilución tuvo un efecto positivo y se observó un aumento del volumen de ruptura de hasta 31 y 45.5 mL, respectivamente. Los mejores resultados se observaron con la mistela desalcoholizada donde el volumen de ruptura fue de 240 y 750 mL, respectivamente.

Con el fin de determinar el volumen de elución, la mistela desalcoholizada se percoló en un cartucho C18 de 10 g y se eluyó con etanol. Se recogieron fracciones cada 50 mL y se analizaron los taninos y antocianos, así como un análisis indirecto mediante hidrólisis ácida de los precursores glicosídicos presentes en las fracciones. Solo en las 2 primeras fracciones se detectó liberación de aromas. Además, el 98% de antocianos y más de 90% de taninos estuvieron presentes en las dos primeras fracciones. Se consideraron 100 mL como el volumen de elución. Por lo tanto, el método de extracción finalmente obtenido permitió extraer más del 90% de los polifenoles de la uva y prácticamente todos los precursores que liberan aromas por hidrólisis ácida.

4.3.2. Condiciones de la hidrólisis

4.3.2.1. Hidrólisis acelerada a 45 ºC

Una vez desarrollado el método de extracción de las PAFs, se desarrolló un método de hidrólisis acelerada para la liberación de los compuestos aromáticos procedentes de los precursores glicosídicos. Las PAFs se reconstituyeron en vino sintético y se envejecieron a 45 ºC en condiciones anóxicas estrictas en diferentes tiempos (2, 4 y 7 semanas). Con el fin de estudiar el efecto del azúcar y las pérdidas producidas durante el proceso de extracción,

la mistela, así como la rPAF enriquecida en azúcar (rsPAF) también fueron envejecidas. Para evaluar el efecto del oxígeno, una muestra de cada tipo se envejeció durante 7 semanas sin condiciones de anoxia. Se estudiaron los compuestos liberados de los precursores glicosídicos de manera sensorial y cuantitativamente.

Las mistelas envejecidas desarrollaron fuertes aromas a caramelo y pasas. La adición de azúcares a la PAF, indujo la formación de notas de queroseno atribuidas a TDN. La presencia de oxígeno provocó una fuerte distorsión de los perfiles sensoriales. Por el contrario, la reconstitución de la PAF en vino sintético seguido de envejecimiento anóxico produjo el desarrollo de aromas intensos semejantes a los aromas observados en vinos tintos.

Por lo tanto, las rPAF envejecidas a 45 °C necesitan al menos 7 semanas para liberar los compuestos que producen intensos aromas frutales similares a algunos de los matices del aroma de los vinos más apreciados. Sin embargo, 7 semanas es demasiado tiempo, por ello se estudió minimizar el tiempo mediante el aumento de la temperatura, eligiéndose 75 ºC.

4.3.2.2. Hidrólisis acelerada a 75 ºC

Las rPAF fueron incubadas en condiciones de estricta anoxia durante diferentes tiempos desde 3 a 72 h. A 75 °C, en solo 24 h, había un desarrollo satisfactorio del aroma. Además, los test triangulares realizados mostraron que entre 24, 38 y 48 h de incubación no había diferencias significativas. Las fracciones de aroma desarrolladas fueron estudiadas también por GC-O y GC-MS. Los resultados confirmaron que los hidrolizados obtenidos a 75 ºC en solo 24 h, fueron sensorial, química y olfatométricamente, muy cercanos a los obtenidos después de 7 semanas a 45 °C.

Se identificaron al menos 32 odorantes diferentes a niveles potencialmente relevantes desde el punto de vista sensorial en los hidrolizados. Los odorantes se pueden clasificar en cinco

categorías: i) derivados lipídicos, incluidos *Z*-3-hexenal, 1-octen-3-one, *Z* -1,5-octadien-3-one, *Z*-3-hexenol, *E*-2-octenal, *Z*-2-nonenal, *E*-2-nonenal, *E,Z*-2,6-nonadienal, *E,E*-2,4-nonadienal, γ-decalactona y massoia lactona; ii) fenoles volátiles y vainillinas, incluidos guaiacol, cresoles, eugenol, 2,6-dimetoxifenol, *E*-isoeugenol y vanillina; iii) norisoprenoides y terpenoles, incluido el óxido de linalol (y/o dihidromircenol), linalol, TDN, β-damascenona y β-ionona; iv) derivados de aminoácidos, incluidos metional y sotolón; y v) compuestos misceláneos, incluyendo β-feniletanol, cinamato de etilo, furaneol, 3-mercaptohexanol y tres compuestos no identificados.

Los odorantes de las categorías i y iv probablemente serán transformados por la levadura, por lo que pueden no llegar al vino. Sin embargo, los odorantes en las categorías i, iii y v serán esenciales para el desarrollo del aroma del vino. La presencia de 3-mercaptohexanol es sorprendente, ya que por primera vez se sugiere que este importante compuesto aromático podría formarse a partir del precursor sin la acción de la levadura.

4.3.3. Caracterización del potencial aromático de uvas de Tempranillo y Garnacha

Con el método ya desarrollado, se llevó a cabo la caracterización de 33 lotes de uvas de Tempranillo y Garnacha procedentes de diferentes bodegas, diferentes terroirs y con diferente estado de madurez. Utilizando la metodología desarrollada se prepararon las PAFs que fueron posteriormente reconstituidos en vinos sintético y sometidos a hidrólisis acelerada a 75 ºC durante 24 h. Seguidamente se llevó a cabo la caracterización de estas muestras mediante análisis sensorial, GC-O y GC-MS.

4.3.3.1. Caracterización sensorial

Se realizó un test de agrupación en el que se agruparon las muestras por similitud aromática. Se observaron dos grupos grandes, uno principalmente compuesto por las uvas de la variedad Garnacha que se subdividió en 2 clústeres (clústeres i y ii), y un segundo gran grupo compuesto principalmente por uvas Tempranillo y en el que encontramos los clústeres iii, iv y v. Se observaron 5 clústeres: i) fruta tropical/cítrico, floral; ii) floral, fruta compotada; iii) madera/tostado, fruta roja, fruta compotada y fruta negra; iv) vegetal; y v) vegetal y fruta compotada. De estos grupos se seleccionó una PAF representativa de cada clúster que fue caracterizada sensorialmente en más profundidad mediante perfilado rápido.

En el perfilado rápido, la muestra procedente del clúster i fue descrita principalmente con notas a fruta tropical/cítrico, floral y queroseno; la muestra seleccionada en el clúster iii se describió con notas a tostado-madera, fruta roja y fruta compotada; la muestra perteneciente al clúster iv fue descrita con notas alcohólicas; y las muestras seleccionadas de los clústeres ii y v se describieron principalmente con notas a fruta compotada y alcohólico. Además, todas las rPAFs tuvieron notas vegetales. Por lo tanto, estás muestras fueron descritas de manera muy similar tanto en el perfilado rápido como en el test de agrupamiento. Curiosamente, los datos sensoriales no mostraron ningún efecto relevante sobre la procedencia geográfica de las uvas ni el estado de madurez.

4.3.3.2. GC-O

Con el fin de identificar los odorantes responsables de las descripciones distintivas entre los clústers, las 5 rPAFs seleccionadas y caracterizadas mediante perfilado rápido fueron analizadas por GC-O. El GC-O de las muestras representativas reveló que el 3MH, óxido de linalol y dihridromircenol, 2 odorantes no identificados, fenilacetaldehido y Z-1,5-octadien-3-ona fueron los odorantes más discriminantes. Además, varios derivados

lipídicos incluyendo 7 aldehídos, 2 cetonas y 2 lactonas pudieron ser responsables del fondo vegetal. También se identificaron otros compuestos como 4 fenoles volátiles, cinamato de etilo, β-ionona, β-damascenona, linalol, α-terpineol y furaneol.

4.3.3.3. GC-MS

Los datos cuantitativos mostraron que las concentraciones de algunos compuestos fueron muy similares entre las rPAFs que pertenecieron al mismo clúster en el test de agrupación. Los odorantes que generaron mayores variabilidades a nivel sensorial fueron β-damascenona, TDN, linalol, limoneno, furaneol y 4-vinilfenol. Además, la massoia lactona también mostró un alto potencial discriminante. Por otro lado, β-ionona, geraniol, 1,8-cineol, guaiacol y 4-vinilfenol también podrían inducir diferencias sensoriales.

Las muestras de Garnacha fueron ricas en norisoprenoides (excepto iononas), terpenoles (excepto limoneno) y en derivados de la vainillina, mientras que las muestras de Tempranillo fueron ricas en fenoles volátiles. De los dos clústeres de Garnacha (clústeres i y ii), las muestras del clúster ii contenían altos niveles de β-ionona, β-damascenona, linalol, limoneno y massoia lactona, mientras que las del clúster i tuvieron concentraciones altas de TDN. Estos compuestos explican las diferencias encontradas a nivel sensorial, así como las notas a queroseno observadas en la muestra perteneciente al cluster i, a excepción de las notas a fruta tropical/cítricas que pueden ser explicadas a nivel olfatométrico por la presencia de 3MH, óxido de linalol y dihidromircenol. Por otro lado, en el caso del Tempranillo (clústeres iii, iv y v), las muestras del clúster iv contuvieron concentraciones menores en la mayoría de los compuestos, lo que explica su carácter vegetal. Las muestras del clúster v también tenían concentraciones bajas de la mayoría de los compuestos excepto de iononas y β-damascenona lo que explicaría ese carácter a fruta compotada junto con las

notas vegetales. Las muestras del clúster iii tuvieron niveles más altos de fenoles volátiles lo que explicaría las notas a tostado/madera.

4.4. Conclusiones

La mayoría de los precursores glicosídicos y polifenoles de la uva presentes en 750 mL de mistela pudieron aislarse por SPE a partir de mistela desalcoholizada usando 10 g de C18. Sin embargo, en estos PAF no se extrajeron los aminoácidos, los cationes metálicos y los precursores de DMS. Además, la reconstitución de los PAF en vino sintético seguido de envejecimiento en anoxia durante 7 semanas a 45 °C o durante 24 h a 75 °C condujeron al desarrollo de aromas fuertes que recuerdan algunos matices de olor observados en vinos tintos envejecidos. Por el contrario, las mistelas envejecidas de manera similar desarrollaron fuertes aromas a caramelo y pasas. La adición de azúcares a los PAF, indujo la formación de notas de queroseno atribuidas a TDN. La presencia de oxígeno, provocó una fuerte distorsión de los perfiles sensoriales.

Los 32 odorantes identificados por GC-O fueron similares entre muestras envejecidas a 45 y 75 °C y pertenecieron a 4 categorías principales (derivados de lípidos; fenoles y vainillinas volátiles; y norisoprenoides y terpenos) y un quinto grupo misceláneo que sorprendentemente incluyó el 3MH. Además, los análisis de GC–MS y GC-O de PAF de 7 semanas a 45 °C también fueron relativamente similares a los obtenidos después de 24 h a 75 °C, lo que sugiere que esta hidrólisis rápida de PAFs puede ser una herramienta prometedora para el estudio del aroma potencial de la uva de vinificación.

Además, la caracterización de las 33 muestras de uva permitió identificar cinco perfiles aromáticos diferentes: i) vegetal; ii) vegetal y fruta compotada; iii) floral y fruta compotada; iv) floral y cítrico/fruta tropical; y v) madera/tostado, fruta roja, fruta negra y fruta compotada. GC-O ha revelado que estos perfiles aromáticos se deben a 19 odorantes: i)

Odorantes constitutivos: Ocho aldehídos que proporcionan un fondo "vegetal" común y β-damascenona y massoia lactona que proporcionan un fondo de "fruta compotada" y ii) Odorantes discriminantes: Linalol, óxido de linalol, 3MH, furaneol, guaiacol y metoxieugenol parecen ser responsables de los diferentes matices aromáticos.

Los análisis cuantitativos confirmaron la existencia de los cinco perfiles diferentes. Garnacha fue rica en norisoprenoides, terpenoles y derivados de la vainillina y Tempranillo rico en fenoles volátiles.

Para más información, mirar los artículos:

- **Alegre, Y**.; Arias-Pérez, I.; Hernández-Orte, P.; Ferreira, V., Development of a new strategy for studying the aroma potential of winemaking grapes through the accelerated hydrolysis of phenolic and aromatic fractions (PAFs). Food Research International 2020, 108728.
- **Alegre, Y**.; Sáenz-Navajas, M. P.; Hernández-Orte, P.; Ferreira, V. Sensory, olfactometric and chemical characterization of the aroma potential of Grenache and Tempranillo winemaking grapes. (2020). Food chemistry (Submitted).

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INTRODUCCIÓN GENERAL

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1. EL AROMA DEL VINO

El aroma del vino es una de las características que más influye en la aceptabilidad o preferencia de los consumidores dado que es un producto consumido fundamentalmente por placer sensorial, en el cual el aroma es su mejor carta de presentación.

La formación de este aroma es un proceso complejo en el que están implicados no solo la uva y la levadura, sino también una serie de mecanismos tanto químicos como enzimáticos producidos desde los procesos pre-fermentativos hasta el envejecimiento del vino (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud-Funel, 2006). Por lo tanto, debido a esa gran variabilidad asociada a la elaboración del vino, su aroma es uno de los más complejos y difíciles de caracterizar.

Durante el proceso de fermentación por el que las levaduras convierten los azúcares del mosto en alcohol, también se generan una serie de compuestos químicos que son claves en las propiedades organolépticas que presentará el vino final.

Dentro de estos compuestos químicos se encuentran metabolitos secundarios como alcoholes superiores, ácidos grasos y sus ésteres etílicos, acetoina, diacetilo y acetaldehído. Estos metabolitos secundarios generan el denominado tampón aromático del vino, formado por la mezcla de 27 compuestos procedentes de diferentes familias químicas que se encuentran en concentraciones de unos pocos mg/L en una fermentación alcohólica normal. La presencia de todos estos componentes en cualquier bebida alcohólica afecta a la percepción de los odorantes en nuestro sistema sensorial, siendo responsables de importantes procesos, como por ejemplo de la supresión de algunas notas aromáticas,

particularmente de notas frutales y amaderadas (de-la-Fuente-Blanco, Sáenz-Navajas, & Ferreira, 2016; Escudero, Campo, Fariña, Cacho, & Ferreira, 2007).

La composición del tampón del vino puede cambiar ligeramente ya que depende del metabolismo de la levadura y otras prácticas enológicas, pero, en general, su perfil sensorial no cambia mucho y se describe como "vinoso" (Ferreira, de-la-Fuente-Blanco, & Sáenz-Navajas, 2019). Por suerte, no todos los vinos se describen como “vinosos” sino que hay muchos vinos que poseen notas aromáticas muy diversas.

A pesar de que, a finales de los años 80 se habían identificado más de 800 compuestos presentes en la fracción volátil de los vinos (Maarse & Vischer, 1989), no todas las moléculas presentes tienen relevancia a nivel sensorial. Solo algunas moléculas que están presentes a una concentración suficiente para superar el tampón tienen la capacidad de llegar a generar los atributos sensoriales del aroma que se encuentran en los diferentes vinos.

Está generalmente aceptado que los compuestos derivados de las uvas son los que juegan un papel más decisivo en la expresión de las notas aromáticas características de la variedad. En algunos casos, varios compuestos contribuyen a la percepción de un aroma en particular, como es el caso de los aromas afrutados (San Juan, Ferreira, Cacho, & Escudero, 2011). En otros, un único compuesto es responsable del aroma propio de la variedad, como el linalol en los vinos Moscatel (Ribéreau-Gayon, Boidron, & Terrier, 1975), el *Z*-óxido de rosas en los vinos de la variedad Gewürstraminer (Guth, 1997; Ong & Acree, 1999), o el 4-etilfenol en el carácter Brett del vino (Suárez, Suárez-Lepe, Morata, & Calderón, 2007).

2. PRECURSORES EN LA UVA

En general, la uva de vinificación es un producto de características aromáticas neutras. Los compuestos aromáticos se pueden encontrar libres o ligados (Carro, López, Gunata, Baumes, & Bayonove, 1996; Gunata, Bayonove, Baumes, & Cordonnier, 1986).

La fracción de aroma libre es muy pequeña en la mayoría de las variedades y no contiene ningún compuesto aromático a concentraciones a las que puedan ser considerados compuestos aromáticos impacto. De hecho, en uvas neutras se han encontrado niveles cuantificables de compuestos C_6 junto con niveles bajos de algunos alcoholes, cetonas, ésteres y terpenos (Ferrandino, Carlomagno, Baldassarre, & Schubert, 2012; Genovese, Lamorte, Gambuti, & Moio, 2013; Gómez, Martinez, & Laencina, 1995; Stevens, Bomben, & McFadden, 1967). Esto es debido a que las uvas son frutas ricas en agua y no contienen estructuras vacuolares especiales en las que almacenar de forma segura las moléculas no polares como los compuestos aromáticos. Las moléculas hidrofóbicas presentes en muchos componentes aromáticos, se estabilizan en la pulpa y la piel formando enlaces covalentes con moléculas polares, como azúcares o aminoácidos.

La uva de vinificación contiene un elevado potencial aromático bajo la forma de precursores inodoros (Hewitt, Mackay, Konigsbacher, & Hasselstrom, 1956; Weurman, 1961). Estos precursores pueden ser transformados en compuestos aromáticos durante el proceso de vinificación (Swiegers & Pretorius, 2005). Estos compuestos derivados de las uvas son los que juegan un papel más decisivo en la expresión del aroma final y constituyen el denominado aroma varietal del vino.

Hay dos grandes grupos de precursores en la uva, precursores específicos e inespecíficos. Los precursores de aroma específicos de la uva son moléculas no volátiles e inodoras, que pueden producir un compuesto aromático específico por hidrólisis de un enlace químico,

por reordenamiento químico espontáneo o por una combinación de ambos mecanismos (Delfini, Cocito, Bonino, Schellino, Gaia, & Baiocchi, 2001; López, Ezpeleta, Sánchez, Cacho, & Ferreira, 2004; Tominaga, Peyrot des Gachons, & Dubourdieu, 1998; Waterhouse, Sacks, & Jeffery, 2016). Además, el compuesto aromático también podrá ser formado por simple hidrólisis del conjunto de precursores extraídos de la uva a pH normal del vino, o alternativamente, por hidrólisis en presencia de enzimas.

Por el contrario, los precursores inespecíficos son aquellas moléculas que pueden transformarse en moléculas aromáticas como consecuencia de la acción metabólica compleja de levaduras, bacterias u otros microorganismos (Ferreira & López, 2019). Por ejemplo, el aminoácido leucina puede ser metabolizado por las levaduras *Saccharomyces* dando lugar a diferentes compuestos como ácido isovalérico, alcohol isoamílico y acetato de isoamilo (Hazelwood et al., 2008). Sin embargo, la leucina no puede considerarse como un precursor específico de estos compuestos aromáticos dado que la levadura durante la fermentación puede producir todos esos compuestos incluso si no hay leucina en los mostos de fermentación (Ferreira & López, 2019).

2.1. Precursores específicos de la uva

La glicosidación es la forma más habitual en la que se acumulan los aromas en las frutas. La uva no sólo contiene precursores glicosídicos, sino que contiene otras moléculas no volátiles como polioles, S-metilmetionina (Loscos, Segurel, Dagan, Sommerer, Marlin & Baumes, 2008), ácidos e hidroxiácidos (Sefton, Skouroumounis, Massy-Westropp, & Williams, 1989; Skouroumounis, Massy-Westropp, Sefton, & Williams, 1992; Strauss, Wilson, & Williams, 1988), así como precursores ligados a la cisteína y al glutatión.

2.1.1. Precursores cisteínicos y glutatiónicos

Los precursores no volátiles de algunos mercaptanos polifuncionales están formados por el mercaptano polifuncional (4MMP y 3MH) unido a una molécula de cisteína y/o glutatión (Fedrizzi, Pardon, Sefton, Elsey, & Jeffery, 2009; Murat, Tominaga, & Dubourdieu, 2001; Peyrot des Gachons, Tominaga, & Dubourdieu, 2000, 2002; Tominaga, Darriet, & Dubourdieu, 1996; Tominaga, Peyrot des Gachons, et al., 1998) formando los precursores cisteínicos y glutatiónicos: cisteína-3-mercaptohexanol (CYSMH), cisteína-4-mercapto-4-metil-2-pentanona (CYSMP), glutatión-3-mercaptohexanol (GLUMH), glutatión-4-mercapto-4-metil-2-pentanona (GLUMP) (Peyrot des Gachons et al., 2000, 2002; Tominaga, Peyrot des Gachons, et al., 1998). Además, también se han identificado conjugados de dipéptidos de 3-MH, S-3-hexan-1-ol-cisteinil-glicina (CysGly-3MH) y S-3-hexan-1-ol-γ-glutamil-cisteína (γGluCys-3MH) (Bonnaffoux, Roland, Rémond, Delpech, Schneider, & Cavelier, 2017; Capone, Pardon, Cordente, & Jeffery, 2011; Cordente, Capone, & Curtin, 2015) y de 4MMP, S-4-mercapto-4-metil-2-pentanona-l-cisteinil-glicina (CysGly-4MMP) y S-4-mercapto-4-metil-2-pentanona-N-(l-γ-glutamil)-l-cisteína (γGluCys-4MMP) (Bonnaffoux, Delpech, Rémond, Schneider, Roland, & Cavelier, 2018; Bonnaffoux et al., 2017).

Los precursores de la 4MMP están presentes en concentraciones menores en comparación con los precursores del 3MH (Concejero, Hernández-Orte, Astrain, Lacau, Baron, & Ferreira, 2016), siendo GLUMH el precursor más abundante (Capone, Sefton, Hayasaka, & Jeffery, 2010). La localización de estas moléculas en la baya (piel y/o pulpa) modula su extracción durante la elaboración del vino, dado que algunos procesos de vinificación, como el contacto con la piel y el prensado, influyen en la extracción de los mismos (Maggu, Winz, Kilmartin, Trought, & Nicolau, 2007). Además, la maduración afecta directamente a la concentración de precursores aumentando desde el envero hasta el momento de la

vendimia (Peyrot des Gachons et al., 2000; Roland, Vialaret, Razungles, Rigou, & Schneider, 2010).

La piel de la uva protege a la pulpa de la oxidación, pero cuando la uva es procesada durante las etapas pre-fermentativas, los precursores se ven expuestos al oxígeno (Roland, Vialaret, et al., 2010). Estos precursores tienen enlaces tioéter carbono-azufre (C-S) por lo que son bastante estables frente a la oxidación.

2.1.1.1. Formación de los mercaptanos polifuncionales

La biotransformación de estos precursores en los mercaptanos polifuncionales correspondientes implica su absorción en la levadura mediante el uso de transportadores de aminoácidos y glutatión (GSH) presentes en la membrana plasmática (Cordente et al., 2015; Subileau, Schneider, Salmon, & Degryse, 2008a, 2008b). Una vez dentro del citoplasma, el GLUMH se escinde a CysGly-3MH en la vacuola de la levadura por acción de la γ-glutamato transpeptidasa ECM38, que también podría catalizar la formación de CYSMH a partir del dipéptido precursor γGluCys-3MH (Cordente et al., 2015; Santiago & Gardner, 2015). A día de hoy, aún queda por identificar la carboxipeptidasa responsable de la escisión del dipéptido CysGly-3MH en CYSMH, así como, la ruta de degradación de GLUMP para producir 4MMP.

Una vez que los precursores cisteínicos se asimilan a partir del mosto de uva, o se forman a través de la degradación de sus precursores glutatiónicos y/o dipéptidos, éstos son escindidos por los enzimas de la levadura con actividad β-liasa. Estos enzimas rompen el enlace carbono-azufre dando lugar a la liberación del mercaptano polifuncional correspondiente y, por ende, a la liberación del aroma (Tominaga, Peyrot des Gachons, et al., 1998) (Figura 1).

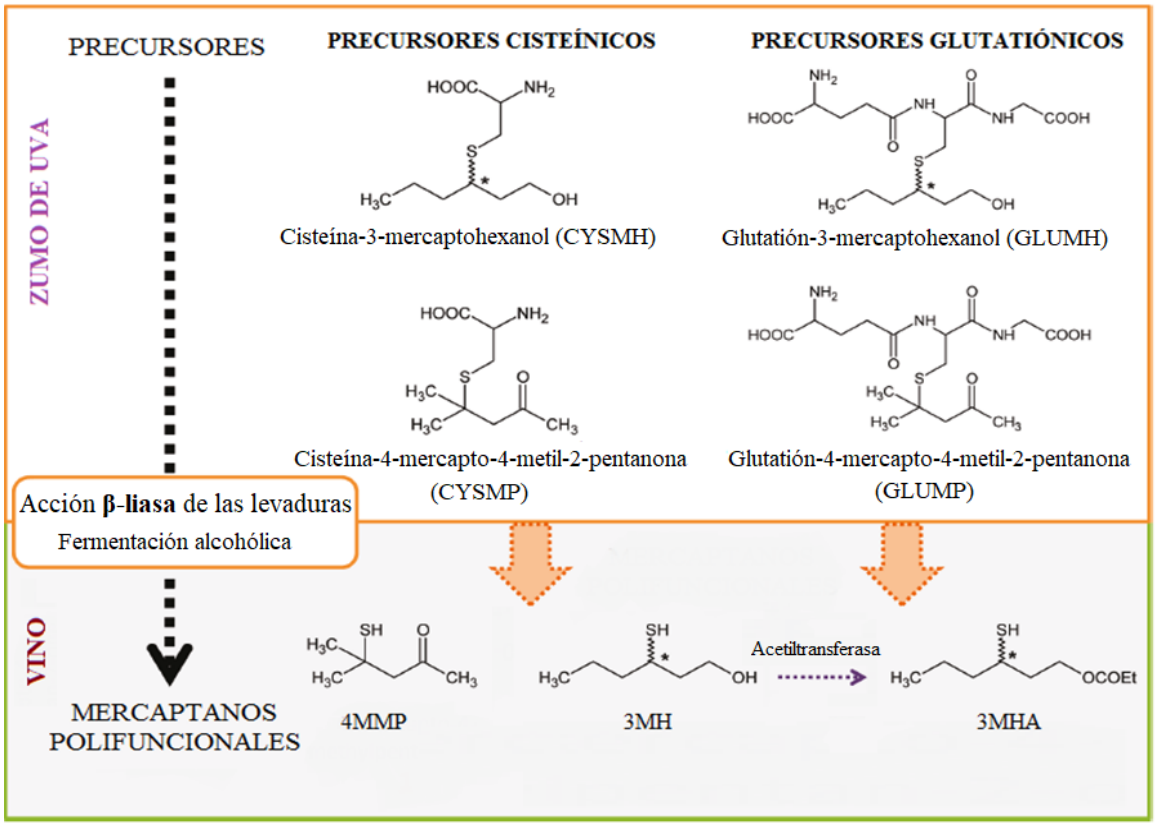

Figura 1. Formación de la 4-mercapto-4-metil-2-pentanona (4MMP) y 3-mercaptohexanol (3MH) a partir de sus precursores cisteínicos y glutatiónicos. Formación del y acetato de 3-mercaptohexilo (3MHA) a partir de la acetilación del 3MH. Adaptado de (Roland, Schneider, Razungles, & Cavelier, 2011).

El mecanismo de liberación de los mercaptanos polifuncionales se investigó por primera vez para la 4MMP mediante la eliminación de genes que codifican las liasas carbono-azufre de la levadura *Saccharomyces cerevisiae*. Los resultados del trabajo mostraron la existencia de cuatro genes, BNA3, CYS3, GLO1 e IRC7, que parecían estar implicados en la liberación de 4MMP (Howell, Klein, Swiegers, Hayasaka, Elsey, Fleet, Høj, Pretorius, & de Barros Lopes, 2005). Sin embargo, Thibon et al., demostraron que solo la β-liasa Irc7p era capaz de convertir CYSMP en 4MMP (Thibon, Marullo, Claisse, Cullin, Dubourdieu, & Tominaga, 2008). Posteriormente, se relacionó la variación en la capacidad de liberación de tioles volátiles entre cepas de levaduras con la presencia o ausencia de la versión completa de la enzima (Belda, Ruiz, Navascues, Marquina, & Santos, 2016; Dufour, Zimmer, Thibon, & Marullo, 2013; Santiago & Gardner, 2015). Sin embargo, Belda et al.,

y Ronconi et al., (Belda et al., 2016; Roncoroni, Santiago, Hooks, Moroney, Harsch, Lee, Richards, Nicolau, & Gardner, 2011) observaron que una deleción de 38 pares de bases encontrada en muchas cepas de vino producen una versión truncada de la enzima Irc7p que es inactiva. Holt et al., (Holt, Cordente, Williams, Capone, Jitjaroen, Menz, Curtin, & Anderson, 2011) demostraron que la enzima Str3p también libera los mercaptanos polifuncionales de los precursores cisteínicos. Por lo tanto, en *Saccharomyces cerevisiae*, existen al menos dos genes, IRC7 y STR3 que codifican las enzimas β-liasa Irc7p y Str3p que liberan los mercaptanos polifuncionales de los conjugados de cisteína (Holt et al., 2011; Roncoroni et al., 2011). Además, existe una vía alternativa de formación del 3MH en la que compuestos C_6 insaturados, como *E*-2-hexenal, se unen a compuestos azufrados (Schneider, Charrier, Razungles, & Baumes, 2006).

Los mercaptanos polifuncionales se encuentran en el vino en concentraciones de ng/L, mientras que los precursores están presentes en la uva y mosto en el rango de µg/L. Es decir, la concentración de los compuestos aromáticos es hasta 1000 veces inferior que la de sus precursores. Estudios previos (Bonnaffoux et al., 2018; Concejero et al., 2016; Peyrot des Gachons et al., 2000; Roland, Schneider, Razungles, Le Guerneve, & Cavelier, 2010) han demostrado que una parte de los precursores está directamente relacionada con el aroma generado, pero solo una pequeña parte de estos liberan el tiol volátil aromático durante la fermentación. El factor de conversión de los precursores en los mercaptanos polifuncionales es menor del 5%.

2.1.2. Precursores glicosídicos

Desde el punto de vista cuantitativo, la fracción de precursores glicosídicos es la más importante y, de hecho, fue la primera en descubrirse hace más de 40 años (Cordonnier & Bayonove, 1974).

Algunos compuestos aromáticos relevantes del vino que pertenecen a los grupos de terpenoles, como linalol y geraniol; norisoprenoides, como β-damascenona, β-ionona y 1,1,6-trimetil-1,2-dihidronaftaleno (TDN); misceláneos como cinamato de etilo; fenoles volátiles, como guaiacol, eugenol, 4-vinilfenol y 4-vinilguaiacol; o las vainillinas, como vainillina y acetovanillona, derivan de diferentes precursores glicosídicos presentes en la uva (Hjelmeland & Ebeler, 2015). Estos precursores glicosídicos están formados por una o varias gliconas (molécula de azúcar) unida a una aglicona. La dificultad de su estudio se debe a que la aglicona en algunos casos es el compuesto aromático, como por ejemplo el linalol (Strauss, Wilson, Gooley, & Williams, 1986; Wilson, Strauss, & Williams, 1984), pero en muchos otros casos, especialmente en el caso de los norisoprenoides (Waterhouse et al., 2016), la aglicona es una molécula que solo después de diferentes reordenamientos químicos espontáneos lentos formará el compuesto aromático.

En la mayoría de los casos, las agliconas estan unidas con β-D-glucosa formando monosacáridos, o más frecuentemente, disacáridos de cuatro tipos principales (Williams, Strauss, Wilson, & Massy-Westropp, 1982a; Voirin, Baumes, Bitteur, Gunata, & Bayonove, 1990) (6-O-β-d-malonil-β-d-glucopiranosidos, α-L-arabinofuranosil-β-D-glucopiranósido, α-L-ramnopiranosil-β-D-glucopiranósido y β-D-apiofuranosil-β-D-glucopiranósido) (Figura 2). Recientemente, también se han identificado dos trisacáridos en uvas (Godshaw, Hjelmeland, Zweigenbaum, & Ebeler, 2019; Hjelmeland, Zweigenbaum, & Ebeler, 2015). Esto hace que el número final de moléculas precursoras sea demasiado alto para ser monitoreado directamente.

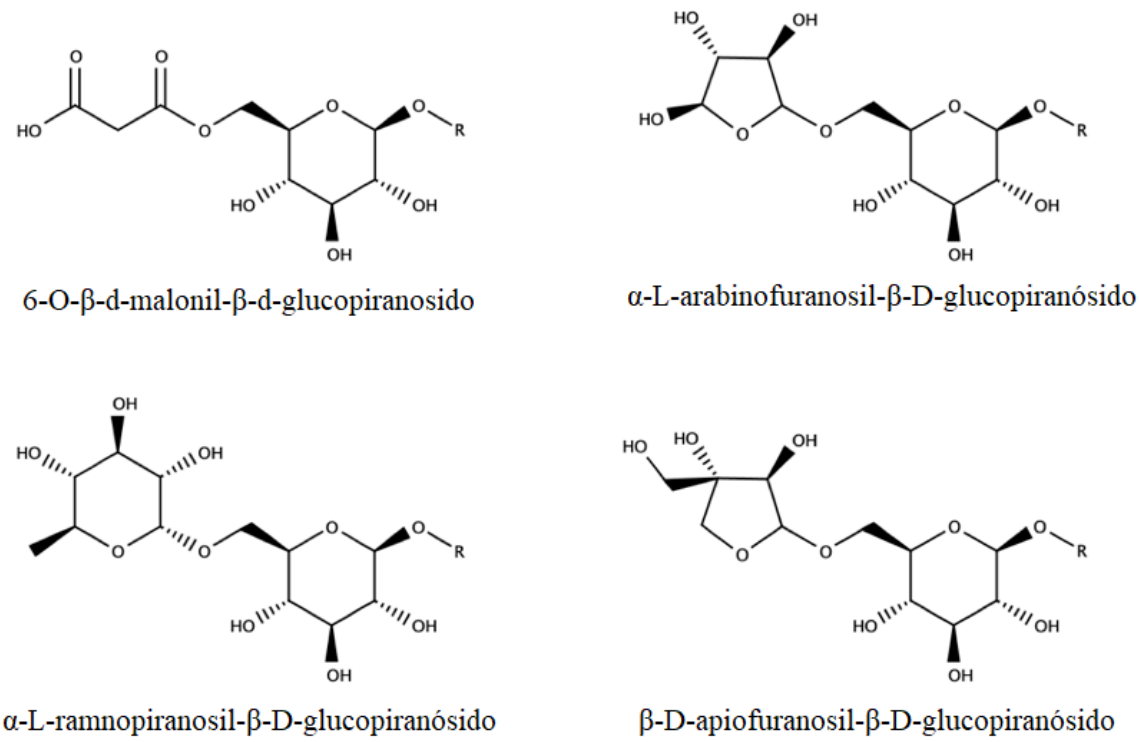

Figura 2. Estructura de gliconas de los precursores glicosídicos. Adaptada de Hjelmeland & Ebeler, 2015.

2.1.2.1. Liberación de los compuestos aromáticos

La liberación de la aglicona del glucósido se produce durante el proceso de vinificación por la acción de las levaduras (Delfini et al., 2001; Fernández-González & Di Stefano, 2004; Hernández-Orte, Cersosimo, Loscos, Cacho, García-Moruno, & Ferreira, 2008), mediante glicosidasas exógenas o endógenas (Gunata, Bayonove, Tapiero, & Cordonnier, 1990; Sánchez-Palomo, Díaz-Maroto Hidalgo, González-Viñas, & Pérez-Coello, 2005), o por hidrólisis ácida lenta (López et al., 2004; Skouroumounis & Sefton, 2000).

2.1.2.2. Tipos de agliconas

En general, se han identificado más de 100 agliconas diferentes (Schneider, Razungles, Augier, & Baumes, 2001; Williams, Sefton, & Wilson, 1989; Williams, Strauss, Wilson, & Massy-Westropp, 1982b; Winterhalter, 1992) clasificadas en las siguientes categorías:

i) Terpenoles

Los terpenoles incluyen importantes compuestos como linalol, α-terpineol, nerol, geraniol y sus óxidos, así como polioles como el 3,7-dimetiloct-1-eno-3,7-diol que mediante reordenamientos químicos producen diferentes terpenoles (Williams, Strauss, & Wilson, 1980). Los terpenoles pueden formarse directamente por la acción de glicosidasas de la levadura, o mediante transformaciones espontáneas al pH del vino, como se observa en la Figura 3. Además, los polioles también pueden estar presentes como precursores glicosídicos (Strauss et al., 1988).

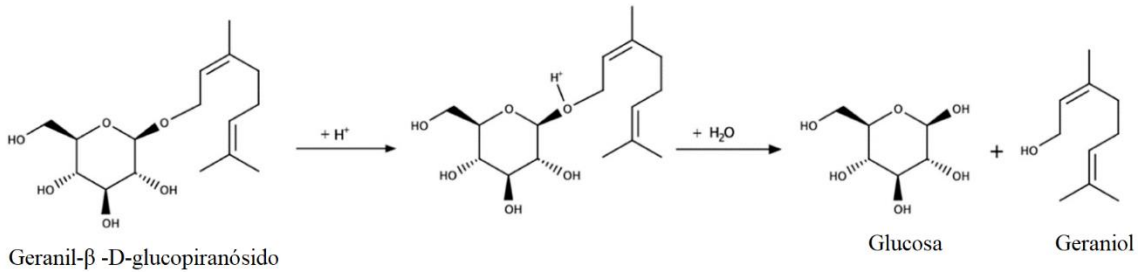

Figura 3. Formación de geraniol mediante la hidrólisis ácida del geranil-β-D-glucopiranósido. Adaptado de (Ferreira & López, 2019; Waterhouse et al., 2016).

ii) Norisoprenoides

En el caso de los norisoprenoides encontramos compuestos como β-damascenona, β-ionona o TDN, así como otras moléculas aromáticas con menor importancia, como α-ionona, Riesling acetal y vitispiranos. Estos compuestos no se encuentran en forma de agliconas como tal, y por lo tanto no son liberados directamente de los precursores, sino que se forman a partir de otras moléculas. Las principales agliconas son 3-hidroxi-β-damascona, dihidro-β-ionona y diferentes ionoles, particularmente 3-oxo-a-ionol y vomifoliol (Cabrita, Freitas, Laureano, & Di Stefano, 2006; García-Muñoz, Asproudi, Cabello, & Borsa, 2011).

Los norisoprenoides desde el punto de vista químico se dividen en dos formas: megastigmanas y no megastigmanas. Las formas megastigmanas son norisoprenoides

oxigenados en el carbono 7 (serie de las damascenonas) o en el carbono 9 (serie de las iononas). Además, algunos polioles C_{13} con estructura megastigmana pueden dar lugar a compuestos como TDN, vitispiranos y β-damascenona (Sefton et al., 1989; Strauss, Dimitriadis, Wilson, & Williams, 1986) como se ve en la Figura 4.

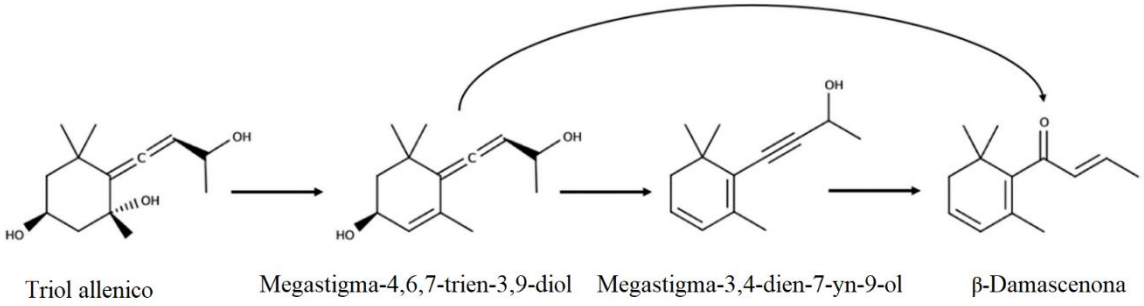

Figura 4. Formación de la β-damascenona a partir de poliol C_{13} con estructura megastigmana. Adaptado de (Daniel, Puglisi, Capone, Elsey, & Sefton, 2008).

iii) Lactonas

Las lactonas como γ-decalactona y massoia lactona se pueden formar a partir de los hidroxiácidos correspondientes que se encuentran en forma de precursores glicosídicos (Gracia-Moreno, López, & Ferreira, 2015).

iv) Fenoles volátiles

Los fenoles volátiles como guaiacol, eugenol, isoeugenol, 2,6-dimethioxifenol, 4-vinilguaiacol y 4-vinilfenol pueden ser extraídos de la madera (Kennison, Gibberd, Pollnitz, & Wilkinson, 2008), pero pequeñas cantidades de estos volátiles también están presentes en forma glicosilada en las uvas.

v) Derivados de la vainillina

Al igual que los fenoles volátiles, las vainillinas pueden ser extraídas de la madera pero también están presentes en forma glicosilada en las uvas. La vainillina podría formarse

también por oxidación del 4-vinilguaiacol (Vanbeneden, Saison, Delvaux, & Delvaux, 2008).

vi) Compuestos misceláneos

Los ésteres etílicos como cinamato de etilo, ciclohexanoato de etilo y 4-metilpentanoato de etilo proceden de los hidroxiácidos correspondientes (Gracia-Moreno et al., 2015). El cinamato de etilo, que se forma a partir del precursor glicosídico del ácido cinámico (Cho, Kim, Lee, & Moon, 2014). Asimismo, también se puede considerar el furaneol, cuyo precursor glicosídico ha sido recientemente identificado (Sasaki, Takase, Tanzawa, Kobayashi, Saito, Matsuo, & Takata, 2015) y debido a su alta polaridad no se cuantifica de manera habitual (Oliveira, 2019).

2.2. Precursores inespecíficos de la uva

Además de estos precursores específicos, en la uva existen también los precursores inespecíficos. Los principales precursores no específicos de la uva son los aminoácidos (Albers, Larsson, Lidén, Niklasson, & Gustafsson, 1996; Hernández-Orte, Cacho, & Ferreira, 2002). Estos precursores inespecíficos están directamente relacionados con la formación de compuestos aromáticos importantes producidos durante la fermentación alcohólica (alcoholes superiores, ácidos volátiles, aldehídos y ésteres).

2.2.1. Aminoácidos

Los aminoácidos son los elementos de uva más conocidos relacionados con los odorantes. Ferreira et al., (Ferreira, López, & Cacho, 2000) encontraron que los niveles de alcoholes superiores, sus acetatos, los ácidos ramificados y sus ésteres etílicos estaban vinculados a la variedad de uva con la que se había elaborado el vino. Más tarde, se demostró que la fermentación de mostos sintéticos que contenían los perfiles de aminoácidos característicos

de cada variedad, generaron perfiles aromáticos propios de la variedad (Hernández-Orte et al., 2002).

2.2.1.1. Formación de alcoholes superiores y ácidos volátiles

Además del etanol, los alcoholes superiores (también conocidos como alcoholes de fusel) son los principales alcoholes que aportan características sensoriales al vino, incluyendo propanol, isobutanol, alcohol amílico activo, alcohol isoamílico y alcohol 2-feniletílico (o β-feniletanol). Los alcoholes superiores son producidos por las levaduras durante la fermentación alcohólica mediante la conversión de los aminoácidos presentes en el medio (valina, leucina, isoleucina, metionina, treonina y fenilalanina) siguiendo la denominada ruta de Ehrlich o anabólicamente a partir del azúcar (Figura 5) (Bell & Henschke, 2005; Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008).

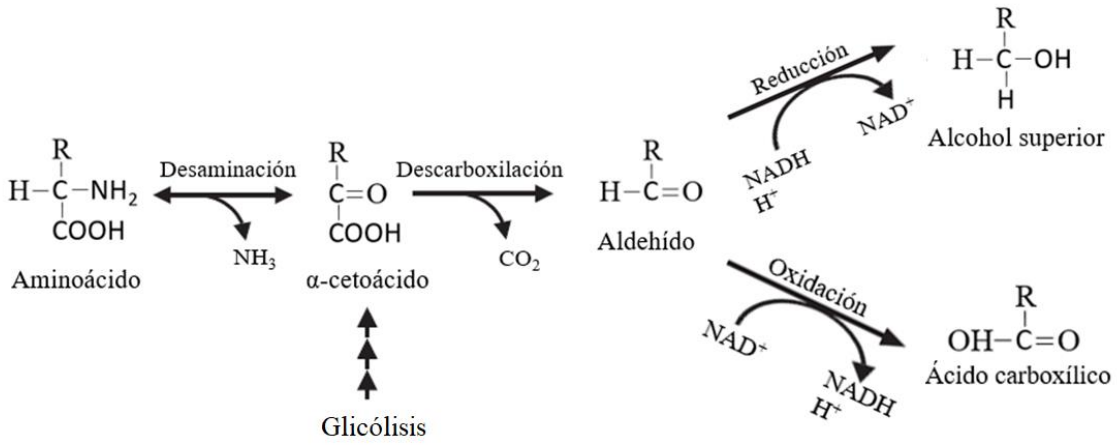

Figura 5. Catabolismo de aminoácidos para la formación de alcoholes superiores y ácidos mediante la ruta Ehrlich, y anabólicamente, a partir de los azúcares mediante glicólisis. Adaptado de (Bell & Henschke, 2005; Hazelwood et al., 2008).

El primer paso de esta ruta consiste en una reacción de transaminación en el que el grupo amino del aminoácido se transfiere a un α-cetoglutarato para formar un α-cetoácido y glutamato. Posteriormente, el α-cetoácido por descarboxilación da lugar al aldehído. El aldehído o bien se puede reducir a través de una reacción dependiente de NADH a su

respectivo alcohol superior o puede ser oxidado mediante una reacción dependiente de NAD^+ a su correspondiente ácido volátil (Figura 5) (Bell & Henschke, 2005; Hazelwood et al., 2008).

Los aminoácidos aromáticos fenilalanina y tirosina son metabolizados a través de la ruta de Ehrlich dando lugar al β-feniletanol, y al tirosol (ρ-hidroxifeniletanol), respectivamente. La metionina es metabolizada de manera similar originando el metionol. La metionina es un aminoácido que contiene azufre y, en paralelo a la ruta de Ehrlich, puede originar metanotiol y α-cetobutirato debido a la acción de una demetiolasa (Hazelwood et al., 2008; Perpète, Duthoit, De Maeyer, Imray, Lawton, Stavropoulos, Gironga, Hewlins, & Richard Dickinson, 2006). Además, también puede producir cisteína a través del oxaloacetato, que está altamente relacionado con la formación de compuestos de azufre volátiles, como el sulfuro de hidrógeno (Moreira, Mendes, Pereira, Guedes de Pinho, Hogg, & Vasconcelos, 2002). En la Tabla 1 se pueden ver los diferentes alcoholes superiores, aldehídos y ácidos volátiles formados mediante la ruta de Ethrlich a partir de sus aminoácidos correspondientes.

Tabla 1: Compuestos precursores e intermedios en la formación de los alcoholes superiores y los ácidos volátiles a partir de sus aminoácidos correspondientes a través de la Ruta Ehrlich. Modificado de (Hazelwood et al., 2008; Styger, Prior, & Bauer, 2011)

Aminoácido	α-cetoácido	Aldehído	Alcohol	Ácido
Ala	Ácido 2-cetopropiónico	Acetaldehído	Etanol	Ácido acético
Leu	α-cetoisocaproato	3-metilbutanal	Alcohol isoamílico	Ácido isovalérico
Val	α-cetoisovalerato	isobutanal	isobutanol	Ácido isobutírico
Ile	α-cetometilvalerato	2-metilbutanal	Alcohol amílico activo	Ácido 2-metilbutírico
Phe	2-fenilpiruvato	2-fenilacetaldehído	β-feniletanol	Ácido fenilacético
Tyr	ρ-OH-fenilpiruvato	ρ-OH-fenilacetaldehído	ρ-OH-feniletanol	Ácido ρ-OH-fenilacético
Met	α-ceto-γ(metiltio)butirato	metional	metionol	Ácido 3-(metiltio)propiónico
Thr o α-ABA	Ácido α-cetobutírico	propionaldehído	propanol	

Ala, alanina; Leu, leucina; Val, valina; Ile, isoleucina; Phe, fenilalanina; Tys, tirosina; Trp, triptófano; Met, metionina; The, treonina, α-ABA, ácido α-aminobutírico; OH, hidroxi.

La formación del propanol está directamente relacionada con la concentración de nitrógeno y el crecimiento de la levadura (Bell & Henschke, 2005), y no parece estar relacionada con la concentración del aminoácido treonina o ácido α-aminobutírico. Sin embargo, el metionol que se forma a partir de la metionina por la ruta de Ehrlich, muestra una relación directa con la concentración inicial de metionina (Etschmann, Kötter, Hauf, Bluemke, Entian, & Schrader, 2008; Hernández-Orte, Ibarz, Cacho, & Ferreira, 2005; Perpète et al., 2006).

Dado que la cantidad de los aminoácidos valina y leucina en el mosto es baja, la obtención de alcoholes superiores y ácidos volátiles (isobutanol, alcohol isoamílico, ácido isobutírico y ácido isovalérico) a través de la degradación de aminoácidos puede ser insignificante. Por lo tanto, algunos alcoholes y ácidos también se forman anabólicamente a partir del azúcar (mediante la formación de α-cetoácidos a partir del piruvato) (Figura 5) (Bell & Henschke, 2005; Hazelwood et al., 2008; Styger et al., 2011). Parte de las rutas enzimáticas necesarias para la formación de los alcoholes superiores a partir de sus aminoácidos correspondientes son comunes con las rutas anabólicas de formación de alcoholes superiores a partir de los azúcares.

2.2.1.2. Formación de aldehídos

Los aldehídos son compuestos intermedios en la ruta de Ehrlich (Tabla 1). El acetaldehído se forma durante la fermentación alcohólica en el proceso de glucólisis y mayoritariamente es transformado en etanol por la acción de los enzimas alcohol deshidrogenasas. Además, el acetaldehído se puede oxidar a ácido acético (Tabla 1) generando acetil coenzima A (acetil-CoA), que es usado en el metabolismo de lípidos o aminoácidos, entre otros (Peddie, 1990; Swiegers, Bartowsky, Henschke, & Pretorius, 2005). Al igual que los alcoholes superiores y los ácidos volátiles, cuando hay déficit de aminoácidos, los aldehídos se

pueden formar por la descarboxilación del α-cetoácido correspondiente procedente del azúcar por la ruta anabólica (Figura 5).

Otra ruta de formación de los aldehídos es la degradación de Strecker que consiste en la desaminación oxidativa y descarboxilación de un aminoácido en presencia de un compuesto α-dicarbonílico o una quinona derivada de un flavanol (Baert, De Clippeleer, Hughes, De Cooman, & Aerts, 2012; Bueno, Marrufo-Curtido, Carrascón, Fernández-Zurbano, Escudero, & Ferreira, 2018; Rizzi, 2006; Wietstock, Kunz, & Methner, 2016). Los aminoácidos de Strecker son la valina, leucina, isoleucina, metionina y fenilalanina. La reacción conduce a la formación de aldehídos de Strecker, que poseen un átomo de carbono menos que el aminoácido original, y a una α-aminocetona.

Estos aldehídos son moléculas aromáticas importantes en el aroma del vino, como es el caso del metional y del fenilacetaldehído que son responsables de las notas a verdura cocida y miel en los vinos oxidados (Culleré, Cacho, & Ferreira, 2007; Escudero, Cacho, & Ferreira, 2000; Silva Ferreira, Hogg, & Guedes de Pinho, 2003). Sin embargo, la mayoría de estos aldehídos se reducen a sus alcoholes correspondientes y sus concentraciones suelen ser muy bajas.

2.2.1.3. Formación de ésteres

Los ésteres en el vino son producidos principalmente durante la fermentación alcohólica por el metabolismo de las levaduras mediante la esterificación química de alcoholes y ácidos.

Los ésteres de acetato se generan mediante una reacción de condensación de los alcoholes superiores o etanol con ácido acético. Esta reacción es catalizada por las enzimas alcohol acetiltransferasas (AAT) y requiere acetil-coA. La esterificación es altamente dependiente de la cepa ya que se han identificado varios genes que codifican la enzima requerida en *S.*

cerevisiae (Peddie, 1990; Swiegers & Pretorius, 2005). El principal acetato es el acetato de etilo, con un fuerte olor a pegamento, debido a la alta concentración de ácido acético y etanol producidos durante la fermentación. Por otro lado, otros acetatos como el acetato de isoamilo y el acetato de feniletilo también suelen estar presentes en niveles superiores a sus umbrales de olfacción en el vino, contribuyendo con notas aromáticas a plátano y rosa, respectivamente (Styger et al., 2011).

Los ésteres etílicos también son producidos por una reacción química de esterificación de los ácidos correspondientes. Durante la fermentación, la levadura produce ácidos grasos de cadena media. A medida que se reduce el metabolismo celular, hay un exceso de ácidos grasos, por lo que se produce su esterificación con etanol produciendo ésteres etílicos de ácidos grasos (Peddie, 1990; Saerens, Delvaux, Verstrepen, Van Dijck, Thevelein, & Delvaux, 2008). Los ésteres etílicos pueden proceder de ácidos grasos lineales, dando lugar a ésteres como: decanoato de etilo, octanoato de etilo, hexanoato de etilo, butirato de etilo y propanoato de etilo; o de ácidos grasos de cadena ramificada formando: ciclohexanoato de etilo, 4-metilpentanoato de etilo, 3-metilpentanoato de etilo, 2-metilpentanoato de etilo, 3-metilbutirato de etilo, 2-metilbutirato de etilo, 3-hidroxibutirato de etilo e isobutirato de etilo (San Juan, Cacho, Ferreira, & Escudero, 2012; Styger et al., 2011).

Además, también existen rutas alternativas para los catabolismos de aminoácidos como ácido aspártico (asp), treonina (thr), valina (val) y metionina (met), que conducen a la formación de compuestos como acetoina, diacetilo, metanotiol, metionol y acetaldehído (Bell & Henschke, 2005; Rauhut, 1993).

3. LA LEVADURA

La liberación de aromas tiene lugar durante la fermentación gracias a la acción de las levaduras y, la intensidad y cualidad de los aromas obtenidos varía en función de la levadura utilizada para la fermentación.

Tradicionalmente, la fermentación de vinos se ha llevado a cabo por cepas de levaduras endémicas presentes tanto en las uvas como en las bodegas (Barata, Malfeito-Ferreira, & Loureiro, 2012; Escalante-Minakata & Ibarra-Junquera, 2007; Fleet, 2003). La variabilidad entre zonas geográficas, condiciones atmosféricas y presencia de otros microorganismos competidores hacen que la microbiota presente en la uva sea muy variable (Lúquez Bibiloni, Formento, & Díaz Peralta, 2007). *Saccharomyces cerevisiae* es conocida como la "levadura del vino" debido a su alto poder fermentativo y resistencia no solo a otros microorganismos competidores sino también al etanol y a antisépticos como dióxido de azufre (SO_2) (Fleet, 2003; Henick-Kling, Edinger, Daniel, & Monk, 1998; Jolly, Varela, & Pretorius, 2014). Además, en las uvas existen otros géneros de levadura, las levaduras *no-Saccharomyces*, que son las responsables de aromas muy especiales que generan un carácter diferenciador y único al vino final.

La fermentación con levaduras endémicas es denominada fermentación espontánea y es realmente importante debido a que gracias a ella se consiguen características organolépticas típicas de la zona (Escalante-Minakata & Ibarra-Junquera, 2007). Las levaduras *no-Saccharomyces* aparecen en fases iniciales de la fermentación y después, debido a su lento crecimiento y a la inhibición producida por los efectos combinados de etanol, bajo pH, y deficiencia de oxígeno, son sustituidas por las del género *Saccharomyces* (Combina, Elia, Mercado, Catania, Ganga, & Martínez, 2005; Henick-Kling et al., 1998).

No todas las levaduras son consideradas positivas ya que algunas son a menudo asociadas con la generación de aromas negativos (Jolly et al., 2014). Además, esta variabilidad entre levaduras genera una gran incertidumbre en la elaboración del vino ya que pone en riesgo la estabilidad y reproducibilidad del proceso de fermentación deseado año tras año. Por ello, para garantizar la fermentación completa, actualmente muchas bodegas inoculan en sus mostos una cantidad conocida y controlada de levadura comercial. La adición de un gran número de células al mosto asegura que no haya crecimiento espontáneo de microorganismos, obteniendo así una fermentación controlada y evitando problemas en la fermentación. Sin embargo, la oferta de levaduras comerciales es muy escasa y centrada principalmente en el género *Saccharomyces*, dejando de lado las levaduras *no-Saccharomyces*, que son responsables de gran parte del éxito de los vinos diferenciados en el mercado. Esta centralización en la inoculación de levaduras *Saccharomyces* ha llevado a un alto control en el proceso fermentativo, pero también a una homogenización organoléptica del vino, ya que frente a diferentes uvas, zonas geográficas y añadas los perfiles aromáticos expresados son similares.

La contribución de las levaduras al aroma del vino depende de la concentración de los compuestos formados, lo cual a su vez depende de las características del mosto a nivel de nutrientes, temperatura y cantidad de azúcares. La formación de compuestos volátiles a través del metabolismo de las levaduras es dependiente de sus actividades enzimáticas y, por ende, de sus genes. Por ello, la intervención de las levaduras *no-Saccharomyces* en la elaboración del vino es una importante fuente de aroma (Hernández-Orte et al., 2008; Loscos, Hernández-Orte, Cacho, & Ferreira, 2007; Padilla, Gil, & Manzanares, 2016).

Se han aislado en mostos de uva más de 40 especies *no-Saccharomyces* (Ciani, Comitini, Mannazzu, & Domizio, 2010; Jolly, Augustyn, & Pretorius, 2006). A pesar de todas las variables que influyen en la elaboración del vino y en la vendimia, las especies de levadura

encontradas tanto en mosto como en vino son similares en todo el mundo (Jolly et al., 2006). Sin embargo, la cantidad y cualidad de las diferentes levaduras que participan en la fermentación de cualquier bodega año tras año es cambiante y condicionada a los factores externos que modulan su crecimiento en los viñedos.

Actualmente se conoce el potencial beneficioso que tienen las *no-Saccharomyces* para la calidad del vino. Asimismo, se han desarrollado varios cultivos de levaduras *no-Saccharomyces* y se han implantado los protocolos para la inoculación secuencial y/o co-inoculación con *Saccharomyces cerevisiae* (Benito, Calderón, Palomero, & Benito, 2015; Gobbi, Comitini, Domizio, Romani, Lencioni, Mannazzu, & Maurizio, 2013; Zott, Thibon, Bely, Lonvaud-Funel, Dubourdieu, & Masneuf-Pomarede, 2011). Sin embargo, debido a la elevada biodiversidad de este grupo de levaduras, todavía quedan muchas oportunidades para su explotación en la producción de vino, estudiando no solo las levaduras *no-Saccharomyces* sino también su interacción con las levaduras *Saccharomyces*.

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OBJECTIVES

OBJECTIVES

This thesis has focused on the study of the formation of the different aromatic compounds present in the wine from the different precursors present in the grape. The thesis is composed by 3 chapters that deal with each type of precursor: amino acids (chapter I), cysteinylated and glutathionylated precursors (chapter II) and glycosidic precursors (chapter III).

The main objective of chapter I is to study the effect of must composition, especially of the amino acids and the cysteinylated and glutathionylated precursors, on the aromatic profile generated by different *non-Saccharomyces* yeasts. This main objective is divided into different sub-objectives:

i) Develop two rapid sensory-directed methodologies:
   a. The first one for the screening and selection of wine samples with diverse aroma profiles, generated by a wide range of *non-Saccharomyces* yeasts.
   b. The second for the identification of the key compounds responsible for the generation of the different aromatic profiles among a large number of similar samples.

ii) Select *non-Saccharomyces* yeasts capable of generating similar aromatic profiles among musts from different terroir.

The main objective of chapter II is to study the reason for the low conversion factor between polyfunctional mercaptans and their precursors. It is intended to verify if the reason is the need for yeast to use these precursors as a source of some nutrient. In addition, we want to

determine how the addition of different nutrients to the must affects the release of polyfunctional mercaptans. For this, this objective is divided into sub-objectives:

i) Study the effect of the amino acid profile on the release of polyfunctional mercaptans from their precursors.

ii) Determine if the yeast has a preference for some type of precursor (cysteinylated and glutathionylated).

iii) Determine if the yeast uses the precursors as a source of sulfur.

iv) Determine the effect of cysteine and GSH in the genes expression related to the metabolism of polyfunctional mercaptan precursors

The objective of chapter III has been the development of a new methodological strategy to measure the aromatic potential of wine grapes. This main objective is divided into the following sub-objectives:

i) Develop a method for the precursor extraction.

ii) Develop an accelerated hydrolysis method to determine the aromatic potential of grapes.

iii) Apply the methodology developed to grapes of different varieties, wineries, terroirs and ripeness states.

CHAPTER I

Study of the effect of must composition on the generation of different aromas profiles by different *non-Saccharomyces* yeasts

CHAPTER I. STUDY OF THE EFFECT OF MUST COMPOSITION ON THE GENERATION OF DIFFERENT AROMAS PROFILES BY DIFFERENT NON-SACCHAROMYCES YEASTS

1. INTRODUCTION

Sensory science works at the service of food industry in that it aims at developing methodologies able to characterize and measure product quality towards product features (Lawless & Heymann, 2010). Among strategies employed for evaluating product quality, categorization task has been successfully applied to identify quality exemplars based on expert´s judgements (Sáenz-Navajas, Ballester, Pêcher, Peyron, & Valentin, 2013). Besides the identification of products linked to quality perception, finding the sensory drivers of quality is essential for the food industry in general and the wine industry in particular. Descriptive sensory methodologies are the most powerful tools used in sensory discipline as they generate descriptive data explaining sensory differences among samples and thus the distinctive sensory character of the final product. Traditional descriptive methods are time and money-consuming mainly due to the long training period that is usually needed for developing vocabulary, references and reaching consensus in the use of descriptors. Thus, there is a trend in food sensory science to develop less time-consuming and more flexible methodologies (Valentin, Chollet, Lelièvre, & Abdi, 2012; Varela & Ares, 2012). These methods tend to replace trained panelists by non-trained consumers based on the assumption that panelists do not differ in their perceptions but solely in the way they describe them (Murray, Delahunty, & Baxter, 2001). These methods allow consumers to choose and use their own vocabulary without being trained in the use of descriptors (Guerrero, Gou, & Arnau, 1997), which deem faster, and more cost-effective (Valentin et al., 2012; Varela & Ares, 2012) than classical conventional descriptive analysis. Another

advantage of carrying out descriptive analysis with non-trained consumers is that the vocabulary they generate is often easily interpreted, which facilitates communication between marketing and scientific departments (Guerrero et al., 1997). Among alternative methods, flash profiling (Dairou & Sieffermann, 2002) is able to provide a product map in a very short time; however the interpretation of sensory terms describing samples is sometimes difficult as they are freely generated by consumers and no consensus in their definition is reached. This absence of consensus can be partly overcome by carrying out the task with semi-trained panelists instead of naïve consumers, which can in most cases provide descriptive profiles similar to non-trained consumers and thus easier to understand and interpret than those of trained panels, and at the same time they are likely to generate descriptions easier to interpret than the consumer panel (Guerrero et al., 1997). Another alternative method is sorting task, which has been successfully applied to sort exemplars with different aromatic profiles based on expert judgements. This task consists of sorting samples into different groups according to sensory similarities and measuring the number of times that each pair of wines are grouped together (Chollet, Lelièvre, Abdi, & Valentin, 2011; Patris, Gufoni, Chollet, & Valentin, 2007). This could be followed by a description of the samples with few descriptors in order to have a brief characterization of the established groups (Sáenz-Navajas, Campo, Avizcuri, Valentin, Fernández-Zurbano, & Ferreira, 2012). This task is rapid and produces little fatigue (Chollet et al., 2011; Patris et al., 2007). Besides the identification of products linked to the perception of different aromas, finding the key compounds of distinctive aromatic profiles is essential for the food industry in general and the wine industry in particular. Descriptive sensory methodologies are the most powerful tools to explain sensory differences among samples (Sáenz-Navajas, Alegre, de-la-Funete-Blanco, Ferreira, García, Eizaguirre, Razquin, & Hernández-Orte, 2016). The sorting task has been used for a variety of beverages such as drinking water

(Falahee & MacRae, 1995, 1997), beer (Chollet et al., 2011) or wine (Campo, Cacho, & Ferreira, 2008; Parr, Valentin, Green, & Dacremont, 2010).

In wine industry, the intrinsic quality of the product, which is related to its organoleptic properties, is dependent on both grape composition and technology used during wine making. Grape must contains all the nutrients necessary for the growth of the yeasts during the fermentation process. Monteiro et al., (Monteiro & Bisson, 1991) found that amino acids are used in the first steps of fermentation, mainly for protein biosynthesis. Although Rapp et al., (Rapp & Versini, 1991) reviewed that amino acid composition is related with the wine aroma, currently, such a correlation is not clear. On the other hand, during wine making, the selection of the suitable fermentation yeast strain is one of the most important factors that affect the flavor quality of the final product (Swiegers, Bartowsky, Henschke, & Pretorius, 2005). In the present time, there is a widely spread tendency among winemakers to inoculate musts with industrial *Saccharomyces* yeasts. This practice has the advantages of assuring reliable and rapid fermentations and reducing the risks of spoilage and unpredictable changes of wine flavor (Romano, Fiore, Paraggio, Caruso, & Capece, 2003). However, the massive culture of commercial yeasts during winemaking can led to the loss of characteristic aromas attributed to certain spontaneous fermentations. The dominance of spontaneous *non-Saccharomyces* yeasts during the early stages of alcoholic fermentation has been associated with the generation of both positive distinctive aromas and negative off-odors. Positive odor compounds such as fruity esters (Clemente-Jimenez, Mingorance-Cazorla, Martínez-Rodríguez, Las Heras-Vázquez, & Rodríguez-Vico, 2004; Renault, Coulon, De Revel, Barbe, & Bely, 2015), acetates (Stribny, Gamero, Pérez-Torrado, & Querol, 2015) or varietal aromas such as norisoprenoids, terpenoids or polyfunctional mecaptans (released from their odorless precursor due to β-glucosidase and β-liase activity of yeasts) (Jolly, Varela, & Pretorius, 2014; Rodríguez, Lopes, Van Broock,

Valles, Ramón, & Caballero, 2004) have been related to the presence of *non-Saccharomyces* yeasts. At the same time, certain strains appearing in uncontrolled fermentations have also been found to produce undesirable off-odors such as acetaldehyde (Englezos, Rantsiou, Torchio, Rolle, Gerbi, & Cocolin, 2015), acetic acid (Ciani & Maccarelli, 1997), ethyl acetate (Jolly, Augustyn, & Pretorius, 2003), higher alcohols (Clemente-Jimenez et al., 2004), diacetyl (by oxidation of acetoin) (Capece, Fiore, & Romano, 2005) or negative sulfur compounds such as hydrogen sulfide (Du Toit, Pretorius, & Lonvaud-Funel, 2005; Mendes-Ferreira, Barbosa, Lage, & Mendes-Faia, 2011). Hence, the selection of *non-Saccharomyces* yeasts producing positive aromas under controlled conditions deems important. The low tolerance of this nonconventional yeasts to alcohol concentration, usually leads to stuck fermentations. For ensuring complete alcoholic fermentation mixed inoculations of selected strains of these species with *Saccharomyces cerevisiae* are usually employed (Archana, Ravi, & Anu-Appaiah, 2015; Belda, Navascués, Marquina, Santon, Calderon, & Benito, 2015; Yamaoka, Kurita, & Kubo, 2014). Hence, inoculating mixed cultures of yeasts generating quality aromas has been revealed as an interesting tool in the wine industry for limiting the potential uniformity of aromatic characteristics of final wine and gaining in sensory complexity (Barbosa, Mendes-Faia, Lage, Mira, & Mendes-Ferreira, 2015; Carrau, Gaggero, & Aguilar, 2015; Ciani, Comitini, Mannazzu, & Domizio, 2010; Lage, Barbosa, Mateus, Vasconcelos, Mendes-Faia, & Mendes-Ferreira, 2014; Steensels & Verstrepen, 2014).

Selection criteria of *non-Saccharomyces* yeasts producing quality aromas are usually based on their capacity to produce individual volatile compounds with positive aroma descriptors. Even at trace concentrations, some of these compounds have powerful odors and have been demonstrated to contribute directly to wine aroma. They are considered to have a key impact, since they are often found at concentrations far above their olfactory perception

thresholds (Allen, Lacey, & Boyd, 1995; Tominaga, Baltenweck-Guyot, Peyrot des Gachons, & Dubourdieu, 2000). Therefore, a limited number of individual volatiles with known either positive sensory activity, such as esters or acetates, or negative such as acetaldehyde, volatile acids or higher alcohols, are usually quantified and their contribution to overall wine flavor is discussed based on their concentration (Clemente-Jimenez et al., 2004; Furdíková, Makyšová, Ďurčanská, Špánik, & Malík, 2014; Malík, Ďurčanská, Hronská, & Malík, 2014; Steensels, Meersman, Snoek, Saels, & Verstrepen, 2014). This methodology is bound to lose important information related to impact aroma compounds, because it is limited to the study of a reduced list of volatiles ignoring others. Besides, the sensory role of individual compounds based on their concentration is often misinterpreted. As an example, the rose-like higher alcohol β-phenylethanol has been suggested to contribute positively to the floral aroma of wines (Clemente-Jimenez et al., 2004; Furdíková et al., 2014) and thus yeasts producing higher amounts of it are reported to be superior exemplars. However, studies carried out in our laboratory in complex matrices, have demonstrated that the presence of this compound at concentrations (of even 300 mg/L) higher than their sensory threshold (14 mg/L) do not have any significant sensory role in the overall wine flavor (Escudero, Gogorza, Melús, Ortín, Cacho, & Ferreira, 2004; Ferreira, Ortín, Escudero, López, & Cacho, 2002). This suggests that most usual methodologies aimed at finding quality aromas based on the quantification of a limited number of volatiles would either misinterpret or lose valuable information, especially when optimizing any technical process (such as the selection of the appropriate fermentative yeast) during wine elaboration. Moreover, compounds not only interact between them but also, due to the great amount of compounds present in wine and the difference in their concentrations, ultratrace aroma compounds are often masked by more prevalent compounds which may be present at concentrations above 100 mg/L. So that, it is difficult

to identify the compounds responsible of the different notes. Thus, the implementation of sensory strategies able to directly measure the sensory impact of the product on consumer perception can improve the identification of quality exemplars and provide valuable information to producers. These strategies linked to the use of sequential techniques can led to the identification of key compounds that generate differences between similar wines. Ferreira et al., (Ferreira, Hernández-Orte, Escudero, López, & Cacho, 1999) developed a method for obtaining fractions in diluted alcohol solutions, using reversed-phase HPLC (High Performance Liquid Chromatography) fractionation. The fractions collected can be easily described by direct olfaction, after the addition of water or synthetic wine to adjust the alcohol content to 11-14% in each fraction (Ferreira et al., 1999; Pineau, Barbe, Van Leeuwen, & Dubourdieu, 2009). This aims to simplify the identification of compounds through the isolation of groups of compounds present in wines for further gas chromatography-olfactometry (GC-O) analysis.

In this context, the present work aimed at developing two rapid sensory-directed methodologies. The first one for the screening and selection of wine samples, with diverse aroma profiles, generated by a wide range of *non-Saccharomyces* yeasts based on their capacity to generate quality and distinctive aromas. The second for the identification of the key compounds responsible for the generation of the different aromatic profiles among a large number of similar samples. Additionally, the effect of *non-Saccharomyces* and *Saccharomyces* yeasts co-inoculation is also studied. For these purposes, the methodological approaches combined: i) categorization task for the selection of quality exemplars followed by descriptive flash profiling with GC-O analysis for identifying chemical odorants driving main sensory differences among wine samples and related to quality aroma profiles; and ii) the sorting task with a description step for the selection of the exemplars with different aromatic profiles followed by fractionation and further GC-O

analysis for identifying the chemical odorants that produce the main sensory differences among wine samples.

2. MATERIALS AND METHODS

2.1. Reagents and standards

HPLC quality Dichloromethane was supplied by Fisher Scientific (Loughborough, UK). LiChrosolv quality methanol, LiChrolut EN resins, HPLC quality ethanol and diethyl ether (EMSURE) were supplied by Merck (Darmstadt, Germany). *n*-Hexane for organic trace analysis (UniSolv). Anhydrous sodium sulfate of analysis ACS-ISO quality was purchased from Panreac (Barcelona, Spain). Ethylenediaminetetraacetic acid disodium salt 2-hydrate (EDTA), L-cysteine hydrochloride hydrate 99%, 1,4-dithioerythritol, octafluoronaphthalene 96% (OFN), and 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) were supplied by Aldrich (Steinheim, Germany). O-methylhydroxylamine hydrochloride purum > 98%, 2,3,4,5,6-pentafluorobenzylbromide (PFBBr) and 4-methoxy-α-toluenethiol were obtained from Fluka (Buchs, Switzerland). 4-mercapto-4-methyl-2-pentanone (4MMP) 98% and 3-mercaptohexanol (3MH) > 95% were purchased from Alfa Aesar (Karlsruhe, Germany). 3-mercaptohexyl acetate (3MHA) was obtained from Oxford Chemical (Hartlepool, U.K.). Pure standards of the four precursors cysteine-3-mercaptohexan-1-ol (CYSMH), cysteine-4-mercapto-4-methylpentan-2-one (CYSMP), glutathione-3-mercaptohexan-1-ol (GLUMH) and glutathione-4-mercapto-4-methylpentan-2-one (GLUMP) were synthesized by Roowin (Riom, France), having a purity ≥ 95%. Bond Elut-ENV resins, prepacked in a 50 mg cartridge (1 mL total volume) and a semiautomated solid-phase extraction (SPE) Vac Elut 20 station, were supplied by Varian (Walnut Creek, CA). Liquid chromatography coupled to mass spectrometry (LC–MS) grade acetonitrile and formic acid obtained from Scharlau (Barcelona, Spain) were used as mobile phases. Pure water was obtained from a Milli-Q purification system (Millipore, USA). The standards used for identifications were supplied by Aldrich (Steinheim, Germany), Merck,

ChemService (West Chester, PA), Fluka (Buchs, Switzerland), Sigma (St. Louis, MO), PolyScience (Niles, IL), Lancaster (Strasbourg, France), Alfa Aesar (Karlsruhe, Germany), Panreac (Barcelona, Spain), SAFC (Steinheim, Germany), and Oxford Chemicals (Hartlepool, U.K.). β-Damascenone was a gift from Firmenich (Geneva, Switzerland).

2.2. Samples

Three set of fermentations were carried out. Forty-eight *non-Saccharomyces* strains from the yeast culture collection of LEV2050 (Pamplona, Spain) were used for red (Tempranillo) and white (Verdejo) grapes in the first set of fermentations (Figure I.1a). All of them were non-commercial. Along with *non-Saccharomyces* yeasts, three *Saccharomyces* strains were used as reference for red (R18, R19, R20) and two for white musts (W38, W39). Thus, a total of one hundred and one yeast strains were studied (50 for red and 51 for white musts).

Then, five *non-Saccharomyces* yeast strains were selected for each variety and were used in the second fermentations set as can be observed in Figure I.1b: W5, W10, W20, W36 and W47 for white musts and R14, R22, R24, R27 and R30 for red musts. This second set was composed by 60 fermentations. Thirty samples were taken in the case of white wines (25 samples obtained from the fermentation of five different musts with each selected yeast strain and 5 samples from the fermentation of the five musts with one of the yeast strains (W36) as a control of the fermentation). The same number and treatment was used in the case of red wines (with R22 as a control).

From these yeasts, two *non-Saccharomyces* yeasts for each variety were selected (W20, W36, R22 and R27) and were used in the third set of fermentations with and without the co-inoculation with *Saccharomyces cerevisiae* (Figure I.1c).

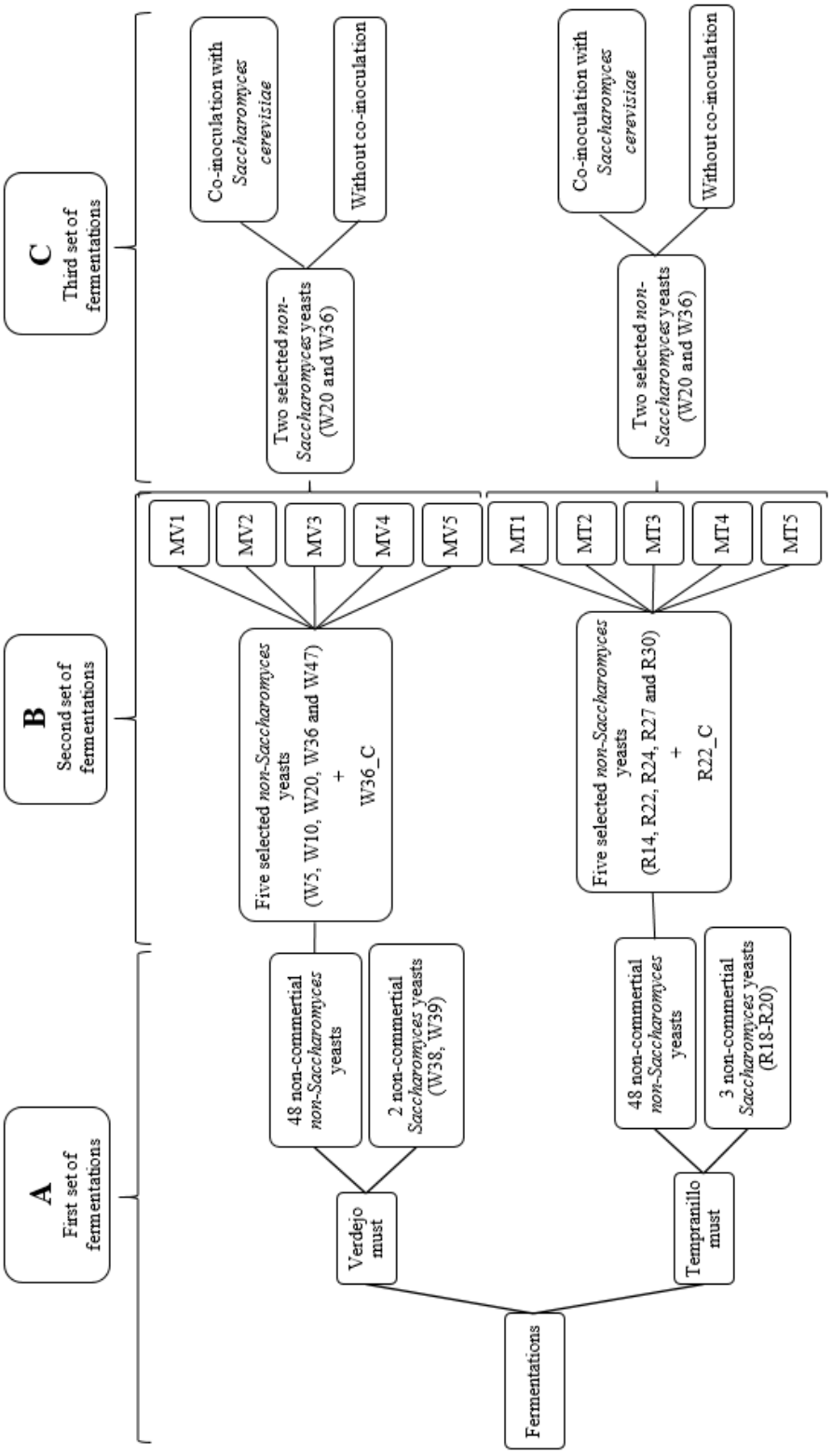

Figure I.1. Three set of fermentations: a) first set carried out with 100 yeast strains (fifty for Verdejo and fifty for Tempranillo musts); b) second set composed by 10 *non-Saccharomyces* yeasts (five for Verdejo and five for Tempranillo must) and 10 different musts (5 Verdejo and 5 Tempranillo musts).; and c) Third set of fermentations carried out with 4 *non-Saccharomyces yeasts* (two for each variety) and with and without the co-inoculation with *Saccharomyces cerevisiae.*

2.3. Microvinification processes

The first set of fermentations was carried out with an unique Tempranillo red grape must and Verdejo white grape must collected during October 2014 from DOCa Rioja and DO Rueda regions in Spain, respectively. Then, after the *non-Saccharomyces* yeasts selection, the second set of fermentation was done with five different Verdejo white grape musts (MV1, MV2, MV3, MV4 and MV5) from different terroirs in DO Rueda region in Spain and five different Tempranillo red grape musts (MT1, MT2, MT3, MT4 and MT5) taken from the DOCa Rioja, Ribera del Duero, DO Rueda and DO Manchuela regions in Spain. Verdejo and Tempranillo grapes were taken during October 2015 and 2016. Afterwards, the third set of fermentation was done with the four selected *non-Saccharomyces* yeasts and 2 different Verdejo white grape musts (MV3 in the case of W20 and MV4 in the case of W36) and two different Tempranillo red grape musts (MT3 in the case of R22 and MT2 in the case of R27). Verdejo and Tempranillo grapes were taken during October 2016 from DO Rueda in the case of Verdejo grape musts and DO Manchuela and DO Ribera del Duero in the case of MT3 and MT2, respectively.

Tempranillo grapes were frozen at -20 ºC until the fermentation process was carried out. Verdejo grapes were removed from the stems, crushed, and pressed. Then, the must was sulfited (3 g/hL), racked off and stored at -20 ºC until winemaking.

Tempranillo red grapes and Verdejo white musts were defrosted at room temperature during 48h. Tempranillo grapes were pressed, sulfited with potassium metabisulfite (4 g/hL) and distributed to the different tanks. In the different set of fermentation, red must was firstly supplemented with diammonium phosphate to reach 180 mg/L of yeast assimilable nitrogen content to avoid nitrogen deficiencies during alcoholic fermentation as recommended in literature (Bely, Sablayrolles, & Barre, 1990), while white must was

not supplemented as it already contained between 187-245 mg/L. Then, musts were distributed to 2-liter-containers equipped with vent bungs. Prior to inoculation and/or co-inoculation, they were pasteurized and controlled (inoculation in YM-agar plates during 48 h at 28 ºC) to assure the dominance during fermentation of the strains object of study. Pasteurized musts were inoculated at the rate of 10^6 cfu/mL.

Fermentations were carried out at 20 ºC and 16 ºC for red and white wines, respectively. They were controlled by measuring the content in reducing sugars by refractometry. Alcoholic fermentation took place in the range of 4-8 and 3-11 days, for red and white musts, respectively. Once fermentation concluded (no variation in refractive index in two consecutive days), samples were stored at 4 ºC during 48 h to permit the sedimentation of gross lees and then were racked off again. The sulfur dioxide content was adjusted to reach 30 mg/L of free sulfur dioxide (SO_2) and samples were stored at 5 ºC to favor the sedimentation of fine lees.

2.4. Sensory analyses

Panelists. Different panels of wine experts took part in the sensory analyses as can be observed in Table I.1. The panelists fitted the category of wine-science researchers and teaching staff who were regularly involved in wine-making and/or wine evaluation. They were all considered wine experts according to Parr, Heatherbell (Parr, Heatherbell, & White, 2002) specifications. All assessments were conducted in individual tasting booths. Sensory analysis tasks were carried out in Zaragoza (Spain) at Laboratory for Analysis of Aroma and Enology (LAAE).

Table I.1. Panelists who participated in the different sensory analyses.

	Sensory analysis	Number of participants	Men (%)	Women (%)	Lower age (years)	Higher age (years)	Median (years)
a	Categorization task (White wines)	17	51.8	48.2	19	67	39.5
b	Categorization task (Red wines)	15	40.0	60.0	25	74	36.8
c	Flash profiling (White and red wines)	15	51.8	48.2	19	67	39.5
d	Sorting task- Effect of must composition (White and red wines in 2015)	15	33.0	67.0	24	60	35.0
e	Sorting task- Effect of must composition (White and red wines in 2016)	15	33.0	67.0	24	44	30.4
f	Sorting task- Effect of co-inoculation	16	37.5	62.5	26	74	35.8

In all cases, one hour before formal tasting, samples were removed from the 5 ºC cold room and 20 mL of samples were served in dark approved wine glasses (ISO NORM 3591, 1977) labelled with 3-digit random codes and covered by plastic Petri dishes according to a random arrangement different for each assessor. All samples were served at room temperature and were evaluated in individual booths. Panelists were not informed about the nature of the samples to be evaluated. They were only told that they were either young white or young red wines.

White and red wines from the first set of fermentations (Figure I.1a) were separately submitted to two different sensory tasks. Firstly, a categorization task was carried out to select exemplars with higher aroma quality according to a panel of wine experts. These samples were further sensory described (flash profiling) by a panel of semitrained panelists.

2.4.1. Categorization task (CT)

Both in white and red samples, the pre-selection of yeasts was conducted in two sessions within the same day (one at 10 a.m. and the second at 16 p.m.). In both cases, two control commercial wines (high quality (HQ_W) and low quality (LQ_W) for white samples, and HQ_R and LQ_R for red wines) were used. These control samples were presented in both sessions for examining panel performance. LQ_W and LQ_R were neutral white and red wines, respectively, spiked with 70 mg/L of acetaldehyde to generate a low-quality wines (acetaldehyde is related to wine oxidation), while HQ_W and HQ_R were commercial white and red wines expected to have higher aroma quality than LQ_W and LQ_R.

Samples. For each variety, the study took part in two sessions. In the case of white samples, twenty-seven wines were included in each session: twenty-five different samples together with two control commercial wines (HQ_W and LQ_W presented in both sessions), making a total of fifty-four white samples. For red wines, a total of fifty-five samples were categorized in terms of aroma quality by the wine experts: 25 and 26 samples were included in the first and second sessions, respectively. Within each session the same two control wines (HQ_R, LQ_R) were included to control panel performance.

Procedure. The participants had to examine in each session the samples exclusively in terms of orthonasal aroma quality and sort them in five quality groups: ‘‘very high’’, ‘‘high’’, “average”, ‘‘low’’ or ‘‘very low’’. These five categories were easily interpretable by participants. Once they had formed the groups on the table, participants were provided with a pencil and a sheet in order to write down their responses. Then, participants were asked to associate to each of the five groups a maximum of 2-3 attributes. Participants were presented with the following instructions:

''Twenty-seven glasses of young white wines are presented on the table. Each glass is coded by a three-digit number. You are asked to orthonasally smell the twenty-seven wines firstly from left to right and then to form five groups (according to the following categories: very high, high, average, low or very low) on the table according to your perceived aroma quality''.

Data analysis. The number of times each wine was classified by participants in each of the five quality groups was counted. Three categories were finally considered in data analysis for simplifying the presentation of results. The "very high" and "high" as well as "low" and "very low" quality categories were jointly considered. Data were encoded in a wine (50) x quality level (3) contingency table, in which each cell represented the frequency of the categorization of a wine in one category level. Correspondence Analysis (CA) was performed on the contingency table. Hierarchical Cluster Analysis (HCA) with the Ward criteria was finally applied to all the factors derived from CA. The quality category ("very high/high", "average" and "low/very low") best defining the resulting clusters were identified by computing their probability of characterizing a cluster (Lebart, Piron, & Morineau, 1995). Analyses were carried out with SPAD software (version 5.5).

2.4.2. Flash profiling (FP)

Samples mostly included in both "high" and "very high" quality categories in the categorization task (nine white and seven red samples) were further submitted to descriptive analysis by means of flash profiling methodology.

Samples. For white samples, the descriptive task was carried out with a total of 13 samples: nine selected in the categorization task and two blind control samples (Marques de Riscal (MR_W) and Los Molinos (LM_W) both commercial white wines elaborated with Verdejo) in duplicate. For red wines, descriptive task was carried out with a total of 11 red

samples: seven selected in the categorization task and two blind commercial control samples (Borja (BJ_R) and Los Molinos (LM_R) elaborated with Grenache and Tempranillo, respectively) in duplicate. Control samples were included for examining panel performance.

Procedure. Flash profiling (for both white and red wines) involved two sessions separated by an inter-session. In *the first session* the 13 white samples (or the 11 samples for red samples) were presented simultaneously to each assessor. They were firstly given an explanation about the procedure. Then, they were asked to individually generate the aroma descriptors that differentiated the wine set. They were asked to avoid hedonic terms and to use exclusively descriptive terms. They were free to generate as many attributes as they wanted and to take as much time as needed. During *the inter-session*, the experimenter pooled all the generated attributes to form a global list that was provided to the assessors in the second session. This global list was presented as an aid tool to allow assessors to update their own list if desired but it was not aimed at reaching a consensus. With this global list they could either add to their list a few terms they thought were relevant but did not generate themselves or replace some of their own terms by terms they thought were more adapted. In *the second session*, assessors were asked to rank order the samples on each of their chosen attributes. Sensory attributes were evaluated using a nonstructured 10 cm continuous length scale anchored with the words “absence” and “high intensity” on the left and right ends, respectively, being ties allowed.

Data analysis. Individual assessors´ rank data were firstly collected in a matrix built for each participant (wines in rows and terms in columns). The global data matrix formed by the individual matrices generated by the assessors was submitted to Generalized Procruster Analysis (GPA). Descriptors mentioned by at least three assessors (20% of the panel) were

used to visualize the relationships between samples and attributes. Analyses were carried out with XLSTAT software (version 2014.2.02).

2.4.3. Sorting task

From flash profiling, three *non-Saccharomyces* yeasts for each variety were selected. Additionally, two *non-Saccharomyces* yeasts for each variety were selected by a sensory analysis carried out in LEV2050, making a total of 5 yeasts for each variety. These yeasts were used in the second set of fermentations (Figure I.1b) to ferment 5 different Verdejo and Tempranillo musts from different Spanish areas and from 2 consecutive vintages (2015 and 2016). In order to study the effect of the must composition, the samples were submitted to 4 sorting tasks (two for white samples in the two vintages, and two for red samples in the 2 vintages).

Moreover, from these sorting tasks, two *non-Saccharomyces* yeasts for each variety were selected. These yeasts were used in the third set of fermentations (Figure I.1c) to ferment Verdejo and Tempranillo musts with the co-inoculation of *Saccharomyces cerevisiae*. Each fermentation was done in duplicate. In order to check the effect of the co-inoculation with *Saccharomyces cerevisiae* yeast, an additional sorting task was carried out.

Samples. For studying the effect of must composition, samples obtained in the second set of fermentations (Figure I.1b) were studied. Thirty-two white wines and 32 red wines were submitted to the sorting tasks. Twenty-five were taken from wines produced by the fermentation of the five different musts with each of the 5 selected *non-Saccharomyces* yeast strains, and five control wines from each of the five different musts inoculated with one of the yeasts (W36 and R22). These control samples were presented for examining the yeast reproducibility. Two replicate samples were introduced to assure the reproducibility of the panel, making up the 32 samples. On the other hand, for studying the effect of co-

inoculation, samples obtained in the third set of fermentations (Figure I.1c) were used. Seventeen white and red samples were submitted to one sorting task, nine white wines and eight red wines. Four came from the fermentation of musts with the four selected *non-Saccharomyces* yeasts, and the other four came from the co-inoculation of these *non-Saccharomyces* with *Saccharomyces cerevisiae.* These fermentations were carried out in duplicate. Moreover, one replicate white sample was presented to assure the panel reproducibility, making up the 17 samples.

Procedure. Sorting task was carried out to select exemplars with similar aromas according to a panel of wine experts. The panelists were asked to sort the wines into groups based on odor similarities. They could make as many groups as they wished. Once they had formed the groups on the table, the panelists were provided with a pencil and paper in order to write down their responses. They were then asked to provide 1-3 free descriptors to describe each of the groups.

Data analysis. For each subject, results were encoded in an individual similarity matrix (wines x wines) with each cell indicating if two wines were put in different groups or in the same group (0 and 1, respectively), as described by Sáenz-Navajas et al., (Sáenz-Navajas et al., 2012). These individual matrices were summed across subjects; the resulting co-occurrence matrix represents the global similarity matrix where larger numbers mean higher similarity between samples. Thus, samples grouped together are more similar than samples sorted into different groups. The assumption underlying this method is that samples grouped together are more similar than samples sorted into different groups (Sáenz-Navajas et al., 2012).

A multidimensional scaling (MDS) analysis was performed with the co-occurrence matrix in order to obtain a spatial representation of the wines (Schiffman, Reynolds, & Young, 1981). This technique aims to create a spatial map representing the relative similarities and

differences between samples. A non-parametric scaling algorithm was used (alternating least-square scaling). This procedure seeks iteratively a solution preserving the rank-order of the perceived similarities between products. The quality of fit was measured by the stress value, which is based on the sum of the squares of distances between objects observed in the raw data and objects observed in the p-dimensional MDS space (Sáenz-Navajas et al., 2012). The stress varies from 0 (perfect fit) to 1 (worst possible fit), a value bellow 0.2 is generally considered as a good agreement between the initial and final configurations (Cox & Cox, 2001).

For cluster analysis, the coordinates obtained from the MDS were submitted to a hierarchical cluster analysis (HCA) with the Ward criterion (Murtagh, 1983). The MDS and HCA analyses were performed using XLSTAT software (version 2014.2.02). HCA makes it possible to identify the wines that belong to the same cluster and check the spatial arrangement of the MDS data (Lebart et al., 1995).

For analysis of vocabulary, each panelist described each group of wines with one to three free descriptors. For each panelist, the terms given for a group of samples were associated to each wine of the group. The underlying assumption within this approach is that all the samples belonging to the same group were described by the terms in the same way. Thus, the frequency of quotation of each descriptor was calculated and only descriptors cited by at least three panelists (20% of the panel) were considered.

2.5. HPLC fractionation

2.5.1. Preparation of the sample: Solid phase extraction (SPE)

Total wine extracts were obtained by direct solid phase extraction (SPE) as described by López et al., (López, Aznar, Cacho, & Ferreira, 2002), with some modifications. Thus, 750

mL of sample was passed through commercial cartridges of 1000 mg of LiChrolut EN resin. Aroma extracts were obtained by elution with 10 mL of ethanol.

In order to obtain a more concentrated extract, a demixture was carried out following the method proposed by Ferreira et al., (Ferreira, Escudero, López, & Cacho, 1998), with some modifications. 8.6 g H_2NaPO_4-H_2O and 35.3 g $(NH_4)_2SO_4$ was placed in a 100 mL volumetric flask. Ten mL of the ethanol extract was added over the salt, and the required amount of water was added to adjust the alcoholic content of the mixture to 13%. After ensuring the separation of the phases, two milliliters of the organic phase were pipetted and transferred to a vial. A 1:4 dilution of this extract with Milli-Q water was used for the subsequent fractionation.

2.5.2. HPLC fractionation

The procedure was based on the method described by Ferreira et al., (Ferreira et al., 1999). The HPLC system was supplied by Varian (Walnut Creek, CA, USA), with a manual injector, an automated gradient controller, and a diode array 990 UV detector. The column used was a C18 Kromasil 5 µm, 25 cm x 10 mm I.D., supplied by Análisis Vínicos (Tomelloso, Spain). The column is protected by a 2 cm precolumn of the same phase. The chromatographic conditions included: flow-rate of 2 mL/min, detection at 254 nm and injection volume of 2 mL of the water-ethanol (1:4) extract. The program gradient involved phase A, water, and phase B, ethanol; 0-10 min, 10% B; 10-40 min, from 10% B to 100% B and 40-45 min, 100% B. All the samples were filtered through a 0.45 µm filter before injection. Twelve fractions of 6 mL were collected.

The fractions eluted from the HPLC procedure were evaluated by 3 Spanish wine experts. A 50 µL amount of the fraction was added to an odor strip. After the ethanol was removed, the odor strips were submitted to a description task by the judges. The fractions whose

attributes were similar to the descriptors of the wines by means of the sorting task were selected.

2.6. Identification of key odorants by GC-O study

Two gas chromatography-olfactometry analyses were carried out: i) wines selected in the flash profiling (5 white and 5 red wines); and ii) selected fractions in the sensory analyses (eight fractions from white wines and the same number for red samples).

2.6.1. Samples extracted for GC-O analysis

2.6.1.1. Samples extracted from selected wines

Total wine extracts were obtained by direct solid phase extraction (SPE) as described by López et al., (López et al., 2002), with some modifications. Therefore, 100 mL of sample was passed through commercial cartridges of 100 mg of resin LiChrolut EN. Aroma extracts were obtained by elution with 1 mL of ethanol. Extracts were stored at -20 ºC until GC-O analyses.

Total wine aroma extracts were reconstituted in synthetic wine (5 g/L of tartaric acid and 9% ethanol, pH 3.2 and 3.5 for white and red wines, respectively) by adding one mL of extract to 99 mL of synthetic wine.

A dynamic headspace sampling technique designed to obtain representative extracts for olfactometry analysis was used to capture wine aroma (San Juan, Pet'ka, Cacho, Ferreira, & Escudero, 2010). Therefore, a standard SPE cartridge (0.8 cm internal diameter, 3 mL internal volume) filled with 400 mg of LiChrolut EN resins was first washed with 20 mL of dichloromethane and then dried by letting air pass through (negative pressure of 0.6 bar, 10 min). The Lichrolut EN cartridge was placed on the top of a bubbler flask near the liquid surface (80 mL of reconstituted wine), which was continuously stirred with a magnetic stir

bar and kept at a constant temperature of 37 ºC by immersion in a water bath. A controlled stream of nitrogen (500 mL/min) was passed through the sample for 100 min. The volatile wine constituents released in the headspace were trapped in the cartridge containing the sorbent. After 100 min, the cartridge was removed and dried by letting nitrogen (N_2) pass through; then, analytes were eluted with 3.2 mL of dichloromethane with 5% methanol. After this, the extract was concentrated under a stream of pure nitrogen to a final volume of 200 µL.

2.6.1.2. Samples extracted from selected fractions

The fractions selected were diluted with acid water (5 g/L of tartaric acid, pH 3.5) to adjust their alcoholic content to 11% (v/v), and finally extracted by SPE as described by López et al., (López et al., 2002), with some modifications. Thus, 30-55 mL of sample adjusted to 11% was passed through commercial cartridges of 50 mg of LiChrolut EN resin. Aroma extracts were obtained by elution with 600 µL of dichloromethane with 5% methanol. The extracts were stored at -20 ºC until GC-O analysis.

2.6.2. Gas chromatography-Olfactometry (GC-O)

GC-O analyses with the extracts prepared were carried out with a Trace GC gas chromatograph (ThermoQuest, Milan, Italy) with a flame ionization detector (FID) and a sniffing port ODO-I from SGE (Ringwood, Australia). The capillary column used was a DB-WAX (polyethylenglycol) supplied by J&W (Folsom, CA, USA), 30 m x 0.32 mm i.d. x 0.5 mm film thickness, and a deactivated precolumn (3 m x 0.32 mm i.d.) from Supelco (Bellefonte, PA). Hydrogen was used as carrier gas at a constant flow rate of 3.5 mL/min.

The injection was conducted in splitless mode (60 s splitless time). The injection volume was 1 µL. The injector and detector temperature was 250 ºC. The sniffing port was heated using a thermostat made in the laboratory to prevent the condensation of high boiling point

compounds, and it was equipped with a humidifier of deionized water. The temperature program used for analysis of the sample was 40 ºC for 2 min, increased by 20 ºC/min to 130 ºC and then 4 ºC/min to 220 ºC, maintaining this temperature for 10 min.

The olfactometric analysis was carried out by a panel of 6 trained judges in the case of selected wines and 3 trained judges for selected fractions belonging to the laboratory staff. Each olfactometry was performed in one 25-min session (within the range of 3-28 min of the GC-O). Sniffers indicated the time, description and odor intensity when an aroma was detected. A 7-point structured category scale was used for measuring perceived odor intensity (anchored with 0 = not detected; 1 = weak odor, 2 = clear odor; 3= extremely strong odor), and allowing intermediate values (0.5, 1.5 and 2.5).

The identification of the odorants was carried out by a comparison of their odors, chromatographic retention index in the DB-Wax column and chromatographic retention index in the DB-5 column with those of pure reference compounds, when available.

2.6.3. Multidimensional gas chromatography- olfactometry –mass spectrometry (GC-GC-O-MS)

In order to identify the compounds that could not be identified by their retention index in both columns, multidimensional gas chromatography-olfactometry-mass spectrometry was used.

The analysis was performed using a multidimensional gas chromatograph supplied by Varian (Walnut Creek, CA), which constituted two independent gas chromatographs interconnected by means of a transfer line kept at 200 °C.

Chromatograph 1 (GC-1): The chromatograph was a CP 3800 model equipped with a 1079 PTV (Programmable Temperature Vaporization) injector, a flame ionization detection (FID) system and an olfactometric port (ODO-II obtained from SGE, Ringwood, Australia)

both connected by a flow splitter to the column exit, so that simultaneous FID and sniffing monitoring of the effluent from the first column was possible. This GC was retrofitted with a Deans pressure-driven switching valve (Valco Instruments, Houston, TX), which enables selective transfer of heart cuts eluting from the first column directly onto the analytical column placed in the second chromatograph. The carrier gas (He) was delivered at a constant pressure of 30 psi. The column was a DB-WAX supplied by J&W (Folsom, CA), 30 m × 0.32 mm i.d. with 0.50 μm film thickness. An uncoated, deactivated fused-silica column obtained from Supelco was used as an interface between the Deans switching valve and the FID and ODO detectors. The oven temperature program was 40 °C for 2 min, increased by 20 ºC/min to 130 ºC and then 4 ºC/min to 220 ºC, maintaining this temperature for 10 min. The FID was kept at 300 °C.

Chromatograph 2 (GC-2): This chromatograph was a Varian CP 3800 model coupled to an ion trap mass spectrometric-detector (Saturn, 2000). The system was equipped with a CO_2 cryotrapping unit and an olfactometric port (ODO-II from SGE) at the end of the column, so that simultaneous olfactometry and MS scanning was possible. A make-up flow was diverted through a flow splitter placed at the end of the column and a flow restrictor was placed between the flow splitter and the MS detector. The column was a Factor Four VF-5MS supplied by Varian (30 m × 0.32 mm × 1 μm film thickness). The column was directly connected to the Deans valve placed in the first chromatograph *via* the thermostated transfer line. The first centimeters of the column in the second GC crossed the cryofocusing unit (CO_2), and the end of the column was linked to a splitter connected to both the MS and ODO detectors. Two minutes after the heart-cutting, the CO_2 flow was removed at the same time that the temperature program of the second oven was activated (at first at 50 °C, then raised by 4 °C/min up to 200 °C, by 100 °C/min up to 300 °C and finally this temperature was maintained for 10 min). The MS parameters were: transfer line at 170 °C, ion trap at

150 °C and trap emission current 30 μA. The global run time was recorded in full scan mode (*m/z* 25–250 mass range), at 0.5 scans per second. FID and MS data were registered and processed with Workstation 6.30 software equipped with the NIST MS library (NIST, Gaithersburg, MD).

Inside the PTV injector of the first chromatograph there was a 3.4 mm i.d. insert filled with carbofrit (Restek, Bellefonte, PA) previously washed with dichloromethane and dried with a stream of nitrogen. Forty microliters of the concentrated extracts were injected. The injection was carried out in the large volume mode typical of the PTV injector. The initial injector temperature was 40 °C. The split valve was closed after 0.4 min of solvent evaporation and the injector was then heated to 250 °C at 200 °C/min. After 3 min the split valve was opened again.

For an odorant with retention time t_s (in minutes), the heart-cutting interval was set at $[t_s - 0.9] \pm 0.1$ min, 0.9 being the delay time and ±0.1 min the interval required to guarantee a quantitative transfer of the analyte from the first to the second column.

Forty microliters of the extracts were first monitored by GC–O in the first chromatograph. In further chromatographic runs, selective heart-cuttings were performed to isolate the odorants of interest which were transferred to the second oven and monitored by olfactometry with simultaneous MS detection. Thus, coelutions were avoided in the second column. The identity of the odorants was determined through the mass spectra and the linear retention indices on both columns.

2.6.4. Data analysis

The GC-O data were processed taking into account the frequency of citation (F) and the intensity of each odor zone (I), obtaining the modified frequency percentage (% MF) from the formula given by Dravnieks (Dravnieks, 1985):

$$\%MF = \sqrt{\%F \; x \; \%I}$$

where F (%) is the detection frequency of an aromatic stimulus expressed as a percentage and I (%) is the average intensity expressed as percentage of the maximum intensity. For the sake of simplicity, those odorants not reaching a maximum GC-O score of 30% MF in any of the studied samples were eliminated and considered as noise.

A two-way analysis of variance (ANOVA) in which sample was the fix factor and judges random factor was performed on the intensity scores of each of the olfactory areas for assessing their discrimination ability. Further Fischer´s post-hoc pairwise comparisons (95%) were carried out for significant effects.

2.7. Quantitative analysis of aroma precursors and volatile odorants

Both the aminoacids and the cysteinylated and glutathionylated precursors of the Verdejo and Tempranillo musts from the 5 different terroirs from 2015 and 2016 were quantified. In addition, the aromas released during the microfermentations with the 2 selected yeasts co-inoculated with *Saccharomyces cerevisia* for each variety were also quantified.

2.7.1. Amino acids quantification

The amino acids alanine (ala), arginine (arg), asparagine (asn), aspartic acid (asp), glycine (gly), glutamic acid (glu), glutamine (gln), cysteine (cys), histidine (his), isoleucine (ile), leucine (leu), lysine (lys), methionine (met), ornithine (orn), phenylalanine (phe), proline (pro), serine (ser), threonine (thr), tyrosine (tyr), valine (val) and γ-aminobutyric acid (GABA) were determined by HPLC with fluorescence detector according to the method reported by Hernández-Orte et al., (Hernández-Orte, Ibarz, Cacho, & Ferreira, 2003) based on the derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). The HPLC used were a Waters Alliance system consisting of a 2695 separation model, a

thermostat-controlled column oven (Waters, TCM), a system interface module (Waters) and a programmable fluorescence detector (model 1046 A, HP). A Maxima 820 Chromatography Manager was connected to the system for data acquisition and management. Separation was carried out using a 20 x 4.6 mm sentry guard column connected to a 250 x 4.6 mm column (Luna C18 bonded silica) from Analytical Phenomenex (Torrance, CA, USA). Detection was carried out by fluorescence with excitation at 250 nm and emission at 395 nm. Injection volume: 20 µL. A quaternary gradient system was used. To obtain the concentration data, the corresponding compound peak areas relative to a selected internal standard (α-ABA, α-aminobutyric acid) were calculated and calibration curves were used.

2.7.2. Cysteinylated and glutathionylated precursors

The precursors CYSMH, CYSMP, GLUMH and GLUMP were analyzed following the procedure validated by Concejero et al., (Concejero, Peña-Gallego, Fernández-Zurbano, Hernández-Orte, & Ferreira, 2014). For this purpose, 5 mL of must were centrifuged and the supernatant was filtered with two tandem filters of 0.45 µm and 0.20 µm.

The analyses were performed on a liquid chromatograph Shimadzu Nexera 30 CE (Shimadzu Corporation, Japan) coupled to a mass spectrometer QTRAP AB Sciex 3200 (AB SCIEX, Massachusetts, USA), consisting of a triple quadrupole with an electrospray as ionization source (ESI Turbo V™ Source).

The ultrahigh liquid performance chromatography (UHPLC) conditions were: the column was ACQUITY UPLC BEH C18, 130 °A, 1.7 µm particle diameter, 2.1 mm × 100 mm, from Waters (Ireland) ACQUITY UPLC a filter in-line filter column from Waters (Ireland). The solvents were: mobile phase A 0.1% formic acid in water and mobile phase B 0.1% formic acid in acetonitrile with a flow of 0.62 mL/min. The gradient profile started from

5.0% B, increased to 7.0% B in 1.94 min, maintained 0.97 min and then increased to 42% B in 1.59 min. After, it increased to 90% B in 0.2 min and maintained 1 min and finally returned to initial conditions 5.0%.

2.7.3. Major compounds

Quantitative analysis of the major compounds was carried out using the method proposed and validated by Ortega et al., (Ortega, López, Cacho, & Ferreira, 2001), based on a liquid-liquid microextraction with dichloromethane (DCM). In a screw-crapped centrifuge tube were prepared 4.1 g of $(NH_4)_2SO_4$, 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 were agitated horizontally for 90 min and afterwards centrifuged for 10 min at 2500 rpm. The aqueous phase formed was discarded and the organic phase collected with syringe. The organic phase was further analyzed by Gas Chromatography with Flame Ionization Detection (GC-FID). Two microliters of sample were injected at 250 ºC with a split ratio of 1/20. The column was kept initially at 40 ºC for 5 min and then the temperature starts increasing until 120 ºC at 4 ºC/min, then at 2 ºC/min until 112 ºC, then at 3 ºC/min until 125 ºC and hold for 5 min. Temperature increases until 160 ºC at 3 ºC/min and finally until 200 ºC at 6 ºC/min and hold for 30 min. The H_2 flow was 2.2 mL/min. The internal standards were 2-butanol, 4-methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanone and 2-octanol.

2.7.4. Minor compounds

This analysis was carried out using the method proposed and validated by López, et al., (López et al., 2002) with the following modifications: Standard SPE cartridges (1 mL total volume) filled with 65 mg of LiChrolut EN resins were placed in the vacuum manifold extraction system (Varian Sample preparation products) and the sorbent was conditioned

by rinsing the cartridges with 2 mL of dichloromethane, 2 mL of methanol and, finally, with 2 mL of a water-ethanol mixture (12%, v/v). The cartridges were then loaded with 15 mL of wine sample and 100 µL of ethanol solution of internal standards (2-octanol, 3-octanone and 3,4-dimethylphenol). This mixture was passed through the SPE cartridges (2 mL/min), followed by a washing step using 1.5 mL of a 30% water-methanol (v/v), 1% $NaHCO_3$ (w/v) solution. The resins were then dried by letting air pass through them (negative pressure of 0.6 bar, 10 min). Analytes were recovered in a 2 mL vial by elution with 0.6 mL of dichlromethane containing 5% methanol (v/v). The extract was then analyzed by GC with ion trap MS detection (GC-450 gas chromatograph fitted to a Varian Saturn 2200 ion trap MS). The column was DB-WAX from J &W Scientific (Folsom, CA, USA), 60 m × 0.25 mm i.d., with 0.25 µm film thickness. The column initial temperature was 55 °C for 0.40 min, heated to 300 at 200 °C/min remaining at that temperature for 50 min, then decreased until 200 ºC at 200 ºC/min and hold at this temperature. The ion source was operated in NCI mode using methane at 3 bar as reagent gas. The temperature of the ion source was 220 °C, and the interface was kept at 270 °C. The analytes and internal standards ions are acquired in the single ion monitoring (SIM) mode from minute 5.5 to minute 17 at 0.18 s/point. The carrier gas was He at 1.5 mL/min.

2.7.5. Polyfunctional mercaptans

The analysis of 4-methyl-4-mercapto-2-pentanone (4MMP), 3-mercaptohexyl acetate (3MHA) and 3-mercaptohexanol (3MH) in the samples was performed according to a previously validated method (Mateo-Vivaracho, Zapata, Cacho, & Ferreira, 2010). For this purpose, in a 24 mL screw-capped vial, 23 mL of wine were added with 0.2 g of EDTA (5 g/L) and 0.6 g of L-cysteine hydrochloride hydrate (0.1 M) and it was stirred for 2 min. After this, the wine was transferred to a 20 mL volumetric flask, and 15 µL of an ethanolic solution containing 1400 µg/L of 2-phenylethanethiol as internal standard was added. This

volume was transferred to a 24 mL screw-capped vial to which 0.2 g of O-methylhydroxylamine was added, the mixture was stirred for 15 s, and the vial was incubated at 55 °C for 45 min. Six milliliters of this incubated sample were then loaded onto a 50 mg BondElut-ENV SPE cartridge (previously conditioned with 1 mL of dichloromethane, 1 mL of methanol, and 1 mL of water). Some wine major volatiles were removed by rinsing with 4 mL of a 40% methanol/water solution 0.2 M in phosphate buffer at pH 7.7 and, after this, with 1 mL of water. A second internal standard was added to the cartridge; 20 μL of an ethanolic solution containing 150 μg/L of 4-methoxy-α-toluenethiol and 200 μL of water were mixed and loaded onto the cartridge. Mercaptans retained in the cartridge were directly derivatized by passing first 1 mL of an aqueous solution of DBU (6.7%) and later 50 μL of a 2000 mg/L solution of PFBBr in hexane, and letting the cartridge imbibe with the reagent for 20 min at room temperature (25 °C). Excess of reagent was removed by adding 100 μL of a 2000 mg/L solution of mercaptoglycerol in 6.7% DBU aqueous solution, and letting the cartridge react again for 20 min at room temperature. The cartridge is then rinsed with 4 mL of a 40% methanol/water solution 0.2 M in H_3PO_4 and with 1 mL of water. Derivatized analytes were finally eluted with 600 μL of a solvent mixture (hexane 25% in diethyl ether), and then 10 μL of the chromatographic internal standard solution (OFN 22.5 μg/L in hexane) was added to the extract. The eluate is finally washed with five 1 mL volumes of brine (200 g/L NaCl water solution), transferred to a 2 mL vial, and spiked with a small amount of anhydrous sodium sulfate. Four microliters of this sample was directly injected in cold splitless mode into the Gas chromatography coupled to mass spectrometry with negative chemical ionization (GC–MS-NCI) system. The apparatus was a Shimadzu QP-2010Plus gas chromatograph with a quadrupole mass spectrometric detection system. Four microliters of extract was injected in a split/splitless liner packed with silanized glass wool in a Shimadzu Programmed Temperature Vaporizing

(PTV) injector. The initial temperature of the injector was 65 °C, and after 25 s, it was heated at 16 °C/s to 260 °C, remaining at this temperature until the end of the analysis. The carrier gas was He, flowing through the column during the splitless time (4.15 min) at 2.69 mL/min and then, when the split valve was opened, the flow was fixed at 1.44 mL/min. The column was a Factor Four capillary column DB-5 from J &W Scientific, 20 m × 0.18 mm i.d., with 0.18 μm film thickness. The column initial temperature was 40 °C for 4.15 min, heated to 140 at 25 °C/min, then to 180 at 15 °C/min, then to 210 at 30 °C/min, and finally to 280 at 250 °C/min, remaining at that temperature for 10 min. The ion source was operated in NCI mode using methane at 3 bar as reagent gas. The temperature of the ion source was 220 °C, and the interface was kept at 270 °C. The analytes and internal standards ions are acquired in the single ion monitoring (SIM) mode from minute 5.5 to minute 17 at 0.18 s/point.

2.7.6. Statistical analysis

In order to establish the significant differences among samples obtained with or without co-inoculation for each volatile compound, analysis of variance (ANOVA) were carried out. Compounds in bold express significant differences (significance level of 95%). These analyses were performed using SPSS (SPSS Inc., Chicago, IL) for Windows, version 19.

3. RESULTS AND DISCUSSION

A total of 100 yeasts (50 for white and 50 for red musts) were studied: Forty-eight non-commercial *non-Saccharomyces* yeasts and two *saccharomyces* yeasts as reference were used for Verdejo and Tempranillo musts fermentation.

3.1. Selection of *non-Saccharomyces* yeasts by sensory analyses

In order to select samples with higher aroma quality, wine samples were separately submitted to a categorization task. Then, these selected samples were further submitted to a second sensory analysis (flash profiling) in order to get a deeper description of the aroma.

3.1.1. Pre-selection of high quality yeasts by Categorization task (CT)

Panel control. Control samples (for whites: HQ_W1/HQ_W2 and LQ_W1/LQ_W2; for reds: HQ_R1/HQ_R2 and LQ_R1/LQ_R2) were included in the categorization task aimed at (i) evaluating panel reproducibility and (ii) covering a relatively wide range of aroma quality for evaluating panel discrimination ability. Concerning reproducibility, in both cases, duplicated samples (for whites: HQ_W1/HQ_W2 and LQ_W1/LQ_W2; for reds: HQ_R1/HQ_R2 and LQ_R1/LQ_R2) presented in different sessions were projected close together in the maps (Figures I.2 and I.3), which suggests that the panel was globally reproducible. With regard to discrimination ability of the panel, LQ_W1/LQ_W2 for white and LQ_R1/LQ_R2 for red sample sets were wines spiked with 70 mg/L of acetaldehyde to decrease aroma quality, while samples HQ_W1/HQ_W2 and HQ_R1/HQ_R2 were commercial wines in absence of defaults. For white wines, samples LQ_W1/LQ_W2 were included in the low/very low quality category, while HQ_W1/HQ_W2 in the high/very high quality group, which would demonstrate the discrimination ability in terms of aroma quality of the panel of experts. For red wines, both pairs of samples (LQ_R1/LQ_R2 and

HQ_R1/HQ_R2) were mainly classified in the average quality group, but they were differently perceived in terms of quality as HQ_R1/HQ_R2 were included by 47% of experts in the highest quality category, while LQ_R1/LQ_R2 by 23% of participants. Thus, even if control samples did not show important quality differences, the discrimination ability of the panel can be confirmed as there were samples such as R39 (included by 100% of the panel in the very low/low quality category) and R20_sacch (included by 93% of the panel in the high/very high quality category) which were clearly classified in different quality categories (Figure I.3). This suggests that the panel of experts was able to classify in different quality categories both white and red wines with different aroma quality, which confirmed the discrimination ability of the panel for both sample sets.

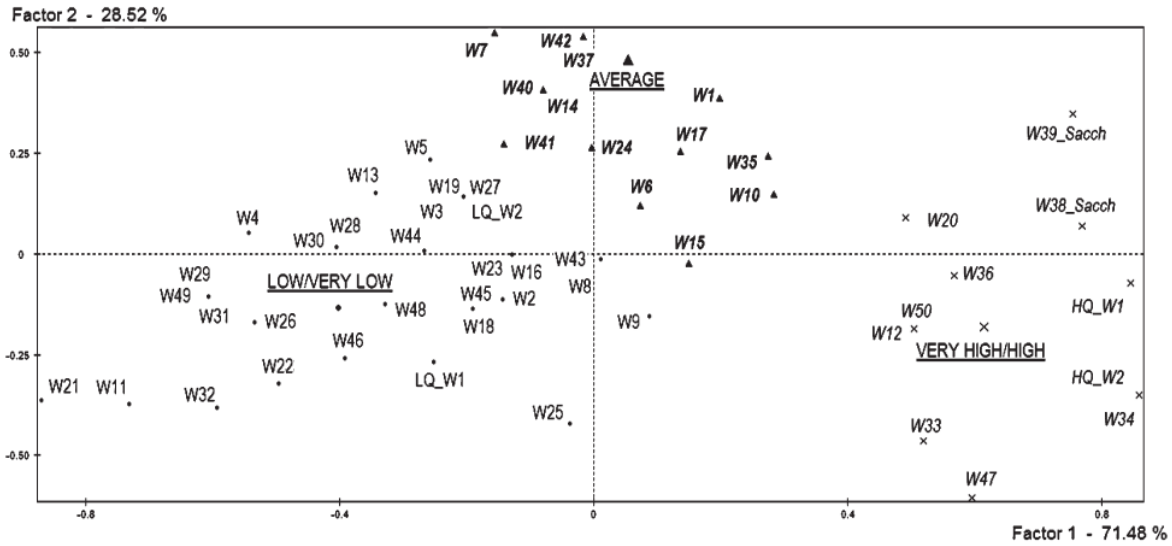

Figure I.2. Projection of the three quality categories (low/very low, average and very high/high) and 54 white samples on the bi-dimensional CA map yielded from the categorisation task based on orthonasal aroma quality perception of a panel of experts. Cluster 1: low/very low quality represented by a dot; Cluster 2: average quality represented by a triangle; and Cluster 3: very high/high quality represented by a cross.

Categorization task. In both sample sets (white and red wines), the first dimensions of the CA maps (Figures I.2 and I.3), which represent most variability, could be interpreted as the quality perceived by experts. Wines mostly included in the highest quality category (very high/high) are projected on the right part of the plot and just in the opposite side are samples

categorized in the lowest quality group (very low/low). In the middle of the plot are samples belonging to the "average" quality category. According to hierarchical cluster analysis (HCA) calculated on all the CA factors, 54% of white wine samples belonged to the lowest quality category, 26% to "average" and 20% were categorized in the highest quality group (W20, W50, W12, W33, W36, W47, W39_sacch, W38_sacch, W34 and two control samples: HQ_W1, HQ_W2) as it can be observed in Figure I.2. For red wines (Figure I.3), less number of samples than for white wines was included in the lowest quality group (30% for red vs 54% for white wines). Most red samples were included in the average quality cluster (57%), while only 13% of samples formed part of the very high/high quality category (R45, R24, R22, R27, R47, R19_sacch, R20_sacch).

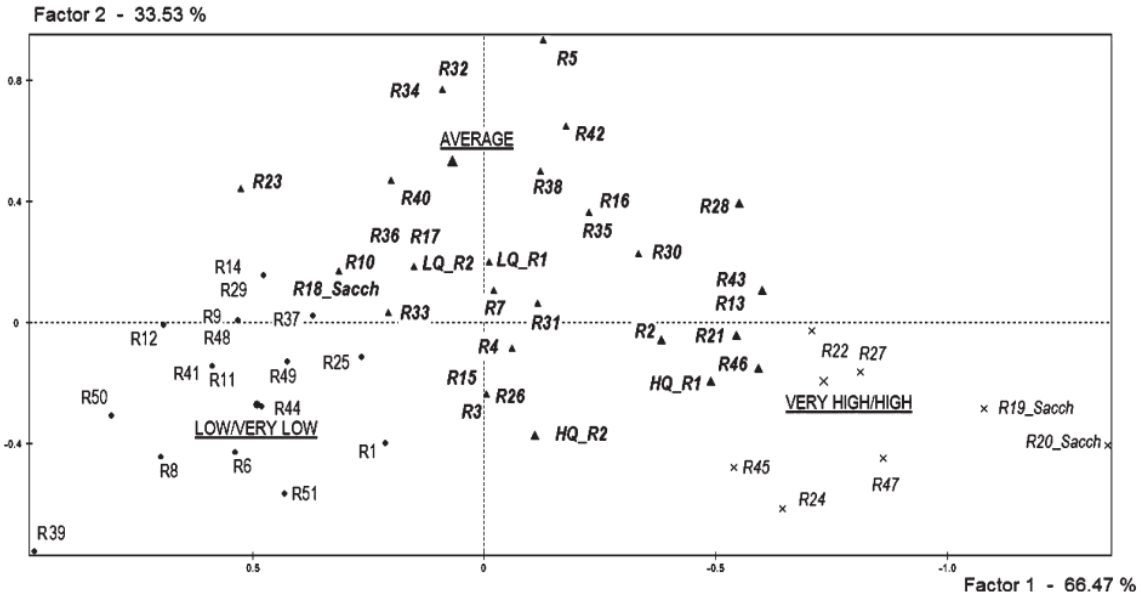

Figure I.3. Projection of the three quality categories (low/very low, average and very high/high) and 55 red samples on the bi-dimensional CA map yielded from the categorisation task based on orthonasal aroma quality perception of a panel of experts. Cluster 1: low/very low quality represented by a dot; Cluster 2: average quality represented by a triangle; and Cluster 3: very high/high quality represented by a cross.

It is worth mentioning, that for white wines, both reference samples fermented with *S. cerevisiae* (W39_sacch, W38_sacch) were mainly included in the highest quality category. Similarly, for red wines, two out of the three samples (R19_sacch, R20_sacch) inoculated with *S. cerevisiae* formed part of the high/very high quality group.

Attributes. After categorization task, participants were instructed to cite a maximum of three terms describing samples belonging to each of the quality categories, which allowed having a raw association of quality categories and aroma descriptors. Results showed that most cited (> 20% of the panel) attributes for the highest quality category of white wines were *fruit* (41%), *tropical fruit* (41%) and *floral* (35%) and for red wines, *lactic* and *caramel* (both cited by 33% of experts), followed by *fresh fruit*, *red fruit*, *strawberry*, *banana*, *floral* and *toffee* (all of them cited by 27% of the panel). On the contrary, *dirty aroma* (41%), followed by *sewer* (24%) and *reduction* (24%) were mainly used for characterize white wines within the lowest quality category and *rotten eggs-hydrogen sulfide* (40%), *reduction* (33%) and *sewer* (27%) for red samples. These results confirmed the suggested that the presence of reductive-related aroma were common in low quality exemplars fermented with certain *non-Saccharomyces* yeasts in white and red sample sets.

3.1.2. Descriptive analysis of the high quality selected yeasts by Flash profiling (FP)

Leaving aside control samples used in the previous sensory task, nine white and seven red wines categorized in the highest quality group were further submitted to orthonasal descriptive analysis by means flash profiling with a panel of semi-trained assessors.

Generation of attributes. In the first session, wine experts quickly generated their own list of discriminant attributes (in less than 30 min in all cases) given their familiarity with wine aroma description, citing between 3 and 15 attributes for both white and red wines. For the second session, assessors retained between 3 and 10 attributes in both cases. For a total of 99 and 83 sensory terms, 49 and 33 terms were semantically different for white and red wines, respectively. Among them, 8 for white wines (Table I.2a) and 12 for red wines (Table I.2b) were used by at least three assessors (20% of the panel), being *fruit in syrup*

(53% of assessors), *tropical fruits* (47%), *citrus fruits* (40%), and *banana* (40%) the attributes mostly cited for white samples and *red fruit* (47%), *fruit in syrup* (47%) and *caramel-toffee* (47%) for red samples. Interestingly, the term *fruit in syrup* appeared in both sample sets.

Table I.2. Frequency of citation (expressed as %) of attributes rated by at least 20% of judges in the flash profiling task carried out with the 13 white wine wines and 11 red wines selected in the categorisation task

a. White samples		**b**. Red samples	
Attribute	Frequency of citation (%)	Attribute	Frequency of citation (%)
Fruit in syrup	53	Red fruit	47
Tropical fruits	47	Fruit in syrup	47
Citrus fruits	40	Caramel–toffee	47
Banana	40	Strawberry yogurt	33
Boxtree–vegetal	27	Banana	33
Wet grain–hay	27	Spicy	27
Green–herbal	27	Vegetal–green	27
Apple	20	White fruit	27
		Floral	20
		Tropical fruit	20
		Alcohol	20
		Dried fruit	20

It is interesting to point out that most attributes cited in categorization task were further used in flash profiling, even if lower number of descriptive terms, and less specific, were generated in categorization task than in flash profiling. This difference was especially important for white sample set. Thus, in categorization only three terms were cited by at least 20% of the panel (fruit, tropical fruit and floral), while eight in flash profiling (Table I.2a). Among them, the fruity category, which involved exclusively fruity and tropical fruit in white sample categorization, it was unfolded into five different terms in flash profiling (fruit in syrup, tropical fruit, citrus, banana and apple). In both sample sets, new attributes

appeared in flash profiling, which were not cited in categorization task. This could be attributed to the fact that in flash profiling the sensory space was more specific (only samples included in the highest quality category) than in categorization task, where samples ranging from very low to very high quality were evaluated.

Ranking. It is noteworthy that for the sample set of white wines, participants declared that ranking the samples for all attributes was difficult. However, for the group of red wines (carried out with the same participants and one week later), they stated that the task was easier mainly because they had already develop their own strategy for performing flash profiling. This could illustrate the difficulty of performing this sensory task for the first time. Notwithstanding all participants finished the ranking task in less than 60 minutes for both white and red sample sets, suggesting that it is a feasible task for describing wine samples by semitrained judges.

Figures I.4a and I.5a show the projection of white wine samples on the first and second principal components of the Generalized Procruster Analysis (GPA) maps representing, respectively, 58% and 13% of the original variance for white wines, and 54% and 27% for described red wines. Control samples presented in duplicate (commercial white wines: Marques de Riscal, MR_1/MR_2, and Los Molinos, LMW_1/LMW_2; commercial red wines: Borja, BJ_1/BJ_2, and Los Molinos, LMR_1/LMR_2) are plotted close together in the map, which suggests that the panel can be globally considered as repeatable.

Hierarchical cluster analysis (HCA) calculated on all the GPA dimensions yielded two main groups of white samples: Cluster 1 and Cluster 2. The first component (PC1) opposed both groups (Figure I.4a). Cluster 1 (positive values of PC1) was formed by the four commercial wines (MR_1, MR_2, LMW_1, LMW_2), two samples fermented with commercial *S. cerevisiae* yeasts (W38_Sacch, W39_Sacch) and one sample fermented with *non-Saccharomyces* (W20).

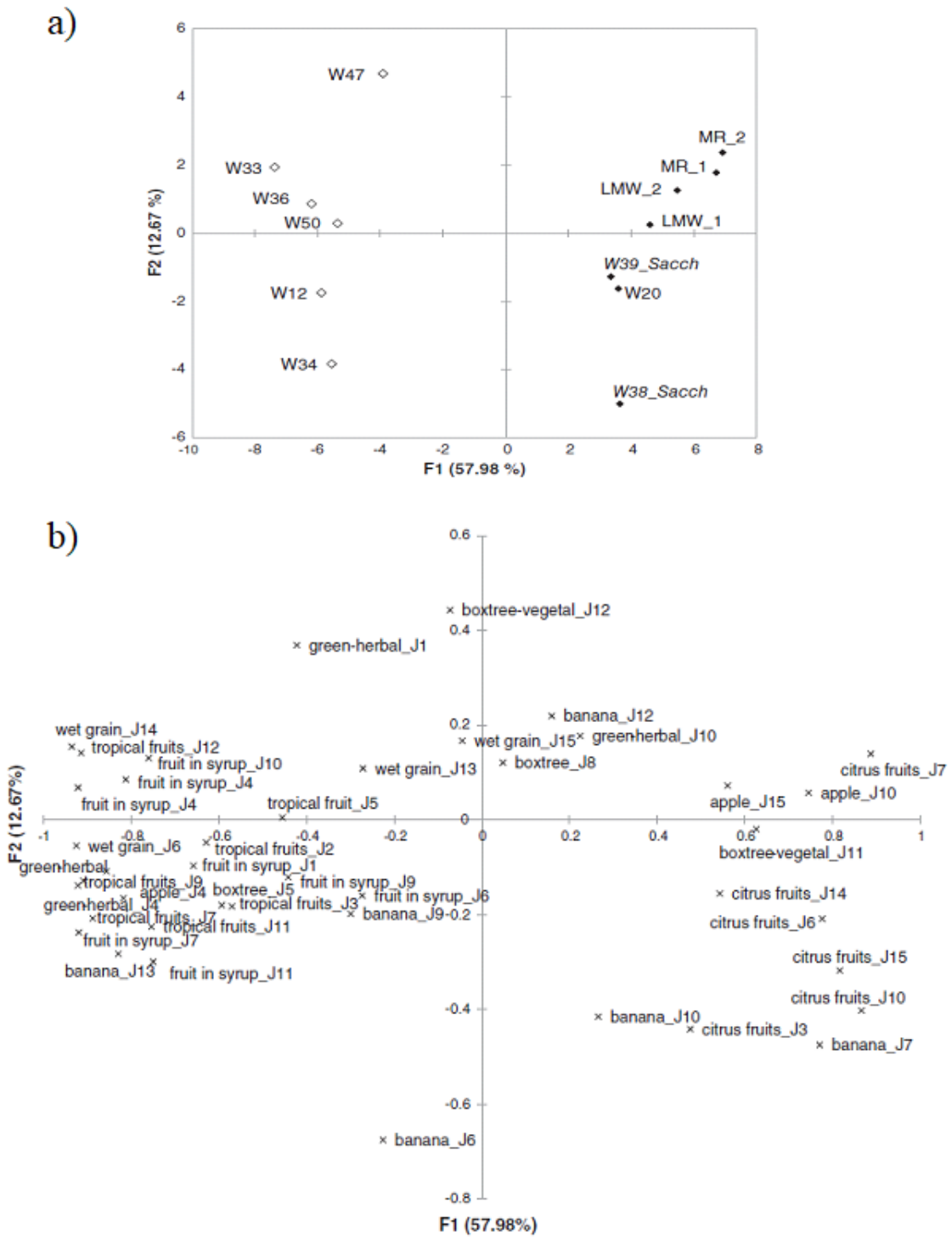

Figure I.4. Projection of (a) white samples (samples belonging to Cluster 1 and Cluster 2 are represented with different symbols), and (b) individual descriptors (given by each of the 15 judges: J1–J15) on the consensus space obtained using generalised procruster analysis (GPA) over the aroma profile derived from flash profiling.

According to Figure I.4b, the panel agreed in mainly attributing a *citrus fruit* aroma character to this group of samples. Cluster 2 (negative values of PC1) was composed of exclusively samples fermented with *non-Saccharomyces* yeasts (W47, W33, W36, W50, W12 and W34). According to Figure I.4b, these samples were consensually characterized by the following terms: *fruit in syrup* and *tropical fruits*. Even if samples belonging to cluster 2 shared these aroma attributes, they were spread along PC2, being sample W47 opposed to W34 and W12. Sample W47 was described with less sweet aromas such as *fruit in syrup*, while with more fresh character such as *box tree-vegetal* and *green-herbal*. Contrary, W34 and W12 would have sweeter nuances related to *fruit in syrup*. Samples W33, W36 and W50, which were plotted close in the map (Figure I.4a) were also characterized by the term *wet grain* as can be observed in Figure I.4b.

On the other hand, the projection of red wine samples on the first two dimensions of the GPA and the projection of most cited terms are shown in Figures I.5a and I.5b, respectively. Cluster analysis yielded three groups of samples. Cluster 1 was formed by the four commercial wines used as control samples: BJ_1/BJ_2 and LM_1/LM_2. Attending to the descriptions shown in Figure I.5b, the panel agreed in describing samples BJ_1/BJ_2 as *spicy*, which seems logical as these samples were aged in oak barrels which could contribute to this nuance. On the contrary, sample LM_1/LM_2 did not seem to be clearly associated to any of the generated descriptors as no term is located close to it (Figure I.5b). This supports the idea that this wine was selected to be a quite neutral sample in terms of aroma properties. The second cluster, formed by two samples fermented with commercial *Saccharomyces cerevisiae* yeasts (R19_sacch and R20_sacch), was plotted on the top part of the map (positive values for PC2) and associated to attributes such as *strawberry yogurt*, *red fruits* and *toffee* (Figure I.5b).

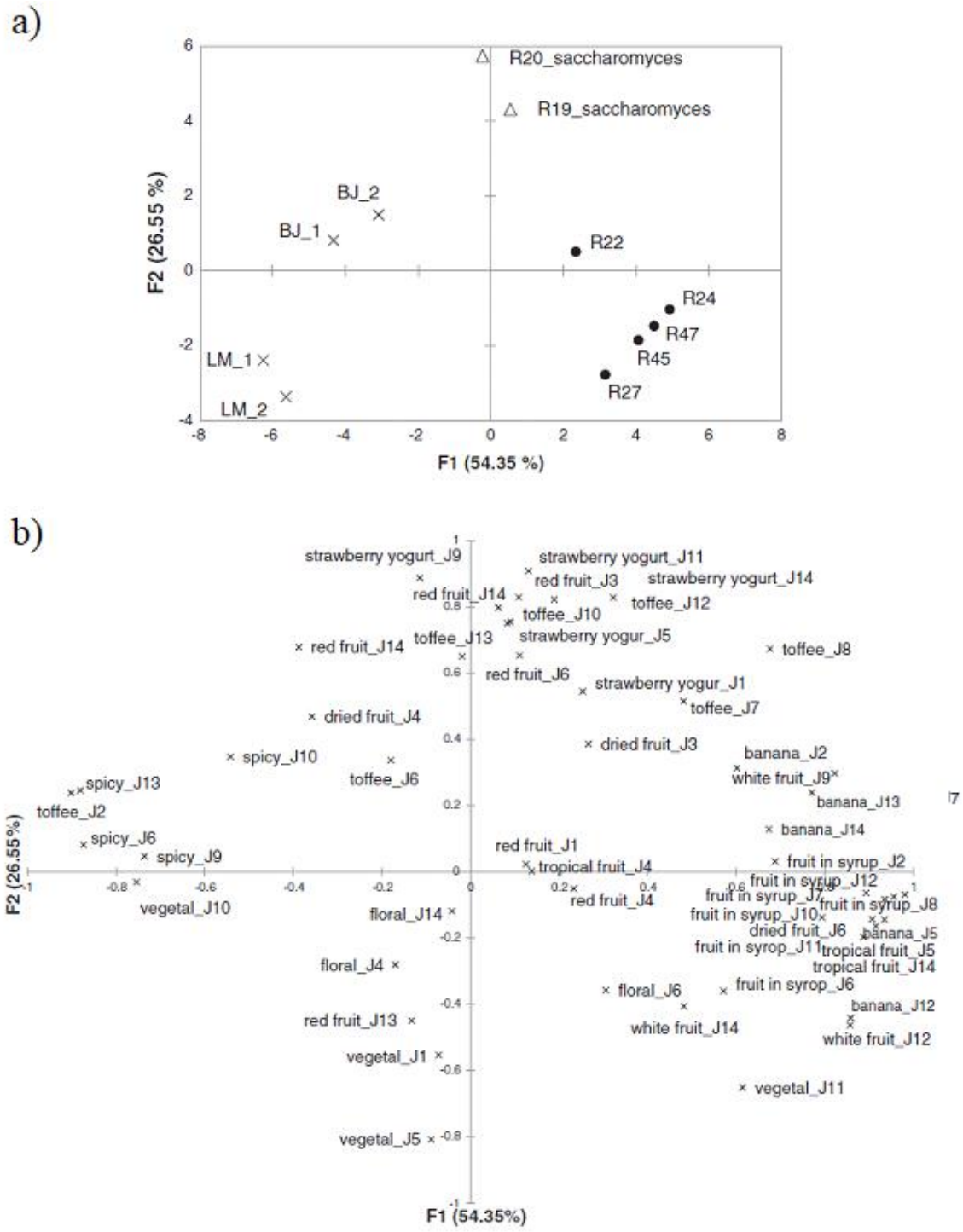

Figure I.5. Projection of (a) red samples (samples belonging to Cluster 1, Cluster 2 and Cluster 3 are represented with different symbols), and (b) individual descriptors (given by each of the 15 judges: J1–J15) on the consensus space obtained using generalised procruster analysis (GPA) over the aroma profile derived from flash profiling.

The third cluster, which is formed by five samples fermented with *non-Saccharomyces* yeasts (R22, R24, R47, R45, R27), presented common aroma nuances related to *fruit in syrup*, *white fruits* and *banana*. Within this cluster of samples, sample R22 acquired the lowest value of PC1, which would suggest that this last sample was less intense in these sweet aromas. Besides, it is the unique sample within the cluster that was located in the positive direction of PC2, suggesting that it was richer in aromas related to *red fruits* and *strawberry yogurt* than the rest of samples belonging to this cluster. All this showed that among the *non-Saccharomyces* samples, R22 would yield the most different aroma profile showing intermediate characteristics between *saccharomyces* and *non-Saccharomyces* samples.

With these results, the yeasts that generated the most different aroma profiles were selected and the samples were further characterized by GC-O. Among white samples, five exemplars were analyzed: W39_sacch (*citrus fruit*), W20 (*citrus fruit*), W47 (*box tree* and *tropical fruits*), W36 (*fruit in syrup*, *tropical fruit* and *wet grain*) and W12 (*fruit in syrup*). The following red samples were submitted to GC-O analyses: R20_sacch (*strawberry yogurt*, *red fruit* and *toffee*), R22 (*red fruit*, *strawberry yogurt*, *fruit in syrup*, *white fruits* and *banana*), R24, R45 and R27 (*fruit in syrup*, *white fruits* and *banana*).

3.2. Identification of key odorants of the selected samples by GC-O analysis

In order to identify the odorants responsible for the distinctive aroma profiles generated by the selected yeasts, the 10 samples (five samples for each variety) were submitted to GC-O. These olfactometric analyses were carried out by a panel of 6 trained judges. A summary of the results from the GC-O analyses of both white and red wines can be seen in Tables I.3 and I.4, respectively.

Table I.3. Odorants identified by GC-O in the five white wines selected. Gas chromatographic data, olfactory description, chemical identity, modified frequency (MF) expressed as %, significance (*P*) and maximum %MF for each compound. Compounds in bold letters present significant differences (P < 0.1) according to two-way ANOVA (samples as fix factor and judges as random factor) and maximum minus minimum values >50% (max-min >50%). Different letters indicate significant differences according to Fischer post-hoc test.

RI	Odour description	Chemical identity	W39	W20	W47	W36	W12	*P*	max-min
978	Butter, fruity, strawberry	diacetyl+ ethyl isobutyrate	24	40	0	7	20	ns	40
1015	Sweet, fruity, solvent	isobutyl acetate	10	55	62	58	53	ns	52
1043	**Fruity, strawberry**	**ethyl butyrate**	**62^{a}**	**67^{a}**	**15^{b}**	**10^{b}**	**24^{b}**	**<0.01**	**57**
1078	Sweet, strawberry	ethyl 3-methylbutyrate	30	0	0	0	0	ns	30
1119	Tabacco, green, herbal	1-hexen-3-one	55	10	10	0	0	ns	55
1136	**Fruity, banana**	**isoamyl acetate**	**83^{a}**	**65^{b}**	**75^{b}**	**10^{c}**	**7^{c}**	**<0.001**	**75**
1218	Solvent	isoamyl alcohol	75	65	66	20	74	ns	55
1248	**Fruity, strawberry**	**ethyl hexanoate**	**64^{b}**	**75^{b}**	**0^{a}**	**7^{a}**	**28^{a}**	**<0.1**	**75**
1285	Fruity, anise	hexyl acetate	7	7	19	0	34	ns	34
1320	Earthy, musty, roasted	2,5-dimethylpirazine	33^{ab}	0^{b}	19^{ab}	40^{b}	26^{ab}	<0.1	40
1333	**Roasted, spicy, bready, barbecue**	**2,6-dimethylpyrazine**	**69^{a}**	**29^{b}**	**0^{b}**	**0^{b}**	**25^{b}**	**<0.01**	**69**
1445	Floral, green, medicinal	*E*-2-octenal	37	27	0	18	17	ns	37
1454	Green, tabacco, earthy	2-isopropyl-3-methoxypyrazine	0^{b}	0^{b}	49^{a}	0^{b}	0^{b}	<0.001	49
1455	Green, earthy, dusty	3,5-dimethyl-2-methoxypyrazine*	0^{b}	0^{b}	0^{b}	43^{a}	0^{b}	<0.01	43
1456	**Roasted, coffee**	**2-furfurylthiol**	**71^{a}**	**7^{b}**	**0^{b}**	**0^{b}**	**37^{b}**	**<0.001**	**71**
1559	Wet cardboard, dusty	2-methylpropanoic acid	18^{b}	17^{b}	0^{b}	47^{a}	0^{b}	<0.1	47
1658	Burnt fur, roasted	2-acetylpyrazine	48	44	50	54	57	ns	13
1741	**Tropical, citrus, grapefruit**	**3-mercaptohexyl acetate**	**71^{a}**	**48^{b}**	**81^{a}**	**76^{a}**	**25^{b}**	**<0.05**	**57**
1753	Spicy, saffron	n.i. 1753	8	0	0	0	31	0.333	31
1842	**Sweet, cooked apple**	**β-damascenone**	**12**	**33**	**65**	**38**	**64**	**<0.1**	**53**
1847	Green, fruity, sulfury	3-mercaptohexanol	22	0	33	0	45	0.265	45
1881	Spicy, sweet, medicinal, smoke	guaiacol	7	10	25	59	24	0.166	42
1933	Floral, roses	β-phenylethanol	24	30	10	10	30	0.252	20
2025	Metalic, green, caustic	n.i. 2025	0	0	0	35	0	0.118	35
2055	Caramel, sweet, strawberry	furaneol	10	7	29	0	37	0.353	37
2217	Spicy, clove, curry	sotolon	47	40	14	58	0	0.146	58

RI Retention index on polar capillary column (DB-WAX)
n.i. Not identified (compound did not produce any clear signal in the mass spectrometer)

As can be observed in Table I.3, twenty-six odorants for white wines have been identified. Among these, seven compounds presented %MF significantly different (P < 0.05) and four compounds were close to significance, showing a trend (P < 0.1). The difference between the maximum MF and the minimum (max-min) can be taken as a criterion for

differentiability. Compounds reaching values above 50% in this parameter and presenting significant ($P < 0.05$) or close to significance ($P < 0.1$) differences among the studied wines are marked in bold letters in the corresponding column of Table I.3. As can be seen in the table, seven compounds: three fruity esters (ethyl butyrate, isoamyl acetate and ethyl hexanoate), one pyrazine (2,6-dimethylpyrazine), two polyfunctional mercaptans (2-furfurylthiol and 3-mercaptohexyl acetate) and one norisoprenoid (β-damascenone) presented significant ($P < 0.1$) and values above 50%. The samples W39 and W20 presented similar MF for the three ethyl esters and the pyrazine, which is consistent with the results observed in the sensory analysis (Figure I.4a) in which these samples were plotted close together in the plot.

From these results, two-dimensional PCA plot calculated with the average %MF (of scores given by the 6 sniffers) for each sample and compound that presented significant differences ($P < 0.1$) and the difference between maximum MF and minimum was above 50% is shown in Figure I.6.

As can be observed in Figure I.6, data derived from GC-O were able to differentiate samples (similar projection of samples) as did sensory profiling (Figure I.4a). The first PC of Figure I.6, which explained almost 60% of variability, confronted samples W39_sacch and W20 (positive values on PC1) from samples W47, W36 and W12 (negative values on PC1). The first group of samples (W39 and W20), which were mainly characterized with fresh fruity aroma (citrus fruit) according to flash profiling (Figure I.4b), were richer in two linear fruity-like esters (ethyl butyrate and hexanoate), 2,6-dimethylpyrazine (described with terms such as roasted, spicy, bready and barbecue by the panel of sniffers) and 2-furfurylthiol (roasted/coffee-like odor) (Figure I.6). It is difficult to explain the citrus character of these samples by the presence of exclusively these compounds, which would indicate that more complex sensory interactions are involved in the formation of such fresh

character. The sweet character (such as fruit in syrup) attributed to samples plotted on the left part of Figure I.6 (W47, W36, W12) could be explained by the presence of β-damascenone. This norisoprenoid has been demonstrated to be involved in the formation of the sweet-fruity aroma (and contrary to fresh aroma) of wines (San Juan, Ferreira, Cacho, & Escudero, 2011). The second PC, explaining 22% of variability, was mainly driven by 3-mercaptohexyl acetate (3MHA) (Figure I.6), which could explain the fact that sample W47 (higher value on PC2) was described with a more fresh character (boxtree-vegetal or green-herbal) than samples W36 and especially W12. This volatile thiol was already demonstrated to be responsible of the tropical fruit and box tree character (Mateo-Vivaracho et al., 2010) of white and rosé wines at concentrations above 50 ng/L. In line with these results, differences in concentration of 3MHA have been already attributed to different yeasts (Masneuf-Pomarede, Mansour, Murat, Tominaga, & Dubourdieu, 2006).

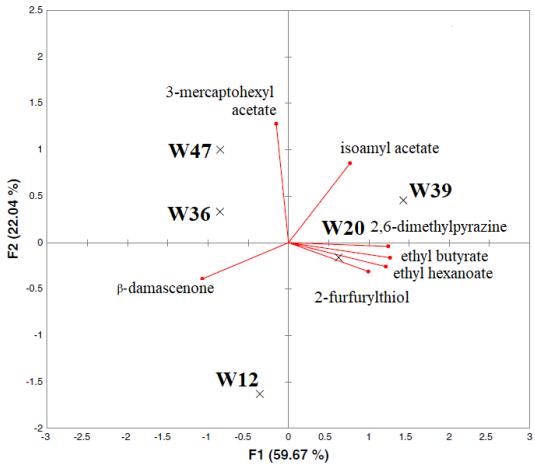

Figure I.6. Projection of selected wine samples and compounds derived from GC-O analysis.

In the case of red samples, thirty-one odorants have been identified. Eleven compounds presented %MF significantly different ($P < 0.05$) and three compounds were close to significance, showing a trend ($P < 0.1$). Six compounds reached values above 50% and presented significant ($P < 0.1$) differences among the studied wines as can be seen in Table I.4: ethyl butyrate, ethyl dihydrocinnamate, 2-phenylethyl acetate, isoamyl acetate, isobutanol, and one non-identified compound: n.i. 1458. Although diacetyl did not show

significant ($P < 0.05$) differences, the presence of high MF of this compound in R20 and R22 samples could be one of the responsible of the strawberry-yogurt notes found in these samples in Figure I.5b.

Table I.4. Odorants identified by GC-O in the five red wines selected. Gas chromatographic data, olfactory description, chemical identity, modified frequency (MF) expressed as %, significance (*P*) and maximum %MF for each compound. Compounds in bold letters present significant differences ($P < 0.1$) according to two-way ANOVA (samples as fix factor and judges as random factor). Different letters indicate significant differences according to Fischer post-hoc test.

RI	Odour description	Chemical identity	R20	R22	R24	R45	R27	*P*	max
978	Butter, fruity, strawberry	diacetyl+ethyl isobutyrate	40	74	14	25	10	0.175	40
1017	Sweet, fruity, solvent	isobutyl acetate	52	25	54	29	56	0.510	54
1043	**Fruity, strawberry**	**ethyl butyrate**	**65^{ab}**	**76^{a}**	**23^{bc}**	**0^{c}**	**31^{bc}**	**<0.05**	**76**
1064	Fruity, anise, strawberry	ethyl 2-methylbutyrate	14	14	0	14	40	0.375	40
1112	**Solvent**	**isobutanol**	**13^{b}**	**13^{b}**	**0^{b}**	**65^{a}**	**47^{a}**	**<0.01**	**65**
1134	**Banana**	**isoamyl acetate**	**83^{a}**	**18^{b}**	**80^{a}**	**91^{a}**	**87^{a}**	**<0.05**	**91**
1219	Solvent	isoamyl alcohol	75	84	68	41	64	0.797	84
1248	Fruity, strawberry	ethyl hexanoate	65	52	32	0	29	0.249	65
1306	Floral, green, medicinal	*E*-2-octenal	0^{b}	0^{b}	43^{a}	14^{ab}	0^{b}	<0.05	43
1309	Roasted, barbecue	2-methyl-3-furanthiol	0^{b}	43^{a}	0^{b}	0^{b}	0^{b}	<0.01	43
1319	Mushroom, solvent	n.i.1319	0	59	32	35	50	0.287	59
1453	Earthy, green pepper	3-isopropyl-2-methoxypyrazine	14	68	53	18	71	0.126	71
1458	**Meaty, cardboard**	**n.i.1458**	**53^{a}**	**0^{b}**	**0^{b}**	**0^{b}**	**0^{b}**	**<0.01**	**53**
1470	Nut, cardboard, artichoke, earth	n.i.1470	54	10	0	35	0	0.236	54
1516	Floral, soap	decanal	43^{a}	0^{b}	0^{b}	0^{b}	0^{b}	<0.01	43
1560	Green, carboard, rancid	*E-2*-nonenal	35	38	50	38	61	0.729	61
1605	Mouldy, cooked vegetable	n.i.1605	48^{a}	0^{b}	0^{b}	29^{a}	0^{b}	<0.05	48
1610	Grass, green, fresh	acetaldehyde	0^{b}	0^{b}	0^{b}	43^{a}	0^{b}	<0.05	43
1654	Burnt fur, roasted	2-acetylpyrazine	68	29	47	74	35	0.478	74
1832	Rancid, floral, green	*E*,*E*-2,4-decadienal	35	0	59	50	10	0.138	59
1839	**Floral, rose**	**2-phenylethyl acetate**	**35^{ab}**	**0^{b}**	**53^{a}**	**0^{b}**	**68^{a}**	**<0.05**	**68**
1847	Sweet, cooked apple	**β-damascenone**	50	19	29	29	14	0.852	50
1880	Spicy, sweet, medicinal, smoke	guaiacol	14	74	74	41	40	0.420	74
1907	**Sweet, floral**	**Ethyl dihydrocinnamate**	**52^{a}**	**0^{b}**	**0^{b}**	**10^{b}**	**0^{b}**	**<0.05**	**52**
1934	Floral, rose	β-phenylethanol	48	23	45	10	14	0.495	48
1964	Caramel, spicy	n.i.1964	14	41	38	0	14	0.269	41
2023	Caramel, solvent, vegetal	n.i. 2023	20	10	40	14	25	0.591	40
2053	Caramel, sweet, strawberry	furaneol	52	37	29	0	20	0.553	52
2155	Sweet, floral	ethyl cinnamate	0	0	46	0	14	<0.1	46
2206	Animal, leather	4-ethylphenol	13	0	47	0	29	<0.1	47
2219	Spicy, clove, curry, burnt	sotolon	14	0	46	20	0	<0.1	46

RI Retention index on polar capillary column (DB-WAX)
n.i. Not identified (compound did not produce any clear signal in the mass spectrometer)

Moreover, two-dimensional PCA plot calculated with the average %MF for each sample and compound that presented significant differences ($P < 0.1$) and the difference between maximum MF and minimum was above 50% is shown in Figure I.7.

For red samples, as for white wines, Figure I.7 shows that data derived from GC-O were able to differentiate samples (similar projection of samples) as did sensory profiling (Figure I.5a). The first PC of Figure I.7, which retained more than 42% of variability, separated samples R20 (*Saccharomyces* yeast) and R22 (*non-Saccharomyces*) from the rest of samples (R27, R45, R24), all of them being the result of fermentation with *non-Saccharomyces* yeasts. In Table I.4 it can be observed that samples R20_*sacch* and R22 presented higher MF values for ethyl butyrate (strawberry aroma), which could explain their distinctive red-fruity aroma described by the panel of experts. These samples were confronted to the sweet aroma (fruit in syrup) characterizing the other three *non-Saccharomyces* samples (R27, R45, R24). These samples presented high MF values for the sweet-like compounds such as isoamyl acetate and 2-phenylethyl acetate (R24 and R27), which could the responsible for their fruit in syrup aroma.

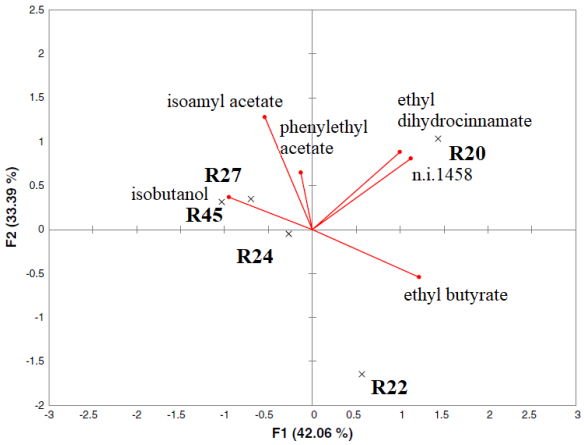

Figure I.7. Projection of selected red samples and compounds derived from GC-O analysis

Due to the results observed both in sensory and GC-O analyses, the samples W20, W36 and W47 in the case of white wines, and R22, R24 and R27 in the case of red wines, were selected for studying the effect of must composition on the *non-Saccharomyces* yeasts.

Besides these samples, the company LEV2050 chose two more yeasts for each variety (W5 and W10 for Verdejo musts and R14 and R30 for Tempranillo).

3.3. Effect of must composition: different terroirs

To study the effect of must composition, the selected yeasts were used for fermenting 5 Verdejo musts and 5 Tempranillo musts from different terroirs. Then, the obtained wines were submitted to a sorting task analysis in order to obtain a cluster of the wines with a similar aromatic profile. Thirty-two wines from each variety (25 wines obtained with the 5 musts fermented with each one of the 5 yeast strains; 5 control wines (C) from the fermentation of the 5 different musts with the W36 yeast for white and the R22 yeast for red musts, and 2 replicate (R) samples) were submitted to sorting task analyses by a panel of wine experts in order to sort them into different groups according to the odor similarities. The panelists were then instructed to cite 1 to 3 terms to describe the samples belonging to each group. The results obtained in the sorting tasks are summarized in Figures I.8 and I.9 for white and red wines, respectively.

The results showed that the replicate wines (W47MV3 and W47MV3_R, and W5MV1 and W5MV1_R for white wines; and R27MT1 and R27MT1_R, and R30MT2 and R30MT2_R for red wines) were projected close to each other in the dendrogram derived from the cluster analysis (Figures I.8 and I.9). In addition, control samples (W36 and W36_C for with wines and R22 and R22_C for red wines) were plotted together in the dendrograma, which suggest that the yeast strains were repeatable, except for the samples R22MT1_C and R22MT2_C that appeared in different clusters. Hence, the participants were able to classify samples with the same aromatic profile within the same group, and in general, the yeast strains generated the same aromatic profile in two separate fermentation processes.

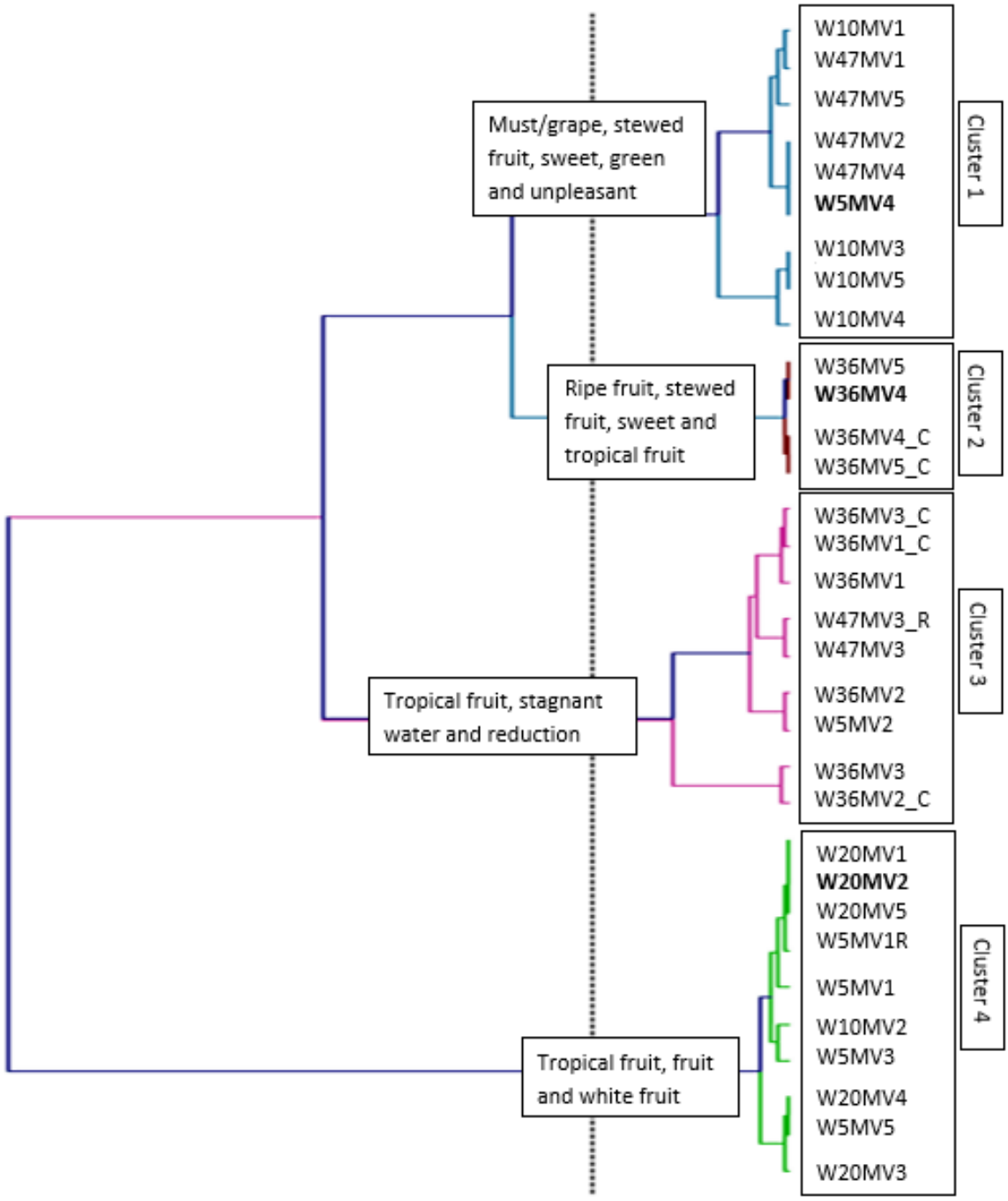

Figure I.8. Dendrogram illustrating the results of the Verdejo wine sorting task. Wines in bold are those selected for further fractionation. Yeasts (W5, W10, W20, W36, W47), Verdejo musts (MV1–MV5), replicate samples (R) and control samples (C).

For white wines, the cluster analysis (Figure I.8) shows that the panelists made 4 groups of samples. The most frequently cited (>20% of the panel) descriptors for samples belonging to cluster 1 were *stewed fruit*, *sweet*, *green, must/grape* and *unpleasant*; for cluster 2, *tropical fruit, stewed fruit*, *ripe fruit* and *sweet;* for cluster 3, *tropical fruit, reduction* and *stagnant water;* and cluster 4, *tropical fruit, white fruit* and *fruit*.

The wines fermented with the yeast **W20** was plotted in the same group, while wines fermented with the yeasts **W47**, **W10**, **W36** and the control (W36_C) were mainly grouped in the same cluster with the exception of some musts. In the case of samples belonging to W36 and W47 that were plotted in cluster 3, their description to reduction could be because of the time passed between the fermentation processes (October 2015) and sensory analysis (June 2016). Thus, W20, W36 and W47 were not as affected by the composition of the must as **W5** that was plotted in 3 different clusters, which indicate that the composition of the must affected W5 and caused the generation of different aromatic profiles.

For red samples, as can be observed in Figure I.9, the panelists also sorted the samples into 4 groups. The samples belonging to cluster 1 were described such as *reduction, rotten eggs*, *sewer* and *fruit*; cluster 2 had descriptors such as *red fruit, lactic caramel* and *sweet* aromas*;* cluster 3 was described as *stewed fruit, white fruit, green, cereal* and *tropical fruit;* and cluster 4 showed descriptors such as *tropical fruit*, *white fruit* and *stewed fruit*.

The wines fermented with the yeasts **R27**, **R30**, **R14** and **R22** were mainly grouped together with the exception of R14MT2 and R22MT1. By contrast, **R24** were plotted in three different groups with different aromatic profiles.

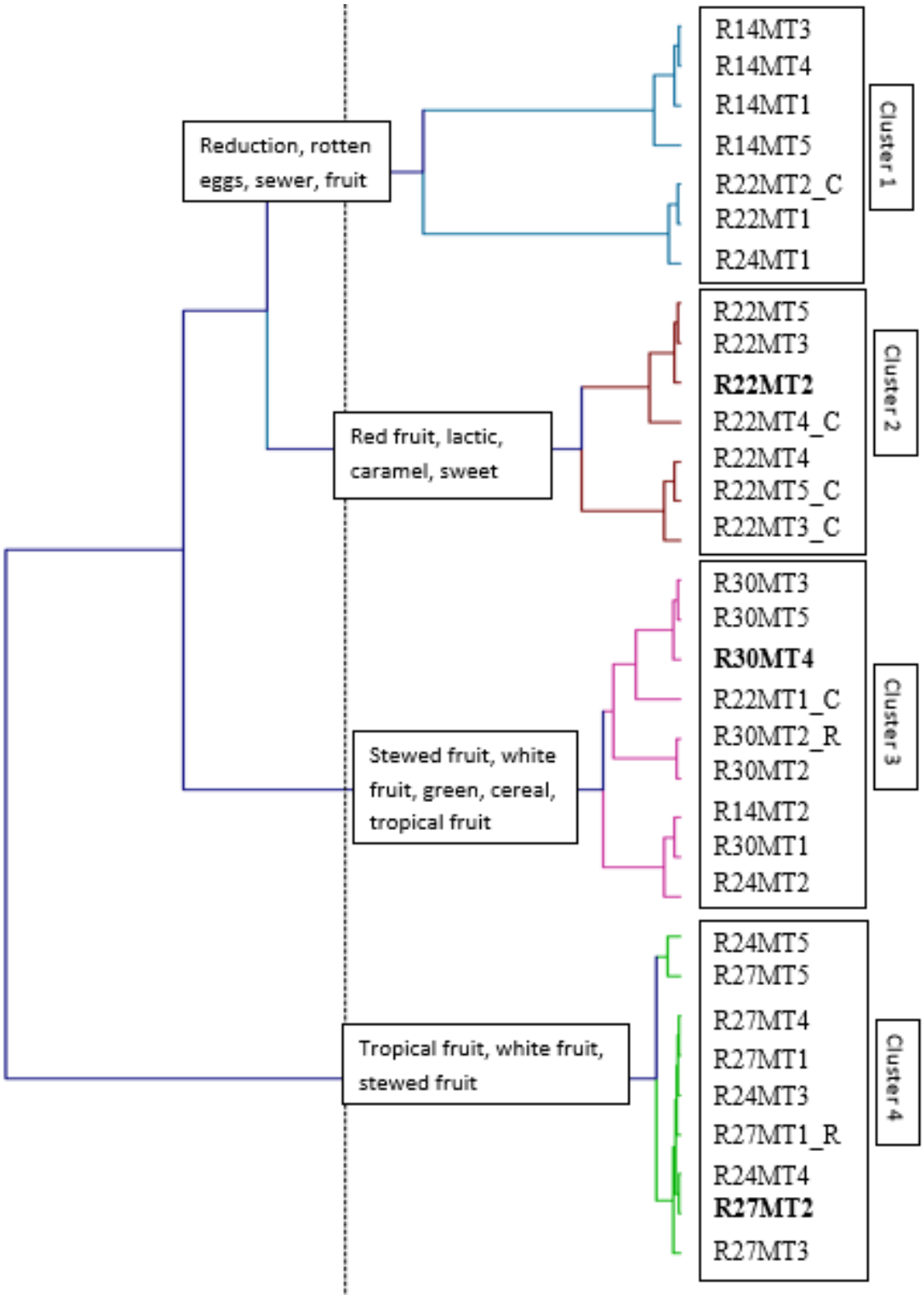

Figure I.9. Dendrogram illustrating the results of the Tempranillo wine sorting task. Wines in bold are those selected for further fractionation. Yeasts (R14, R22, R24, R27, R30), Tempranillo musts (MT1–MT5), replicate samples (R) and control samples (C).

Therefore, *non-Saccharomyces* yeasts (with the exception of W5 in the case of white wines and R24 for red wines) were, in general, able to generate a similar aromatic profile when fermenting must from the same variety but from different terroirs. However, the must that was the exception was not the same for all the yeasts, being for white musts: MV2 for W10, MV3 for W47 and MV4 and MV5 in the case of W36; and for red musts MT1 for R22 and MT2 for R14 and the control (R22_C).

From these results, two different strategies were followed. On the one hand, in order to identify the compounds present in the different musts that could affect the generation of different aromatic profiles by *non-Saccharomyces* yeasts, the cysteinylated and glutathionylated precursors, as well as amino acids from each of the musts were analyzed. On the other hand, to know the key compounds that generate positive aromas in Verdejo and Tempranillo wines, a sample of each cluster were further submitted to HPLC fractionation.

3.3.1. Verdejo musts

The results obtained in the quantitative analyses of the amino acids and cysteinylated and glutathionylated precursors present in the musts from the different terroirs can be observed in Tables I.5 and I.6, respectively.

3.3.1.1. Amino acids

As can be observed in Table I.5, the musts MV4 and MV5 had lower concentration in most of the amino acids, particularly MV4. By contrast, the must MV2 had higher concentration in most of the amino acids with the exception of aspartic acid, asparagine, glutamic acid and γ-aminobutyric acid. Samples obtained with W10 yeast were plotted in the same cluster in the dendrogram (cluster 1 in Figure I.8) with the exception of W10MV2, so the higher

concentration in most of the amino acids observed in MV2 could led to the different aroma profile observed in W10MV2.

Table I.5. Concentration of the amino acids (mg/L) present in the musts from different terroirs from Verdejo variety.

Amino acids	MV1	MV2	MV3	MV4	MV5
Asp	43.8	31.9	34.5	16.0	20.0
Asn	45.0	35.5	39.5	42.1	39.5
Ser	35.4	36.4	35.2	17.3	29.7
Glu	92.4	98.8	85.2	59.5	68.7
His	24.4	26.7	23.9	13.0	19.5
Gly	3.89	5.18	0.74	4.58	< D.L.
Ala	90.5	113	97.7	52.1	80.5
GABA	70.9	80.4	78.3	45.1	81.5
Cys	1.34	5.21	2.63	0.73	2.72
Tyr	10.4	12.4	10.5	5.82	8.62
Val	21.5	24.7	21.5	11.6	17.0
Met	3.85	4.50	3.64	1.87	2.43
Lys	4.41	5.99	4.42	3.88	3.62
Ile	10.7	12.3	10.7	5.75	8.36
Leu	12.5	14.5	12.6	6.59	9.84
Phe	19.1	22.6	21.8	9.87	17.1

<D.L, under detection limit; asp, aspartic acid; asn, asparagine; ser, serine; glu, glutamic acid; his, histidine; gly, glycine; ala, alanine; GABA, γ-amino butyric acid; cys, cysteine; tyr, tyrosine; val, valine; met, methionine; lys, lysine; ile, isoleucine; leu, leucine; phe, phenylalanine.

Moreover, this must had higher concentration in the amino acids related to the aromatic compounds. It is well known that during the alcoholic fermentation, yeasts produce higher alcohols through the conversion of the amino acids present in the medium through the Ehrlich pathway or catabolic formation of the higher alcohols (Bell & Henschke, 2005; Hazelwood, Daran, van Maris, Pronk, & Dinckinson, 2008; Zoecklein, Fugelsang, Gump, & Nury, 1995). Thus, β-phenylethanol, ethanol, isoamyl alcohol, isobutanol and methionol could be formed from their respective amino acids phenylalanine, alanine, leucine, valine and methionine, respectively. Thereafter, through chemical esterification of these alcohols

mediated by the yeasts, esters such as phenylethyl acetate, isoamyl acetate and isobutyl acetate can be formed (Lambrechts & Pretorius, 2000; Maarse, 1991; Rapp & Versini, 1991; Swiegers et al., 2005). Therefore, the fruity character observed in W10MV2 (cluster 4 in Figure I.8) could be due to the higher formation of isoamyl acetate, phenylethyl acetate and isobutyl acetate from their respective amino acids (leucine, phenylalanine and valine, respectively) (Bell & Henschke, 2005; Lambrechts & Pretorius, 2000).

By contrast, when W36 yeast was used to ferment the musts MV1, MV2 and MV3, reduction aromas were observed (cluster 3 in Figure I.8). These aromas could be due to the time passed between the fermentation process and the sensory analysis as was previously mentioned. However, these notes to reduction were not observed when W36 yeast fermented MV4 and MV5. This fact could be due to the lower concentration of methionine observed in MV4 and MV5 musts, and also to the lower amount of cysteine in the case of MV4 must (Table I.5). It is well know that these sulfur amino acids are related with formation of sulfur compounds that form the reduction aromas (De Mora, Eschenbruch, Knowles, & Spedding, 1986; Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006).

3.3.1.2. Cysteinylated and glutathionylated precursors

Regarding the concentration of cysteinylated and glutathionylated precursors present in the different musts (Tables I.6), it can be observed that the precursors of the 4-mercapto-4-methyl-2-pentanone (CYSMP and GLUMP) were under the detection limit.

As can be seen in Table I.6, the must that had higher concentration in CYSMH was MV2, opposite to MV1 that had the lowest concentration. By contrast, in the case of GLUMH, the must with the highest concentration was MV1, and the must with the lowest amount was MV4. Taking into account the sum of 3MH precursors (CYSMH and GLUMH), the must with the lowest amount of 3MH precursors was MV4, while MV1 was the must with

higher concentration. Samples obtained with W5 yeast although were plotted in different clusters in the dendrograma (Figure I.8), were described with tropical fruit notes, except the sample W5MV4. This lack of tropical fruit aromas in the sample obtained with MV4 could be due to the lower concentration in 3MH precursors in this must (Fedrizzi, Pardon, Sefton, Elsey, & Jeffery, 2009; Peyrot des Gachons, Tominaga, & Dubourdieu, 2002; Tominaga, Peyrot des Gachons, & Dubourdieu, 1998) (Table I.6).

Table I.6. Concentration (μg/L) of the cysteinylated and glutathionylated precursors present in the musts from different terroirs from Verdejo variety.

	MV1	**MV2**	**MV3**	**MV4**	**MV5**
CYSMP	< D.L	< D.L	< D.L	< D.L	< D.L
CYSMH	73.8	229	106	95.4	165
GLUMP	< D.L	< D.L	< D.L	< D.L	< D.L
GLUMH	4734	3447	3642	2624	3981
Sum of 3MH precursors	4808	3676	3750	2719	4147

<D.L, under detection limit.

3.3.2. Tempranillo musts

The results obtained in the quantitative analyses of the amino acids and cysteinylated and glutathionylated precursors present in Tempranillo musts can be observed in Tables I.7 and I.8, respectively.

3.3.2.1. Amino acids

As can be seen in Table I.7, MT2 had higher concentration in most of the amino acids. This could be the reason why, in the case of samples obtained with R14 yeast, all samples developed reduction aromas (cluster1 in Figure I.9) except those obtained with MT2 (cluster 3 in Figure I.9) that developed fruit aromas. However, this could not be explained by the presence of lower amount of sulfur amino acids (Table I.7), but for the presence of higher concentration of alanine and phenylalanine that could form higher concentrations of

ethyl esters and phenylethyl acetate (Bell & Henschke, 2005; Lambrechts & Pretorius, 2000), respectively. On the other hand, MT3 was the must that had the lowest concentration in most of the amino acids.

Table I.7. Concentration of the amino acids (mg/L) present in the musts from different terroirs from Tempranillo variety.

Amino acids	**MT1**	**MT2**	**MT3**	**MT4**	**MT5**
Asp	32.7	26.8	7.28	20.4	14.1
Asn	44.9	38.8	41.9	47.1	40.7
Ser	22.1	25.81	8.81	19.4	16.9
Glu	121	123	32.6	84.2	57.6
His	30.4	60.7	6.11	38.7	43.2
Gly	20.6	9.95	< D.L.	2.41	2.22
Ala	98.1	109	45.1	90.4	77.8
GABA	152	232	51.1	116	93.1
Cys	4.66	5.84	2.65	4.96	3.25
Tyr	3.25	6.66	0.58	2.26	3.67
Val	21.8	22.2	4.36	16.7	51.6
Met	0.75	2.07	< D.L	0.31	10.9
Lys	5.19	4.87	2.18	3.58	4.25
Ile	13.0	8.57	2.14	6.88	34.3
Leu	12.6	13.6	2.71	8.95	40.9
Phe	8.63	14.1	3.03	13.0	12.8

<D.L, under detection limit; asp, aspartic acid; asn, asparagine; ser, serine; glu, glutamic acid; his, histidine; gly, glycine; ala, alanine; GABA, γ-amino butyric acid; cys, cysteine; tyr, tyrosine; val, valine; met, methionine; lys, lysine; ile, isoleucine; leu, leucine; phe, phenylalanine

3.3.2.2. Cysteinylated and glutathionylated precursors

The concentration of cysteinylated and glutathionylated precursors present in the different musts can be observed in Table I.8. As can be observed, the concentration of these precursors in Tempranillo musts was lower than those observed in Verdejo musts (Table I.6), and the precursors of the 4-mercapto-4-methyl-2-pentanone (CYSMP and GLUMP) were under the detection limit in both varieties.

In the case of Tempranillo musts (Table I.8), the musts with higher concentration in 3MH precursors were MT5 and MT4, while MT3 was the must with the lowest concentration.

However, unlike that observed in white wines, in the red wines it was not possible to explain the lack of tropical fruit aromas in some samples due to the lower concentration of polyfunctional mercaptans precursors in some musts.

Table I.8. Concentration (μg/L) of the cysteinylated and glutathionylated precursors present in the musts from different terroirs from Tempranillo variety.

	MT1	**MT2**	**MT3**	**MT4**	**MT5**
CYSMP	< D.L	< D.L	< D.L	< D.L	< D.L
CYSMH	72.8	128	69.4	134	73.3
GLUMP	< D.L	< D.L	< D.L	< D.L	< D.L
GLUMH	1335	2322	1007	2900	3037
Sum of 3MH precursors	1408	2450	1077	3034	3110

<D.L., under detection limit.

Therefore, although the different *non-Saccharomyces* yeasts were able, in general, to generate similar aromatic profiles when they fermented different musts (Figures I.8 and I.9), it has been observed that in some cases the composition of the must can influence the aromatic profile. As is the case of *non-Saccharomyces* yeasts W10, W36 and W5, in which it has been seen that:

- The higher concentration of amino acids present in the musts can lead to the formation of fruity aromas by W10.
- The absence of reduction notes in the samples belonging to W36 could be due to a lower concentration of sulfur amino acids.
- The absence of tropical fruit notes in W5MV4 could be due to a lower concentration in the cysteinylated and glutathionylated precursors.

However, in the case of Tempranillo musts, the influence of must composition was not as clear as that observed in the Verdejo musts.

3.4. HPLC fractionation

3.4.1. Selection of samples for fractionation

From the data obtained by sorting tasks (Figures I.8 and I.9), 4 different clusters were observed in both cases. From these clusters, were only considered for further fractionation those whose predominant descriptors were positive, due to the main aim was to determine the key compounds able of generate positive aromas. For white wines (Figure I.8), the cluster 3 was not selected because predominant aromas were negative descriptors, and tropical fruit aroma was the descriptor least intense. As for white wines also for red wines (Figure I.9) cluster 1 was not selected because of its negative descriptors.

Due to the similarity in the aromatic profile of wines within each cluster formed in the sorting task (Figures I.8 for white and I.9 for red wines), the HPLC fractionation was carried out only on 1 sample of each cluster. The criteria followed for the selection of the sample was the presence of higher quotation frequencies in the descriptors that characterized the group (Table I.9).

Thus, for white wines from cluster 4, the selected sample was W20MV2 because of its fresh fruit aromas; from cluster 2, W36MV4 was the selected sample due to its sweeter fruit aromas; and W5MV4 was the selected sample from cluster 1 because it was described with green and unpleasant aromas. For red wines from cluster 2, the selected sample was R22MT2 because of its red fruit aromas; from cluster 4, R27MT2 was the selected sample due to its fruity aromas; and R30MT4 was the selected sample from cluster 10 because it was described with green and cereal aromas.

Table I.9. Descriptors given to each sample from Verdejo and Tempranillo varieties.

Cluster	Sample	Descriptors	Frequency
		White samples	
1	**W5MV4**	**Sweet, stewed fruit, green, unpleasant, must/grape**	**19-25**
1	W10MV3	Must/grape	19
1	W10MV4	Must/grape, sweet, unpleasant	19
1	W10MV5	Sweet, stewed fruit, green	19
1	W47MV1	Stewed fruit, unpleasant, green	19
1	W47MV2	Sweet, stewed fruit, ripe fruit, green	19-25
1	W47MV4	Sweet, stewed fruit, unpleasant	19-25
1	W47MV5	Stewed fruit, green, must/grape	19-31
2	**W36MV4**	**Sweet, stewed fruit, tropical fruit, ripe fruit**	**19-25**
2	W36MV5	Sweet, white fruit, tropical fruit, ripe fruit	19
4	W5MV1	Tropical fruit, white fruit	19-38
4	W5MV3	Tropical fruit	38
4	W5MV5	Tropical fruit, fruit	19-31
4	W10MV2	Stewed fruit, fruit, tropical fruit	19
4	W20MV1	Tropical fruit, fruit, White fruit	19-38
4	**W20MV2**	**Tropical fruit, White fruit, fruit**	**19-50**
4	W20MV3	Tropical fruit, fruit, fresh fruit	19-38
4	W20MV4	Tropical fruit, fruit	19-38
4	W20MV5	Tropical fruit, White fruit	19-38
		Red samples	
2	**R22MT2**	**Red fruit, lactic, caramel, sweet**	**19-38**
2	R22MT3	Caramel, sweet, red fruit, lactic	19-31
2	R22MT4	Caramel, sweet, red fruit, lactic	19
2	R22MT5	Red fruit, lactic, caramel	25-31
3	R30MT1	White fruit, cereal, green	19
3	R30MT2	White fruit, cereal, tropical fruit	19-25
3	R30MT3	Green, cereal, white fruit, stewed fruit	19-31
3	**R30MT4**	**White fruit, green, cereal, stewed fruit, tropical fruit**	**19-31**
3	R30MT1	White fruit, cereal, green	19
3	R30MT2	White fruit, cereal, tropical fruit	19-25
3	R30MT3	Green, cereal, white fruit, stewed fruit	19-31
3	**R30MT4**	**White fruit, green, cereal, stewed fruit, tropical fruit**	**19-31**
3	R30MT5	Green, stewed fruit, white fruit, cereal	19-31
3	R24MT2	Stewed fruit, tropical fruit	19
4	R27MT1	Tropical fruit, White fruit, stewed fruit	19-31
4	**R27MT2**	White fruit, tropical fruit, stewed fruit	31
4	R27MT3	Tropical fruit, white fruit	25-38
4	R27MT4	White fruit, tropical fruit	19
4	R27MT5	White fruit, stewed fruit, tropical fruit	19
4	R24MT3	White fruit, stewed fruit, tropical fruit	19
4	R24MT4	Stewed fruit, tropical fruit	19
4	R24MT5	Tropical fruit, fruit, stewed fruit	19

In bold selected wines

3.4.2. HPLC fractionation of selected samples

Using the fractionation process, the aromatic compounds are able to be separated into several groups depending on their polarity (Ferreira et al., 1999). Hence, in each fraction obtained, the number of aromatic compounds is lower than in a total extraction. This facilitates the identification of the compounds that generate the key aromas.

From each of the wine samples selected, 12 fractions were collected and analyzed in a sensory manner. To that end, 50 µL of each fraction were put in an odor strip and each odor strip was presented to three trained judges. The judges were asked to describe each fraction with 1-5 different attributes. Fractions with similar attributes to the descriptors of wines previously selected by means of the sorting task were chosen as can be observed in Tables I.10 and I.11.

In the case of white wines (Figure I.8), for the samples belonging to the cluster with sweeter fruit aromas (cluster 2), W36MV4 was the selected wine and the W36MV4_9, W36MV4_10 and W36MV4_11 fractions were also taken because of their sweet character (Table I.10). Regarding the samples from the cluster associated with fresh fruit notes (cluster 4), the W20MV2 wine was selected and the W20MV2_9, W20MV2_10 and W20MV2_11 fractions were also chosen for possessing these fresh fruit notes that characterized this wine. In the case of the cluster 1, the selected wine was W5MV4. The main difference between this cluster and the other clusters was that cluster 1 showed green and unpleasant aromas which were also identified in the fractions W5MV4_7 and W5MV4_9 (Table I.10).

Table I.10. Attributes given to each selected white wine and their fractions. Underlined attributes are similar to the descriptors found in the selected wines by means of the sorting task.

		Description
Selected wine	W36MV4	Tropical fruit, stewed fruit, ripe fruit and sweet
Fractions	W36MV4_1	No aroma
	W36MV4_2	No aroma
	W36MV4_3	Flower little intense
	W36MV4_4	No aroma
	W36MV4_5	Sweet little intense
	W36MV4_6	No aroma
	W36MV4_7	Coffee little intense
	W36MV4_8	Flower, roses and straw
	W36MV4_9*	Flower, grapefruit skin, tropical fruit, green, rancid, straw and stewed fruit
	W36MV4_10*	Tropical fruit, banana and stewed fruit
	W36MV4_11*	Fruit, sweet, fried and dirty background
	W36MV4_12	No aroma
Selected wine	W20MV2	Tropical fruit, white fruit and fruit
Fractions	W20MV2_1	No aroma
	W20MV2_2	No aroma
	W20MV2_3	No aroma
	W20MV2_4	No aroma
	W20MV2_5	No aroma
	W20MV2_6	Toast, rancid and sunflower seeds
	W20MV2_7	Rancid, sunflower seeds and sweet
	W20MV2_8	Unpleasant and roses
	W20MV2_9*	Orange skin, dead flower, toast and vinylphenol
	W20MV2_10*	Pear, dirty background, fruit and honey
	W20MV2_11*	Peach, tropical fruit, sweet, glue and dirty background
	W20MV2_12	No aroma
Selected wine	W5MV4	Stewed fruit, sweet, green, must/grape and unpleasant
Fractions	W5MV4_1	No aroma
	W5MV4_2	Paper
	W5MV4_3	Cheese
	W5MV4_4	No aroma
	W5MV4_5	No aroma
	W5MV4_6	No aroma
	W5MV4_7*	Coffee, toast and unpleasant
	W5MV4_8	Roses and floral
	W5MV4_9*	Dead flower, green, toast, grapefruit skin, vinylphenol and dirty
	W5MV4_10	Glue and wax
	W5MV4_11	Acid fruit and solvent
	W5MV4_12	No aroma

*: Selected fractions

Table I.11. Attributes given to each selected red wine and their fractions. Underlined attributes are similar to the descriptors found in the selected wines by means of the sorting task.

		Description
Selected wine	R27MT2	Tropical fruit, white fruit and stewed fruit
Selected fraction	R27MT2_1	No aroma
	R27MT2_2	Sweet little intense
	R27MT2_3	Sweet little intense
	R27MT2_4	No aroma
	R27MT2_5	No aroma
	R27MT2_6	No aroma
	R27MT2_7	Unpleasant little intense
	R27MT2_8*	Tropical fruit, sweet and ester
	R27MT2_9*	Peach, fruit, sweet, banana, white fruit, apple and pear
	R27MT2_10	No aroma
	R27MT2_11	No aroma
	R27MT2_12	No aroma
Selected wine	R30MT4	Stewed fruit, white fruit, green, cereal and tropical fruit
Selected fraction	R30MT4_1	No aroma
	R30MT4_2	Sweet little intense
	R30MT4_3	Strawberry candy
	R30MT4_4	No aroma
	R30MT4_5	No aroma
	R30MT4_6	solvent
	R30MT4_7	Solvent, acetone
	R30MT4_8*	Fruit, toast bread, green, roses, unpleasant, sweet fruit and burned hair
	R30MT4_9*	Flower, fruit, rancid, cereal, tropical fruit, ripe fruit, green, vinylphenol and toast
	R30MT4_10	Nail polish, acetone and fruit little intense
	R30MT4_11	Unpleasant, rotten fruit and nail polish
	R30MT4_12	No aroma
Selected wine	R22MT2	Red fruit, lactic, caramel and sweet
Selected fraction	R22MT2_1	No aroma
	R22MT2_2	No aroma
	R22MT2_3	Green
	R22MT2_4	No aroma
	R22MT2_5*	Sweet, strawberry candy, toast and strawberry
	R22MT2_6	Burned hair, paperboard
	R22MT2_7	Toast little intense and floral
	R22MT2_8	Floral and pollen
	R22MT2_9*	Dead flower, grapefruit skin, green, fresh, strawberry and fruit
	R22MT2_10*	Ripe fruit, fruit in alcohol, aldehyde, caramel and roses
	R22MT2_11*	Sweet fruit, alcohol, fruit and strawberry candy
	R22MT2_12	No aroma

*: Selected fractions.

Regarding the red wines (Figure I.9), the samples described with fruity aromas were found in cluster 4 and the selected wine was R27MT2. The R27MT2_8 and R27MT2_9 fractions were characterized as the most fruity (Table I.11). These fruity aromas were accounted as the main difference with the other groups in Figure I.9. Regarding the cluster 3, the wine selected was R30MT4. The main difference between this cluster and the other clusters was the presence of descriptors such as green and cereal. The fractions which contained these aromas were R30MT4_8 and R30MT4_9. For the samples from the cluster characterized with red fruit and lactic aromas (cluster 2 Figure I.9), the selected wine was R22MT2, and the R22MT2_5, R22MT2_9, R22MT2_10 and R22MT2_11 fractions were taken because they had these kinds of aromas (Table I.11).

3.4.3. Identification of key odorants of the selected samples by GC-O analysis

The selected fractions belonging to wines whose descriptors corresponded to the different groups formed in the sorting task were further characterized by GC-O. The main target of this analysis was to find the key chemical molecules responsible for the distinctive notes between the wines (Ferreira, San Juan, Escudero, Culleré, Fernández-Zurbano, Sáenz-Navajas, & Cacho, 2009). A summary of the results from the GC-O analyses of both white and red wines can be seen in Tables I.12 and I.13, respectively. Twenty-one odorants for white wines and twenty-seven for red wines were identified.

For white wines, the tropical fruit aromas found in the samples W36MV4 and W20MV2 in the sorting task (Figure I.8) were mainly related with 3-mercaptohexyl acetate and 3-mercaptohexanol, with MFs higher than 68% in the GC-O analysis (Table I.12). As some authors have described, 3-mercaptohexyl acetate and 3-mercaptohexanol are responsible for the tropical fruit character (Lee, Chang, Yu, Lai, & Lin, 2013; Mateo-Vivaracho et al.,

2010; Pinu, Edwards, Jouanneau, Kilmartin, Gardner, & Villas-Boas, 2014). The sweeter fruit aromas which were described in the wine W36MV4 (cluster 2 Figure I.8) could be linked with isoamyl acetate (89% of MF), 2-phenylethyl acetate (84%), benzyl acetate (68%), un unknown in a RI 2469 (79%) and β-damascenone (76%), whose main descriptors were banana, floral, honey, sweet and baked apple, respectively.

It is worth noting that β-damascenone and isoamyl acetate generate wines with a sweeter fruit character. San Juan et al., and Ferreira et al., (Ferreira, Sáenz-Navajas, Campo, Herrero, de-la-Fuente-Blanco, & Fernández-Zurbano, 2016; San Juan et al., 2011) demonstrated that β-damascenone is involved in the formation of the sweet-fruity aroma (and contrary to fresh aroma) of wines. Thus, the sweet fruit character attributed to wines plotted in cluster 2 in Figure I.8 could be explained by the presence of these compounds.

On the other hand, the white wine W20MV2 (cluster 4 in Figure I.8) that was characterized by fresh fruit aromas showed high MFs in isobutyl acetate (68%), ethyl hexanoate (71%) and dihydromyrcenol + linalool oxide (74%). Some authors have previously demonstrated that these esters are involved in the formation of the fresh-fruity aroma of wines (Bowen & Reynolds, 2012; Lee et al., 2013).

The odorant responsible for the odor zone at RI = 1497 (grapefruit skin and green aromas) could not be identified by CG-O. This compound is important due to their high MFs in some fractions and their potential to modify the aroma of wine (Table I.12). The identification of these compounds was carried out by dual GC–GC with simultaneous olfactometric and mass spectrometric detections, which made the isolation of this compound possible as well as their further identification by mass spectrometry. For instance, dihydromyrcenol and linalool oxide (RI 1497) appeared together in the GC-O analysis and were linked with the green and fresh fruit character.

Table I.12. Odorants identified by GC-O in the selected fractions (3, 3 and 2 fractions) for the three white wines (Y4MV4, Y1MV1 and Y1MV4). Gas chromatographic data, olfactory description, chemical identity, modified frequency (MF) expressed as %. Compounds in bold are important for the differences between the three wines.

RI		Descriptor	Compound	Cluster 2 Stewed fruit, ripe fruit, tropical fruit			Cluster 4 White fruit, fruit, tropical fruit			Cluster 1 Green, unpleasant	
DB-WAX	DB-5			W36MV4_9	W36MV4_10	W36MV4_11	W20MV1_9	W20MV1_10	W20MV1_11	W5MV4_7	W5MV4_9
1016	823	Solvent, strawberries, sweet	Isobutyl acetate[1]	46	58	46	46	**68**	65	0	20
1134	877	Banana, sweet	Isoamyl acetate[1]	0	**89**	41	0	68	0	0	0
1168	950	Sweet, fruit	Ethyl 3-methylpentanoate[2]	65	35	29	25	20	0	0	0
1219	741	Cheese, solvent, unpleasant	Isoamyl alcohol[1]	0	0	0	65	0	0	0	0
1246	999	Strawberry candy	Ethyl hexanoato[1]	0	0	58	0	0	**71**	0	0
1331	872	Fried, coconut, fruit, sweet, toasted	2-methyl-3-furanthiol[2]	25	50	35	74	0	35	0	74
1497	1050	Grapefruit skin, citric, acid, rancid	Dihydromyrcenol[3]*+ linalool oxide[3]	50	0	20	**74**	29	20	65	**68**
1528	1151	Rancid, sunflower seeds, unpleasant	Z-2-nonenal[1]	71	61	54	58	0	0	46	50
1658	1096	Burning hair	Benzyl mercaptan[1]	0	58	35	58	41	41	65	54
1668	1054	Floral, honey	Phenylacetaldehyde[1]	65	0	0	0	0	0	0	46
1681	837	Cheese	3-methylbutiryc acid[1]	0	0	0	0	0	0	**89**	0
1740	1254	Tropical fruit, fruit	3-mercaptohexyl acetate[1]	**79**	**74**	**71**	54	**84**	**74**	0	61
1751		Sweet, blackberry	Benzyl acetate[1]	68	0	0	29	0	0	32	0
1838	1262	Floral, roses	2-phenylethyl acetate[1]	41	**84**	0	0	76	0	0	20
1845	1393	Baked apple, stewed fruit	β-damascenone[1]	**76**	**71**	**76**	65	65	65	65	58
1861	1134	Grapefruit skin	3-mercaptohexanol[1]	**91**	**68**	**71**	**94**	61	38	58	**87**
1933	1124	Floral, wine, roses	β-phenylethanol[1]	68	0	0	65	0	0	32	29
2078	1187	Unpleasant, Straw, dust, flower	Octanoic acid[1]	0	61	29	0	82	35	0	0
2216	1107	Roasted, spicy, curry	Sotolon[1]	61	25	0	68	0	0	68	68
2293		Unpleasant	Decanoic acid[1]	0	0	82	0	0	41	0	0
2469	1347	Floral, pollen, honey, roses	Indole[3]	79	0	0	0	0	0	0	58

RI: Retention Index on polar capillary column (DB-WAX) and non-polar capillary column (DB-5); [1]: Identification by injection of pure reference standard; [2]: Identification by Retention Index on both columns; [3]: Identification by GC-GC-O-MS; *: Identified for the first time in wines; n.i.: Not identified (compounds did not produce any clear signal in the mass spectrometer).

Moreover, dihydromyrcenol had a high MF (Table I.12), mainly in the wines W20MV2 (74% MF) and W5MV4 (68% MF), and could generate green and fresh fruit aromas in wines. Davies et al., (Davies, 1990) and Píry et al., (Píry, Príbela, Ďurčanská, & Farkaš, 1995) reported that dihydromyrcenol was involved in the formation of citrus and herbal aromas in essential oils and blackcurrant.

The white wine W5MV4 was characterized by aromas such as green, must/grape, sweet, stewed fruit and unpleasant (Figure I.8 and Table I.10), the green and unpleasant aromas representing the main difference between this wine (cluster 1 in Figure I.8) and other wines belonging to the other clusters. The GC-O analysis (Table I.12) showed high MFs in 3-mercaptohexanol (87%) and dihydromyrcenol + linalool oxide (68% MF) (green aroma), and 3-methylbutyric acid (89%) (unpleasant odour).

The difference between W5MV4 and the other two wines is the presence of 3-methylbutiryc acid, responsible for the formation of unpleasant aromas in wines (Charles, Martin, Ginies, Etièvant, Coste, & Guichard, 2000) and the absence of a high MF for esters that generate fruit notes.

The red wine R27MT2 (cluster 4 in Figure I.9) that was characterized by fruit aromas (tropical, white and stewed fruit) showed high MFs (Table I.13) in isoamyl acetate (84%), ethyl hexanoate (76%), 3-mercaptohexyl acetate (68%), 2-phenylethyl acetate (68%) and β-damascenone (74%). These compounds generate fruit notes in red wines (Bowen & Reynolds, 2012; Ferreira et al., 2002; Ferreira et al., 2009; Mateo-Vivaracho et al., 2010; Pineau, Barbe, Van Leeuwen, & Dubourdieu, 2007; San Juan et al., 2011).

Table I.13. Odorants identified by GC-O in the selected fractions (2, 2 and 4 fractions) for the three red wines (R27MT2, R30MT4 and R22MT2). Gas chromatographic data, olfactory description, chemical identity, modified frequency (MF) expressed as %. Compounds in bold are important for the differences between the three wines.

RI		Description	Compound	Cluster 4. White, tropical and stewed fruit		Cluster 3. Green and cereal		Cluster 2. Red fruit, lactic and caramel			
DB-WAX	DB-5			R27MT2_8	R27MT2_9	R30MT4_8	R310MT4_9	R22MT2_5	R22MT2_9	R22MT2_10	R22MT2_11
1015	823	Strawberry, sweet, solvent	Isobutyl acetate[1]	20	58	58	29	29	29	**71**	46
1060		Unpleasant	n.i. 1060	0	25	0	0	0	0	65	58
1094		Green	Hexanal[t]	0	0	29	29	0	65	20	0
1131	877	Banana, fruit	Isoamyl acetate[1]	**84**	**82**	0	0	0	0	0	0
1154	802	Green	Z-3-hexenal[1]*	0	0	**76**	**76**	25	76	58	58
1216	741	Cheese	Isoamyl alcohol[1]	0	0	68	0	0	25	0	0
1247	999	Fruit, acid fruit	Ethyl hexanoate[1]	0	**76**	0	20	0	0	0	61
1329	872	Fried, cooked, nuts	2-methyl-3-furanthiol[2]	41	0	76	82	76	61	54	58
1393	861	Green, flower, vegetable	Z-3-hexenol[2]	0	0	0	0	0	68	0	0
1452		Straw, cereal, green pepper	2-isopropyl-3-methoxypyrazine[1]	29	20	25	65	33	29	54	50
1494	1050	Boj, grapefruit skin	Dihydromyrcenol[3]*+ linalool oxide[3]	0	0	**71**	65	25	0	25	0
1581		Green, cucumber, flower	E,Z-2,6-nonadienal[t]	0	0	0	74	0	61	20	0
1658	1096	Burning hair, toasted	Benzylmercaptan[1]	35	50	58	65	46	25	29	20
1666	1054	Flower, roses	Phenylacetaldehyde[1]	0	0	**79**	0	0	0	0	0
1681	837	Yeast, manure	3-methylbutiric acid[1]	0	0	71	0	0	0	0	0
1710	1180	Tropical fruit, flower	α-terpineol[2]	0	0	68	0	0	0	0	0
1740	1254	Tropical fruit, fruit, flower	3-mercaptohexyl acetate[1]	0	**68**	0	61	0	0	0	0
1837	1262	Roses, flower, wax	2-phenylethyl acetate[1]	**68**	0	25	54	0	29	54	0
1845	1393	Sweet, cooked apple, fruit, quince	β-damascenone[1]	0	**74**	25	65	0	65	**71**	**71**
1860	1134+ 979	Grapefruit skin + unpleasant, rancid	3-Mercaptohexanol[1] + hexanoic acid[1]	0	0	**68**	**84**	58	87	61	35
1934	1124	Flower, roses, dead flower	β-phenylethanol[1]	0	0	84	58	20	29	0	32
2052	1075	Sweet, strawberry candy	Furaneol[1]	0	0	35	58	38	**68**	32	0
2176	1472	Sweet, cinnamon	γ-decalactone[1]	0	0	20	35	65	41	0	0
2217	1107	Curry, toasted	Sotolon[1]	0	0	65	82	71	32	20	29
2240	1320	Honey	Methylantranilate[2]	0	0	**79**	0	0	0	0	0
2247	1365	Toasted, spicy	4-vinylguaiacol[1]	0	0	0	68	0	0	0	0
2293		Unpleasant, rubber	Decanoic acid[1]	0	38	32	0	0	29	20	79

RI: Retention Index on polar capillary column (DB-WAX) and non-polar capillary column (DB-5); [t]: Tentative identification; [1]: Identification by injection of pure reference standard.
[2]: Identification by Retention Index on both columns; [3]: Identification by GC-GC-O-MS; *: Identified for the first time in wines; n.i.: Not identified (compounds did not produce any clear signal in the mass spectrometer).

The wines belonging to cluster 3, besides having the same notes as the wines from cluster 4, were also characterized by green and cereal notes (Figure I.9). As can be seen in Table I.13, the fractions belonging to R30MT4 (selected as representative of cluster 3) showed high MFs in *Z*-3-hexenal (76%) and dihydromyrcenol + linalool oxide (71%), which generated green aromas, 3-mercaptohexanol (84%) (tropical fruit aroma), α-terpineol (68%) (fruity notes), methylantranilate (79%) and phenylacetaldehyde (79%) (stewed fruit aromas). The green aromas could thus be explained by the presence of *Z*-3-hexenal and dihydromyrcenol + linalool oxide in the red wines. Notwithstanding the cereal aromas could not be attributed to any compound, they could be related to the presence of 2-isopropyl-3-methoxypyrazine that has a MF of 65% in this wine.

Moreover, *Z*-3-hexenal had a high MF, mainly in the wine R30MT4 (Table I.13), and could generate green aroma in red wines. The identification of this compound was made by dual GC–GC and injection of pure reference standard, which made possible the identification of *Z*-3-hexenal. Seideneck et al., (Seideneck & Schieberle, 2011) reported that *Z*-3-hexenal was involved in the formation of green aromas in orange juice.

The red fruit, lactic and caramel character identified in the red wines from cluster 2 in Figure I.9 (R22MT2) could be linked to isobutyl acetate (71%), β-damascenone (71%) and furaneol (68%) (Table I.13). Furaneol produces red fruit notes in wines (Ferreira et al., 2016), while β-damascenone behaves as an enhancer of fruit notes from ethyl hexanoate and caramel notes (Ferreira et al., 2002; Pineau et al., 2007).

Besides, there are aromas that appeared in the fractions (Tables I.10 for white and I.11 for red wines) which were not perceived in the wines themselves (Table I.9). These aromas could be explained by the presence of compounds with MFs higher than 68% in the GC-O analysis (Tables I.12 and I.13). Toast and coffee aromas present in some fractions (W20MV2_9, W5MV4_7 and W5MV4_9 for white wines and R30MT4_8, R30MT4_9

and R22MT2_5 for red wines) could be due to the presence of compounds such as 2-methyl-3-furanthiol and/or sotolon, which were present at high MFs. The rancid and dirty background notes could be linked to a MF higher than 71% in *Z*-2-nonenal for white wines (W36MV4_9) and *E,Z*-2,6-nonadienal for red wines (R30MT4_9). The fraction W20MV2_10 that had attributes as honey, showed a high MF in 2-phenylethyl acetate. Green aromas present in R22MT2_9 fraction could be due to high MFs in *Z*-3-hexenal and *Z*-3-hexenol compounds. The fractions R30MT4_8 and R30MT4_9 described with attributes such as unpleasant and vinylphenol, had high MFs in 3-methylbutiric acid (71%) and 4-vinylguaiacol (68%), respectively.

In addition, many studies have been carried out to determine the volatile compounds responsible for the most important aromatic nuances of each wine. In some cases, a single compound is capable of transmitting its own aroma, such as linalool in Moscatel wines or 4-ethylphenol in the Brett character of wine (Suárez, Suárez-Lepe, Morata, & Calderón, 2007). In others, the situation is more complex and various molecules influence and interact in the perception of a particular note, as has been observed with fruity aromas (Francis & Newton, 2005; San Juan et al., 2011). Odorants can interact, showing either additive or competitive effects, which may even turn into synergistic or antagonistic effects (Ferreira, 2012). Thus, phenylacetaldehyde (in W36MV4 sample in Table I.12) and fatty acids such as decanoic (in W36MV4 and R22MT2 samples in Tables I.12 and I.13, respectively), octanoic (in W20MV2 sample in Table I.12) and hexanoic acids (in R22MT2 sample in Table I.13) contributed positively to the perception of fruitiness. According to San Juan et al., (San Juan et al., 2011) the presence of these fatty acids can also contribute to the suppression of the perception of vegetable notes, as can be seen in the W5MV4 wine which showed a green character (Figure I.8 and Table I.9) and where no fatty acids were observed with MFs higher than 20% (Table I.12). On the contrary, R22MT2 wine which showed

high MFs for compounds that generate green aromas (*Z*-3-hexenol and *Z*-3-hexenal) (Table I.13), these notes were not perceived because of the high MFs of these fatty acids. In the same way, β-damascenone enhances the fruity notes of ethyl hexanoate in hydroalcoholic solution, as can be seen in the W20MV2 sample in Table I.12 and the R22MT2 sample in Table I.13 (Pineau et al., 2007). The enolones, such as furaneol, enhance the red fruit character (Ferreira et al., 2016), while β-phenylethanol has not got any effect on the aroma of wine (de-la-Fuente-Blanco, Sáenz-Navajas, & Ferreira, 2016). On the other hand, isoamyl alcohol could suppress the perception of the fruit character in R30MT4 wine (Ferreira et al., 2016) and the red fruit character in R22MT2 wine (de-la-Fuente-Blanco et al., 2016). As can be seen in the R22MT2 sample where this alcohol was not observed.

3.5. Effect of must composition: different vintages

In order to study the behavior of the selected *non-Saccharomyces* yeasts (W5, W10, W20, W36 and W47 for white and R14, R22, R24, R27 and R30 for red samples) in two consecutive vintages, grapes from Verdejo and Tempranillo varieties from the 5 terroirs that were used in the vintage of 2015, were also taken in the vintage of 2016. Five fermentation with each yeast were also carried out. As in 2015, sorting tasks consisting on sort the wines according to odor similarities were also carried out.

This study let us to prove if the selected *non-Saccharomyces* yeasts were able to generate a similar aromatic profile not only when fermenting musts from different terroirs, but also in the two consecutive vintages.

Thirty-two wines from each variety (25 wines obtained with the 5 musts fermented with each yeast, 5 control wines (C) from the fermentation of the 5 different musts with one of the yeasts (W36 for white and R22 for red musts), and 2 replicate (R) samples) were submitted to sorting task analyses.

As can be seen in Figures I.10 and I.11 for white and red samples, respectively, replicate (W36MV5 and W36MV5_R and W47MV3 and W47MV3_R for white, and R14MT3 and R14MT3_R and R30MT2 and R30MT2_R for red wines) and control wines (W36_C and R22_C for white and red wines, respectively) were projected close to each other in the dendrogram derived from the cluster analysis.

In the case of white wines, as can be observed in Figure I.10, the panelists formed 5 groups of samples. Musts fermented with the *non-Saccharomyces* yeasts **W20** were grouped in cluster 1 with *tropical fruit, white fruit*, *fruit* and *green* aromas; in the case of **W36** yeast, the five wines were also grouped together in cluster 5 and were described as *tropical fruit, ripe fruit, stewed fruit* and *white fruit*; samples belonging to **W10** yeast were sorted in cluster 2 described as *rotten eggs* and *green*, with the exception of W10MV3 (plotted in cluster 1); musts fermented with **W47** yeast were clustered in 2 different groups, in cluster 4 described with c*ooked vegetables, green,* and *sweet* and in cluster 5; and the samples obtained with **W5** were sorted into 2 different groups, cluster 3 described as *caramel*, and cluster 4.

Thus, the *non-Saccharomyces* yeasts **W20** and **W36** were able to generate the same aromatic profile when fermenting 5 musts from different terroirs. Therefore, although some samples belonging to W36 from the vintage of 2015 were plotted in a different cluster with attributes to reduction (Figure I.8), which could due to the evolution in time of unstable samples, W36 and W20 yeasts were also able to generate a similar aromatic profile in the two consecutive vintages. In both vintages these yeasts generated aromas such as *tropical fruit, fruit* and *white fruit* in the case of the samples obtained with W20 yeast, and aromas such as *tropical fruit, stewed fruit* and *ripe fruit* in the case of W36 yeast.

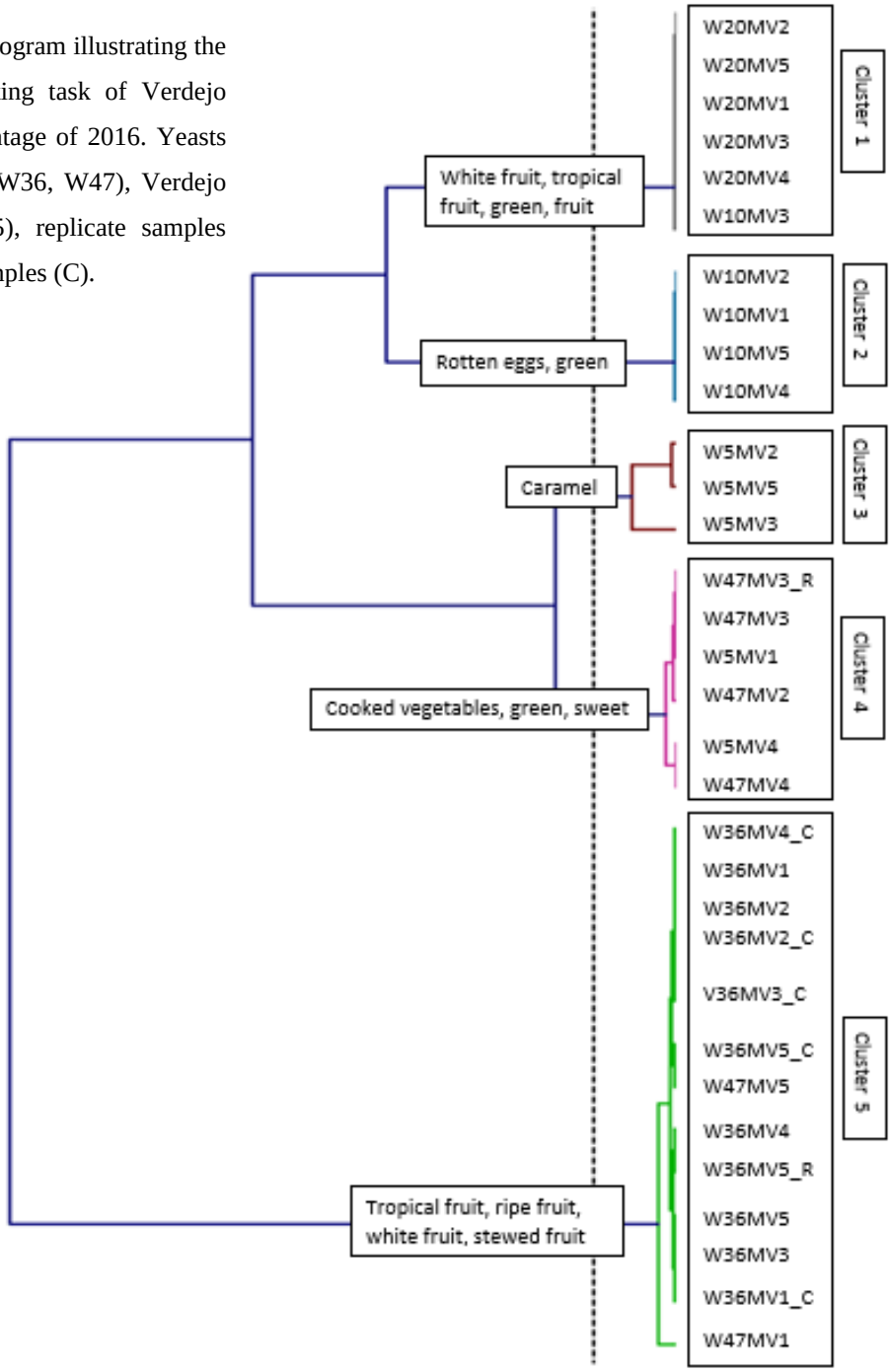

Figure I.10. Dendrogram illustrating the results of the sorting task of Verdejo wines from the vintage of 2016. Yeasts (W5, W10, W20, W36, W47), Verdejo musts (MV1–MV5), replicate samples (R) and control samples (C).

Thus, must composition did not affect the metabolism of W20 and W36 as affected the metabolism of W47, W5 and W10 yeasts, which generated different aromatic profiles not only between the different musts, but also between the different vintages.

In the case of red wines, as can be observed in Figure I.11, the panelists formed 3 groups of samples. Musts fermented with the *non-Saccharomyces* yeasts **R22** were grouped in cluster 1 described as *red fruit*; in the case of **R24** and **R27** yeasts, the five wines were also grouped together in cluster 2 and were described as *white fruit, tropical fruit* and *stewed fruit*; samples belonging to **R14** and **R30** yeasts were sorted in cluster 3 described as *stewed fruit, must/grape* and *white fruit*.

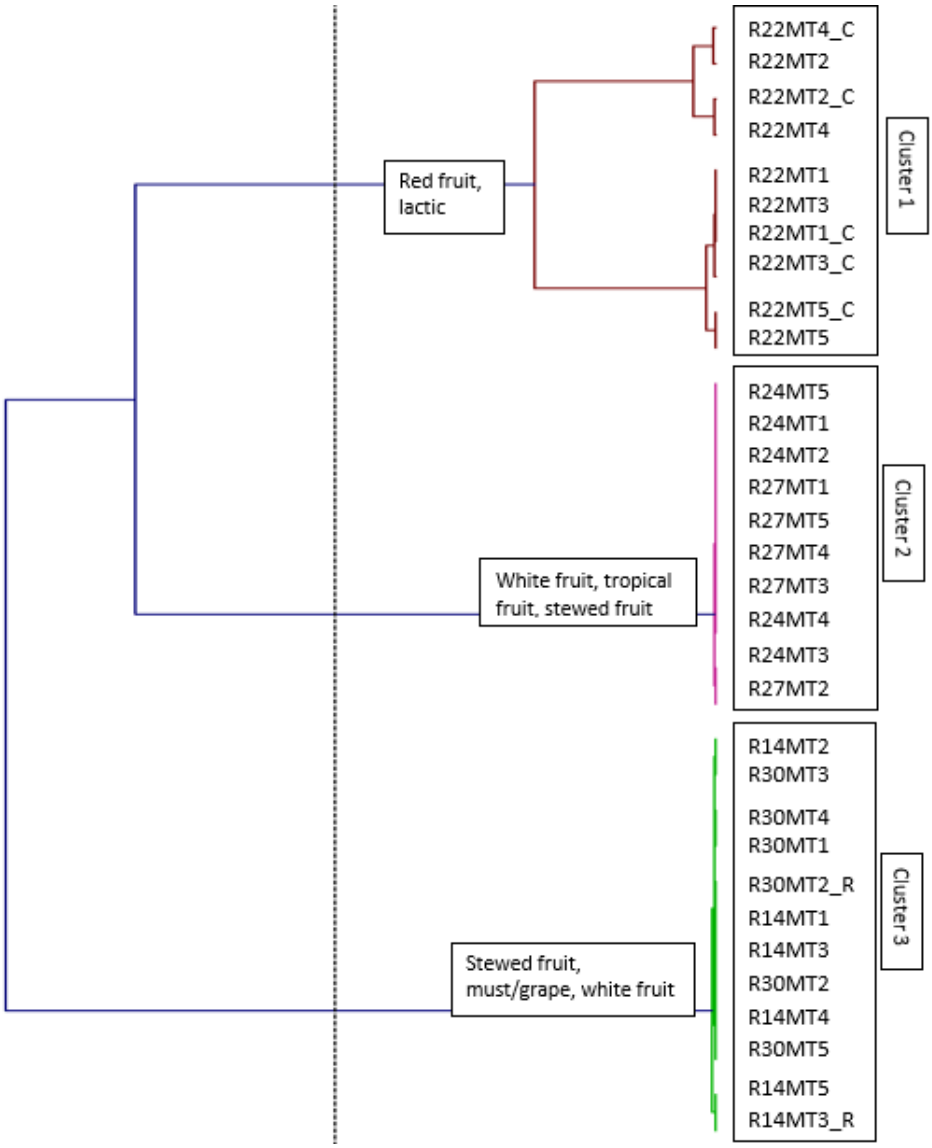

Figure I.11. Dendrogram illustrating the results of the sorting task of Tempranillo wines from the vintage of 2016. Yeasts (R14, R22, R24, R27, R30), Verdejo musts (MT1–MT5), replicate samples (R) and control samples (C).

Thus, in 2016, the *non-Saccharomyces* yeasts studied in the case of Tempranillo musts were able to generate the same aromatic profile when fermenting 5 musts from different terroirs. In addition, **R22** and R**27** yeasts were able to generate the same aromatic profile when fermenting musts from different vintages, 2015 and 2016 (Figures I.9 and I.11, respectively).

As in the vintage of 2015, in 2016 the amino acids and cysteinylated and glutathionylated precursors present in the musts were also analyzed to compare between the two vintages.

3.5.1. Verdejo musts

In Tables I.14 and I.15, it can be observed the results of the amino acids and cysteinylated and glutathionylated precursors, respectively, present in the 5 different musts of Verdejo variety from the vintage of 2016 used to carry out the fermentations with the selected yeasts.

3.5.1.1. Amino acids

As can be observed in Table I.14, amino acids were, in general, at higher concentration in the musts from the vintage of 2016 than in the vintage of 2015 (Table I.5). However, the relationship between terroirs was not maintained between the two years of study, since in 2016 (Table I.14), the must that had the highest concentration in most of the amino acids was MV3 while the one with the lowest concentration was MV1. This higher concentration in amino acids related to aroma compounds in MV3 could be the reason why, in the case of samples obtained with W10 yeast, all samples developed rotten eggs aromas (cluster 2 in Figure I.10) except those obtained with MV3 (cluster 1 in Figure I.10) that developed fruit aromas. The presence of higher concentration of valine, leucine, phenylalanine could form higher concentrations of isobutyl acetate, acetate isoamilo and phenylethyl acetate, respectively (Bell & Henschke, 2005; Lambrechts & Pretorius, 2000), respectively.

Table I.14. Concentration of amino acids (mg/L) in the musts from Verdejo variety from five different terroirs form the vintage of 2016.

	MV1 2016	MV2 2016	MV3 2016	MV4 2016	MV5 2016
Asp	70.7	106	120	99.4	92.0
Asn	38.2	30.3	26.8	35.1	34.9
Ser	38.6	67.5	77.3	61.7	53.1
Glu	114	167	195	156	139
His	27.5	41.0	46.2	38.5	32.4
Gly	5.94	1.87	3.88	2.78	2.34
Ala	83.9	177	175	136	113
GABA	65.0	93.1	112	93.4	80.4
Cys	5.11	8.05	9.90	8.67	5.66
Tyr	7.26	11.1	12.8	10.2	9.21
Val	24.8	34.2	39.3	33.4	30.4
Met	3.59	6.77	7.08	5.69	5.02
Lys	5.25	6.03	7.20	5.88	5.07
Ile	14.2	21.2	25.3	20.0	18.0
Leu	16.5	24.9	28.4	23.3	20.7
Phe	17.9	31.1	31.6	32.3	30.4

<D.L, under detection limit; asp, aspartic acid; asn, asparagine; ser, serine; glu, glutamic acid; his, histidine; gly, glycine; ala, alanine; GABA, γ-amino butyric acid; cys, cysteine; tyr, tyrosine; val, valine; met, methionine; lys, lysine; ile, isoleucine; leu, leucine; phe, phenylalanine.

3.5.1.2. Cysteinylated and glutathionylated precursors

As in the case of amino acids, the precursors of polyfunctional mercaptans were at higher concentration in the musts from the vintage of 2016 (Table I.15) than in the vintage of 2015 (Table I.6).

Table I.15. Concentration (μg/L) of the cysteinylated and glutathionylated precursors present in the musts from different terroirs from Verdejo variety from the vintage of 2016.

	MV1 2016	MV2 2016	MV3 2016	MV4 2016	MV5 2016
CYSMP	< D.L.	< D.L.	< D.L.	0.44	< D.L.
CYSMH	59.6	109	158	73.2	87.3
GLUMP	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.
GLUMH	5125	12708	11818	8126	10753
Sum of precursors	5185	12817	11976	8199	10840

<D.L., under detection limit.

Moreover, the relationship between terroirs was not kept in the two years of study, since in 2016 the must with the highest concentration of cysteinylated and glutathionylated

precursors was MV2 while the one with the lowest concentration was MV1. However, this lowest concentration of 3MH precursors in MV1 is opposed to the results obtained by means of sorting task (Figure I.10) in which it was observed that the samples W47MV1 and W47MV5 were described with tropical fruit aroma, while the other samples obtained with W47 were not described with this aroma.

3.5.2. Tempranillo musts

In Tables I.16 and I.17, it can be observed the results of the amino acids and cysteinylated and glutathionylated precursors, respectively, present in the 5 different musts of Tempranillo variety from the vintage of 2016.

3.5.2.1. Amino acids

In the case of the musts from Tempranillo variety (Table I.16), there was no clear trend in the concentration of amino acids in the different musts from the vintage of 2016.

Table I.16. Concentration of amino acids (mg/L) in the musts from Tempranillo variety from five different terroirs form the vintage of 2016.

	MT1 2016	MT2 2016	MT3 2016	MT4 2016	MT5 2016
Asp	15.7	10.6	5.93	14.6	18.0
Asn	34.0	26.8	44.9	39.4	35.4
Ser	25.6	8.78	14.0	10.5	16.9
Glu	67.8	31.8	95.1	37.8	61.4
His	41.0	25.5	32.6	22.5	38.0
Gly	1.45	< D.L.	3.62	< D.L.	0.41
Ala	87.4	40.5	72.2	48.8	78.9
GABA	189	58.4	108	74.7	117
Cys	3.29	2.11	8.70	1.89	4.32
Tyr	8.33	2.98	1.53	3.29	6.10
Val	13.1	15.8	33.4	17.3	12.0
Met	3.85	4.02	3.10	4.80	1.41
Lys	3.55	2.07	3.33	1.87	3.23
Ile	6.84	10.3	23.7	14.3	5.61
Leu	10.4	13.9	32.6	16.3	8.71
Phe	9.10	6.74	8.10	6.67	9.31

<D.L, under detection limit; asp, aspartic acid; asn, asparagine; ser, serine; glu, glutamic acid; his, histidine; gly, glycine; ala, alanine; GABA, γ-amino butyric acid; cys, cysteine; tyr, tyrosine; val, valine; met, methionine; lys, lysine; ile, isoleucine; leu, leucine; phe, phenylalanine.

Only MT3 must had the amino acids at higher concentration compared to the musts of the vintage of 2015 (Table I.7). Therefore, as in the case of Verdejo musts, the relationship between the different terroirs was not kept between the two vintages.

3.5.2.2. Cysteinylated and glutathionylated precursors

Regarding cysteinylated and glutathionylated precursors, in the case of musts from Tempranillo variety (Table I.17), there was no clear trend among vintages, since there were musts with higher concentration of polyfunctional mercaptan precursors in the vintage of 2015 (MT4 and MT5) (Table I.8), and musts with the highest concentration of these precursors in 2016 (MT1 and MT2). However, these differences in 3MH precursors concentrations cannot explain the tropical fruit aroma found in the samples plotted in cluster in the dendrograma (Figure I.11) obtained through the sorting task analysis.

Table I.17. Concentration (μg/L) of the cysteinylated and glutathionylated precursors present in the musts from different terroirs from Tempranillo variety from the vintage of 2016.

	MT1 2016	**MT2 2016**	**MT3 2016**	**MT4 2016**	**MT5 2016**
CYSMP	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.
CYSMH	105	154	145	231	147
GLUMP	< D.L.	< D.L.	< D.L.	< D.L.	< D.L.
GLUMH	3075	2972	1793	2436	2710
Sum of precursors	3180	3126	1938	2667	2857

<D.L., under detection limit.

Thus, in the case of both amino acids and cysteinylated and glutathionylated precursors, the relationship between the different terroirs was not maintained between the two vintages for the two varieties. Nevertheless, it is worth noting that in both vintages, the *non-Saccharomyces* yeast W10 was influenced by the must composition generating in both cases fruit aromas when the must used was those with higher concentration in amino acids.

Therefore, due to the *non-Saccharomyces* yeasts **W20** and **W36** for white and **R22** and **R27** for red musts were able to produce a similar aromatic profile (Figures I.8, I.9, I.10 and I.11) after the fermentation of musts from different terroirs and different vintages, it could be said that these *non-Saccharomyces* yeasts were rather independent of the must composition, and for this reason these yeasts were selected.

3.6. Effect of co-inoculation with *Saccharomyces cerevisiae*

In order to study the effect of co-inoculation with *Saccharomyces cerevisiae* and to prove if the co-inoculation generated some change on the aroma formed by the *non-Saccharomyces* yeasts (W20, W36, R22 and R27), these *non-Saccharomyces* yeasts were used to ferment Verdejo and Tempranillo musts with and without the co-inoculation with *Saccharomyces cerevisiae*. Thereafter, the four wines obtained from each variety (2 wines obtained from the fermentation with the *non-Saccharomyces* yeasts, and 2 from the fermentation with *non-Saccharomyces* yeasts with co-inoculation with *Saccharomyces cerevisiae)* were submitted to a sensory analysis and quantitative analyses.

3.6.1. Sensory analyses

To test if the two wines obtained for each yeast (with and without co-inoculation of *Saccharomyces cerevisiae*) developed the same aroma profile, the wines were submitted to a sorting task analysis. In addition, a replicate sample was introduced to assure the reproducibility of the panel. The result can be observed in Figure I.12.

As can be seen in Figure I.12, the replicate samples (W36 + *Sacch*_1 and W36 + *Sacch*_1_R) were projected close to each other in the dendrogram. There were a clear separation between white and red wines, with 4 clusters in the case of white, and 3 clusters in the case of red wines. In the case of white wines, there was a clear separation between samples obtained with *non-Saccharomyces* yeasts, forming two groups (one for the samples

obtained with the *non-Saccharomyces* yeast W36 and other for the samples fermented with W20). Within each of these two groups, two subgroups were formed that differentiated between the samples obtained with the co-inoculation and the samples without it. By contrast, in the case of red wines, three groups were formed, one composed by samples obtained with the *non-Saccharomyces* yeast R22, other with samples obtained with the *non-Saccharomyces* yeast R27 and the third group composed by samples obtained with the co-inoculation.

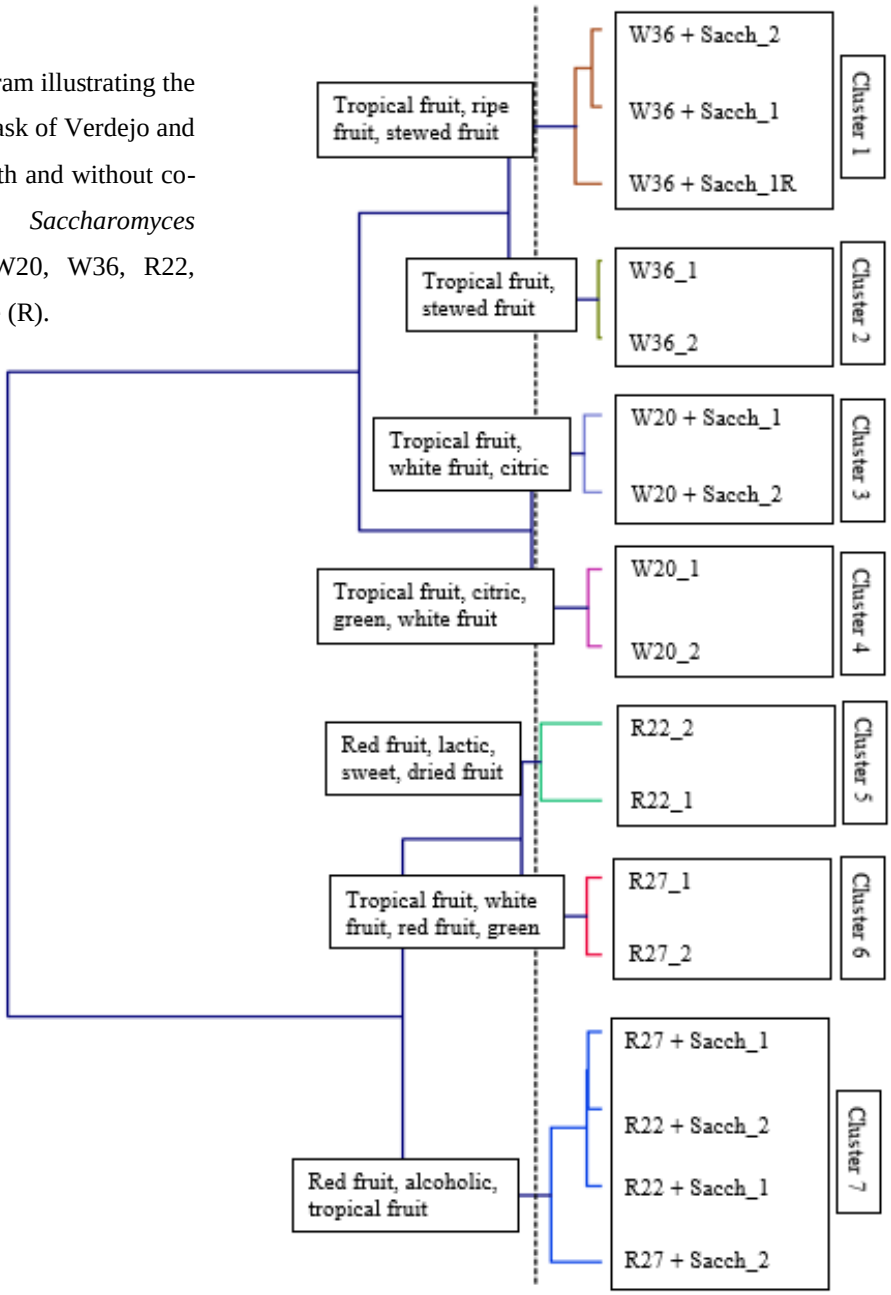

Figure I.12. Dendrogram illustrating the results of the sorting task of Verdejo and Tempranillo wines with and without co-inoculation with *Saccharomyces cerevisiae*. Yeasts (W20, W36, R22, R27), replicate sample (R).

Therefore, W36, W20, R22 and R27 are *non-Saccharomyces* yeasts capable of generating a similar aromatic profile when fermenting musts from different terroirs and from different vintages. Nevertheless, when these *non-Saccharomyces* yeasts are co-inoculated with *Saccharomyces cerevisiae*, two different cases may be occur:

i) The aromatic profile is determined by the *non-Saccharomyces* yeast and, therefore, it continues being close similar to the aromatic profiles observed previously when fermenting musts from different terroirs from 2015 and 2016.

ii) After co-inoculation, there is no difference between the aromatic profiles generated by the different *non-Saccharomyces* yeasts, but the aroma is mainly determined by the *Saccharomyces cerevisiae*.

The first case was observed for the *non-Saccharomyces* yeasts from white samples (W36 and W20), in which, each *non-Saccharomyces* yeast was able to generate a different aromatic profile and, therefore, there were plotted into 2 different groups (Figure I.12). Within the first group (formed by the samples fermented with the *non-Saccharomyces* yeast W36), two clusters were observed: cluster 1 formed by W36 co-inoculated with *Saccharomyces cerevisiae*, and described with attributes such as *tropical*, *ripe* and *stewed fruit*; and cluster 2 formed by the *non-Saccharomyces yeast* W36 without co-inoculation and described with *tropical* and *ripe fruit* notes. On the other hand, within the group formed by the samples fermented with the *non-Saccharomyces* yeast W20, two clusters were also observed (Figure I.12): cluster 3 formed by W20 with co-inoculation and described as *tropical fruit, white fruit and citric*; and cluster 4 formed by the W20 without co-inoculation and described with *tropical fruit, white fruit, citric and green* aromas. Thus, despite co-inoculation, the *non-Saccharomyces yeast* W36 and W20 were able to keep a similar aromatic profile to those previously observed (Figures I.8 and I.10).

By contrast, in the case of *non-Saccharomyces* yeasts from red samples (R22 and R27), the second case occurred in which the aromatic profile was determined by *Saccharomyces cerevisiae* (Figure I.12). As can be seen in Figure I.12, the samples obtained with the *non-Saccharomyces* yeasts R22 and R27 were plotted into 3 different groups. The first two groups were formed by samples fermented with R22 (cluster 5) and R27 (cluster 6) without inoculation with *Saccharomyces cerevisiae*. Cluster 5 was describe as *red fruit, lactic, sweet* and *dried fruit*, and cluster 6 was described with *tropical fruit, white fruit, red fruit* and *green* notes (Figure I.12). Thus, these *non-Saccharomyces* yeasts were capable of generating a similar aromatic profiles to those previously obtained (Figures I.9 and I.11). However, the third group (cluster 7) was formed by the *non-Saccharomyces* yeasts with co-inoculation of *Saccharomyces cerevisiae*, and it was described as *red fruit, alcoholic* and *tropical fruit*. Hence, in the case of the *non-Saccharomyces* yeasts R22 and R27, although without co-inoculation there were able to generate a similar aromatic profile to those previously generated, with co-inoculation the aroma is mainly determined by the *Saccharomyces cerevisiae* and there is no difference between *non-Saccharomyces* yeasts used.

Therefore, while in red wines the aromatic profile was determined by the *Saccharomyces cerevisiae,* in the case of white samples, the aromatic profile was determine by *non-Saccharomyces* yeasts W36 and W20 that kept their own aromatic profile despite co-inoculation.

3.6.2. Quantitative analyses

In order to study whether these differences observed in the sensory analysis were explained with concentration of the different aroma compounds, the two wines obtained for each yeast (with and without co-inoculation with *Saccharomyces cerevisiae*) were submitted to

different quantitative analyses: major compounds, minor compounds and polyfunctional mercaptans. The results can be observed in Table I.18, I.19 and I.20, respectively.

Table I.18. Quantitative data for major compounds (mg/L) analyzed in the eight samples obtained after the fermentation with 4 *non-Saccharomyces* yeasts (W20, W36, R22 and R27) with and without co-inoculation with *Saccharomyces cerevisiae*. Compounds in bold present significant differences ($P < 0.05$) according to one-way ANOVA.

	White samples				Red samples			
	W20	**W20+ Sacch**	**W36**	**W36+ Sacch**	**R22**	**R22+ Sacch**	**R27**	**R27+ Sacch**
Acetaldehyde	**14.8 ± 1.22**	**23.7 ± 0.17**	11.6 ± 0.46	16.1 ± 5.64	16.4 ± 6.65	11.7 ± 1.24	**28.7 ± 2.64**	**11.9 ± 0.30**
Diacetyl	< D.L	< D.L	< D.L	< D.L	< D.L	0.62 ± 0.61	0.73 ± 0.01	0.79 ± 0.29
Acetoin	3.42 ± 0.57	2.24 ± 0.04	0.68 ± 0.28	1.05 ± 0.07	1.68 ± 0.71	2.37 ± 0.45	**21.6 ± 2.53**	**1.87 ± 0.03**
Ethyl acetate	**22.5 ± 0.13**	**27.6 ± 0.18**	**11.6 ± 0.71**	**45.3 ± 1.10**	38.8 ± 11.9	44.8 ± 1.11	**42.6 ± 0.01**	**47.9 ± 0.58**
Isoamyl acetate	**1.43 ± 0.04**	**1.04 ± 0.02**	2.23 ± 0.23	2.73 ± 0.01	2.47 ± 3.45	4.73 ± 0.23	**2.48 ± 0.08**	**4.84 ± 0.44**
Hexyl acetate	0.20 ± 0.04	0.18 ± 0.03	**1.72 ± 0.01**	**0.34 ± 0.08**	0.09 ± 0.01	0.09 ± 0.02	0.08 ± 0.00	0.09 ± 0.04
Ethyl propanoate	< D.L	< D.L	< D.L	< D.L	0.08 ± 0.01	< D.L	0.05 ± 0.00	< D.L
Ethyl butyrate	0.45 ± 0.04	0.40 ± 0.01	**< D.L**	**0.40 ± 0.01**	0.30 ± 0.35	0.56 ± 0.07	**0.09 ± 0.01**	**0.47 ± 0.06**
Ethyl hexanoate	1.32 ± 0.16	1.07 ± 0.06	**< D.L**	**0.70 ± 0.13**	0.70 ± 0.87	1.10 ± 0.16	0.25 ± 0.06	0.77 ± 0.18
Ethyl octanoate	0.63 ± 0.17	0.68 ± 0.08	**< D.L**	**0.47 ± 0.10**	0.31 ± 0.37	0.40 ± 0.11	**0.10 ± 0.02**	**0.38 ± 0.01**
Ethyl decanoate	0.11 ± 0.01	0.09 ± 0.01	**< D.L**	**0.05 ± 0.01**	0.06 ± 0.02	0.06 ± 0.01	**0.02 ± 0.00**	**0.09 ± 0.01**
Isobutanol	**7.98 ± 0.17**	**9.70 ± 0.31**	**1.01 ± 0.21**	**8.22 ± 2.24**	18.2 ± 8.20	24.4 ± 1.53	29.1 ± 4.55	30.4 ± 0.92
1-Butanol	**0.33 ± 0.00**	**0.52 ± 0.01**	**< D.L**	**0.68 ± 0.08**	0.35 ± 0.35	0.68 ± 0.01	**0.18 ± 0.01**	**0.63 ± 0.02**
Isoamyl alcohol	77.3 ± 1.32	88.7 ± 4.21	**1.05 ± 0.57**	**101 ± 11.4**	119 ± 90.9	181 ± 10.9	**57.9 ± 7.06**	**196 ± 21.0**
1-Hexanol	**0.55 ± 0.04**	**0.79 ± 0.01**	**0.10 ± 0.01**	**0.52 ± 0.04**	0.28 ± 0.03	0.29 ± 0.01	**0.68 ± 0.03**	**0.31 ± 0.02**
Z-3-Hexenol	0.12 ± 0.01	0.11 ± 0.01	**0.01 ± 0.00**	**0.08 ± 0.00**	0.14 ± 0.01	0.14 ± 0.01	**0.10 ± 0.00**	**0.09 ± 0.00**
Methionol	**0.26 ± 0.01**	**0.37 ± 0.01**	**0.14 ± 0.04**	**0.53 ± 0.06**	1.17 ± 0.84	1.94 ± 0.16	**0.93 ± 0.02**	**2.46 ± 0.28**
Benzyl alcohol	**0.08 ± 0.01**	**0.12 ± 0.01**	**0.02 ± 0.00**	**0.09 ± 0.00**	0.05 ± 0.01	0.06 ± 0.00	0.05 ± 0.01	0.06 ± 0.01
β-Phenylethanol	**5.39 ± 0.38**	**6.94 ± 0.03**	**0.08 ± 0.04**	**10.6 ± 1.85**	30.4 ± 26.6	55.7 ± 3.16	**9.89 ± 2.18**	**47.2 ± 0.69**
Ethyl lactate	**0.24 ± 0.04**	**0.74 ± 0.01**	**< D.L**	**0.95 ± 0.19**	0.25 ± 0.24	0.41 ± 0.01	**0.19 ± 0.05**	**0.63 ± 0.07**
Diethyl succinate	**< D.L**	**0.12 ± 0.03**	**< D.L**	**0.05 ± 0.01**	0.06 ± 0.01	0.07 ± 0.01	0.03 ± 0.00	0.07 ± 0.01
γ-Butyrolactone	**1.52 ± 0.08**	**3.23 ± 0.19**	**0.41 ± 0.01**	**1.60 ± 0.16**	0.81 ± 0.37	1.06 ± 0.07	1.62 ± 0.08	1.45 ± 0.20
Butyric acid	**1.57 ± 0.05**	**2.39 ± 0.03**	**0.66 ± 0.03**	**1.70 ± 0.03**	1.31 ± 1.00	2.05 ± 0.14	**0.22 ± 0.01**	**1.51 ± 0.07**
Isobutyric acid	**0.42 ± 0.04**	**0.66 ± 0.04**	**0.19 ± 0.01**	**0.68 ± 0.02**	0.72 ± 0.42	0.91 ± 0.01	**0.32 ± 0.00**	**0.84 ± 0.09**
3-methylbutyric acid	**0.31 ± 0.01**	**0.50 ± 0.01**	**0.05 ± 0.01**	**0.74 ± 0.10**	0.39 ± 0.33	0.64 ± 0.01	**0.08 ± 0.01**	**0.52 ± 0.02**
Hexanoic acid	7.20 ± 0.26	7.53 ± 0.20	**0.23 ± 0.01**	**3.98 ± 0.09**	3.10 ± 2.76	5.20 ± 0.09	**1.67 ± 0.15**	**4.57 ± 0.47**
Octanoic acid	**18.3 ± 0.11**	**13.6 ± 0.42**	**0.09 ± 0.04**	**8.48 ± 0.76**	7.24 ± 7.00	13.4 ± 0.24	**4.23 ± 0.17**	**12.0 ± 1.38**
Decanoic acid	2.57 ± 0.35	1.70 ± 0.20	**0.06 ± 0.00**	**1.46 ± 0.30**	1.84 ± 1.08	1.74 ± 0.29	1.60 ± 0.41	1.71 ± 0.71

< D.L, under detection limit.

As can be observed in Tables I.18, there was higher concentration in most of the major compounds obtained when W20, W36 and R27 were co-inoculated with *Saccharomyces cerevisiae* than when fermentations were carried out without co-inoculation. This is in accordance with previous works that have observed that mixed fermentations (*Saccharomyces cerevisiae/non-Saccharomyces* yeasts) produced higher contents of different compounds (Zhang, Luan, Duan, & Yan, 2018). Only, isoamyl acetate and octanoic acid in the case of W20, hexyl acetate for W36 and acetaldehyde, acetoin, 1-hexanol and *Z*-3-hexenol for R27 were present at higher concentration when *Saccharomyces cerevisiae* was not used. On the other hand, in the case of the *non-Saccharomyces* yeast R22, there were no significant differences between fermentation with or without co-inoculation. Regarding minor compounds (Table I.19), although there were fewer significant differences between the samples obtained with or without co-inoculation than those observed in the major compounds, the use of *Saccharomyces cerevisiae* in Verdejo musts provided samples with higher concentration in some minor compound. By contrast, in the case of Tempranillo samples, co-inoculation only led to a higher concentration in butyl acetate and phenylethyl acetate in both *non-Saccharomyces* yeasts, ethyl isovalerate, isobutyl acetate and β-citronellol in R22 and ethyl vanillate in R27. Nevertheless, without co-inoculation, ethyl 2-methylbutyrate and ethyl dihydrocinnamate in the case of R22 and phenylethyl acetate, 4-vinylguaiacol and methyl vanillate for R27 were present in a higher concentration.

It is interesting that, in the case of white samples, most of the major and minor compounds (Tables I.18 and I.19, respectively) had higher concentration in samples fermented with W20 than in those fermented with W36. This could be because this *non-Saccharomyces* yeast was more similar to *Saccharomyces cerevisiae* than other *non-Saccharomyces* yeast

as was observed in Figure I.4, in which wine fermented with W20 was plotted close to commercial wines and wines fermented with *Saccharomyces cerevisiae*.

Table I.19. Quantitative data for minor compounds (µg/L) analyzed in the eight samples obtained after the fermentation with 4 *non-Saccharomyces* yeasts (W20, W36, R22 and R27) with and without co-inoculation with *Saccharomyces cerevisiae*. Compounds in bold present significant differences ($P < 0.05$) according to one-way ANOVA.

	White samples				Red samples			
	W20	**W20+ Sacch**	**W36**	**W36+ Sacch**	**R22**	**R22+ Sacch**	**R27**	**R27+ Sacch**
Ethyl isobutyrate	2.38 ± 0.00	4.29 ± 0.18	2.09 ± 2.07	4.24 ± 0.10	11.6 ± 2.84	15.5 ± 0.96	4.81 ± 2.58	6.65 ± 0.35
Ethyl 2-methylbutyrate	< D.L	< D.L	< D.L	< D.L	**0.97 ± 0.00**	**0.85 ± 0.00**	0.55 ± 0.00	< D.L
Ethyl isovalerate	2.41 ± 0.00	2.55 ± 0.00	1.48 ± 1.46	< D.L	**< D.L**	**15.0 ± 0.00**	19.4 ± 1.45	10.2 ± 0.00
Isobutyl acetate	**13.1 ± 0.35**	**8.90 ± 0.57**	**70.2 ± 4.31**	**18.7 ± 2.47**	**3.20 ± 0.42**	**42.2 ± 0.85**	42.2 ± 5.44	56.3 ± 2.47
Butyl acetate	2.45 ± 0.35	2.55 ± 0.35	**27.1 ± 1.70**	**6.95 ± 0.64**	**2.35 ± 0.78**	**5.15 ± 0.07**	**3.10 ± 0.14**	**5.15 ± 0.64**
Phenylethyl acetate	**49.9 ± 0.64**	**56.5 ± 0.07**	257 ± 26.2	301 ± 7.85	**23.3 ± 2.62**	**691 ± 26.8**	**953 ± 78.1**	**683 ± 40.0**
Benzaldehyde	**51.2 ± 0.13**	**24.0 ± 0.47**	**5.87 ± 1.64**	**43.0 ± 5.43**	5.31 ± 0.18	4.15 ± 0.57	3.04 ± 0.58	2.71 ± 0.06
Linalool	2.75 ± 0.05	2.68 ± 0.10	2.23 ± 0.09	2.99 ± 0.33	2.68 ± 0.38	3.16 ± 0.34	6.65 ± 1.04	5.01 ± 0.26
Linalool acetate	**0.21 ± 0.00**	**0.45 ± 0.04**	0.21 ± 0.05	0.24 ± 0.01	1.48 ± 0.25	1.19 ± 0.25	1.10 ± 0.21	0.79 ± 0.00
α-Terpineol	1.78 ± 0.08	1.75 ± 0.28	1.35 ± 0.02	1.88 ± 0.18	0.86 ± 0.06	0.81 ± 0.13	0.95 ± 0.03	0.85 ± 0.16
β-Citronelol	**< D.L**	**1.53 ± 0.07**	**< D.L**	**2.87 ± 0.33**	**0.78 ± 0.00**	**3.19 ± 0.01**	2.02 ± 0.99	3.08 ± 0.30
Geraniol	3.54 ± 0.24	3.30 ± 0.01	1.57 ± 0.60	2.49 ± 0.39	3.22 ± 0.00	3.70 ± 0.00	9.34 ± 3.19	2.83 ± 0.00
β-Damascenone	**19.5 ± 1.15**	**15.0 ± 0.54**	15.1 ± 0.76	14.4 ± 0.45	5.59 ± 0.32	6.78 ± 0.69	7.70 ± 0.33	8.44 ± 0.97
α-Ionone	0.21 ± 0.06	0.34 ± 0.04	0.16 ± 0.03	0.19 ± 0.01	0.35 ± 0.08	0.31 ± 0.01	0.45 ± 0.04	0.31 ± 0.07
β-Ionone	**0.13 ± 0.01**	**0.36 ± 0.01**	0.12 ± 0.02	0.11 ± 0.01	0.58 ± 0.04	0.50 ± 0.10	0.54 ± 0.07	0.44 ± 0.04
Guaiacol	8.89 ± 0.42	8.41 ± 0.22	**0.67 ± 0.35**	**6.72 ± 0.74**	3.82 ± 1.89	16.8 ± 9.02	6.09 ± 0.66	13.0 ± 5.83
o-Cresol	0.91 ± 0.18	0.92 ± 0.02	0.99 ± 0.06	1.03 ± 0.13	< D.L	< D.L	< D.L	< D.L
4-Ethylguaiacol	**0.26 ± 0.00**	**0.31 ± 0.00**	**< D.L**	**0.22 ± 0.05**	**< D.L**	**0.11 ± 0.07**	1.16 ± 0.03	1.04 ± 0.14
Eugenol	0.31 ± 0.01	0.30 ± 0.01	**0.17 ± 0.04**	**0.34 ± 0.02**	0.85 ± 0.06	0.95 ± 0.23	0.16 ± 0.00	< D.L
4-Ethylphenol	0.13 ± 0.01	0.19 ± 0.04	0.15 ± 0.06	0.18 ± 0.06	0.22 ± 0.01	0.11 ± 0.00	12.3 ± 0.45	36.1 ± 6.53
4-Vinylguaiacol	**504 ± 3.68**	**810 ± 15.6**	**18.8 ± 6.08**	**1048 ± 11.1**	5.48 ± 1.83	24.5 ± 8.70	**1.84 ± 0.02**	**1.77 ± 0.62**
E-Isoeugenol	1.51 ± 0.10	1.19 ± 0.08	**0.51 ± 0.14**	**1.40 ± 0.23**	2.38 ± 0.30	1.61 ± 0.45	29.9 ± 3.75	44.4 ± 22.6
2,6-Dimethoxyphenol	8.15 ± 0.64	8.90 ± 0.57	**0.70 ± 0.42**	**7.20 ± 0.00**	14.2 ± 5.66	40.6 ± 21.2	14.4 ± 1.34	28.7 ± 6.36
4-Vinylphenol	**225 ± 6.86**	**257 ± 1.98**	**7.45 ± 0.78**	**277 ± 31.5**	6.80 ± 2.97	16.9 ± 7.14	2.25 ± 0.11	2.75 ± 0.12
Methoxyeugenol	0.89 ± 0.10	0.89 ± 0.08	0.40 ± 0.30	0.93 ± 0.25	1.91 ± 0.33	2.27 ± 0.72	0.42 ± 0.00	0.48 ± 0.04
Ethyl dihydrocinnamate	0.13 ± 0.00	0.28 ± 0.01	0.35 ± 0.01	0.36 ± 0.01	**7.41 ± 0.60**	**0.54 ± 0.07**	1.16 ± 0.03	1.04 ± 0.14
E-Whiskylactone	0.36 ± 0.11	0.47 ± 0.01	**< D.L**	**0.35 ± 0.02**	< D.L	< D.L	< D.L	< D.L
γ-Nonalactone	**2.74 ± 0.11**	**4.36 ± 0.14**	**1.67 ± 0.20**	**4.30 ± 0.25**	2.62 ± 0.09	2.81 ± 0.40	2.20 ± 0.45	2.46 ± 0.45
Vanillin	2.38 ± 0.35	2.63 ± 0.05	1.57 ± 0.28	1.14 ± 0.09	3.53 ± 1.41	3.80 ± 1.61	2.44 ± 0.11	1.92 ± 0.45
Methyl vanillate	8.95 ± 0.07	9.55 ± 0.49	**0.95 ± 0.21**	**9.30 ± 0.99**	4.35 ± 0.07	4.20 ± 0.57	**7.65 ± 0.35**	**4.90 ± 0.71**
Ethyl vanillate	**< D.L**	**0.42 ± 0.32**	0.36 ± 0.36	< D.L	2.29 ± 0.08	2.65 ± 0.68	**1.24 ± 0.47**	**8.75 ± 2.14**
Acetovanillone	69.7 ± 0.42	72.9 ± 3.25	**9.20 ± 0.28**	**70.6 ± 7.28**	30.1 ± 2.83	29.7 ± 4.45	52.5 ± 9.12	41.5 ± 5.16

< D.L, under detection limit

In the case of polyfunctional mercaptans (Table I.20), the co-inoculation brought to higher concentration of 3-mercaptohexanol (3MH) in both *non-Saccharomyces* yeasts from Verdejo variety (W20 and W36).

Table I.20. Quantitative data for polyfunctional mercaptans (ng/L) analyzed in the four samples obtained after the fermentation with 2 *non-Saccharomyces* yeasts (W20 and W36) from Verdejo variety with and without co-inoculation with *Saccharomyces cerevisiae*. Compounds in bold present significant differences ($P < 0.05$) according to one-way ANOVA.

	W20	W20+Sacch	W36	W36+Sacch
4-mercapto-4-metil-2-pentanona	< L.D.	< L.D.	< L.D.	< L.D.
3-mercaptohexyl acetate	82.9 ± 29.8	54.3 ± 0.37	162 ± 24.9	132 ± 31.9
3-mercaptohexanol	**386 ± 49.8**	**591 ± 22.65**	**192 ± 14.0**	**639 ± 20.0**

Moreover, regarding to the different aroma profiles obtained in the sorting task (Figure I.12), the tropical nuances observed in white wines could be due to the presence of high concentration of 3MH and 3MHA. As some authors have described, these compounds are responsible for the tropical fruit character (Lee et al., 2013; Lund, Thompson, Benkwitz, Wohler, Triggs, Gardner, Heymann, & Nicolau, 2009; Mestres, Busto, & Guasch, 2000; Pinu et al., 2014; Swiegers & Pretorius, 2007; Tominaga, Murat, & Dubourdieu, 1998). The sweeter character observed in wines obtained with W36 could be linked to the presence of higher concentration of isoamyl acetate, isobutyl acetate, butyl acetate, phenylethyl acetate (Culleré, Escudero, Cacho, & Ferreira, 2004; Noguerol-Pato, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2009; Yu, Xie, Xie, Ai, & Tian, 2019), ethyl dihydrocinnamate (ethyl-3-phenylpropanoate) (Moio & Etièvant, 1995) and 3-mercaptohexyl acetate (Lund et al., 2009). On the other hand, the fresher character observed in wines from W20 could be due to the higher concentrations of ethyl hexanoate, octanoate and decanoate, ethyl isovalerate (Bowen & Reynolds, 2012; Lee et al., 2013; Noguerol-Pato et al., 2009; Pineau et al., 2009; Yu et al., 2019) and geraniol, and the green notes to

the presence of higher concentration in hexanol and *Z*-3-hexenol (Culleré et al., 2004; Mozzon, Savini, Boselli, & Thorngate, 2016).

By contrast, in the case of red wines, the red fruit notes, that appeared in the three groups of the dendrograma (Figure I.12), does not seem to be determined by a particular compound but by the interactions between several compounds. As discussed by Francis and Newton (Francis & Newton, 2005), wine aroma is not just the sum of individual constituents, but the result of complex interactions between a large number of chemical compounds. On the other hand, the green aroma observed in R27 could be due to the presence of higher concentration of *Z*-3-hexenol (Culleré et al., 2004; Mozzon et al., 2016).

Therefore, in the case of white samples, although the co-inoculation with *Saccharomyces cerevisiae* led to significantly higher concentrations in most of the compounds analyzed, it did not generate a significant change in the aromatic profile obtained (Figure I.12). Both *non-Saccharomyces* yeasts (W20 and W36) were able to generate a similar aromatic profile with and without *Saccharomyces cerevisiae*, observing fresher notes in the wines obtained with W20 and sweeter notes in those fermented with W36. In addition, these different nuances could be explained by the presence of higher concentration in some aroma compound (Tables I.18, I.19 and I.20). Conversely, in the case of red wines, the co-inoculation brought about to a different aromatic profile. However, this separation in a different cluster in the dendrograma (Figure I.12) cannot be explained quantitatively by the compounds analyzed, so the different aromas observed could be due either to other compounds that have not been analyzed, or to interactions between the different compounds present in the samples.

4. CONCLUSIONS

Categorization task followed by flash profiling and GC-O analysis has revealed to be a fast and effective sensory-directed methodology for the selection of high quality aroma wines. This method allowed identifying seven Verdejo (**W12, W20, W33, W34, W36, W47** and **W50**) and five Tempranillo (**R22, R24, R27 R45** and **R47**) samples fermented with different *non-Saccharomyces* yeasts and producing high quality aroma profiles according to a panel of Spanish wine professionals. Among quality exemplars, different aroma profiles could be identified such as citrus, fruit in syrup, boxtree/vegetal, tropical or wet grain aromas for Verdejo and red fruit or fruit in syrup for Tempranillo. GC-O analyses identified β-damascenone, 3-mercaptohexyl acetate and ethyl butyrate as distinctive quality compounds linked to dried, tropical and red fruit aromas, respectively.

Moreover, a sorting task followed by fractionation and GC-O analysis has also proven to be an effective and rapid sensory-directed methodology for the identification of compounds responsible for the distinctive aromas of wines. This methodology allowed identifying wines with distinctive aromas such as: sweet fruit, fresh fruit or green and unpleasant for Verdejo wines, and fruity character, red fruit or green and cereal for Tempranillo wines. GC-O analyses identified isoamyl acetate, β-damascenone, 2-phenylethyl acetate and un unknown in the RI 2469 as compounds linked to ripe and stewed fruit aromas; 3-mercaptohexyl acetate and 3-mercaptohexanol as compounds capable of generating tropical fruit aromas; isobutyl acetate, ethyl hexanoate and dihydromyrcenol as compounds capable of producing white fruit aromas; and 3-methylbutiric acid as a compound responsible for unpleasant aromas in Verdejo wines. In the case of Tempranillo wines, *Z*-3-hexenal was identified as a compound linked to green aromas; isoamyl acetate, ethyl hexanoate, 3-mercaptohexyl acetate, 2-phenylethyl acetate and β-damascenone as

compounds able to produce fruity notes in red wines; and isobutyl acetate, furaneol and β-damascenone as compounds that can generate caramel and red fruit aromas.

In addition, this methodology also allowed identifying four *non-Saccharomyces* yeasts (**W20** and **W36** in the case of Verdejo wines and **R22** and **R27** in the case of Tempranillo) that were able to generate wines with similar aromatic profile when fermenting musts not only from different terroirs, but also from different vintages. Thus, these *non-Saccharomyces* yeasts were no dependent on the must composition.

However, the co-inoculation of these *non-Saccharomyces* yeasts with *Saccharomyces cerevisiae* led to different results. In the case of white samples, the aromatic profile was determine by *non-Saccharomyces* yeasts W36 and W20 that kept their own aromatic profile despite co-inoculation. By contrast, in the case of red wines, the aromatic profile was determined by the *Saccharomyces cerevisiae,* and there was no difference if the *non-Saccharomyces* yeast used was R22 or R27.

Therefore, these sensory methodologies are presented as effective and rapid tools in the screening and characterization of quality aroma profiles. The wine industry could benefit from the use of these methodologies as a complementary tool for identifying and characterizing quality exemplars obtained under different technical procedures.

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CHAPTER II

Release of polyfunctional mercaptans from their precursors under different conditions

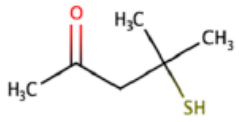
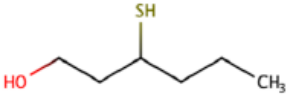
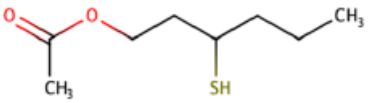
CHAPTER II. RELEASE OF POLYFUNCTIONAL MERCAPTANS FROM THEIR PRECURSORS UNDER DIFFERENT CONDITIONS

1. INTRODUCTION

Sulfur compounds, especially polyfunctional mercaptans, may play an important role in the aroma of many products, both fresh (plants, vegetables, fruit, etc.) and processed (roasted coffee, wine, etc.) (Harsch & Gardner, 2013; Tominaga, Murat, & Dubourdieu, 1998). Polyfunctional mercaptans are varietal aroma compounds that contribute to the aroma characteristics of white and rosé wines, and also in some red wines (Harsch & Gardner, 2013; Roland, Schneider, Razungles, & Cavelier, 2011). The main aromatic polyfunctional mercaptans are 3-mercaptohexan-1-ol (3MH) associated with grapefruit and citrus zest notes, 3-mercaptohexyl acetate (3MHA) associated with notes of passion fruit and guava, and 4-mercapto-4-methyl-pentan-2-one (4MMP), typically characterized as boxwood, blackcurrant, broom and passion fruit aromas (Lund, Thompson, Benkwitz, Wohler, Triggs, Gardner, Heymann, & Nicolau, 2009; Mestres, Busto, & Guasch, 2000; Swiegers & Pretorius, 2007; Tominaga, Murat, et al., 1998). These compounds are considered to have a key impact, since they are often found in concentrations far above their olfactory perception thresholds (Table II.1). These compounds have been identified in a wide range of varietal wines (Campo, Ferreira, Escudero, & Cacho, 2005; Tominaga, Baltenweck-Guyot, Peyrot des Gachons, & Dubourdieu, 2000; Tominaga, Darriet, & Dubourdieu, 1996). They were primarily identified as responsible for the typical notes of Sauvignon Blanc (SB) wines and they contribute positively to the fruity notes of young wines (Roland et al., 2011; Tominaga, Murat, et al., 1998). Moreover, Escudero et al., (Escudero, Gogorza, Melús, Ortín, Cacho, & Ferreira, 2004) observed that 4MMP is responsible for the perception of fresh notes in Macabeo wines. Then, in 2010, Mateo-Vivaracho et al.,

(Mateo-Vivaracho, Zapata, Cacho, & Ferreira, 2010) observed that 4MMP, 3MH and 3MHA are essential compounds in the aroma of many white wines. Furthermore, 4MMP is also a key compound in the aroma of the German variety Scheurebe (Guth, 1997) and the Slovak variety Devin (Peťka, Ferreira, González-Viñas, & Cacho, 2006). On the other hand, Murat et al., and Ferreira et al., (Ferreira, Ortín, Escudero, López, & Cacho, 2002; Murat, Tominaga, & Dubourdieu, 2001) found that 3MH is an essential compound in the aroma of wines in Grenache, Merlot and Cabernet Sauvignon varieties, as well as Campo et al., and Sarrazin et al., (Campo, Cacho, & Ferreira, 2008; Sarrazin, Shinkaruk, Tominaga, Bennetau, Frerot, & Dubourdieu, 2007) observed that this compound is also a key compound in the aroma of botrytized Sauternes wines. These studies suggest that polyfunctional mercaptans have an important role in the aroma of wines from different grape varieties. Nevertheless, the influence of the variety like amino acid profile is not clear.

Table II.1. Structures, threshold and occurrence of varietal thiols.

Compound	Structure	Threshold in model wine*	Range of occurrence in wine**
4-mercapto-4-methyl-2-pentanone		0.8	n.d. to 90
3-mercaptohexanol		60	n.d. to 7300
3-mercaptohexyl acetate		4	n.d. to 440

n.d.: not detected
* (Tominaga, Murat, et al., 1998)
** (Mateo-Vivaracho et al., 2010)

The biogenesis pathways of 3MH and 4MMP involve cysteinylated precursors such as cysteine-3-mercaptohexan-1-ol (CYSMH) and cysteine-4-mercapto-4-methylpentan-2-one (CYSMP) (Tominaga, Peyrot des Gachons, & Dubourdieu, 1998), and glutathionylated

precursors as glutathione-3-mercaptohexan-1-ol (GLUMH) and glutathione-4-mercapto-4-methylpentan-2-one (GLUMP) (Fedrizzi, Pardon, Sefton, Elsey, & Jeffery, 2009; Peyrot des Gachons, Tominaga, & Dubourdieu, 2002). Aromatic thiols are released from their precursors during alcoholic fermentation (AF) when the β-lyase enzymatic action of yeast produces the cleavage of the carbon-sulfur bond (Tominaga, Peyrot des Gachons, et al., 1998). The synthesis of 3MHA during must fermentation consists of the acetylation of the volatile thiol 3MH by the action of the yeast alcohol acetyltransferase (Swiegers & Pretorius, 2007; Swiegers, Willmott, Hill-Ling, Capone, Pardon, Elsey, Howell, de Barros Lopes, Sefton, Lilly, & Pretorius, 2006). In addition, in 2002, Peyrot des Gachons et al., (Peyrot des Gachons et al., 2002) demonstrated the possibility of enzymatic conversion of GLUMH into CYSMH. Hence, GLUMH could act both as a pro-precursor of CYSMH and as a precursor of 3MH (Grant-Preece, Pardon, Capone, Cordente, Sefton, Jeffery, & Elsey, 2010; Thibon, Cluzet, Merillon, Darriet, & Dubourdieu, 2011). Another alternative biogenetic pathway for the generation of 3MH involve C6 unsaturated compounds, such as *E*-2-hexenal, which experiment a sulfur addition during AF (Schneider, Charrier, Razungles, & Baumes, 2006).

Nevertheless, the polyfunctional mercaptan concentration in the resulting wines is not directly correlated to the precursor concentrations in the starting musts (Coetzee & du Toit, 2012) and in fact, the mean level of conversion of the cysteinylated and glutathionylated precursors into their corresponding thiol is between 0.17 and 4.2% (Bonnaffoux, Delpech, Rémond, Schneider, Roland, & Cavelier, 2018; Concejero, Hernández-Orte, Astrain, Lacau, Baron, & Ferreira, 2016; Peyrot des Gachons, Tominaga, & Dubourdieu, 2000; Roland, Schneider, Razungles, Le Guerneve, & Cavelier, 2010). These facts suggest that there are many aspects related to the uptake and use of these precursors by yeast which

remain poorly understood. Moreover, to the best of our knowledge, the ability of yeasts to use each of the precursors during AF, particularly 4MMP precursors, has not been studied.

The abundance of precursors seems to be linked to the grape variety (Coetzee & du Toit, 2012) and yeast assimilable nitrogen (YAN) (Chone, Lavigne-Cruege, Tominaga, Van Leeuwen, Castagnede, Saucier, & Dubourdieu, 2006). In addition, YAN is also very important for the yeast growth and fermentation metabolism. The main sources of YAN in grape juice are ammonium ions and amino acids (Henschke & Jiranek, 1993). During alcoholic fermentation, yeast strains can use these nitrogen sources in several ways, particularly for protein synthesis and growth (Bauer & Pretorius, 2000; Styger, Prior, & Bauer, 2011; Vilanova, Ugliano, Varela, Siebert, Pretorius, & Henschke, 2007). As low concentrations of yeast assimilable nitrogen (YAN) can increase the risk of stuck fermentations (Bely, Sablayrolles, & Barre, 1990), it is usual to supplement grape musts with di-ammonium phosphate (DAP). Supplementation with amino acids is an alternative approach but this can modulate the aroma profile of the final wine (Hernández-Orte, Ibarz, Cacho, & Ferreira, 2006). The amino acid profile may affect the order in which different amino acids are used by yeast, and it may also affect the production of volatile compounds (Hernández-Orte, Cacho, & Ferreira, 2002).

In addition, sulfur plays an important role in many metabolisms. It is involved in the formation of sulfur amino acids (methionine and cysteine), in the protection against oxidative stress by means of glutathione (GSH) (Hébert, Casaregola, & Beckerich, 2011) and in the formation of polyamines that are essential for the growth of yeasts, among others (Hamasaki-Katagiri, Tabor, & Tabor, 1997). Some authors have studied the effect of the addition of cysteine and GSH to SB must on the release of polyfunctional mercaptans upon fermentation. While, in the case of cysteine, it has been observed that this addition produces an increase in the concentration of 3MH and 3MHA (Harsch & Gardner, 2013), in the case

of GSH, the results are contradictory. Patel et al., (Patel, Herbst-Johnstone, Lee, Gardner, Weaver, Nicolau, & Kilmartin, 2010) observed a decrease in the 3MH and 3MHA levels, while Wegmann-Herr et al., (Wegmann-Herr, Ullrich, Schmarr, & Durner, 2016) observed an increase in the concentration of 3MH. In addition, there are no studies on the effect of the addition of different sulfur compounds and different addition concentrations on the metabolization of polyfunctional mercaptan precursors.

Therefore, this chapter has 4 different aims:

i) To test whether the different amino acid profiles influence the use of polyfunctional mercaptan precursors by yeast during alcoholic fermentation.

ii) To identify the precursors preferred by the yeast.

iii) To test whether different concentrations and sources of sulfur can cause a different consumption of the polyfunctional mercaptans precursors, as well as a different release of the polyfunctional mercaptans.

iv) Study the effect of the addition of cysteine and glutathione on the genes related with the metabolism of polyfunctional mercaptans precursors.

For the first purpose, synthetic juice with different amino acid profiles simulating different grape varieties were fermented. For the second purpose, fermentations of model solutions with precursors added separately with different amounts of YAN were also carried out. For the third and fourth, synthetic juice containing different levels and sources of sulfur compounds were fermented. In the final wines, polyfunctional mercaptans and their precursors were analyzed and the correlation between them studied. In addition, for the fourth purpose, transcriptomic studies were also carried out.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Pure standards of the four precursors GLUMP, GLUMH, CYSMP and CYSMH were synthesized by Roowin (Riom, France), having a purity ≥95%. Liquid chromatography coupled to mass spectrometry (LC–MS) grade acetonitrile and formic acid obtained from Scharlau (Barcelona, Spain) were used as mobile phases. Spectrophotometer UV Nanodrop ND-1000, average speed centrifuge and Trizol were supplied from Thermo Fisher Scientific (Waltham, USA), while high-speed centrifuge with a JA20 rotor, Multisizer 3 coulter counter and Isoton were obtained from Beckman Coulter (California, USA). Chloroforme was obtained by Merck (Darmstadt, Germany). RNeasy mini Kit and RNase-Free DNasa set were purchased by Qiagen (Hilden, Germany). Bioanalyzer 2100, RNA 6000 Nano LabChip Kit, One-Color RNA Spike-In Kit, Low Input Quick Amp Labeling Kit one-color, Gene Expression Hybridization kit and Hybridization Gasket slide kit were obtained from Agilent Technologies (Santa Clara, USA). GenePix 4000B Microarray Scanner from Molecular Devices LLC (California, USA).

2.2. Culture conditions

2.2.1. Synthetic juice

The synthetic juice used (pH 3.5) contained 105 g/L of glucose and 105 g/L of fructose. This juice was adapted from that described by Bely et al., (Bely et al., 1990) with the following modifications to carry out the different experiments. The experiments were performed with different YAN (120, 150 and 200 mg/L of nitrogen) expressed as ammoniacal nitrogen ($(NH_4)_2HPO_4$) 114.6 mg/L (corresponding to 24.32 mg/L of nitrogen), amino acids with fixed concentration, and amino acids with variable

concentrations to simulate amino acid profiles of different grape varieties (Table II.2). In all the experiments, the amino acids with fixed concentrations (expressed in mg/L) were the same: alanine 60.1, leucine 20.2, valine 20.1, isoleucine 20.2, tyrosine 20.3 and cysteine 5.07).

Table II.2. Composition (in milligrams per liter) of amino acids related to 7 grape varieties-like profiles and 2 versions of one of these varieties adjusted to reach 150 mg/L of total nitrogen.

	Asp	Glu	Ser	Gly	His	Thr	Arg	Pro	Met	Phe	Lys	Gln	GABA
Cabernet Sauvignon-like profile	15.6	32.8	24.6	2.91	221	27.9	56.0	1207	30.7	3.94	0.00	50.3	35.1
Grenache-like profile	26.7	47.5	16.3	0.85	83.9	14.4	153	185	22.9	11.2	2.56	80.5	38.4
Tempranillo-like profile	27.7	27.3	19.3	2.07	44.0	23.2	215	96.9	8.08	2.41	4.39	56.8	16.0
Chardonnay-like profile	33.6	98.1	74.0	2.58	33.9	56.4	137	360	9.21	24.4	8.38	72.3	43.0
Carignan-like profile	29.0	96.2	34.9	0.00	68.0	27.6	154	539	14.3	5.54	0.85	54.6	50.0
Macabeo-like profile	31.7	31.8	25.4	3.71	55.3	22.7	143	126	17.8	13.3	0.00	142	22.0
SB-like profile	22.6	76.6	32.7	1.14	18.6	31.5	204	160	4.23	16.4	5.44	66.6	22.9
SBv1-like profile	20.8	70.5	30.1	1.05	17.1	29.0	188	147	3.89	15.1	5.00	61.3	84.1
SBv2-like profile	13.7	186	19.8	0.69	11.3	19.1	124	97.6	2.57	9.98	3.30	161	55.5

Asp, aspartic acid; glu, glutamic acid; ser, serine; gly, glycine; his, histidine; thr, threonine; arg, arginine; pro, proline; met, methionine; phe, phenylalanine; lys, lysine; gln, glutamine; GABA, γ-amino butyric acid.

Experiment 1. Effect of the amino acid profile of different grape varieties. All fermentations were carried out with the same amount of nitrogen (150 mg N/L), being the amino acid profiles the only difference between samples (Table II.2). The amino acid profiles of seven different grape varieties (Sauvignon blanc (SB), Chardonnay, Macabeo, Carignan, Grenache, Tempranillo and Cabernet Sauvignon) were selected (Hernández-Orte et al., 2002). The amino acid profile of the SB-like profile was modified twice, SB version 1 (SBv1) and SB version 2 (SBv2), through the modification of three amino acids (γ-aminobutyric acid (GABA), glutamic acid and glutamine) based on the previous study of Pinu et al., concerning the contribution of these nitrogenous compounds to volatile thiol development (Pinu, Edwards, Jouanneau, Kilmartin, Gardner, & Villas-Boas, 2014).

Experiment 2. Effect of the precursors added separately. Two different amounts of nitrogen (150 mg N/L and 120 mg N/L) were used. Both with the same ammoniacal nitrogen and amino acids with fixed concentrations as mentioned above, whereas the amino acids with variable concentrations were obtained by adjusting the percentages of the amino acid of the Chardonnay-like profile (without any change in the case of 150 mg N/L, and the decrease of 28% of the concentration of amino acids but keeping the relative concentrations in the case of 120 mg N/L) (Table II.2). A low amount of YAN (120 mg N/L) was chosen in order to try to force the use of polyfunctional mercaptan precursors by the yeast and 150 mg N/L as a sufficient amount to complete the AF (Bely et al., 1990).

For the experiments 3-6, the synthetic juice had the same composition. In order to assure that there was no nitrogen deficiency, a high amount of YAN (200 mg N/L) was chosen. The high amount of nitrogen was obtained by adjusting the percentages of the amino acid profile of the Chardonnay-like profile (increasing the concentration of amino acids by 46%). To this synthetic juice, different compounds have been added individually from stock solutions previously prepared 100 times concentrated by dissolving each compound in milliQ water (with the exception of elemental sulfur that was added directly). In addition, in all cases, a control must without additions was also prepared.

Experiment 3. Effect of sulfur compounds. Seven aliquots of the synthetic juice were prepared to which different sulfur compounds were added individually: elemental sulfur (1 mg/L), GSH (50 and 70 mg/L), methionine (30 and 50 mg/L), cysteine (20 mg/L) and sulfur dioxide (SO_2) (20 mg/L).

Experiment 4. Effect of amino acids that make up the glutathione (GSH). The synthetic juice was divided into 5 aliquots to which cysteine (10, 20 and 30 mg/L), glycine (10 mg/L) and glutamic acid (50 mg/L) were added.

Experiment 5. Effect of sulfur dioxide (SO_2). The synthetic juice was divided into four aliquots to which different concentrations of SO_2 (20, 30, 50 and 70 mg/L) were added.

Experiment 6. Transcriptomic study during fermentation. The synthetic juice was divided into 5 aliquots to which GSH (50 and 70 mg/L) and cysteine (10, 20 and 30 mg/L) were added. In this experiment, three set of fermentations were performed, the first one in order to identify the moment in which the polyfunctional mercaptans are released from their precursors, and the second one to evaluate the growth of yeast population. With the results obtained in the first sets of fermentations, the times in which the transcriptomic analyses would be carried out in the third set of fermentations were decided. The third set of fermentations was carried out for the transcriptomic study.

2.2.2. Polyfunctional mercaptan precursors

The odorless precursors, GLUMP, GLUMH, CYSMP, and CYSMH were added together as stock solution. The stock was prepared by dissolving the precursors 1000 times concentrated in milliQ water. Then, an aliquot with the necessary volume to obtain final concentrations of 50 µg/L for GLUMP and CYSMP, 1000 µg/L for GLUMH and 100 µg/L for CYSMH were added to the synthetic juice.

Moreover, for the experiment 2, the cysteinylated precursors (with the same aforementioned concentrations) were added to a synthetic must of Chardonnay-like profile which had 120 and 150 mg N/L, and the same was done with the glutathionylated precursors.

2.2.3. Yeast strain

The yeast used was *Saccharomyces cerevisiae* Zymaflore X5. This yeast strain was chosen because it has previously been demonstrated to have a high aptitude for releasing

polyfunctional mercaptans from their precursors (Masneuf-Pomarede, Mansour, Murat, Tominaga, & Dubourdieu, 2006). The fermenters were inoculated with 10^6 cells/mL previously grown at 37 ºC for 20 minutes in milliQ water and for 10 minutes in the synthetic juice.

2.2.4. Fermentations

The experiments 1-5 were carried out in small fermenters (100 mL), while the experiment 6 was carried out in fermenters of 1L, both with fermentation locks (Muller valves). All fermentations were done at 20 °C in triplicate. The monitoring of the fermentation was based on the release of CO_2 until reaching constant weight (Bely et al., 1990). The resulting synthetic wines were centrifuged at 4500 rpm for 15 minutes and then stored at 4 ºC for further analyses of polyfunctional mercaptans and their precursors.

Moreover, samples obtained in the experiment 6 were also analyzed by transcriptomic analysis. To that end, a volume of sample corresponding to 10^9 cells was taken and cold centrifuged (4 ºC), 2 min at 3000 rpm. The cell pellet was rapidly washed in 750 µL of colled (4 ºC) DEPC-treated water (water treated overnight with diethylpyrocarbonate and then sterilized for 15 min at 120 °C) and cooled in the ice. After cold centrifugation (4 ºC) 15 seconds at 13000 rpm, the supernatant is removed and the cells are frozen rapidly in a -80 ºC methanol bath.

2.3. Analyses

At the end of the different fermentations, the polyfunctional mercaptans, as well as their precursors were analyzed.

In addition, in the case of the experiment 6, samples were taken at different moments during the fermentation. In the first set of fermentations, for the analyses of the polyfunctional

mercaptans and their precursors, samples were taken at 15, 46, 159, 255, 328 hours and at the end of alcoholic fermentation. In the second set of fermentations, for counting the cell population, samples were collected each 4 hours during 4 times per day for the first 72 h, and then every 24 h until the fermentation was completed.

On the other hand, in the third set of fermentations of the experiment 6, samples were collected at 26 h and 150 h, in each moment transcriptomic analyses were performed.

2.3.1. Counting of the cell population

The cell populations were evaluated using an electronic particle counter (Multisizer 3 Coulter Counter, Beckman Coulter) (Allen, 1990). The samples were previously diluted to remain in the linear range between 20000 and 80000 cells/mL. The cell aggregates were then destroyed by sonication with a generator of utra-sounds (Branson Sonifier, model 250). This measurement allows us to obtain the number of cellules per mL, as well as to know the average volume of yeasts (μm^3).

2.3.2. Cysteinylated and glutathionylated precursor analyses

The precursors CYSMH, CYSMP, GLUMH and GLUMP were analyzed following the procedure validated by Concejero et al., (Concejero, Peña-Gallego, Fernández-Zurbano, Hernández-Orte, & Ferreira, 2014) as was previously described in the section 2.7.2 of chapter I.

2.3.3. Polyfunctional mercaptans measurement

The analysis of 4MMP, 3MHA and 3MH in the samples was carried out following the method proposed and validated by Mateo-Vivaracho et al., (Mateo-Vivaracho et al., 2010). As was previously described in the section 2.7.5 of chapter I.

2.3.4. Transcriptomic analyses

The study of gene expression is based on the analysis of the transcriptome consisting of all the messenger RNAs (mRNAs) present in a cell at a given time and in a given situation. The technique used here implements DNA chips or microarray of Agilent type 8x15 in single color. These chips consist of glass plates on which strands of single-stranded DNA complementary (cDNA) to an mRNA are attached.

Sampling. Previous any transcriptomic analysis, and in order to determine the volume of sample necessary to have 10^9 cellules, samples were taken and the cell population was counted following the procedure described previously in the section 2.3.1 of chapter II. Then, aliquots of 10^9 cellules were collected at the selected time-points during AF (26 h and 150 h) and were treated and stored as was previously indicate in the section 2.2.4 of chapter II.

Transcriptomic analysis. The cell cultures were collected using the Trizol method described by Chomczynski et al., (Chomczynski & Sacchi, 1987). The mRNAs were extracted from cells and undergone retro-transcription and then transcription in the presence of nucleotides labeled with a fluorochrome. The labeled complementary RNAs (cRNAs) were purified and hybridized on DNA chips. This is a glass plate divided into 8 hybridization zones (or gasket wells), each containing approximately 15000 probes. Each sample was placed in a gasket well, and then the array (slide carrying the DNA chips) was put down onto the gasket slide. The assembly thus formed was placed in the hybridization oven and incubated at 65 ºC for 17 h. The labeled cRNA fragments bind to the DNA strands by complementarity of bases. After incubation, the slide was washed, dried and then scanned on the GenePix 4000B Microarray Scanner from Molecular Devices LLC

(California, USA). Differences in gene expression are evaluated based on the intensity of the fluorescence (Duc, Pradal, Sanchez, Noble, Ternière, & Blondin, 2017).

Quantification of RNA. The RNAs were quantified by measuring absorbance at 260 nm with a NanoDrop apparatus.

Quality control. In order to validate the quantity and quality of extracted and purified RNAs, capillary electrophoresis were performed before carrying out the microarray labeling and after the labeling of mRNAs. Capillary electrophoresis were carried out using the Bioanalyzer 2100 RNA 6000 Nano Kit from Agilent Technologies (Santa Clara, USA).

Statistical analyses of microarray data. The R software with version R.3.6.1 version was used for statistical analyses (R Core Team, 2014). The raw microarray data were imported and normalized with the quantile method for normalization between arrays using the limma package (Smyth & Speed, 2003) and the PCA analyses were done using the FactoMineR package (Lê, Josse, & Husson, 2008). The differential analysis was performed using the limma package, in which for each gene, a linear model is applied to assess whether the gene is statistically differentially expressed (over-or under-expression). This model takes into account the duplicate probes on each slide. Some genes are quadriplicats and not duplicates but these genes have two different identifications (IDs). Sorting takes place by ID, they are considered as two different genes. Only genes with significant changes between the samples (an adjusted p-value threshold of 0.05) were selected.

For a functional analysis of the defined genes, the list of genes was analyzed using the web-based tool GeneCodis (http://genecodis.cnb.csic.es/; adjusted p-value = 0.05). GeneCodis is a tool for finding biological annotations that frequently co-occur in a set of genes from different sources (for example, KEGG pathways, GO, Swiss-Prot keywords, and InterPro motifs) and rank them by statistical significance (Carmona-Saez, Chagoyen, Tirado,

Carazo, & Pascual-Montano, 2007; Nogales-Cadenas, Carmona-Saez, Vazquez, Vicente, Yang, Tirado, Carazo, & Pascual-Montano, 2009; Tabas-Madrid, Nogales-Cadenas, & Pascual-Montano, 2012). The genes were input into the web-based tool and analyzed the annotations in regard to the biological processes and metabolic pathways using the GO database (Robinson, Grigull, Mohammad, & Hughes, 2002) and KEGG database (Kyoto Encyclopedia of Genes and Genomes) (https://www.genome.jp/kegg/pathway.html).

2.4. Statistical analyses

Analysis of variance (ANOVA) followed by Duncan's post-hoc test were used to establish the significant differences among fermentations for each precursor and polyfunctional mercaptan. The analyses were carried out using SPSS software (SPSS Inc., Chicago, IL) for Windows, version 19. Different letters express significant differences with a significance level of 95%.

Simple correlation studies between polyfunctional mercaptan concentrations and precursor concentrations in the fermentations were carried out using Excel 2013 (Microsoft).

Furthermore, principal component analysis (PCA) using XLSTAT software (version 2014.2.02) was carried out to illustrate the influence of the amino acid profile on the concentration of the mercaptans.

3. RESULTS AND DISCUSSION

3.1. Experiment 1. Effect of the amino acid profile of different grape varieties.

To study the effect of complete amino acid profiles on the release of polyfunctional mercaptans, nine synthetic juice fermentations (in triplicate) whose composition resemble grape juice and simulate the amino acid profile of different grape varieties (Sauvignon Blanc, Chardonnay, Macabeo, Carignan, Grenache, Tempranillo and Cabernet Sauvignon) were carried out. Fermentations were completed after 21-26 days and at the end of the AF the effect of the amino acid profile on the formation of volatile thiol was evaluated. Synthetic musts with low assimilable nitrogen, but enough to complete the AF (Bely et al., 1990), were prepared in order to try to force the use of cysteinylated and glutathionylated precursors by the yeast.

3.1.1. Effect of amino acid profile on the consumption of the polyfunctional mercaptan precursors

A one-way ANOVA analysis was performed in order to determine the statistical significance of the polyfunctional mercaptan precursors remaining at the end of the alcoholic fermentation. As can be observed in Figure II.1, a significantly higher disappearance of CYSMP was observed in the Chardonnay-like profile wines (63.7%), while a significantly lower disappearance was observed in the Cabernet Sauvignon-like profile wines (10.8%). Likewise, as can be seen in Table II.2, the must with Chardonnay-like profile has 2-3 times more concentration of most of the amino acids in comparison with Cabernet Sauvignon-like profile, except for the amino acids histidine, proline and methionine that have 7, 4 and 3 times more concentration on the must with Cabernet

Sauvignon-like profile, respectively. A similar behavior was observed for the 3MH precursors (Figure II.1) where again there was a significantly higher use in the Chardonnay-like profile wines (99.4% for CYSMH and 98.6% for GLUMH) and a significantly lower disappearance of these precursors in the Cabernet Sauvignon-like profile wines (0% for CYSMH and 96.9% for GLUMH). Moreover, significantly higher consumptions of CYSMH (99.1%) and GLUMH (98.6%) were also observed in the Tempranillo and Grenache-like profile wines, respectively (Figure II.1). These amino acid profiles have lower concentration of serine, proline, methionine, histidine, and threonine compared with Cabernet Sauvignon-like profile (Table II.2) that showed lower consumption of 3MH precursors.

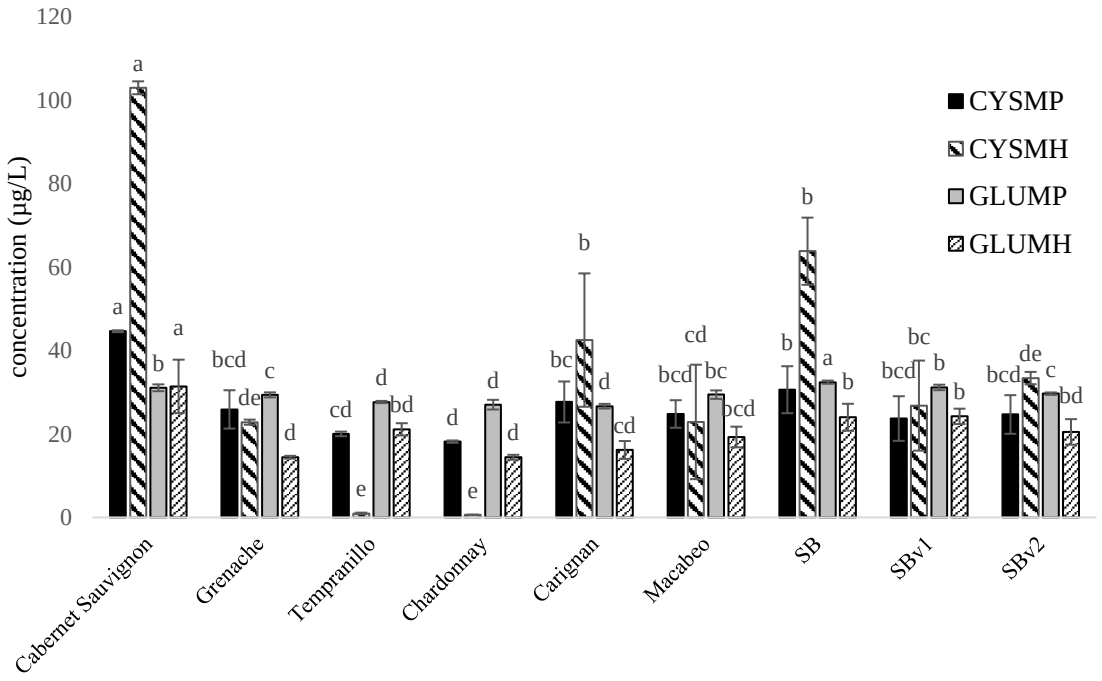


Figure II.1. Polyfunctional mercaptan precursors concentration (µg/L) remaining at the end of the alcoholic fermentation of synthetic mediums simulating 7 different grape variety-like profiles and 2 versions of the SB-like profile. Error bars represent the standard deviations of the means of the triplicates. Different letters indicate significant differences according to Duncan's post-hoc test (significance level 95%).

On the other hand, significantly higher consumptions of GLUMP (44.7 - 46.7%) were observed in the Chardonnay, Tempranillo and Carignan-like profile wines (Figure II.1),

while there was a significantly lower consumption in the SB-like profile wines (35.2%). Furthermore, as can be observed in Table II.2, SB-like profile has 2-4 times lower concentration of the amino acids histidine and methionine than Chardonnay, Tempranillo and Carignan-like profiles, that showed a higher consumption of GLUMP. Hence, with the same must composition and the same YAN, and the amino acid profile being the only difference, the yeast strain did not use the polyfunctional mercaptan precursors during AF in the same manner. Therefore, the necessity of the yeast to use the polyfunctional mercaptan precursors is dependent on the amino acid composition.

3.1.2. Effect of amino acid profile on the release of polyfunctional mercaptans

To test whether the amino acid profile exerts an influence on the release of polyfunctional mercaptans, these compounds were analyzed at the end of the AF. As can be observed in Table II.3, the Chardonnay-like profile wines released significantly more 4MMP (262 ng/L) and 3MH (233 ng/L) than wines with other amino acid profiles (except for Carignan, SBv1, and Macabeo-like profiles that did not show significant differences in the release of 3MH). The Cabernet Sauvignon-like profile wines released a significantly lower amount of 3MH (99.9 ng/L) and the third lowest concentration of 4MMP (80.8 ng/L). Moreover, Carignan and SBv1-like profile wines also showed significantly lower amounts of 4MMP, 72.0 ng/L and 63.2 ng/L respectively. Likewise, these amino acid profiles have lower amount of serine, threonine, phenylalanine, lysine and glutamine than Chardonnay-like profile (Table II.2). For the rest of the wines, as can be observed in Table II.3, the concentrations obtained fell between those of Chardonnay and Cabernet Sauvignon-like profiles. In addition, it is known that oenological and environmental conditions vary the amount of 4MMP and 3MH released in SB wines, there being large differences in the

concentrations of these compounds in different countries. For example, there are higher concentrations in these wines in New Zealand and lower concentrations in Spain or France (Mateo-Vivaracho et al., 2010). This explains why the SB-like profile wines did not generate the highest concentrations of these compounds. In the case of 3MHA, as can be observed in Table II.3, this compound was obtained in a significantly higher concentration in the Tempranillo-like profile wines (7.05 ng/L), whereas the Cabernet Sauvignon, SB and SBv2-like profiles did not generate concentrations of 3MHA above the limit of detection. Tempranillo-like profile have lower concentration of glutamic acid, phenylalanine and GABA than Cabernet Sauvignon, SBv2 and SB-like profile (Table II.2), in which no concentration was observed above the limit of detection. Thus, the amino acid profile modifies the acetylation process, probably by affecting the alcohol acetyltransferase (AAT). Interestingly, the model wines showed lower concentrations of 3MHA compared to real wines, which could mean that the synthetic must may contain some compound that inhibits acetyltransferase, or could mean that real wine may contain some compound that favors acetylation.

Table II.3. Concentration of 4-mercapto-4-methyl-2-pentanone (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) (ng/L) in the wines obtained after alcoholic fermentation of 9 different synthetic musts which simulate the amino acid profiles of different grape varieties.

	4MMP	**3MH**	**AMH**
Olfactory threshold	**0.80***	**60.00***	**4.00***
Cabernet Sauvignon-like profile	80.8 ± 4.58^{de}	99.9 ± 28.1^{d}	< LQ
Grenache-like profile	141 ± 6.35^{b}	16 ± 43.7^{bc}	2.74 ± 0.54^{c}
Tempranillo-like profile	162 ± 5.22^{b}	133 ± 23.5^{cd}	7.05 ± 0.49^{a}
Chardonnay-like profile	262 ± 16.7^{a}	233 ± 21.2^{a}	4.33 ± 0.57^{b}
Carignan-like profile	72.0 ± 7.47^{e}	193 ± 10.9^{ab}	0.65 ± 1.13^{d}
Macabeo-like profile	105 ± 2.27^{cd}	185 ± 20.4^{ab}	2.59 ± 0.71^{c}
SB-like profile	158 ± 5.72^{b}	134 ± 21.3^{cd}	< LQ
SBv1-like profile	63.2 ± 21.5^{e}	193 ± 17.7^{ab}	2.80 ± 1.33^{c}
SBv2-like profile	113 ± 12.9^{c}	114 ± 9.64^{cd}	< LQ

< LQ: below quantification limit.

[a, b. c. d, e] different letters indicate significant differences (significance level 95%) in the different samples of the alcoholic fermentation of each variety.

* Perception thresholds (Tominaga, Murat, et al., 1998).

The multidimensional representation (Principal Component Analysis, PCA) revealed a clear varietal influence on the release of the polyfunctional mercaptans (Figure II.2). The first principal components (PC1 and PC2) accounted for 34.91% and 22.35% of the total variation, respectively. The Principal Component plot shown in Figure II.2 highlights the relevance of the Chardonnay-like profile for the release of 4MMP and 3MH. As can be observed, the Chardonnay-like profile wines, as well as the amino acids aspartic acid, serine, threonine and phenylalanine, appear on the upper right quadrant close to the polyfunctional mercaptans 4MMP and 3MH. In addition, the Tempranillo-like profile wines were placed close to 3MHA.

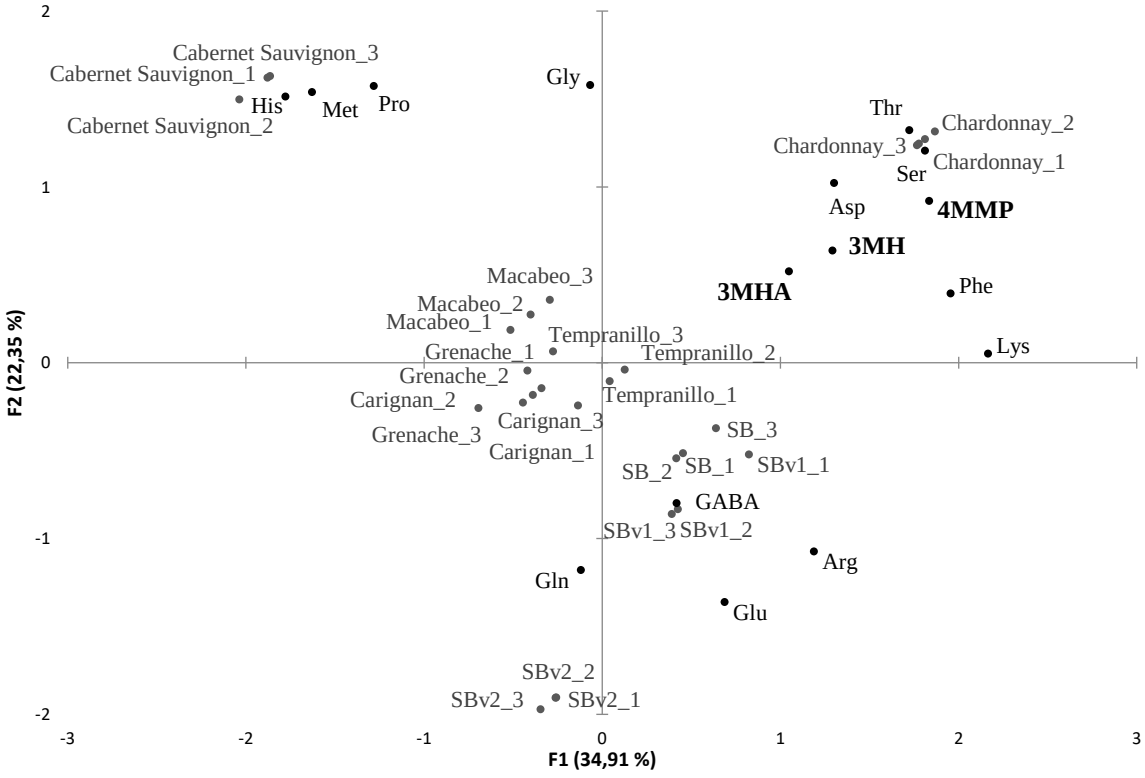


Figure II.2. Projection of the wines with different amino acid profiles simulating 7 different grape variety-like profiles and two versions of the SB-like profile (Cabernet Sauvignon, Macabeo, Grenache, Chardonnay, Tempranillo, Carignan, SB, SBv1 and SBv2) along with the amino acids and polyfunctional mercaptans in the PCA space.

Therefore, the Chardonnay and Tempranillo-like profiles, and in particular the amino acids aspartic acid, serine, threonine and phenylalanine, are related with a higher release of 4MMP, 3MH and 3MHA. On the other hand, the Cabernet Sauvignon-like profile wines appear on the upper left part of the plot opposite the polyfunctional mercaptans (Figure II.2), which could mean that this amino acid profile is related with a lower release of these compounds. Moreover, the SB-like profile wines and its versions (SBv1 and SBv2) were placed close together in the lower quadrant of the plot. These results suggest that the metabolic response of yeast to different amino acid profiles is different and this causes a change in the generation of secondary metabolites, particularly on the release of polyfunctional mercaptans.

3.1.2.1. Effect of the increase in the amounts of GABA, glutamine and glutamic acid on the amino acid profile of the SB variety

Based on previous studies carried out by Pinu et al., (Pinu et al., 2014), the SB-like profile was modified by an increase in the concentrations of the amino acids GABA (obtaining SBv1) and GABA, glutamine and glutamic acid (generating SBv2). The results of the polyfunctional mercaptans released are provided in Table II.3.

Taking SB-like profile wines as the control, the increase in the amino acid GABA in the SBv1-like profile caused significant increases in the concentrations of 3MH and 3MHA, as well as a significant decrease in the release of 4MMP (2.5 times lower) (Table II.3). This decrease in the concentration of 4MMP could indicate that the amino acid GABA exerts a negative effect on the release of this volatile thiol. On the other hand, the increment of the three amino acids in the amino acid profile of the SBv2-like profile also caused a significant decrease in the release of the 4MMP (1.4 times lower) (Table II.3) compared with the SB-like profile wines. This decrease in the concentration of 4MMP in SBv2-like profile wines

was not consistent with previous studies in which glutamine was positively correlated with 4MMP (Pinu et al., 2014). This lack of agreement could be due to the fact that in the case of SBv2-like profile wines, there is not only an increase in glutamine but also in glutamic acid and GABA. As also observed by Pinu et al., the addition of several amino acids together might cause some opposing effects (Pinu et al., 2014), explaining why there was no significant effect on the 3MHA and 3MH. Moreover, this greater decrease in 4MMP in the SBv1-like profile than in the SBv2-like profile wines could be due to an antagonistic effect between GABA and glutamine.

3.1.3. Relation between the release of the polyfunctional mercaptans and the consumption of their precursors

The study of the relationship between polyfunctional mercaptans and their precursors was intended to evaluate whether the disappearance and release of these compounds is also linked to the amino acid profile of the grape variety.

In the Chardonnay-like profile wines, a significantly higher concentrations of 4MMP and one of the highest amount of 3MH (Table II.3) were released compared with other variety like amino acid profiles. Likewise, in these wines, significantly lower concentrations of their precursors at the end of the AF were observed (Figure II.1). Taking into account that the conversion factor was calculated from the precursors added to the must and the polyfunctional mercaptans released, the Chardonnay-like profile wines showed the highest conversion factor (0.525% for CYSMP and GLUMP precursors, 0.225% for CYSMH and 0.023% for GLUMH of the initial precursors). On the other hand, the Cabernet Sauvignon-like profile wines had significantly lower concentrations of 4MMP and 3MH released (Table II.3), with significantly higher concentrations of their precursors at the end of the AF (Figure II.1). Thus, these wines showed lower conversion factors for their precursors

(0.162% for 4MMP precursors, 0.100% for CYSMH and 0.010% for GLUMH). Interestingly, the SB-like profile wines had the lowest disappearance of GLUMP (Figure II.1), but released a significantly greater amount of 4MMP than most of the varieties studied (Table II.3). Thus, these wines showed high conversion factors, 0.315%. Conversely, in the case of the Carignan and Tempranillo-like profile wines for 4MMP, and Grenache and Tempranillo-like profile wines for 3MH, the yeast consumed significantly higher amounts of the precursors (Figure II.1) which did not result in the highest release of 4MMP and 3MH (Table II.3). This could mean that the use of precursors by yeast during AF depends on the amino acid profile, these precursors being used for replacing the lack of some compound in the different varieties. For the rest of the wines with other amino acid profiles, the ranges of conversion between 4MMP and their precursors were 0.210% - 0.323%, and between 3MH and their precursors were 0.114% - 0.193% for CYSMH and 0.011% - 0.019% for GLUMH. The ranges of transformation were similar to those observed in previous studies (Peyrot des Gachons et al., 2000; Roland, Schneider, Le Guerneve, Razungles, & Cavelier, 2010; Roland, Schneider, Razungles, et al., 2010).

Hence, the differences in consumption of both precursors and the release of polyfunctional mercaptans are due to amino acid profiles as well as variations in the ratios of the amino acids. The question, therefore, is what the yeasts need to use the polyfunctional mercaptan precursors in different ways.

3.2. Experiment 2. Effect of the precursors added separately

In the first experiment we observed that more than 40% of the 4MMP precursors and more than 75% of the 3MH precursors (with some exceptions) disappeared, whereas few polyfunctional mercaptans were released. In order to determine whether the total YAN and the type of precursors have an influence on the consumption and release of polyfunctional

mercaptans, musts with different amounts of YAN (120 and 150 mg N/L) were prepared to which cysteinylated and glutathionylated precursors were added in separate fermentations. For both amounts of YAN, a control with the 4 precursors together was used. The experiment was carried out with the Chardonnay-like profile medium because in the fermentations mentioned in the experiment 1 Chardonnay-like profile showed to be the amino acid profile that generated a significantly more 4MMP and a higher, although no significant, concentration of 3MH as well as a high consumption of the precursors. This amino acid profile was adjusted to reach 120 mg N/L and 150 mg N/L in order to test whether in musts with a deficiency of nitrogen (Bely et al., 1990) the yeast uses the precursors as a nitrogen source.

3.2.1. Effect of the use of cysteinylated and glutathionylated precursors added separately on the release of polyfunctional mercaptans

In the case of 4MMP, it was observed that the wines with cysteinylated precursors released practically the same concentrations as the control wines that possessed both types of precursor (Figure II.3).

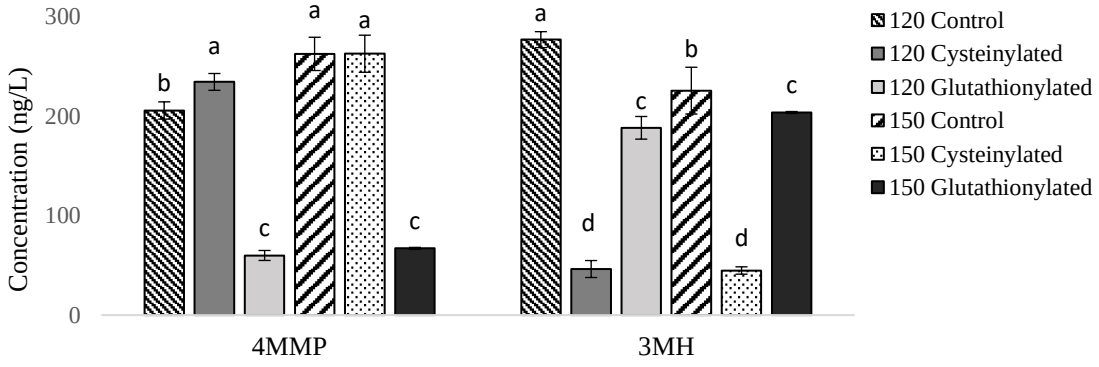


Figure II.3. Concentration of 4MMP and 3MH (ng/L) in the wines obtained after alcoholic fermentation of synthetic juice prepared at two levels of YAN (120 and 150 mg N/L) and with cysteinylated and glutathionylated precursors added separately, as well as two control wines with both types of precursor. Error bars represent the standard deviations of the means of the triplicates. Different letters indicate significant differences according to Duncan's post-hoc test (significance level 95%).

In contrast, when the precursors used were the glutathionylated precursors, the wines generated only 25% of the 4MMP compared to the control wines. Both data were similar at different nitrogen levels, 120 and 150 mg N/L. This indicates that the main precursor of 4MMP is CYSMP. Moreover, to the best of our knowledge, this is the first time that the preference of the yeast for the use of both types of 4MMP precursor has been studied in separate fermentations. In addition, at the end of the AF, the remaining precursors were also analyzed and the results are provided Table II.4. In the case of 4MMP precursors, significantly higher consumptions of CYSMP (52.6% - 55.5%) than GLUMP (16.3% - 8.98%) precursors were observed at both concentrations of nitrogen when the precursors were added in separate fermentations. Likewise, the same results were observed in the control wines with the 4 precursors, with a significantly higher consumption of CYSMP (62.8% - 75.8%) than GLUMP (11.8% - 49.6%) precursors.

Table II.4. Polyfunctional mercaptans precursor concentrations (µg/L) remaining at the end of the alcoholic fermentation of the Chardonnay-like profile wines at two amounts of YAN (120 and 150 mg N/L) with the addition of cysteinylated and glutathionylated precursors together and separately.

	CYSMH	CYSMP	GLUMH	GLUMP
Chardonnay-like profile 150 mg N/L Control CYS/GLU precursors	47.3 ± 0.44^{a}	18.6 ± 2.13^{b}	7.48 ± 1.26^{b}	25.2 ± 3.83^{b}
Chardonnay-like profile 120 mg N/L Control CYS/GLU precursors	0.42 ± 0.13^{b}	12.1 ± 0.22^{c}	57.6 ± 1.80^{a}	44.1 ± 0.68^{a}
Chardonnay-like profile 150 mg N/L Cysteinylated precursors	0.68 ± 0.30^{b}	23.7 ± 1.67^{a}	< LQ	< LQ
Chardonnay-like profile 120 mg N/L Cysteinylated precursors	0.39 ± 0.01^{b}	22.2 ± 0.72^{a}	< LQ	< LQ
Chardonnay-like profile 150 mg N/L Glutathionylated precursors	0.30 ± 0.07^{b}	< LQ	58.3 ± 3.36^{a}	41.9 ± 2.88^{a}
Chardonnay-like profile 120 mg N/L Glutathionylated precursors	0.35 ± 0.06^{b}	< LQ	57.5 ± 3.19^{a}	45.5 ± 1.93^{a}

< LQ: below quantification limit.
$^{a, b, c}$ different letters indicate significant differences (significance level 95 %) in the different samples of the alcoholic fermentation of each variety.

On the other hand, in the case of 3MH, at both nitrogen levels of 120 and 150 mg N/L, amounts close to 90% of those observed in the control wines were released when the glutathionylated precursors were used (Figure II.3). Conversely, the wines with the cysteinylated precursors released only 20% of the 3MH concentrations found in the control wines. Therefore, the main precursor in the case of 3MH is GLUMH, which corroborates previous studies (Subileau, Schneider, Salmon, & Degryse, 2008a).

Likewise, in the case of 3MHA, no significant differences were found. This compound was only generated when the glutathionylated precursors were used (1.45 ng/L for 120 mg N/L and 4.27 ng/L for 150 mg N/L). The absence of significant differences indicates that there is no direct correlation with 3MH precursors, as has been reported previously (Winter, Van Der Westhuizen, Higgins, Curtin, & Ugliano, 2011).

3.3. Experiment 3. Effect of sulfur compounds

The low conversion factor between polyfunctional mercaptans and their precursors, could be due to yeasts use precursors as a source of some nutrient. This nutrient could be a sulfur compound. In order to test it, different fermentations of synthetic juice with the addition of different sulfur compounds were carried out.

3.3.1. Effects of the addition of sulfur compounds on the concentration of polyfunctional mercaptans precursors

Results of the fermentations carried out on synthetic juices to which different S-compounds were spiked, are summarized in Table II.5. The GLUMH metabolization was higher than 62.8% (with the exception of the samples spiked with GSH at both concentrations and methionine at 50 mg/L), while remaining levels of CYSMH and CYSMP were close or higher to their initial concentrations in all cases (between 81.8 and 141 µg/L in the case of

CYSMH and between 39.1 and 50.6 µg/L for CYSMP). This higher metabolization of GLUMH indicates that GLUMH is the main precursor of 3MH, which corroborates the results observed in the experiment 2, as well as previous studies (Subileau, Schneider, Salmon, & Degryse, 2008b), but it is contrary to the results observed by Winter et al., (Winter, Van Der Westhuizen, et al., 2011). On the other hand, remaining GLUMP levels were three times smaller than those of CYSMP, which may suggest that yeast could transform GLUMP into CYSMP. The identification of S-4-mercapto-4-methylpentan-2-one-l-cysteinyl-glycine (CysGly-4MMP) and S-4-mercapto-4-methylpentan-2-one-N-(l-γ-glutamyl)-l-cysteine (γGluCys-4MMP) recently reported by Bonnaffoux et al., supported our findings (Bonnaffoux et al., 2018; Bonnaffoux, Roland, Rémond, Delpech, Schneider, & Cavelier, 2017). It can be observed in Table II.5 that control wines and samples with the addition of elemental sulfur, methionine at 50 mg/L and GSH at 70 mg/L, contained CYSMH above initial levels (100 µg/L), which evidences the active formation of CYSMH from GLUMH, as it was previously demonstrated by Peyrot des Gachons et al., (Peyrot des Gachons et al., 2002).

Table II.5. Polyfunctional mercaptan precursors concentrations (µg/L) remaining at the end of the alcoholic fermentation of wines with the addition of different sulfur compounds: SO_2 (20 mg/L), elemental sulfur (1 mg/L), cysteine (20 mg/L), glutathione (50 and 70 mg/L) and methionine (30 and 50 mg/L), as well as a control without these additions.

	CYSMP	**CYSMH**	**GLUMP**	**GLUMH**
Initial level µg/L	50	100	50	1000
Control	47.4 ± 2.18^{bc}	138 ± 3.83^{ab}	16.4 ± 0.15^{bcd}	225 ± 1.47^{g}
SO_2 (20 mg/L)	39.6 ± 3.38^{e}	94.7 ± 9.68^{de}	15.3 ± 0.51^{d}	164 ± 7.90^{h}
Elemental sulfur	49.7 ± 0.87^{ab}	141 ± 8.54^{a}	16.5 ± 1.00^{bcd}	273 ± 32.9^{f}
Cys (20 mg/L)	39.1 ± 0.38^{e}	85.9 ± 5.69^{ef}	15.8 ± 0.55^{cd}	337 ± 22.4^{e}
GSH (50 mg/L)	41.6 ± 1.87^{de}	81.8 ± 8.26^{f}	17.2 ± 0.46^{b}	754 ± 42.8^{b}
GSH (70 mg/L)	46.1 ± 2.15^{bc}	116 ± 3.23^{c}	18.6 ± 0.97^{a}	844 ± 49.2^{a}
Met (30 mg/L)	44.6 ± 2.56^{cd}	86.1 ± 4.81^{ef}	17.4 ± 0.16^{ab}	372 ± 13.8^{de}
Met (50 mg/L)	50.6 ± 2.51^{a}	128 ± 4.60^{b}	17.5 ± 1.08^{ab}	529 ± 17.2^{c}

^{a,b,c,d,e,f,g,h} Different letters indicate significant differences according to Duncan's post-hoc test (significance level 95%) in the different samples.

It should be also remarked that, although the conversion of cysteinylated precursors from glutathionylated have not been taken into account, remaining levels of CYSMP were significantly correlated with remaining levels of CYSMH (r=0.803; significant at P=0.007) and GLUMP levels with GLUMH levels (r=0.669; significant at P=0.01). Anyway, although one-way ANOVA identified a significant effect associated to the S-source in all cases, the effects were of minor magnitude and surely also of minor relevance for all cases except CYSMP and GLUMH. Only results for these cases will be discussed.

As can be observed in Table II.5, the addition of SO_2, cysteine and GSH at 50 mg/L brought about a decrease in the CYSMP levels (between 16.8 and 21.8% of CYSMP disappearance) compared to the control (5.20%). A major mobilization of amino acids and GSH transporters (Gap1 and Opt1, respectively) could explain this higher metabolization (Cordente, Capone, & Curtin, 2015; Subileau et al., 2008a, 2008b). On the other hand, in the case of GLUMH, as shown in Table II.5, only the addition of SO_2 brought about an increase in the level of metabolization of this precursor (83.6%) compared to the control (77.5%). In all the other cases, the addition of the S-molecule have prevented decreases in postfermentative levels of GLUMH in comparison with the control. In the two cases in which different levels of added S-compound were assayed, there were negative correlations (r=-0.811 and r=-0.989 for samples spiked with GSH and methionine, respectively). Higher concentration of the S-compound added led to a lower metabolization of GLUMH (24.6% vs 15.6% in the case of the GSH additions and 62.8% vs 47.1% in the case of methionine additions) (Table II.5). The higher amounts of GSH and methionine additions could either provide more sulfur, which would cause the yeast not to need the sulfur present in the precursor, or have an inhibitory effect on the metabolization of this precursor (Takahashi & Takahashi, 1968). It is worth noting that the addition of GSH strongly prevented the metabolization of GLUMH (less than 25.0% compared to 77.5% observed in the control),

which suggests that yeasts use this precursor as a source of GSH, which can be used for: i) its protective activity against oxidative stress (Dubourdieu & Lavigne-Cruege, 2004; Hébert et al., 2011; Makhotkina, Dias Araujo, Olejar, Herbst-Johnstone, Fedrizzi, & Kilmartin, 2014; Ugliano, Kwiatkowski, Vidal, Capone, Siebert, Dieval, Aagaard, & Waters, 2011; Wegmann-Herr et al., 2016); ii) as a sulfur source (Vos & Gray, 1979; Winter, Henschke, Higgins, Ugliano, & Curtin, 2011); or iii) to obtain the amino acid residues that constitute the tripeptide GSH (glutamic acid, cys-3MH and glycine) (Bonnaffoux et al., 2017; Capone, Pardon, Cordente, & Jeffery, 2011; Ganguli, Kumar, & Bachhawat, 2007; Jaspers, Gigot, & Penninckx, 1985; Ubiyvovk, Blazhenko, Gigot, Penninckx, & Sibirny, 2006). It could also be possible that GSH influences the gamma-glutamyl transpeptidase (Ecm38p) which, as Cordente et al., have demonstrated, is crucial for initiation of vacuolar GLUMH degradation (Cordente et al., 2015), or the transpeptidase CIS2 which is also required for the conversion of GLUMH into 3MH (Gardner & Santiago, 2015).

3.3.2. Impact of the addition of sulfur compounds on the concentration of polyfunctional mercaptans

Results from the experiment in which a synthetic juice was spiked with different sulfur compounds at different levels are summarized in Figure II.4. A first observation that should be made is that there was significant correlations between the levels of the three volatile compounds, 4MMP, 3MHA and 3MH, in the 8 different treatments ($r=0.622$; 0.731 and 0.840 for the pairs 4MMP-3MH, 4MMP-3MHA and 3MH-3MHA; significant at $P < 0.05$); i.e., the different additions had a similar effect on the different polyfunctional mercaptans. The second observation is that the clearest effect was an increase in the levels of all polyfunctional mercaptans in synthetic juice containing 20 mg/L of SO_2 (355%, 50.9% and

2.31% for 4MMP, 3MH and 3MHA in comparison with the control). These increases are consistent with the fact that in all these samples, the four precursors were metabolized to a greater extent than in the controls (Table II.5), so that, although it may be argued that SO_2 is preventing the oxidation of these compounds (Blanchard, Darriet, & Dubourdieu, 2004; Coetzee, Lisjak, Nicolau, Kilmartin, & du Toit, 2013; Makhotkina, Herbst-Johnstone, Logan, du Toit, & Kilmartin, 2013), higher polyfunctional mercaptan levels due to a higher precursor disappearance seems to be also plausible. Third, the addition of cysteine also brought about a relevant increase in the levels of the three aroma compounds (216%, 30.8% and 1.95% for 4MMP, 3MH and 3MHA, respectively) compared to the control, being the increases particularly noticeable for 4MMP (Figure II.4). Harsch and Gardner also observed higher levels of 3MH and 3MHA when cysteine was added to SB musts (Harsch & Gardner, 2013). This is in agreement with the fact that in the sample added with cysteine, cysteinylated precursors were metabolized to a greater extent than in the control (Table II.5). Fourth, the addition of GSH and methionine followed a dose-dependent effect in which the addition of higher concentrations of added S-compounds led to a lower polyfunctional mercaptan concentrations. This is related to the results observed in the metabolization of the precursors, in which a significantly lower metabolism of GLUMH and of CYSMP is observed at the highest dose of the sulfur compound added (Table II.5). In addition, in both cases the additions caused a significant increase of 4MMP versus the control (with the exception of the addition of 50 mg/L of methionine in which a non-significant decrease was observed). On the other hand, the addition of GSH led to a decrease of 3MH compared to the control, but there was no significant difference in the samples added with methionine (Figure II.4). In the case of 3MHA, no effect was observed. This high amount of 4MMP in the samples spiked with GSH could be due to the fact that GSH prevents the oxidation (Makhotkina et al., 2014; Vaimakis & Roussis, 1996) or

alternatively, it could act as a sulfur donor (Vos & Gray, 1979; Winter, Henschke, et al., 2011). By contrast, the lower concentration of 3MH in the samples added with GSH is contrary to that observed by Ugliano et al., and Makhotkina et al., (Makhotkina et al., 2014; Ugliano et al., 2011) but is in agreement with the results observed by Patel et al., (Patel et al., 2010). It could be due to the significantly lower disappearance of GLUMH in the samples in which GSH was added (Table II.5). On the other hand, the decrease in the concentration of 4MMP in the samples added with 50 mg/L of methionine could be because high concentrations of this compound could have an inhibitory effect on the yeasts (Takahashi & Takahashi, 1968). Finally, the addition of elemental sulfur caused a significant higher formation of 3MHA (1.95%) compared to the control wine, which corroborates previous studies by Araujo et al., (Araujo, Vannevel, Buica, Callerot, Fedrizzi, Kilmartin, & du Toit, 2017) and it indicates that elemental sulfur could have an important role as a sulfur donor in the formation of this thiol.

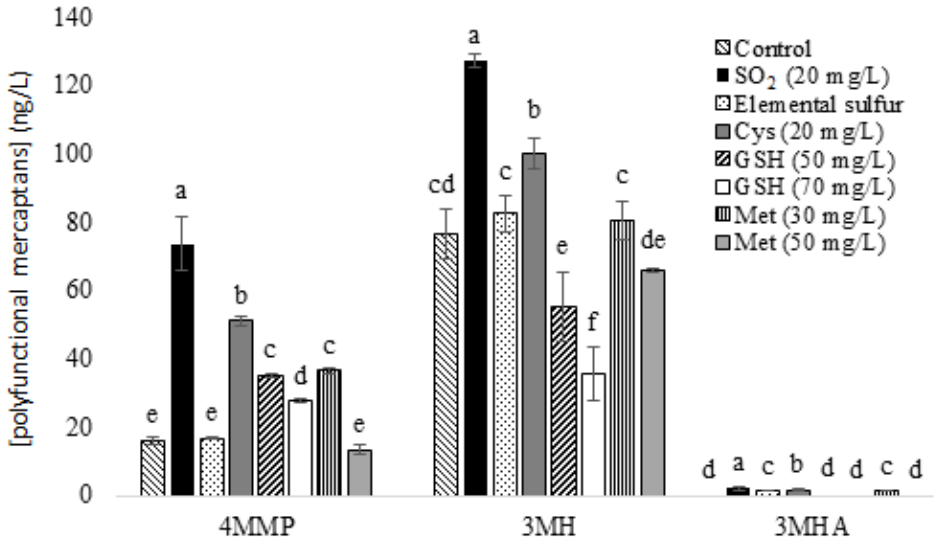


Figure II.4. Concentration of 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexyl acetate (3MHA) and 3-mercaptohexan-1-ol (3MH) (ng/L) in the wines obtained after alcoholic fermentation of synthetic juices prepared with the addition of different sulfur compounds: SO_2 (20 mg/L), elemental sulfur, cysteine (20 mg/L), glutathione (50 and 70 mg/L) and methionine (30 and 50 mg/L), as well as a control without additions. Error bars represent the standard deviations of the means of the triplicates. Different letters indicate significant differences according to Duncan's post-hoc test (significance level 95%).

It is worth noting that the 4MMP levels, 10 to 80 ng/L (Figure II.4), were quite similar to concentrations in commercial wines, < 1 to 60 ng/L (Mateo-Vivaracho et al., 2010; Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud-Funel, 2006; Tominaga et al., 2000), which proves the relevance of 4MMP precursors to the formation of this compound. However, the 3MH levels, 30 to 130 ng/L (Figure II.4), were below the 3MH levels found in commercial wines, 26-18000 ng/L (Lund et al., 2009; Mateo-Vivaracho et al., 2010; Ribéreau-Gayon, Dubourdieu, et al., 2006), despite the fact that GLUMH metabolization, between 150 and 850 µg/L (Table II.5), was quite comparable with the metabolization of this precursor during the fermentation of real wines (Concejero et al., 2016). These results could prove not only that cysteinylated and glutathionylated precursors are not the only source of 3MH in commercial wines, as it was previously observed (Araujo et al., 2017; Harsch & Gardner, 2013; Schneider et al., 2006; Subileau et al., 2008b), but also the presence of other antioxidants that protect polyfunctional mercaptans against oxidation (Blanchard et al., 2004; Coetzee et al., 2013; Dubourdieu & Lavigne-Cruege, 2004; Makhotkina et al., 2014; Nikolantonaki, Chichuc, Teissedre, & Darriet, 2010; Nikolantonaki, Magiatis, & Waterhouse, 2014; Ugliano et al., 2011). On the other hand, the synthetic wines showed lower concentrations of 3MHA (Figure II.4) compared to real wines (Mateo-Vivaracho, Cacho, & Ferreira, 2007; Mateo-Vivaracho et al., 2010), which could be due to the synthetic juices as was previously observed in the experiment 1.

3.3.3. Relationship between remaining precursor levels and polyfunctional mercaptans formed

The amounts of some precursors remaining at the end of the AF and the amounts of polyfunctional mercaptans produced are significantly and negatively correlated, meaning that the amount of polyfunctional mercaptans formed is significantly and positively

correlated to the amount of metabolized precursor (presented in polyfunctional mercaptans equivalents) by yeast, as will be further shown and discussed.

In the case of 4MMP (Figure II.5), its final levels are significantly correlated to the amount of CYSMP metabolized (r=0.889; significant at P=0.0031), suggesting a main role for this precursor, which corroborates the results observed in the experiment 2. The slope of the function indicates that the proportion of metabolized precursor ending in 4MMP is just 0.79%, which corroborates previous studies (Subileau, 2008; Tominaga, Peyrot des Gachons, et al., 1998), while the intercept indicates that there is a constant amount of 4MMP of 12.7 ng/L coming from a different source. Such source should be the other precursor, GLUMP, which was metabolized in all these samples at a fairly constant rate as seen in Table II.5.

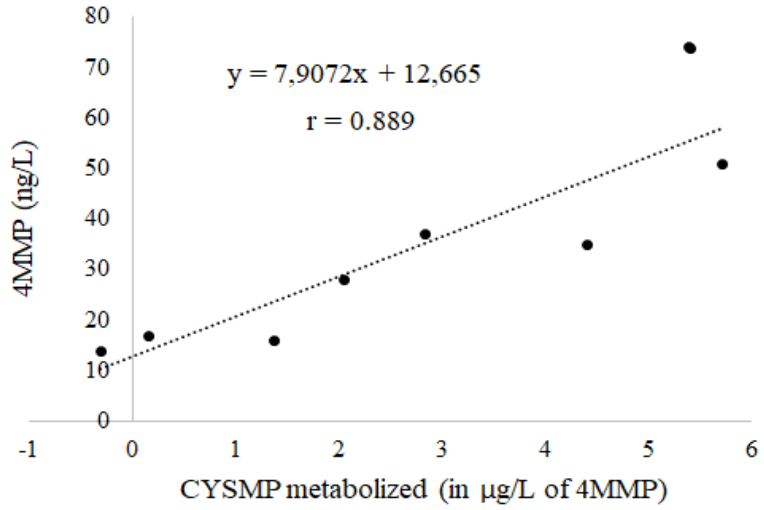


Figure II.5. Correlations between the concentrations of 4MMP with the metabolized CYSMP (presented in 4MMP equivalents).

In the case of 3MH, the most relevant precursor seems to be GLUMH, since it is present at highest levels and there is a highly significant relationship between levels of 3MH and levels of metabolized GLUMH (r=0.862, significant at P=0.0059), as can be seen in Figure II.6a. This is in accordance with Subileau et al., (Subileau et al., 2008b) and with the results observed in the experiment 2 in which it was observed that GLUMH is the main 3MH precursor. However, CYSMH seems to be also relevant, since the correlation 3MH/total

3MH precursor metabolized is still stronger (r=0.923, significant at P=0.0011), as can be seen in Figure II.6b. This could be due to the formation of CYSMH from GLUMH (Peyrot des Gachons et al., 2002). As shown in the figure, the conversion rate is extremely low, just 0.095% of the metabolized precursor ends transformed in 3MH.

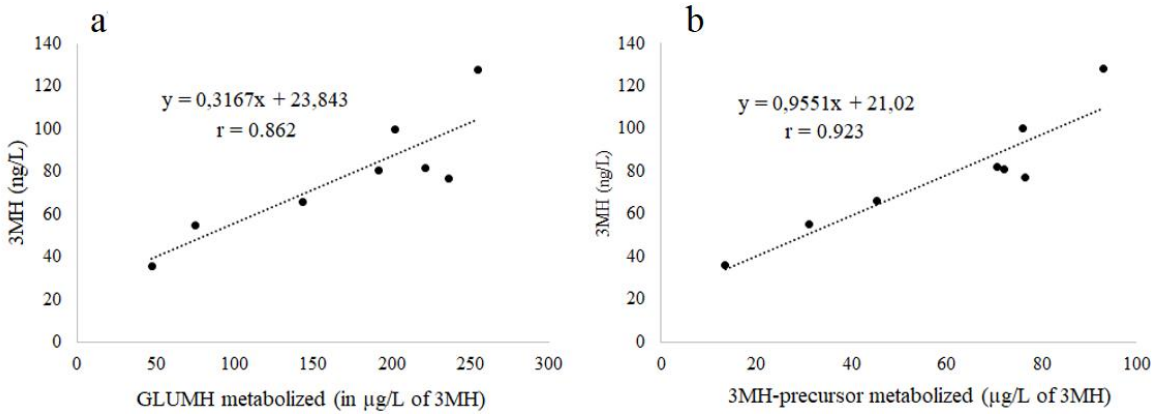


Figure II.6. Correlations between: a) the concentration of 3MH with the metabolized GLUMH (presented in 3MH equivalents); b) 3MH concentrations with metabolized 3MH precursors (presented in 3MH equivalents).

3.4. Experiment 4. Effect of the individual components of glutathione

3.4.1. Effects of the individual components of glutation (GSH) on the concentration of polyfunctional mercaptan precursors

In order to explain why the addition of GSH strongly prevented the metabolization of GLUMH, the effect of the individual amino acid components of GSH was further examined in an individual experiment. Results are summarized in Figure II.7 and reveal that the addition of glutamic acid and glycine did not have any relevant effect on these precursors. Only the addition of glutamic acid induced a decrease in the concentration of cysteinylated precursors, mainly in the case of CYSMH. It is however surprising that the strongest effect was caused by the addition of cysteine on the levels of GLUMH. In addition, previous studies have observed the presence of CysGly-S conjugates and γGluCys-S-conjugates

(Bonnaffoux et al., 2018; Bonnaffoux et al., 2017; Capone, Pardon et al., 2011; Cordente et al., 2015), so yeasts could metabolized glutathionylated precursors in different ways.

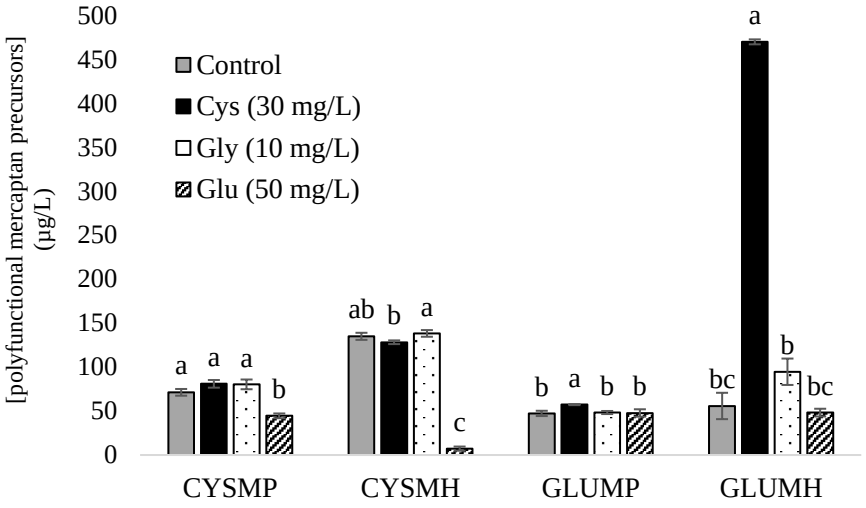


Figure II.7. Concentration of polyfunctional mercaptans precursor (µg/L) remaining at the end of the alcoholic fermentation of musts with the amino acid profile of the Chardonnay-like profile prepared with the addition of the amino acids that are part of the GSH: cysteine (30 mg/L), glycine (10 mg/L) and glutamic acid (50 mg/L), as well as a control without additions. Error bars represent the standard deviations of the means of the triplicates. Different letters indicate significant differences according to Duncan's post-hoc test (significance level 95%).

Due to the presence of significant and relevant differences in the case of cysteine, different levels of cysteine were studied. Results are summarized in Figure II.8 and reveal that the addition of cysteine at 10 and 20 mg/L produced a slight reduction in the remaining levels of CYSMP and CYSMH compared to the control. However, the strongest effect was also caused by the addition of 30 mg/L of cysteine on the levels of GLUMH, observing a lower metabolization of this precursor (53.0%) compared to the control (94.5%), which suggests that yeasts could use this precursor as a source of cysteine, either for the use of the sulfur present in this amino acid, or for the use of the amino acid itself. The contribution of the cysteine present in GSH to H_2S formation previously reported by Winter et al., (Winter, Henschke, et al., 2011) supported our findings. Moreover, previous studies have indicated that cysteine can be used as a component of proteins, as well as a source of organic sulfur

for the synthesis of other sulfur amino acids through transulfuration in homocysteine (Hébert et al., 2011). In fact, it could participate in the formation of methionine and further formation of S-adenosylmethionine (SAM), which is involved in polyamine biosynthesis, such as spermidine that is a polyamine essential for growth (Hamasaki-Katagiri et al., 1997). In addition, this amino acid regulates the transcription of the polypeptide Met4, which not only plays a central role in the regulation of the biosynthetic pathway of sulfur amino acids, but also in the cell cycle (Hansen & Johannesen, 2000).

Although the tendency in both experiments (3 and 4) is the same (the addition of 20 and 30 mg/L of cysteine led to a lower metabolization of GLUMH compared to the control), the GLUMH levels in the experiment 4 were lower than those observed in the experiment 3. This could be due to the fact that, although yeasts were inoculated at 10^6 cells/mL in both cases, the development of the yeast populations could be different.

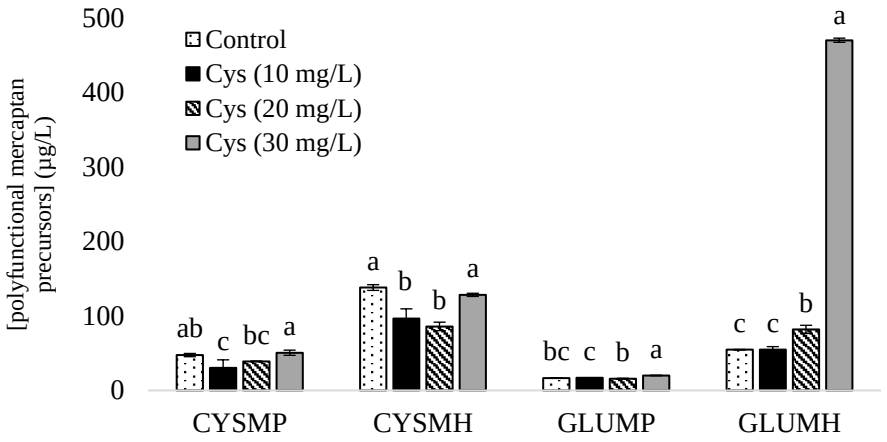


Figure II.8. Concentration of polyfunctional mercaptans precursor (µg/L) remaining at the end of the alcoholic fermentation of synthetic juices prepared with the addition of cysteine at different concentrations: 10, 20 and 30 mg/L. A control without additions was also used. Error bars represent the standard deviations of the means of the triplicates. Different letters indicate significant differences according to Duncan's post-hoc test (significance level 95%).

3.4.2. Impact of the addition of individual components of GSH on the concentration of polyfunctional mercaptans

Regarding to the effect of the addition of different levels of cysteine on the polyfunctional mercaptans concentrations, results are summarized in Figure II.9. There was significant correlations between the levels of 4MMP, 3MH and 3MHA in the different additions (r=0.846; 0.684 and 0.918 for the pairs 4MMP-3MH, 4MMP-3MHA and 3MH-3MHA; significant at $P < 0.05$), and it can be observed a dose-related effect. In the sample spiked with cysteine at 10 mg/L no difference was observed in the concentration of the polyfunctional mercaptans in comparison with the controls.

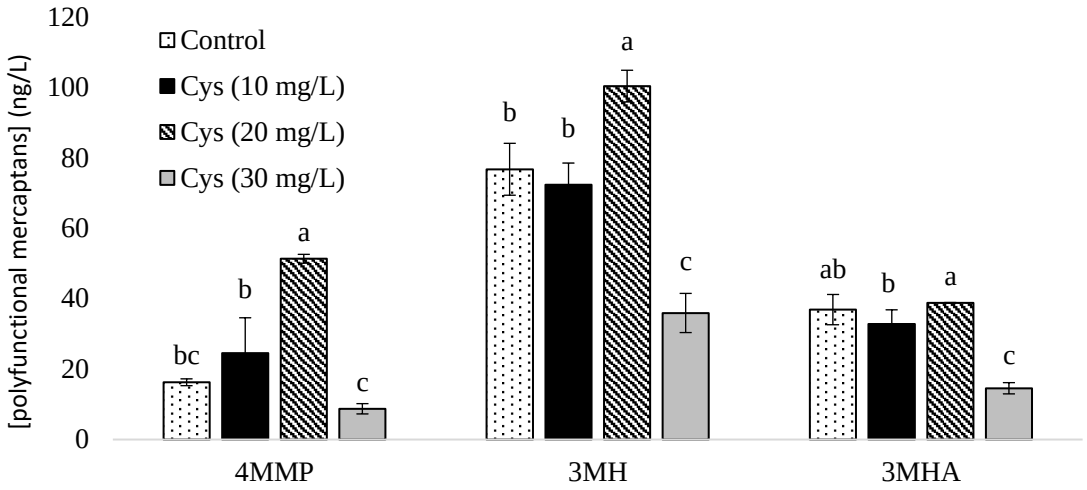


Figure II.9. Concentration of polyfunctional mercaptans (ng/L) in the wines obtained after alcoholic fermentation of synthetic juices prepared with the addition of cysteine at different concentrations: 10, 20 and 30 mg/L, as well as a control without additions. Error bars represent the standard deviations of the means of the triplicates. Different letters indicate significant differences according to Duncan's post-hoc test (significance level 95%).

On the other hand, the addition of cysteine at 20 mg/L led to a significant increase, 215% and 30.8% in the levels of 4MMP and 3MH, respectively, which could be due to the higher metabolization of cysteinylated precursors in the sample with 20 mg/L of cysteine (Figure II.8). Nevertheless, as can be observed in Figure II.9, the addition of 30 mg/L of cysteine

brought about a significant decrease of 3MH (53.2%) and 3MHA (60.5%), while generating a decrease (but not a significant decrease) in the 4MMP concentration in comparison with the control wine. This result is consistent with the fact that when cysteine was added at 30 mg/L, GLUMH precursor was significantly less metabolized than in the controls (Figure II.8). In addition, it could be possible that cysteine at high concentrations inhibits the release of polyfunctional mercaptans.

3.5. Experiment 5. Effect of sulfur dioxide (SO_2)

3.5.1. Effects of SO_2 on the concentration of polyfunctional mercaptan precursors

The surprising effect of SO_2 observed in Table II.5 on the remaining levels of GLUMH was re-examined in an independent experiment in which this antioxidant was added at higher levels. Results are summarized in Figure II.10. As can be seen, the additions of SO_2 at 20 mg/L led to significant lower remaining levels of GLUMH (39.9 µg/L), CYSMH (94.7 µg/L) and CYSMP (39.6 µg/L) compared to the control (54.6, 138.1 and 47.5 µg/L, respectively). However, at 30 and 50 mg/L of addition, the results observed for GLUMH and CYSMH was opposed (Figure II.10). These additions led to a significant lower metabolization of GLUMH compared to the control, while they brought about increases in the CYSMH metabolization levels in comparison with the control. This could mean that SO_2 prevents the formation of CYSMH from GLUMH. The results observed by Capone et al., (Capone & Jeffery, 2011) in which it was observed a decrease in CYSMH levels when a high SO_2 concentration was added, supported our findings. In the case of CYSMP, the addition of SO_2 at 20, 30, 50 and 70 mg/L brought about significant increases in the metabolization level (20.7%, 66.4%, 68.4% and 37.7%, respectively) compared to the control (5.11%).

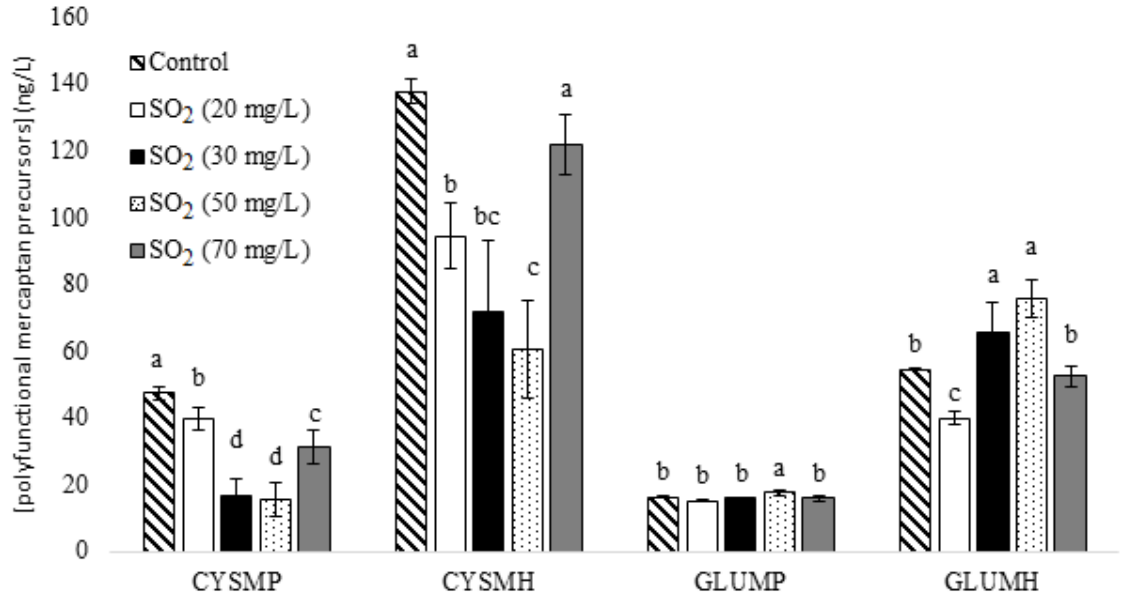


Figure II.10. Concentration of polyfunctional mercaptans precursor (μg/L) remaining at the end of the alcoholic fermentation of synthetic juices prepared with the addition of SO_2 at different concentrations (20, 30, 50 and 70 mg/L). A control without additions was also used. Error bars represent the standard deviations of the means of the triplicates. Different letters indicate significant differences according to Duncan's post-hoc test (significance level 95%).

3.5.2. Impact of the addition of SO_2 on the concentration of polyfunctional mercaptans

Results for polyfunctional mercaptans from the experiment in which SO_2 was added at different levels (20, 30, 50 and 70 mg/L) are summarized in Figure II.11. As can be observed, the addition of 20 mg/L of SO_2 brought about a significant increase, 355% and 66.3% in the 4MMP and 3MH concentrations, respectively. These increases are correlated with the fact that in the sample with the addition of 20 mg/L of SO_2, remaining levels of CYSMP, CYSMH and GLUMH were lower than in the control (Figure II.10). The addition of 50 mg/L levels of SO_2 brought about a significant increase in the concentration of 3MH (30.8%) in comparison with the control wine (Figure II.11). This result is consistent with the fact that in the control, CYSMH was above the initial levels, while when SO_2 was added at 50 mg/L, remaining CYSMH levels was significantly less than in the control (Figure

II.10). It is worth noting that in the samples spiked with SO_2 at 30 and 50 mg/L, 3MH levels were higher compared to the control, but lower compared to the sample added with 20 mg/L of SO_2. This could be due to the fact that in the samples with 20 mg/L of SO_2, GLUMH was metabolized to a greater extent than the control. However, in the samples with SO_2 at 50 and 30 mg/L, the metabolization of GLUMH was lower. On the other hand, in the sample added with 70 mg/L, no significant differences were observed in the 4MMP and 3MH levels, which could be because in this sample, the metabolization of the four precursors was not different compared to the control. Conversely, the effects of the additions on 3MHA concentration were opposite to those observed in the case of 3MH (Figure II.11): in the control sample, the concentration of 3MHA was significantly higher than when different levels of SO_2 were added. This could be due to a lower formation of the acetate from 3MH.

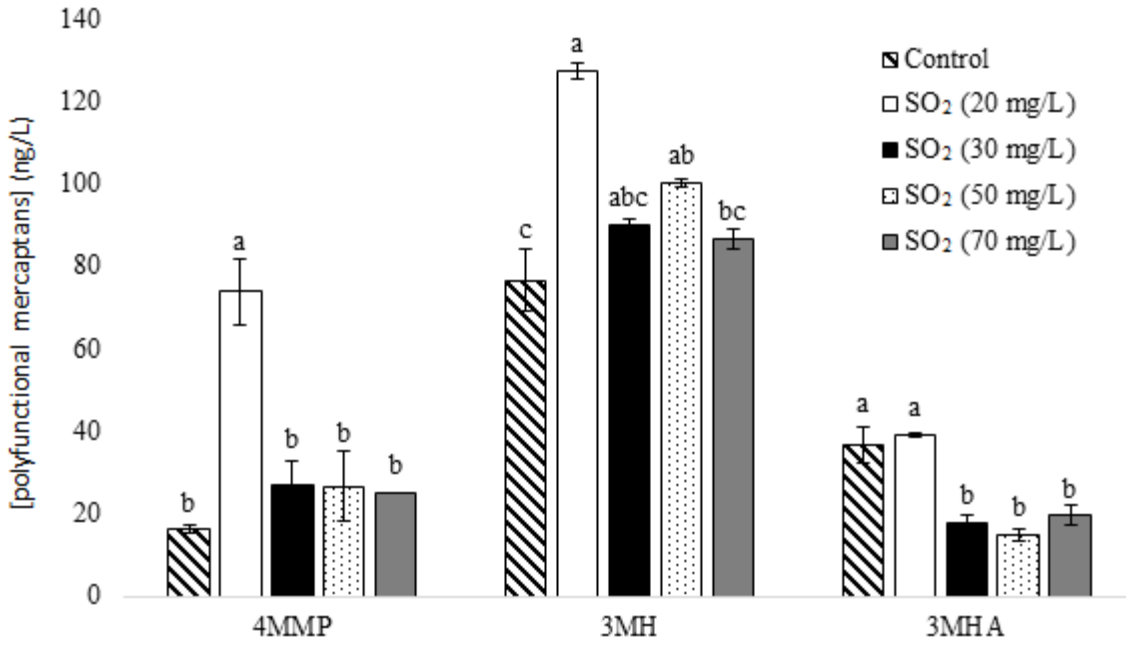


Figure II.11. Concentration of polyfunctional mercaptans (ng/L) in the wines obtained after alcoholic fermentation of synthetic juices prepared with the addition of SO_2 at different concentrations (20, 30, 50 and 70 mg/L), as well as a control without additions. Error bars represent the standard deviations of the means of the triplicates. Different letters indicate significant differences according to Duncan's post-hoc test (significance level 95%).

3.6. Experiment 6. Transcriptomic study during alcoholic fermentation

The lower disappearance of GLUMH when the samples contained GSH and cysteine observed in previous results (Table II.5 and Figure II.8, respectively) led us to think that these differences in the metabolism of the polyfunctional mercaptans precursors could be due to: i) the use of the precursors as a source of some nutrient by the yeast; or ii) a regulation of the yeast genes involved in the metabolic pathway of these precursors. In order to test if these differences were due to an effect on yeast genes, transcriptomic analyses were carried out.

Firstly, in order to determine the moments in which the transcriptomic analysis would be carried out, the evolution of the release of polyfunctional mercaptans from their precursors was first studied, as well as the growth of yeast populations.

3.6.1. Selection of the transcriptomic analyses times

3.6.1.1. Evolution of the release of polyfunctional mercaptans from their precursors during alcoholic fermentation

Synthetic musts were prepared in triplicate to which the cysteinylated and glutathionylated precursors were added at concentrations of (50 µg/L in the case of 4MMP precursors, 100 µg/L for CYSMH and 1000 µg/L for GLUMH). During alcoholic fermentation, in the moments in which there was a change in the speed of the fermentation, different samples were collected at 15, 46, 159, 255, 328 hours and at the end of the alcoholic fermentation. In each of the samples, polyfunctional mercaptans as well as their precursors were analyzed.

Polyfunctional mercaptans. As can be seen in Figure II.12, the concentration of 4MMP increased significantly throughout the alcoholic fermentation, observing its maximum concentration at the end. Two significantly increments in 4MMP can be observed, the first one from 15 to 46 h, and the second one from 159 to 255 h, then the concentration remained practically constant until the end of the AF.

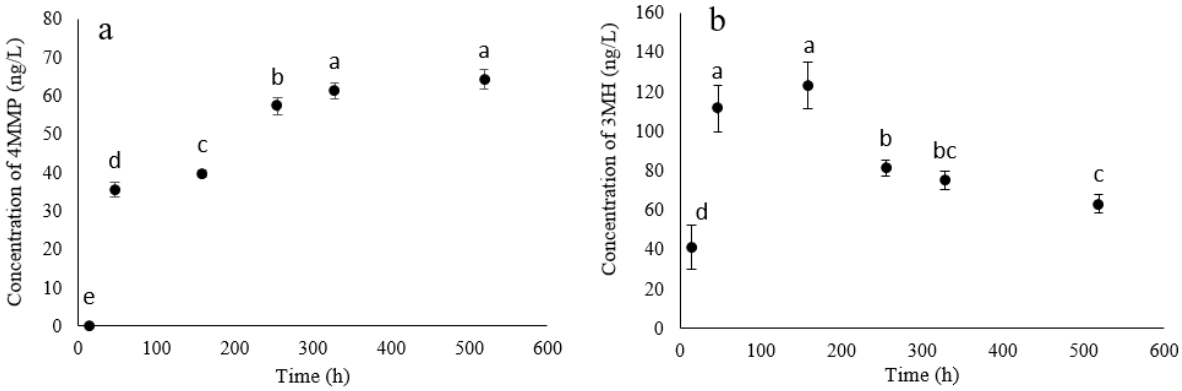


Figure II.12. Evolution of the concentration (ng/L) of 4MMP (a), and 3MH (b), during the alcoholic fermentation of synthetic juice. The different samples were collected at 15, 46, 159, 255, 328 hours and at the end of the fermentation. Error bars represent the standard deviations of the means of the triplicates. Different letters indicate significant differences according to Duncan's post-hoc test (significance level 95%).

However, in the case of 3MH (Figure II.12), the maximum concentration was not observed at the end of AF, but an increase occurred during fermentation followed by a decrease. These results corroborated previous studies in which it was observed that 3MH reached its maximum level during AF not at the end of the AF, unlike 4MMP that reached its maximum concentration at the end of AF (Concejero et al., 2016). In the case of 3MH, from 15 to 46 h its concentration significantly increased, and decreased after 159 hours.

Polyfunctional mercaptans precursors. As can be observed in Figure II.13, the evolution of the concentration of 4MMP precursors decreased during the firsts hours followed by a slightly increment in their concentration (Figures II.13a and b). These results could be due to yeast introduce the precursors in the cells in the firsts steps and then releases them as in the case of amino acids. On the other hand, in the case of 3MH precursors (Figure II.13c

and d) there were significant differences in their concentration throughout AF. In the case of GLUMH, it was observed a significantly decrease from 15 h to 159 h, that could indicate that yeasts metabolized the precursors between these hours. On the other hand, in the case of CYSMH, it was observed a significantly increase between 15 to 159 h, which could be due to the formation of CYSMH from GLUMH (Peyrot des Gachons et al., 2002). Thus, during the firsts steps of AF (between 15 and 159 h) is the time in which yeasts metabolize the precursors.

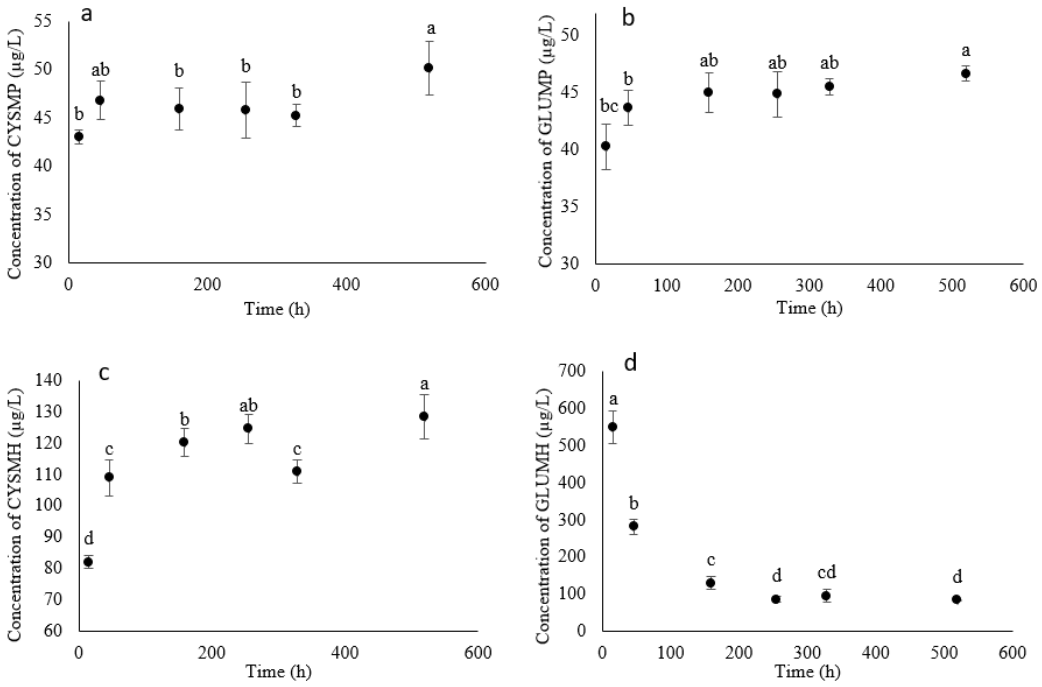


Figure II.13. Evolution of the concentration (µg/L) of CYSMP (a), GLUMP (b), CYSMH (c), and GLUMH (d) during the alcoholic fermentation of synthetic juice. The different samples were collected at 15, 46, 159, 255, 328 hours and at the end of the fermentation. Error bars represent the standard deviations of the means of the triplicates. Different letters indicate significant differences according to Duncan's post-hoc test (significance level 95%).

In addition, taking into account the results of the polyfunctional mercaptans, the decrease in the concentration of the precursors (Figure II.13) coincides with the increase in the concentration of the polyfunctional mercaptans (Figure II.12).

These changes in the concentration of polyfunctional mercaptans could indicate the moments at which the yeast metabolizes the precursors. However, yeast metabolism would

begin before changes in the concentration of both polyfunctional mercaptans and their precursors could be observed. For this reason, and in order to determine the hours at which the transcriptome analyses would be performed, a new fermentation was carried out in which the growth of yeast populations was monitored.

3.6.1.2. Growth of the yeast population during alcoholic fermentation

In order to monitor the growth of the yeast population during alcoholic fermentation, synthetic musts were prepared in duplicate to which cysteine at three concentrations (10, 20 and 30 mg/L) and GSH at 50 and 70 mg/L were added. Moreover, a control without additions were also carried out. During alcoholic fermentation, samples were collected each 4 hours during 4 times per day for the first 72 h, and then every 24 h until the fermentation was completed and the number of cell population were counted.

As can be observed in Figure II.14, from 0 h to 20 h the yeasts were in the latency phase, which is a phase in which yeasts adapt to the environmental conditions (Pérez-Torrado, Carrasco, Aranda, Gimeno-Alcañiz, Pérez-Ortín, Matallana, & del Olmo, 2002). During this phase, the fermentative activity is very low and the composition of the medium is hardly modified (Bataillon, Rico, Sablayrolles, Salmon, & Barre, 1996).

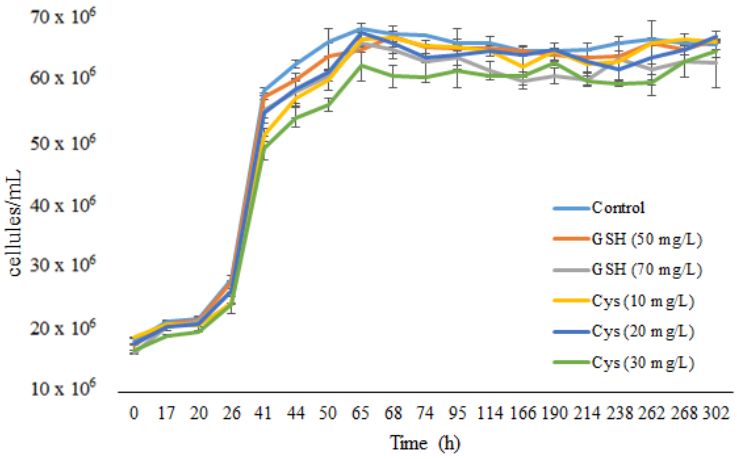


Figure II.14. Evolution of yeast population during the alcoholic fermentation of synthetic must with the addition of GSH (50 and 70 mg/L) and cysteine (10, 20 and 30 mg/L), as well as a control without additions. Amount of cellules per mL (cells/mL) measured each 4 hours during 4 times per day for the first 72 h, and then every 24 h until the end of the fermentation.

The exponential phase begun from 20 h (Figure II.14), but this growth was markedly notably from 26 h to 41 h. This phase of yeasts growth lasts until the maximum population is reached, that in this case was over 65 h (Figure II.14). It is at the end of this phase when the fermentation rate and energy requirements to regulate the fermentation temperature are maximum. However, the changes in the concentration of polyfunctional mercaptans (Figure II.12), as well as in their precursors (Figure II.13) between 47 and 155 hours were hardly significant. At the end of the exponential phase, the yeast population is between 60-68 x 10^6 cells/mL, which is in accordance with previous studies that indicate that the yeast population is between 50 and 250 x 10^6 cells/mL (Bely et al., 1990). Then, from 65 h, the stationary phase begun. During this phase, yeasts populations do not increase but retain the fermentative activity.

Therefore, taking into account the moments studied previously in which it was observed the released of polyfunctional mercaptans from their precursors, it was decided to perform transcriptomic analysis at two different times of alcoholic fermentation: at 26 hours, just at the moment in which there was an intense growth of yeast populations; and at 150 h.

3.6.2. Determination of genes expression by transcriptomic analyses

Transcriptomic analyses were performed on samples collected at 26 and 150 h.

In *Saccharomyces cerevisiae* have been identified 6260 genes. During the alcoholic fermentation, the expression of these genes were analyzed.

With the results of the genes that were expressed in the samples with the addition of cysteine and GSH, as well as in the control without additions, two-dimensional PCA plots were calculated, one for the samples collected at 26 h and the other with the samples collected at 150 h.

The representation of the different samples in the PCA plots indicates the location of these samples according to the genes that were expressed in the different conditions in order to identify the additions that generated more changes in comparison with the control.

3.6.2.1. Changes in genes expression in samples collected at 26 h

As can be observed in Figures II.15, at 26h triplicates were plotted close together, which means that the triplicates were reproducible.

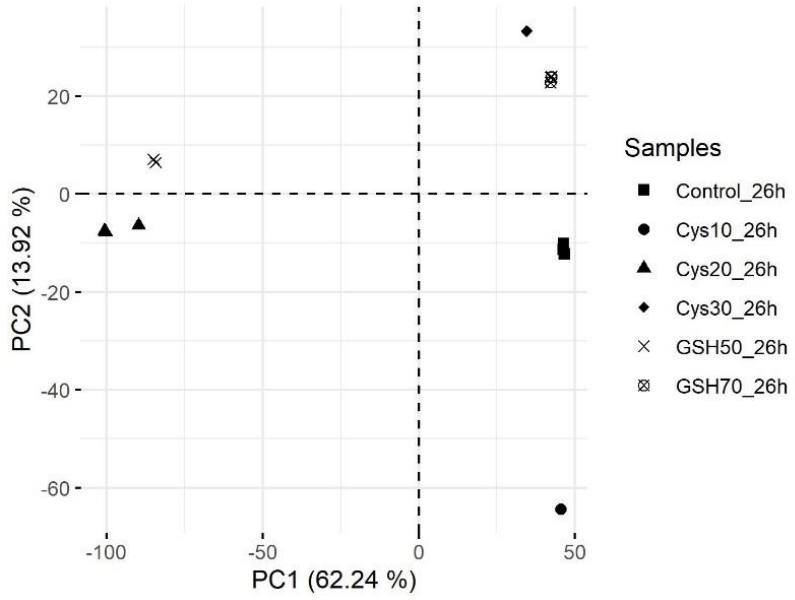


Figure II.15. Projection in a two dimensional PCA plot of samples collected at 26 h according to the genes expressed. Cys10, cysteine at 10mg/L; Cys20, cysteine at 20mg/L; Cys30, cysteine at 30mg/L; GSH50, glutathione at 50 mg/L; GSH70, glutathione at 70 mg/L.

As can be observed in Figure II.15, the first component (PC), which explained 62.2% of variability, confronted control without additions (positive values on PC1) from samples whose synthetic juices were added with the compounds at intermediate concentrations (cysteine at 20 mg/L and GSH at 50 mg/L) (negative values on PC1). This indicates that these additions led to a higher change in the gene expression in comparison with the control.

In order to compare the effect of the different additions, the genes expressed were submitted to differential analyses between each of the addition studied and the control without additions by R software. The number of yeast genes that were differentially ($P < 0.05$) expressed when the samples contained cysteine or GSH compared to the control without additions can be seen in Table II.6. The addition of cysteine at 20 mg/L led to the highest number of genes differentially expressed, 365 genes, followed by the addition of GSH at 50 mg/L, in which it was observed 272 genes. These results could be related with the significant highest concentration of volatile thiols observed previously in the samples with the addition of cysteine at 20 mg/L (Figures II.4 and II.9) and with the higher concentration of 4MMP in the sample added with GSH at 50 mg/L in comparison with the control (Figures II.4).

Table II.6. Number the genes differentially ($P < 0.05$) expressed at 26 h from the beginning of the alcoholic fermentations of synthetic juices with the addition of cysteine (10, 20 and 30 mg/L) and GSH (50 and 70 mg/L) in comparison with the control without additions.

	Cys (10 mg/L)	**Cys (20 mg/L)**	**Cys (30 mg/L)**	**GSH (50 mg/L)**	**GSH (70 mg/L)**
Total genes	6260	6260	6260	6260	6260
Genes differentially expressed	**4**	**365**	**6**	**272**	**3**
Upregulated	0	182	1	133	0
Downregulated	4	183	5	139	3

Regarding the role that these genes have on the yeasts, only those related to the metabolism of cysteine, methionine, GSH, sulfur and cysteinylated and glutathionylated precursors were taking into account.

From all genes that were differentially expressed in comparison with the control, 7 genes in the case of GSH50 and 15 genes in the case of Cys20 were related with the metabolisms

previously cited. Both, the addition of 20 mg/L of cysteine and 50 mg/L of GSH led to a differential expression in some of the genes involved in the metabolic pathways of cysteine and methionine as can be observed in Figure II.16.

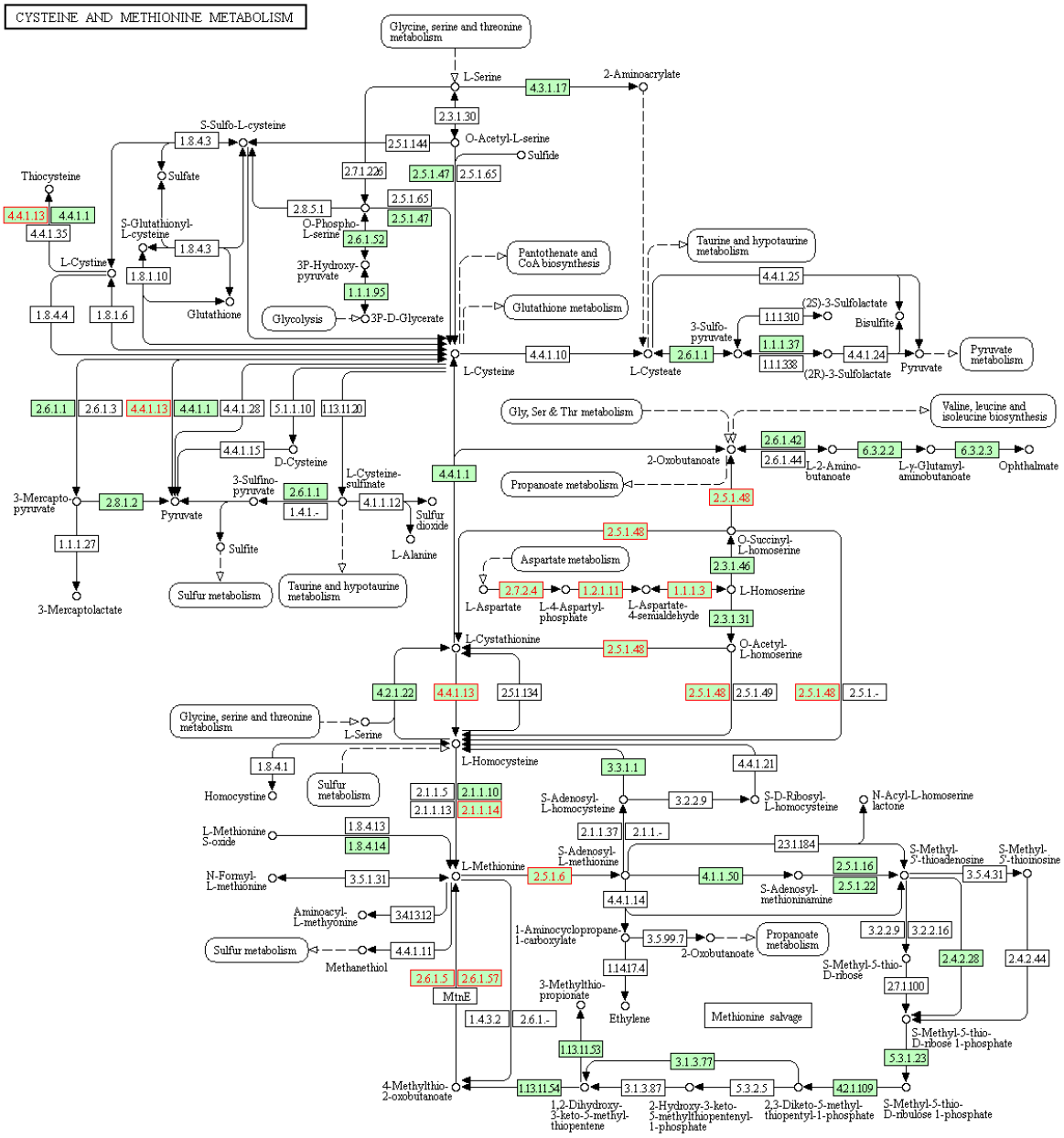


Figure II.16. Pathway of the metabolism of cysteine and methionine amino acids. The genes that have been identified in *Saccharomyces cerevisiae* are marked in green colour. The genes that were differentially ($P < 0.05$) expressed during the alcoholic fermentation of synthetic must with the addition of cysteine or GSH in comparison with the control are indicated by their gene names and with an asterisk (*). The genes that were differentially expressed after the addition of both compounds are marked in red colour, after the addition of cysteine at 20 mg/L are marked in purple colour, and after the addition of GSH at 50 mg/L with blue.

As can be observed in Figure II.16, in the case of the metabolism of sulfur amino acids, there were four genes (HOM3, STR2, MET6 and SAM2) that were differentially ($P < 0.05$) expressed when synthetic juices were added with both compounds (GSH at 50 mg/L and cysteine at 20 mg/L). These four genes were upregulated after both additions and their function is summarized in Table II.7.

Table II.7. Functions of the genes differentially expressed in the samples collected at 26 h during the AF of synthetic musts with the addition of cysteine at 20 mg/L and GSH at 50 mg/L.

Gene name	Differentially express by Cys30	Differentially express by GSH50	Function
CYS3	Upregulated	Not regulated	Cystathionine gamma-lyase that catalyzes one of the two reactions involved in the transsulfuration pathway that yields cysteine from homocysteine[1]
HOM2	Upregulated	Not regulated	Catalyzes the second step in the common pathway for methionine and threonine biosynthesis[2]
HOM3	Upregulated	Upregulated	Catalyzes the first step in the common pathway for methionine and threonine biosynthesis[2]
HOM6	Upregulated	Not regulated	Catalyzes the third step in the common pathway for methionine and threonine biosynthesis[3]
IRC7	Upregulated	Not regulated	β-lyase involved in the production of volatile thiols[4]
MET3	Upregulated	Not regulated	Catalyzes the primary step of intracellular sulfate activation, essential for assimilatory reduction of sulfate to sulfide, and it is involved in methionine metabolism[5]
MET6	Upregulated	Upregulated	Involved in methionine biosynthesis and regeneration[5]
MET10	Upregulated	Not regulated	Converts sulfite into sulfide[5]
MET14	Not regulated	Upregulated	Adenylylsulfate kinase required for sulfate assimilation and involved in methionine metabolism[5]
MET16	Upregulated	Not regulated	Reduces 3'-phosphoadenylyl sulfate to adenosine-3',5'-bisphosphate and free sulfite, it is also involved in sulfate assimilation and methionine metabolism[5]
MET17	Upregulated	Not regulated	Required for methionine and cysteine biosynthesis[5]
MET22	Upregulated	Upregulated	Intermediates of the sulfate assimilation pathway[5]
MMP1	Upregulated	Not regulated	High-affinity S-methylmethionine permease that is required for utilization of S-methylmethionine as a sulfur source[6]
MUP1	Upregulated	Upregulated	High affinity methionine permease that it is also involved in cysteine uptake[7]
SAM2	Upregulated	Upregulated	S-adenosylmethionine synthetase that catalyzes transfer of the adenosyl group of ATP to the sulfur atom of methionine[8]
STR2	Upregulated	Upregulated	Cystathionine γ-synthase important in sulfur metabolism[9]

Cys30, Cysteine at 30 mg/L; GSH50, glutathione at 50 mg/L.
[1](Flavin & Segal, 1964; Howell, Klein, Swiegers, Hayasaka, Elsey, Fleet, Høj, Pretorius, & de Barros Lopes, 2005; Matsuo & Greenberg, 1959).
[2](Mountain, Byström, Larsen, & Korch, 1991).
[3](Robichon-Szulmajster, Surdin, & Mortimer, 1966).
[4](Thibon, Marullo, Claisse, Cullin, Dubourdieu, & Tominaga, 2008)
[5](Masselot & De Robichon-Szulmajster, 1975)
[6](Thomas & Surdin-Kerjan, 1997)
[7](Kosugi, Koizumi, Yanagida, & Udaka, 2001)
[8](Rouillon, Surdin-Kerjan, & Thomas, 1999)
[9](Styger et al., 2011)

It is interesting that in both cases, STR2 was upregulated. This gene could have a similar function of STR3, which had been identified as a gene that encode str3p that is an enzyme the release polyfunctional mercaptans from their cysteinylated precursors (Holt, Cordente, Williams, Capone, Jitjaroen, Menz, Curtin, & Anderson, 2011).

In the case of the addition of 20 mg/L of cysteine, it was also observed that this addition also led to an upregulation of MET17, HOM2, HOM6, CYS3 (also called STR1) and ICR7, that are related in the sulfur amino acids metabolisms and whose functions are indicated in Table II.7.

The most remarkable was the upregulation of IRC7 and CYS3 when the synthetic juices contained cysteine at 20 mg/L. As was previously observed in Figures II.4 and II.9, this addition led to higher concentration of polyfunctional mercaptans. The gene IRC7 encodes for an enzyme with β-lyase activity able of converting CYSMP into 4MMP (Thibon et al., 2008). Moreover, CYS3 is a cystathionine γ-lyase that cleaved a carbon-sulfur bond releasing cysteine (Flavin & Segal, 1964; Matsuo & Greenberg, 1959). Howell et al., (Howell et al., 2005) identified CYS3 as a gene that could be implied in the release of 4MMP.

Therefore, the addition of cysteine at 20 mg/L produces an increase in yeast β-lyase activity, which leads to a higher concentration of polyfunctional mercaptans.

Apart of those genes involved in sulfur amino acids metabolisms, the genes MMP1 and MUP1 were also upregulated by both additions. These genes were not related to sulfur amino acids and/or sulfur metabolisms, but are important due to this methionine permease activity and/or its relation with the uptake of cysteine (Table II.7).

Other genes differentially expressed were those implicated in the metabolism of sulfur as can be observed in Figure II.17, which represents the metabolic pathway of this compound. The functions of these genes are explained in Table II.7.

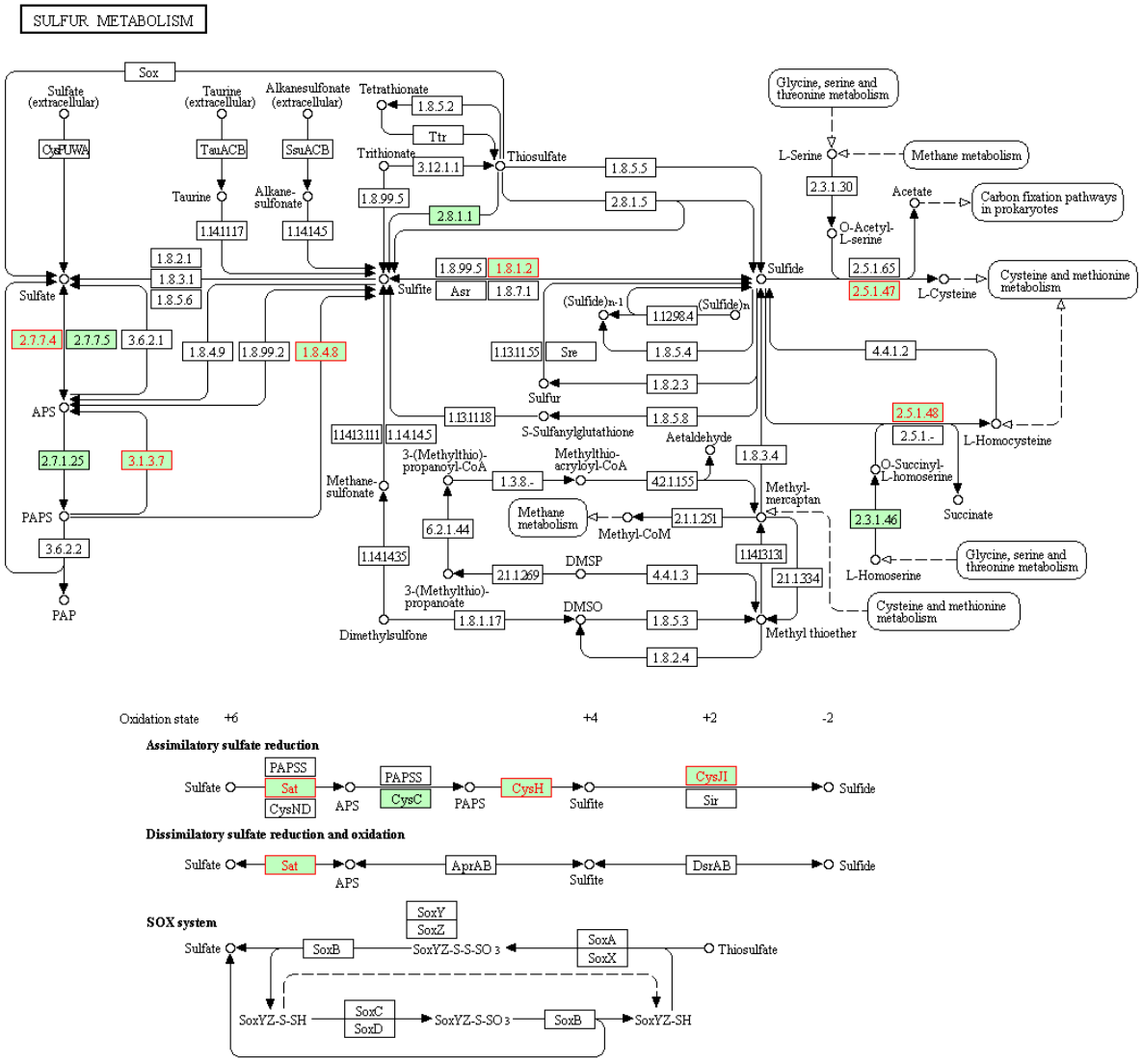


Figure II.17. Pathway of the metabolism of sulfur. The genes that have been identified in *Saccharomyces cerevisiae* are marked in green colour. The genes that were differentially ($P < 0.05$) expressed during the alcoholic fermentation of synthetic must with the addition of cysteine or GSH in comparison with the control are indicated by their gene names and with an asterisk (*). The genes that were differentially expressed after the addition of both compounds are marked in red colour, after the addition of cysteine at 20 mg/L are marked in purple colour, and after the addition of GSH at 50 mg/L with blue.

By contrast, the second component, explaining 13.9% of variability (Figures II.15), confronted samples with the additions of the compounds at the highest concentrations

(cysteine at 30 mg/L and GSH at 70 mg/L) (positive values on PC2) from sample whose synthetic juice was added with cysteine at 10 mg/L (negative values on PC2) leaving the control without additions at values close to 0 of PC2.

On the one hand, the addition of cysteine at 10 mg/L resulted in only 4 genes differentially expressed (Table II.6), which is in accordance with the lack of significant changes on the concentration of polyfunctional mercaptans in the samples added with this compound (Figure II.9). However, none of these genes was related to metabolic pathways of sulfur amino or GSH, sulfur cycle, and/or genes related to the metabolism of polyfunctional mercaptans precursors.

On the other hand, similar results were observed in transcriptomic analyses of wines obtained with the additions of GSH at 70 mg/L and cysteine at 30 mg/L (Table II.6), in which none of the genes differentially expressed was related to the metabolic pathways previously indicated. However, in these cases, it had been previously observed significant changes not only on the polyfunctional mercaptans concentrations (Figure II.4 for GSH at 70 mg/L and Figure II.9 for cys at 30 mg/L), but also on their precursors (Table II.5 in the case of GSH at 70 mg/L and Figure II.8 for cys at 30 mg/L). Therefore, the reason for the lower consumption of GLUMH in the samples with the addition of GSH at 70 mg/L (Table II.5) and cysteine at 30 mg/L (Figure II.8) could be due to a nutritional necessity of the yeast and not to a regulation of the genes.

3.6.2.2. Changes in genes expression in samples collected at 150 h

Regarding to the samples collected at 150h h, in Figure II.18, it can be observed the plotting in a two-dimensional PCA of the samples obtained after the fermentation of synthetic musts with the addition of cysteine and GSH, and a control without additions according to the yeast genes expressed.

As can be observed, the first component (PC), explaining 55.4% of variability, confronted control without additions and cysteine with the addition of 10 mg/L (negative values on PC1) from the rest of the samples (positive values on PC1). This result indicates that the addition of 10 mg/L of cysteine did not generated a differential gene expression, which could explained the results previously observed in Figure II.9, in which 10 mg/L of cysteine did not led a significant change in the polyfunctional mercaptans concentrations.

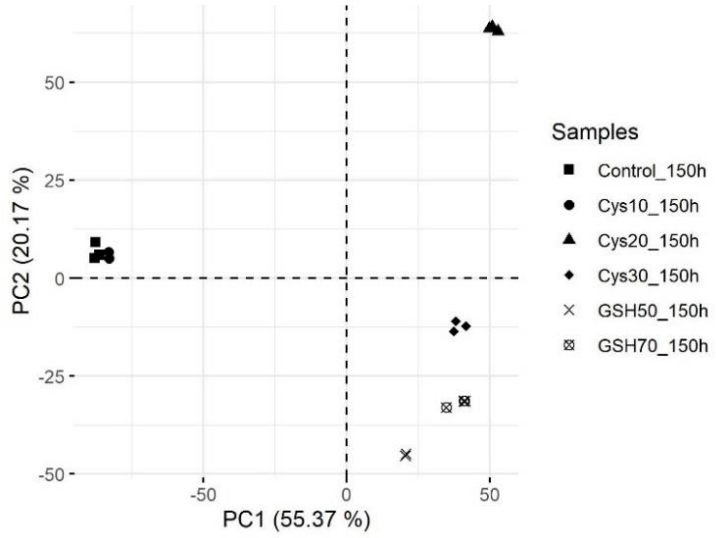


Figure II.18. Projection in a two dimensional PCA plot of samples collected at 150 h according to the genes differentially expressed. Samples with the addition of cysteine at 10, 20 and 30 mg/L, and GSH at 50 and 70 mg/L were compared to the control.

By contrast, the rest of the additions (20 and 30 mg/L of cysteine and 50 and 70 mg/L of GSH) led to a higher numbers of gene expression in comparison with the samples collected at 26 h, particularly in the cases of cys at 30 mg/L and GSH at 70 mg/L as can be observed in Table II.8.

The number of yeast genes that were differentially ($P < 0.05$) expressed when the samples contained cysteine or GSH compared to the control without additions were higher at 150 h (Table II.8) than at 26 h (Table II.6).

Table II.8. Number the genes differentially ($P < 0.05$) expressed at 150 h from the beginning of the alcoholic fermentations of synthetic juices with the addition of cysteine (10, 20 and 30 mg/L) and GSH (50 and 70 mg/L) in comparison with the control without additions.

	Cys10	**Cys20**	**Cys30**	**GSH50**	**GSH70**
Total genes	6260	6260	6260	6260	6260
Genes differentially expressed	**1**	**793**	**716**	**466**	**681**
Upregulated	1	413	375	246	338
Downregulated	0	380	341	220	343

In some cases, the genes regulated were the same that those observed for 26 h, as MET3, MET16, MET22, MMP1, MUP1, SAM2 and STR2. However, these genes were downregulated in the samples collected at 150 h.

Unlike in samples collected at 26 h, in samples at 150 h, genes related to the metabolic pathway of GSH were differentially expressed. The addition of 50 mg/L of GSH upregulated the gene ECM4, while downregulated the gene GSH2. The gene ECM4 encodes for a glutathione transferase and can also act as a cys-thiol transferase (Garcerá, Barreto, Piedrafita, Tamarit, & Herrero, 2006; Xun, Belchik, Xun, Huang, Zhou, Sanchez, Kang, & Board, 2010). The gene GSH2 is involving in the formation of GSH (Grant, MacIver, & Dawes, 1997; Inoue, Sugiyama, Izawa, & Kimura, 1998). The fact that the addition of 50 mg/L of GSH led to an upregulation of the transport of GSH but to a downregulation of the formation of GSH could indicate that the yeast need the GSH (and for this reason the ECM4 is upregulated), but its formation is inhibited because yeast use the GSH added and/or glutathionylated precursors. Moreover, the addition of 30 mg/L of cysteine also led to an upregulation of ECM4. These results are consistent with the data preciously observed in Table II.5 and Figure II.8, in which it was observed a low

metabolism of GLUMH in the samples with the addition of GSH at 50 mg/L and cys at 30 mg/L, respectively. Therefore, yeast use GLUMH as a source of GSH and/or cysteine.

It is interesting that the addition of 20 mg/L led to an upregulation of OPT1, which encode for Opt1p, a GSH transporter. Cordente et al., (Cordente et al., 2015) demonstrated the role of Opt1p as the major transporter responsible for uptake of GLUMH and GLUMP.

Due to the transcriptomic study was carried out between September and December of 2019, the results are still being treated and they will continue to be analyzed after the defense of the thesis.

4. CONCLUSIONS

The main conclusion of this work is that there is a clear effect of the amino acid profile on the consumption of polyfunctional mercaptan precursors as well as on their released compounds. However, not only the amino acid profile, but also the addition of S-compounds have an effect on the direct formation of polyfunctional mercaptans from their precursors.

In the Chardonnay-like profile musts the greatest amount of precursors were consumed, while in the Cabernet Sauvignon-like profile musts the least amount of precursors were consumed. Furthermore, the Chardonnay-like profile wines showed a significantly higher release of 4MMP and a higher, although no significant, concentration of 3MH, while the Tempranillo-like profile wines generated more 3MHA.

In addition, some amino acids showed a positive correlation with the polyfunctional mercaptans, such as aspartic acid, serine, threonine and phenylalanine with 4MMP and 3MH, which indicates that these amino acids could be related with a higher release of these compounds. On the other hand, the increment in the GABA concentration also showed significant increases in the concentrations of 3MH and 3MHA, and a significant decrease in the 4MMP concentration.

It was also observed that the main precursor of 4MMP is CYSMP.

Regarding to the effect of sulfur compounds on the consumption of polyfunctional mercaptan precursors, yeasts could uptake GLUMH in order to obtain the GSH, cysteine and/or sulfur present in this precursor. In addition, according to these results, there is a transformation of GLUMP into CYSMP.

On the other hand, regarding to the effect on the polyfunctional mercaptans, the effect of cysteine and SO_2 depends on the levels of addition, with higher polyfunctional mercaptan levels with the addition of cysteine and SO_2 at 20 mg/L. Conversely, the addition of GSH leads to an increase of 4MMP formation but a decrease on the amount of 3MH.

Although the transformation rate between polyfunctional mercaptans precursors and the aroma formed is very low (0.59% for CYSMP and 0.095% for 3MH precursors), the amount of aroma formed is positively correlated with the amount of metabolized precursors.

The addition of cysteine at 20 mg/L led to an upregulation of the genes IRC7 and CYS3, which encode for enzymes with β-lyase and γ-liase activities, respectively, and OPT1, which encode for a GSH transporter. The overexpression of these genes results in a release of higher concentrations of polyfunctional mercaptans.

Moreover, the addition of 50 mg/L of GSH led to an upregulation of ECM4 and to a downregulation of GSH2. This result could indicate that the yeast need GSH and for this reason upregulate the GSH transferase, but use either the GSH that we have added or the GSH from the precursors, and therefore, yeast inhibits the formation of GSH. Therefore, yeasts could use precursors as a source of GSH and/or cysteine.

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CHAPTER III

The aroma potential of winemaking grapes through the phenolic and aromatic fractions

CHAPTER III. THE AROMA POTENTIAL OF WINEMAKING GRAPES THROUGH THE PHENOLIC AND AROMATIC FRACTIONS

1. INTRODUCTION

The aroma of wine is the result of perceptual interactions between a relatively wide array of aroma compounds. While major wine volatiles are byproducts of yeast fermentation, it has been recently suggested that up to 27 relevant wine aroma compounds have direct origin in grape specific precursors (Ferreira & López, 2019). These specific precursors are mainly glycosides (Gunata, Bitteur, Brillouet, Bayonove, & Cordonnier, 1988; Hjelmeland, Zweigenbaum, & Ebeler, 2015; Williams, Strauss, Wilson, & Massy-Westropp 1982). Aroma molecules derive from these specific precursors at different rates depending on the number and difficulty of the chemical changes required to form the aroma molecule from the precursor. For instance, linalool and geraniol are released from their glycosidic precursors very fast, because only the glycosidic bond between glucose and the aroma molecule has to be broken since the aroma molecule is directly the "aglycone" (Strauss, Wilson, Gooley, & Williams, 1986; Wilson, Strauss, & Williams, 1984). Consequently, these aroma molecules are more easily found in young wines, while aged wines contain decreased levels of these two aroma compounds. In an intermediate category there is, among others, β-damascenone. Its release takes more time because the formation of the aroma molecule requires, at least, a dehydration and a chemical rearrangement, in addition to the cleavage of the glycosidic precursor. This aroma molecule tends to reach maxima levels after some aging (Slaghenaufi & Ugliano, 2018; Waterhouse, Sacks, & Jeffery, 2016). The extreme case is constituted by some other aroma molecules, such as TDN, DMS, guaiacol or vanillin, whose levels increase continuously with aging. In the case of DMS this happens because the cleavage of the precursor is very slow at wine pH. In all the

other cases, it seems that there is a complex net of chemical reactions required to form the aroma molecules.

Although there is previous scientific evidence supporting the existence of a link between wine aromatic quality and content in aroma precursors in grape (Abbott, Coombe, & Williams, 1991; Francis, Sefton, & Williams, 1992), there remain many gaps in our knowledge. This is particularly true in the case of red wines, where some aroma nuances are formed slowly along the aging process from grape aroma precursors. For instance, recent reports have revealed the contribution to mint and fresh notes in aged Bordeaux wines of piperitone and of different lactones derived from menthofuran (Picard, de Revel, & Marchand, 2017; Picard, Franc, de Revel, & Marchand, 2018; Picard, Lytra, Tempere, Barbe, de Revel, & Marchand, 2016) and also the increase with time of tobacco aroma-related compounds derived from norisopresnoids in Valpolicella wines (Slaghenaufi & Ugliano, 2018). Therefore, new strategies able to assess the aromas derived from grape aroma precursor fractions should be sought in order to study the effects of different agronomical or environmental conditions on the grape aroma potential, to assist in the chemical characterization of the precursors and, ultimately, to improve our understanding about the relationship between grape aroma composition and wine aroma properties.

The glycosides of aromatic aglycones are usually isolated on a chromatographic support of C18 type (García-Muñoz, Asproudi, Cabello, & Borsa, 2011) or by a polymeric adsorbent of styrene-divinylbenzene (Gunata, Bayonove, Baumes, & Cordonnier, 1985; Ibarz et al., 2006). Then, they are released by enzymatic or acid hydrolysis (Delfini, Cocito, Bonino, Schellino, Gaia, & Baiocchi, 2001; Loscos, Hernández-Orte, Cacho, & Ferreira, 2009) that can be carried out at different pHs and temperatures.

While enzymatic hydrolysis is far more efficient in terms of breaking the glycosidic bond than acid hydrolysis (Hampel, Robinson, Johnson, & Ebeler, 2014; Liu, Zhu, Ullah, & Tao,

2017), many relevant aroma molecules that are further formed by chemical rearrangement, or esterification such as β-damascenone, TDN or ethyl cinnamates are not even formed via enzymatic hydrolysis (Loscos et al., 2009). Precursors other than glycosides cannot be either determined using this type of hydrolysis. This explains why the sensory properties of acid hydrolyzates obtained at mild temperatures (40-50 ºC) are more intense than those of enzymatic hydrolyzates, and in fact, that only acid hydrolyzates seem to have sensory relevance in wine aroma (Francis et al., 1992; Sefton, Francis, & Williams, 1993). However, in order to speed the process, acid hydrolysis is usually carried out without particular antioxidant precautions and at high temperatures (Loscos et al., 2009), which implies an intense degradation of labile molecules, such as geraniol or linalool (Loscos et al., 2009). In addition, levels of volatile phenols released are also very low and often unrelated to those found by enzymatic hydrolysis. Best results, at least from the sensory point of view, are obtained by slow acid hydrolysis mimicking wine aging (Francis et al., 1992; Loscos, Hernández-Orte, Cacho, & Ferreira, 2010; Sefton et al., 1993). The problem of this strategy is that takes long time and often, aroma notes related to oxidation or to the degradation of carotenoids are noted.

Other analytical strategies make use of the indirect evaluation of the sugar released after hydrolysis, for which a commercial trial has been even proposed (Salinas, de la Hoz, Zalacain, Lara, & Garde-Cerdán, 2012) which may be suitable for making comparisons between grapes from the same type. However, this strategy requires pre-calibration, provides limited information and its real usefulness still requires proper validation. Furthermore, strategies for the direct quantification of the aglycones based on direct HPLC-MS have been recently proposed (Flamini, Rosso, Panighel, Vedova, De Marchi, & Bavaresco, 2014; Godshaw, Hjelmeland, Zweigenbaum, & Ebeler, 2019; Hjelmeland et al., 2015; Schievano, D'Ambrosio, Mazzaretto, Ferrarini, Magno, Mammi, & Favaro, 2013)

but due to the complexity of the number of aglycones and the difficulty in relating them to the aromas revealed, their use has not been extended.

Because of all the complex transformations suffered by grape aroma precursors, it can be argued that the best possible assessment of grape aroma potential will be obtained by hydrolyzing precursors under conditions as close as possible to those observed in real wine aging. For that, it is expected that best results will be obtained if the hydrolysis is carried out in a matrix as similar as possible to real wine regarding alcoholic content, presence of polyphenols, pH and acidity. It can be also anticipated that sugar and amino acids will have to be removed and that the process will have to take place under strict anoxic conditions. All these hypotheses are checked in this thesis in which one of the main goals is to develop a new strategy able to obtain an assessment of the aroma potential of winemaking grapes. Besides applying such strategy to Grenache and Tempranillo winemaking grapes from different origin and states of ripeness.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

LiChrolut EN resin cartridges were obtained from Merck (Darmstadt, Germany), while Sep Pak-C18 resins, prepacked in 10 g cartridges were from Waters (Ireland), and Solid Purple-C18 resins, prepacked in 7 g cartridges, were obtained from Análisis Vínicos (Tomelloso, Spain).

Samples. The study was carried out with Grenache and Tempranillo grapes from different high quality Spanish producers (Dominio Pingus, Bodegas Ramón Bilbao, Bodega Vega Sicilia, Bodega Viñas del Vero, and Bodega Ilurce) belonging to 3 winemaking areas (Ribera del Duero: D, Somontano: S, and Rioja: R). Samples were coded with three identifiers. The first refers to the degree of ripening: unripe (u) were samples taken one week before vintage, ripe (r) samples were harvested at the optimal point of ripeness, and overripe (o) were collected one week after optimal ripeness. The optimal moment of harvest was determined based on Cromoenos® methodology (Bioenos S.I., España) (Kontoudakis, Esteruelas, Fort, Canals, & Zamora, 2010). The second clue identified the variety (T=Tempranillo, G=Grenache), and the third the specific vineyard block belonging to any of the three studied regions: D1-D4 for DO Ribera del Duero, S1-S4 for DO Somontano and R1-R9 for DOCa Rioja vineyard blocks.

2.2. Preparation of ethanolic musts (mistelles)

Ten kilograms of grapes were taken from different areas of north Spain, from two varieties (Grenache and Tempranillo) at one, two of three ripeness states in relation to the optimal date of vintage and depending on climate conditions and vine state. A total number of 33 different lots of grapes were collected (Table III.1).

Table III.1. Thirty-three different lots of grapes collected at three different moments (underripe: u, ripe: r, and overripe: o), two different varieties (Tempranillo: T and Grenache: G) and from three different regions (Ribera del Duero: D, Rioja: R and Somontano, S) and 17 different vineyard plots (D1-D4, R1-R9, S1-S4). In bold samples used for the development of the strategy.

Codes	Ripeness state	Variety	Denomination of Origin	Vineyard plot
uTD1	underripe	Tempranillo	DO Ribera del Duero	D1
rTD1	ripe	Tempranillo	DO Ribera del Duero	D1
oTD1	overripe	Tempranillo	DO Ribera del Duero	D1
uTD2	underripe	Tempranillo	DO Ribera del Duero	D2
rTD2	ripe	Tempranillo	DO Ribera del Duero	D2
oTD2	overripe	Tempranillo	DO Ribera del Duero	D2
rTD3	ripe	Tempranillo	DO Ribera del Duero	D3
rTD4	ripe	Tempranillo	DO Ribera del Duero	D4
uTR1	underripe	Tempranillo	DOCa Rioja	R1
rTR1	ripe	Tempranillo	DOCa Rioja	R1
oTR1	overripe	Tempranillo	DOCa Rioja	R1
uTR2	underripe	Tempranillo	DOCa Rioja	R2
rTR2	ripe	Tempranillo	DOCa Rioja	R2
uGR3	underripe	Grenache	DOCa Rioja	R3
rGR3	ripe	Grenache	DOCa Rioja	R3
oGR3	overripe	Grenache	DOCa Rioja	R3
uGR4	underripe	Grenache	DOCa Rioja	R4
rGR4	ripe	Grenache	DOCa Rioja	R4
rTR5	ripe	Tempranillo	DOCa Rioja	R5
oTR5	overripe	Tempranillo	DOCa Rioja	R5
rTR6	ripe	Tempranillo	DOCa Rioja	R6
oTR6	overripe	Tempranillo	DOCa Rioja	R6
rTR7	ripe	Tempranillo	DOCa Rioja	R7
rGR8	ripe	Grenache	DOCa Rioja	R8
rGR9	ripe	Grenache	DOCa Rioja	R9
uGS1	underripe	Grenache	DO Somontano	S1
rGS1	ripe	Grenache	DO Somontano	S1
uGS2	underripe	Grenache	DO Somontano	S2
rGS2	ripe	Grenache	DO Somontano	S2
uGS3	underripe	Grenache	DO Somontano	S3
rGS3	ripe	Grenache	DO Somontano	S3
uGS4	underripe	Grenache	DO Somontano	S4
rGS4	ripe	Grenache	DO Somontano	S4

Grapes were kept at 5 ºC during the transport from the vineyard to the experimental cellar in the Institute of Grapevine and Wine Sciences (ICVV, Logroño, La Rioja). Grapes were first destemmed and crushed in the presence of 5 g/hL of potassium metabisulfite and 15% (w/w) of ethanol to prevent oxidation and fermentative processes, and to accelerate extraction. After seven days macerating at 13 ºC, the ethanolic must (mistelle) was pressed, filtered and stored at 5 ºC in the dark.

2.3. Optimization of PAFs extraction

2.3.1. Optimization of cartridges and breakthrough volume

For the development of the strategy, two mistelles were used: one from Tempranillo variety from Ribera de Duero (rTD2), and the other from Grenache from Rioja (rGR8). Mistelle was centrifuged at 4500 rpm, 10 ºC for 20 min (Allegra X-22R Beckman Coulter). Then, three different mistelle preparations were used to obtain a higher volume of loaded sample: i) mistelle; ii) mistelle diluted to 50% with milli-Q water at pH 3.5 and iii) mistelle dealcoholized. For the dealcoholization, 750 mL of the mistelle were put into a rotatory evaporator system (Buchi R-215 equipped with a V-700 vacuum pump from Buchi, Flawil, Switzerland) hold at 23 ºC and at pressure of 20 mbar for 3 hours, achieving a final volume around 410 mL containing just 2-3% (v/v) of ethanol as determined by distillation and measurement of density. For extraction, two types of high capacity cartridges were used, Sep Pak-C18 10 g and Solid Purple-C18 7 g.

The 7 and 10 g-C18 cartridges were first conditioned by passing through them 35 and 44 mL of methanol (corresponding to 4 dead volumes of the cartridges of 7 and 10 g) followed by 35 and 44 mL of milli-Q water with 2% ethanol, respectively.

Thereafter, the mistelle, diluted mistelle and dealcoholized mistelle were further passed through the 7 and 10 g-C18 cartridges. After letting the dead volume pass (7 mL and 11 mL in the case of the 7 and 10 g cartridges, respectively), fractions were collected every 5 mL and were analyzed by means of the total polyphenol index (TPI).

Spectrophotometric measurements. TPI was determined as optical density at 280 nm (OD 280) following the method described by Ribéreau-Gayon et al., (Ribéreau-Gayon, Glories, Maujean, A., & Dubourdieu, 2006). The absorbance measurements were done using a UV–vis spectrophotometer UV-1700 Pharma Spec from Shimadzu (Kyoto, Japan).

Determination of breakthrough volume (V_B). The comparison of TPI in each of the fractions obtained from the three types of mistelle with the TPI in each of the initial mistelles (mistelle, diluted mistelle and dealcoholized mistelle) was used to determine the breakthrough volume. A loss of TPI of less than 15% was considered to represent a good V_B.

2.3.2. Optimization of elution volume

After a washing step with 88 mL (corresponding to 8 dead volumes of the 10 g cartridge) of milli-Q water at pH 3.5, pure ethanol was passed through the cartridge. Fractions of 50 mL of pure ethanol were taken and the presence of glycosidic precursors were analyzed in each fraction. In addition, the anthocyanins and total tannins in these fractions were also studied.

Glycosidic precursors analysis. The presence of glycosidic precursors in each fraction was investigated with an indirect method based on the harsh acid hydrolysis of each fraction, following the procedure described by Ibarz et al. (Ibarz, Ferreira, Hernández-Orte, Loscos, & Cacho, 2006), followed by a sensory analysis of the released compounds.

Total anthocyanins content. The determination of anthocyanins content was carried out following the method described by Ribéreau-Gayon et al., (Ribéreau-Gayon et al., 2006).

Total tannins content. The determination of tannins content was carried out following the method described by Ribéreau-Gayon et (Ribéreau-Gayon et al., 2006).

2.4. Extraction of phenolic and aromatic fractions (PAFs)

750 mL of mistelle were dealcoholized in a rotatory evaporator system. The resulting dealcoholized mistelle was passed through a 10 g prepacked Sep Pak C18 cartridge previously conditioned with 44 mL of methanol followed by 44 mL of milli-Q water with

2% of ethanol. The cartridges were then washed with 88 mL of milli-Q water pH 3.5 and dried by letting air pass through them. The polyphenolic and aroma precursor fractions (PAFs) were recovered by elution with 100 mL of absolute ethanol.

2.5. Hydrolysis conditions

Samples. The preparation of the samples was based on the reconstitution of the PAF from Tempranillo (rTD2) and Grenache (rGR8) mistelles in synthetic wine to their original volume (750 mL). The PAF was added to a synthetic wine with 5 g/L of tartaric acid at pH 3.5 and 13.3% (v/v) of PAF (corresponding to 13.3% of ethanol) (rPAF). In addition, to study the effect of sugar, PAF was added to a synthetic wine that also contained sugar (100 g/L of glucose and 100 g/L of fructose) (rsPAFs). Besides, to study the aroma compounds lost during the extraction, mistelle was used and was adjusted at pH 3.5. These three kinds of samples were prepared in duplicate.

Assay. The samples were placed in the anoxic chamber and divided into two 20 mL-vials. The vials were hermetically closed and bagged with two certified oxygen permeability thermos-sealed plastic bags containing an activated charcoal with an oxygen-scavenger (AnaeroGen™ from Thermo Scientific Waltham, Massachusetts, United States) (Vela, Hernández-Orte, Franco-Luesma, & Ferreira, 2017). The bagged samples were then incubated under different conditions.

Accelerated hydrolysis at 45 ºC. The samples were put in a stove at 45 °C for 2, 4 and 7 weeks in the case of rPAFs and mistelles, and only for 7 weeks in the case of rsPAFs. In addition, two 20 mL-vials of each kind of sample (rPAF, rsPAF and mistelle) were used as controls to test the effect of oxygen. The controls were closed hermetically but not bagged, then were incubated at 45 ºC for 7 weeks.

Accelerated hydrolysis at 75 ºC. The rPAF from the Tempranillo (rTD2) variety was incubated at 75 ºC to form arPAFs (accelerated hydrolyzed rPAFs). Two vials were taken out at different times until 72 h (3, 8, 14, 24, 38, 48, 60 and 72 h).

Thereafter, in all cases, the compounds released from glycosidic precursors were analyzed by sensory analysis and gas chromatography-mass spectrometry (GC-MS).

2.6. Sensory analyses of aroma released from accelerated hydrolysis

The hydrolysates obtained from rPAFs of Tempranillo (rTD2) and Grenache (rGR8) in the develop of the strategy were submitted to four different sensory tasks. The first one was carried out to determine the elution volume. The second and third sensory tasks consisted of a descriptive task for both samples obtained from the accelerated hydrolysis at 45 ºC and at 75 ºC. The fourth sensory task consisted of a triangle test for rPAFs obtained at 75 ºC.

Then, once the methodology was developed, a characterization of the aroma potential of 33 lots of Grenache and Tempranillo grapes from different origins, terroirs and state of ripeness (Table III.1) was carried out. For the characterization, two sensory tasks were performed. The first sensory task was a sorting task in order to group the 33 different rPAFs according to odor similarities. The second consisted of a flash profiling to obtain a deeper characterization of the representative rPAFs in each of the groups formed in the sorting task.

Fifteen wine experts in the case of the four firsts analyses (45.5% men and 54.5% women from 26 to 63 years), 22 in the case of the sorting task (27% men and 73% women ranging in age from 25 to 63, with an average of 37 years old), and 12 in the case of the flash profiling (25% men and 75% women ranging in age from 25 to 63, with an average of 35 years old) participated in the sensory analyses. All of them were semi-trained assessors

with experience in the sensory description of wine, considered wine experts according to the specifications of Parr et al. (Parr, Heatherbell, & White, 2002).

In all cases, one hour before the sensory tasks, samples were removed from the 5 ℃ cold room and 10 mL were served at room temperature in dark approved wine glasses (ISO NORM 3591, 1977) labeled with 3-digit random codes and covered by plastic Petri dishes. Besides, for each panelist, samples were presented simultaneously in a different random order.

Elution volume determination. This sensory analysis was carried out to determine the presence/absence of the aroma compounds released from glycosidic precursors in each of the collected fractions. The panelists were asked to smell each hydrolyzed fraction and indicate "yes" if in the smelled fraction there was any aroma or "no" if there was no aroma. They were then asked to indicate one to three free attributes to describe each fraction. The descriptors cited by at least 20% of the panel were used.

Descriptive tasks. In both sessions (one for rPAFs hydrolyzed at 45 ºC and the other for rPAFs hydrolyzed at 75 ºC), the panelists were asked to smell each sample and describe them with 1 to 5 attributes. In addition, they were also asked to indicate the intensity of each of the samples as "low, medium or high intensity". Attributes mentioned by at least 20% of the panel were used.

Triangle test. In addition, rPAFs incubated at 75 ºC during 14, 24, 38 and 48 h were submitted to different triangle tests to identify the presence/absence of significant differences between the pairs: i) arPAF incubated for 14 h and 24 h; ii) arPAF incubated for 24 h and 38 h; and iii) arPAF incubated for 24 h and 48 h. Panelists performed tests in duplicate. In each triangle test, three glasses were presented to each panelist and, based on the orthonasal aroma, they were asked to select the different sample. To identify the

presence of significant differences between the samples incubated during different times, the number of correct answers was compared with the tabulated values.

Sorting task. Thirty-five arPAFs (the 33 arPAFs and 2 replicates) were subjected to a sorting task in which judges were asked to group samples according to odor similarities as it is described in the section 2.4.3 of chapter I.

Flash profiling. One arPAF was selected out of each one of the formed groups by means of sorting task as the most representative one. These five arPAFs were submitted to a more complete sensory description via flash profile methodology as it is described in section 2.4.2 of chapter I. These arPAFs were characterized in duplicate in two sessions held in different days (one replicate by session). However, in this sensory analysis, the panelist were trained during the inter-session in the references of the global list (Table III.2).

Table III.2. Detailed list of references employed during panel training

descriptor	odor references
alcohol	Solution of 15 % (v/v) absolute ethanol in water
dried fruit, fruit in syrup	β-damascenone (0.05 µg/L) + methional (0.5 µg/L) + phenylacetaldehyde (1 µg/L) + furfural (14.1 mg/L)
fresh fruit *(tropical fruit, citrus)*	3-mercaptohexyl acetate (25 ng/L), 3-mercaptohexanol (60 ng/L)
black fruit *(blackberry, blueberry)*	Pool ethyl esters + β-ionone (0.09 µg/L) + 4-mercapto-4-methyl-2-pentanone (0.8 ng/L)
red fruit *(strawberry, raspberry)*	ɣ-decalactone (10 µg/L) + furaneol (5 µg/L)
nuts *(almond, wallnut)**	Reference nº 50 of *Nez du vin*
floral *(white flowers, acacia)*	Linalool (25 µg/L) + ethyl cinnamate (1.1 µg/L) + 2-phenylethyl acetate (250 µg/L)
vegetal-herbaceous *(cut grass, green pepper)*	3-isobutyl-2-metoxipyrazine (2 ng/L); *Z*-3-hexenal (0.25 µg/L)
vegetal-dried herbs *(hay, tobacco)**	Reference nº 50 of *Aromabar of wine scents (premium edition)*
menthol-balsamic	1,8-cineole
lactic *(yoghurt, cheese, cream)*	Diacetyl (100 µg/L)
toasted *(caramel, roasted coffee)*	Furfurylthiol (0.4 ng/L) + furaneol (5 µg/L); benzylmercaptan (0.3 µg/L) + acetylpyrazine (62 µg/L)
animal *(leather, broth)*	4-ethylphenol (35 µg/L)
kerosene	1,1,6-trimethyl-1,2-dihydronaftalen (TDN) (2 µg/L)
moldy	1-octen-3-one (15 ng/L)
oxidation *(backed potato, honey, rotten apple)*	Acetaldehyde (500 µg/L) + methional (0.5 µg/L) + phenylacetaldehyde (1 µg/L)

*references obtained from commercial aroma kits.

References were prepared in ethanolic solutions (15% v/v) and different arPAF matrices to simulate the sensory space studied. During the training, panelists were asked to associate the references to the descriptors in the global list.

Panelists were qualified when they were able to correctly identify at least 80% of the references.

2.7. Aroma compounds quantification

2.7.1. Aroma released from glycosidic precursors

This analysis was carried out using the method proposed and validated by López et al., (López, Aznar, Cacho, & Ferreira, 2002) with the modifications previously described in the section 2.7.4 of chapter I. Two µL of the extract was injected in a QP2010 gas chromatograph equipped with a quadrupole mass spectrometer detector from Shimadzu (Japan) following the method described by Oliveira et al., (Oliveira & Ferreira, 2019). The mass analyzer 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 III.3.

The column, a DB-WAXetr (30 m x 0.25 mm with 0.5 µm film thickness), was from Agilent (USA). Helium (1.26 mL/min) was the carrier gas. The initial oven temperature was 40 °C, kept for 5 min, then raised at 1 °C/min to 65 ºC, then at 2 °C/min to 220 °C and finally hold for 50 min. Injection was made in splitless mode at 250 ºC, splitless time was 1.5 min, and during the injection a pressure pulse of 4 bar was applied.

The quantification was performed by interpolating the SI-normalized peak area in the calibration straight lines containing at least three different concentration levels of each compound.

Table III.3. Mass spectra ions selected to quantify minor and trace compounds using GC-MS.

Compounds	RT	m/z
NORISOPRENOIDS		
β-ionone	74.27	177 , 192
α-ionone	69.67	121, 93, 192
β-damascenone	67.89	69, 19
TDN	63.45	157, 142, 172
Riesling acetal[*1]	57.05	138, 125, 133
Vitispirano A[1]	49.03	121
Vitispirano B[1]	49.33	121
TERPENOIDS		
β-citronellol	65.51	69, 81, 123
Geraniol	69.90	69, 123
Linalool	52.43	71, 93, 121
α-terpineol	61.00	93, 121, 136
Nerol	67.30	93, 68
1,8-cineole	20.96	108, 81
r-limonene	20.81	93, 67
Cis-linalool oxide	44.50	94, 59, 111
Trans-linalool oxide	46.65	94, 59, 111
LACTONES		
massoia lactone	88.50	97, 68
VOLATILE PHENOLS		
Guaiacol	70.32	109, 124
Eugenol	85.49	164, 149
E-isoeugenol	93.58	164, 149
Methoxyeugenol	101.67	194, 119
2,6-dimethoxyphenol	90.05	154, 139
m-cresol	81.98	108, 79
o-cresol	77.83	108, 79
4-ethylguaiacol	79.00	137, 152
4-vinylguaicol	86.77	150, 135
4-vinylphenol	95.57	120, 91
VANILLIN DERIVATIVES		
Acetovanillone	105.43	166, 123
Vanillin	402.46	155, 152, 123
Syringaldehyde	120.38	182, 181, 167
MISCELLANEOUS GROUP		
Furaneol	78.98	57, 128, 85
Ethyl cinnamate	83.76	131, 176
Ethyl 2-hydroxy-4-methylpentanoate	51.93	87, 69

*Compounds tentatively quantified using alkanes to determine the retention index; [1]relative area

2.7.2. Volatile sulfur compounds (VSCs)

To determine if the DMS precursor was extracted in the PAFs from Tempranillo (rTD2) grapes, an accelerated hydrolysis of rPAF and mistelle was carried out. The determination

of DMS was conducted using the method described by Franco-Luesma et al., and López et al., (Franco-Luesma & Ferreira, 2014; López, Lapeña, Cacho, & Ferreira, 2007).

2.7.3. Data analysis

One-way analysis of variance (ANOVA) followed by Duncan's post-hoc test were applied to establish the significant differences among the hydrolyzed samples. The analyses were carried out using SPSS (SPSS Inc., Chicago, IL) for Windows, version 19. Different letters express significant differences with a significance level of 95%.

Furthermore, principal component analysis (PCA) using XLSTAT software (version 2014.2.02; Addinsoft, NY, USA) was carried out to: i) illustrate the quantitative data obtained in the different accelerated hydrolysis during the development of the strategy; and ii) to illustrate the data obtained with the GC-MS analyses using the sensory data (frequency of citation of each attribute). Sensory data were considered simple illustrative variables, but did not take any role in the factorization process.

2.8. Gas Chromatography-Olfactometry (GC-O)

Two GC-O analyses were performed. The first one with the samples obtained during the development of the conditions of the hydrolysis: extracts of rPAF and mistelle from Tempranillo incubated in anoxic conditions during 7 weeks and of rPAF incubated at 75 ºC for 24 h. The second one with the 5 arPAFs selected as representative of each of the formed groups by means of sorting task during the characterization of the 33 rPAFs.

In the first case, the extracts used were those obtained for the GC-MS analysis. However, in the second GC-O analysis, the extract were isolated and preconcentrated using a dynamic headspace sampling technique producing extracts representative of orthonasal olfaction

(San Juan, Pet'ka, Cacho, Ferreira, & Escudero, 2010) as it is detailed in the section 2.6.1.1 of chapter I.

One microliter of the extracts was injected for GC-O analyses with a Trace GC gas chromatograph (ThermoQuest, Milan, Italy) as it is detailed in the section 2.6.2 of chapter I with the following modification. The temperature program used was 40 ºC for 5 min, increased by 4 ºC/min to 100 ºC and then 6 ºC/min to 220 ºC, holding for 10 min.

Sniffing was carried out by 4 trained judges (75% women and 25% men from 25 to 30 years) in the case of the first GC-O, and 6 trained judges (83% women and 17% men from 25 and 34 years) in the case of the second GC-O. All judges from the laboratory staff.

2.9. Amino acids quantification

Amino acids present in rPAF and mistelle from Tempranillo were determined by HPLC with fluorescence detector according to the method reported by Hernández-Orte et al., (Hernández-Orte, Ibarz, Cacho, & Ferreira, 2003) as it is detailed in the section 2.7.1 of chapter I.

2.10. Metal cations quantification

The most abundant and enologically relevant transition metals of musts and wines (Fe, Cu, Mn and Zn) were determined in the rPAF measuring the most abundant isotopes (^{56}Fe, ^{63}Cu, ^{55}Mn and ^{66}Zn) by inductively coupled plasma mass spectrometry using a procedure published by Grindlay et al., (Grindlay, Mora, de Loos-Vollebregt, & Vanhaecke, 2014).

2.11. Characterization of grape by applying the strategy

In order to characterize the 33 lots of grapes (Table III.1), the PAFs were extracted following the method described in the section 2.4 of chaper III. The PAFs were then reconstituted to form a model wine (rPAF) (13.3% ethanol v/v, pH 3.5). Then, 180 mL of

these rPAFs were introduced into the anoxic chamber and distributed into three-60 mL WIT^{TM} (*wine-in-tube*) tubes which were closed in strict anoxic conditions as it is detailed in section 2.5 of chapter III. The WIT tubes were incubated at 75 °C for 24 hours to form the arPAFs. Released aroma compounds were then analyzed by sensory analyses, gas chromatography-mass spectrometry (GC-MS), and gas chromatography-olfactometry (GC-O). As detailed in sections 2.6, 2.7.1 and 2.8 of chapter III, respectively.

3. RESULTS AND DISCUSSION

3.1. Development of the strategy

3.1.1. Solid Phase Extraction

Mistelles are *a priori* suitable matrixes for studying the aroma potential of grapes, since contain all the grape metabolites potentially extracted by physical processes during wine making, are relatively stable from the microbiological point of view, and do not contain all the volatiles produced by yeast. However, they have large amounts of glucose and fructose and significant levels of amino acids and of different metal cations. Since these chemical species form highly reactive systems in which powerful aroma molecules can be formed, such as Strecker aldehydes or Maillard-derived aroma compounds, they likely have to be removed before aroma development. Therefore, a first goal was to separate grape polyphenols and aroma precursors from sugar and amino acids. This was achieved by solid phase extraction on large capacity C18 sorbent beds.

The extraction abilities of different SPE beds or the effect of the different operations carried out on the mistelle on such extraction abilities were studied by plotting the corresponding breakthrough curves. As the most abundant group of grape secondary metabolites are phenols displaying some absorbance at 280 nm, this parameter was selected to monitor the effluent. Given that some phenols are chemically more polar than most aroma precursors, it is expected that absorbance at 280 nm gives quite a conservative assessment about the ability of the SPE bed to extract aroma precursors. Breakthrough curves were built by estimating at each loaded volume the fraction of absorbance not retained in the cartridge (Poole, 2003; Poole, Gunatilleka, & Sethuraman, 2000). Plots of this kind for three different mistelle preparations (diluted mistelle, dealcoholized mistelle and mistelle), and two C18 cartridges (Solid purple 7 g and Sep Pak 10 g) are summarized in Figure III.1.

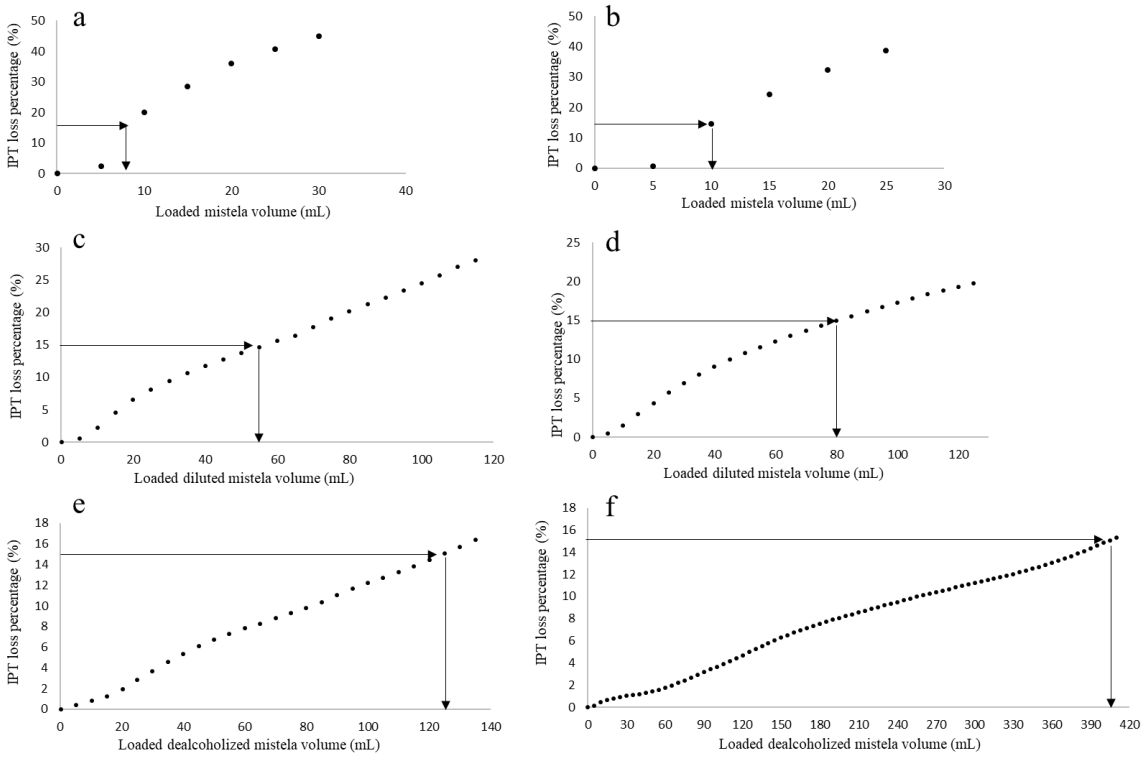

Figure III.1. Plot of the percentage loss of TPI against the corresponding volumes generating the breakthrough volume of the different types of mistelle preparations using two high capacity C18 cartridge: a) mistelle passed through 7 g cartridge, b) mistelle passed through 10 g cartridge, c) diluted mistelle passed through 7 g cartridge, d) diluted mistelle passed through 10 g cartridge, e) dealcoholized mistelle passed through 7 g cartridge, and f) dealcoholized mistelle passed through 10 g cartridge.

As can be seen in Figures III.1a and III.1b, in the case of untreated mistelles, the 15% breakthrough volume is as little as 7 or 10 mL indicating, as expected, that the high presence of ethanol has a pernicious effect on the extraction ability of the bed. In fact, dilution had a very positive effect, as can be seen in Figures III.1c and III.1d, and breakthrough volumes for 1:1 dilutions were 62 and 91 mL, equivalent to 31 and 45.5 mL of the undiluted mistelle, more than 4x larger than the initial ones. The best results were obtained by previous dealcoholization of mistelle, as can be seen in Figures III.1e and III.1f. Breakthrough volumes of 132 mL and 411 mL, equivalent to 240 and 750 mL of the original mistelle, were obtained for the 7 g and the 10 g cartridges, respectively. These last conditions (dealcoholization of 750 mL of mistelle and retention in a 10 g Sep Pak-C18 cartridge)

were retained as optimal, in spite of the fact that most polar precursors, such as those of DMS, could be lost, as will be latter discussed.

In order to optimize elution volume, a cartridge containing the grape extract, was eluted with five consecutive 50 mL-volume fractions of ethanol. Each fraction was analyzed for total anthocyanins, total tannins and aroma precursors. These last were indirectly measured after aroma generation by harsh acid hydrolysis (pH 2.2, 100 ºC, 1 h) using a sensory panel. The results are shown in Table III.4.

Table III.4. a) Presence of aromas released from the glycosidic precursors after harsh acid hydrolysis of the collected fractions at 100 ºC during 1 hour; b) Anthocyanins and total tannins present in the collected fractions. In each fraction, 50 mL are collected.

	Fraction	**1**	**2**	**3**	**4**	**5**
Glycosidic precursors	% of panelists indicating the presence of aromas	100%	100%	6.67%	0%	0%
	Aromas description	Fruit in syrup, sweet and tomato jam notes	Terpenic nuances	-	-	-
Phenols	Anthocyanins (mg/L)	8396	512	133	21.9	18.4
	Anthocyanins (%)	92.2	5.5	1.46	0.24	0.2
	Total tannins (g/L)	8.89	1.41	0.62	0.17	0.15
	Total tannins (%)	79	12.5	5.5	1.55	1.37

Regarding aroma precursors, only the first two fractions produced relevant levels of aromas, the first being more intense, fruity, syrupy and jammy and the second more terpenic (green, herbal). Only one of the judges was able to detect some unspecific aroma in the third fraction. Most anthocyanins (92.4%) were eluted in the first fraction, which also contained 79.1% of total tannins. The second fraction contained 5.64% and 12.5% of anthocyanins and total tannins, respectively. In light of these results, an elution volume of 100 mL of ethanol was chosen.

The characterization of amino acids and transition metal cations (Tables III.5 and III.6, respectively) present in this polyphenolic and aromatic fraction (PAF) reveals that these species are nearly completely lost during the sample treatment. No amino acids were found above the method detection limits in the PAF (Table III.5). The absence of amino acids in the PAF is positive, since these compounds can form powerful aroma compounds, such as phenylacetaldehyde or methional (Bueno, Marrufo-Curtido, Carrascón, Fernández-Zurbano, Escudero, & Ferreira, 2018), but this implies that precursors for DMS will be most surely not present in the PAF.

Table III.5. Amino acids (mg/L) present in mistelle and rPAF from Tempranillo

	Mistelle	**rPAF**
Aspartic acid	25.4	<LD
Asparragine	3.24	<LD
Serine	28.3	<LD
Glutamic acid	34.7	<LD
Histidine	31.8	<LD
Glutamine	<D.L	<LD
Glycine	4.18	<LD
Arginine	63.9	<LD
Threonine and ammonium	237	<LD
Alanine	75.6	<LD
Proline	538	<LD
γ-aminobutyric acid	164	<LD
Tyrosine	5.82	<LD
Valine	14.0	<LD
Methionine	3.03	<LD
Ornitine	13.4	<LD
Lysine	5.69	<LD
Isoleucine	4.76	<LD
Leucine	9.55	<LD
Phenylalanine	11.0	<LD

<L.D, under detection limit

On the other hand, only very little amounts of Fe and Cu, less than 5% and 2% of the initial content of the mistelle, respectively, were found (Table III.6). Two other relevant transition metal cations, Zn and Mn, were also completely lost. The very low levels of metals can in fact be positive, since these compounds, particularly Cu and Fe, are determinant for O_2

consumption (Bueno, Carrascón, & Ferreira, 2016; Carrascón, Bueno, Fernández-Zurbano, & Ferreira, 2017), and seem to be also active catalysts for some reactions in which aroma compounds are formed or degraded (Bueno et al., 2018).

Table III.6. Metal cations (µg/L) present in rPAF

	^{56}Fe	^{63}Cu	^{55}Mn	^{66}Zn
rPAF	51.2	12.6	< D.L	< D.L

<L.D, under detection limit

3.1.2. Aroma development

3.1.2.1. Accelerated hydrolysis at 45 ºC

Assuming that acid hydrolysis provides the best possible assessment of the grape aroma potential, different hydrolysis conditions were studied. In all cases, the PAFs of rTD2 and rGR8 were rediluted with water containing tartaric acid to 13% ethanol and pH 3.5 to form the reconstituted rPAFs. These rPAFs were first hydrolyzed at 45 ºC under strict anoxic conditions at three different times (2, 4 and 7 weeks). In order to assess the relevance of the presence of sugar and also to assess the potential losses of some precursors, the original mistelle and a rPAF enriched in sugars (named rsPAF) were also processed. In order to assess the effect of strict anoxia on aroma development, one of the series was aged 7 weeks in the presence of a little chamber of air and without any special insulation. Aroma compounds released from glycosidic precursors were analyzed by sensory analysis and by GC-MS.

The results of the sensory analysis of the hydrolyzed samples (rPAF, rsPAF and mistelle) reveal that aroma development takes a long time, since intensity and aromatic complexity increased with time. In fact, it was only after 7 weeks of anoxic aging that the samples developed complex and intense aromas, as summarized in the last lines of Tables III.7 and

III.8 for Tempranillo and Grenache, respectively. The most interesting aromas were developed in rPAFs after 7 weeks of anoxic aging at 45 ºC. In the case of Tempranillo variety (Table III.7), the rPAF was described by the panelists as containing fresh fruit, fruit in syrup, sweet, spicy and phenolic notes.

Table III.7. Sensory description of the samples obtained after the accelerated hydrolysis at 45 ºC during 2, 4 and 7 weeks in anoxic conditions and in the presence of oxygen of: i) the reconstituted phenolic and aromatic fractions in synthetic wine (rPAF); ii) the mistelle from Tempranillo grapes; and iii) the reconstituted phenolic and aromatic fractions in synthetic wine and sugar (rsPAF).

	rPAF Tempranillo				Mistelle Tempranillo				rsPAF Tempranillo	
	2w	4w	7w	7wO	2w	4w	7w	7wO	7w	7wO
Sensory descriptors	Sweet, weak	Fruit, fruit in syrup	Fresh fruit, fruit in syrup, spicy, phenolic	Oxidized	Sweet, weak	Sweet, fruit	Tomato marmalade, caramel, syrup, raisin	Oxidized	Sweet, caramel, kerosene	Oxidized

2w, 2 weeks; 4w, 4 weeks; 7w, 7 weeks; O, incubated in the presence of oxygen.

On the other hand, in the case of rPAF from Grenache (Table III.8), the sample was described with fruit in syrup, floral and tea notes. Some of these notes, such as syrupy, sweet, spicy or tea, are typical from hydrolysates obtained from glycosidic precursors (Fischer, 2007; Loscos, Hernández-Orte, Cacho, & Ferreira, 2007), but they were present at much higher intensity and were richer in fruity aromas.

Table III.8. Sensory description of the samples obtained after the accelerated hydrolysis at 45 ºC during 2, 4 and 7 weeks in anoxic conditions and in the presence of oxygen of: i) the reconstituted phenolic an aromatic fractions in synthetic wine (rPAF); ii) the mistelle from Grenache grapes; and iii) the reconstituted phenolic and aromatic fraction in synthetic wine and sugar (rsPAF).

	rPAF Grenache				Mistelle Grenache				rsPAF Grenache	
	2w	4w	7w	7wO	2w	4w	7w	7wO	7w	7wO
Sensory descriptors	Sweet, weak	Fruit in syrup	Fruit in syrup, floral, tea	Oxidized	Sweet, weak	Fruit in syrup	Truffle, syrup, caramel, raisin	Oxidized	Sweet, caramel, kerosene	Oxidized

2w, 2 weeks; 4w, 4 weeks; 7w, 7 weeks; O, incubated in the presence of oxygen.

The presence of sugar in the hydrolytic media had a surprising sensory effect since after 7 weeks of aging, strong kerosene notes were detected in the rsPAF samples (Tables III.7

and III.8). On the other hand, untreated mistelles developed some distinctive notes, such as tomato in the case of Tempranillo (Table III.7) and truffle notes in the case of Grenache (Table III.8), which were attributed to the presence of DMS, further confirming that the precursors for this molecule are lost during the preparation of PAFs. However, untreated mistelles also developed strong and very sweet caramel-like and raisin-like aromas likely related to Strecker degradation and Maillard reaction that masked other varietal aromas. This suggests that untreated mistelles may be not adequate for assessing varietal aroma, which, except for DMS, seems to best expressed in reconstituted PAFs.

The presence of oxygen in all cases was extremely detrimental to aroma development (Tables III.7 and III.8), since samples not stored under strict anoxic conditions developed typical oxygen-related wine off-odors (Chisholm, Guiher, & Zaczkiewicz, 1995; Escudero, Asensio, Cacho, & Ferreira, 2002; Lopes, Silva, Pons, Tominaga, Lavigne, Saucier, Darriet, Teissedre, Dubourdieu, 2009), suffered browning (Cheynier, Basire, & Rigaud, 1989; Fernández-Zurbano, Ferreira, Peña, Escudero, Serrano, & Cacho, 1995; Ma & Waterhouse, 2018) and did not retain any of the typical aromas noted under anoxic conditions.

In order to get a better insight into the chemicals potentially responsible for these differences, two of the samples (together with rPAF at 75 ºC), were subjected to semiquantitative GC-O (Table III.9), and all samples were submitted to quantitative GC-MS of selected odorants (Tables III.10 and III.11 for Tempranillo and Grenache, respectively).

Samples sent to GC-O were the rPAF and mistelle incubated at 45 ºC for 7 weeks from Tempranillo, and rPAF aged at 75 ºC for 24 h, which displayed the strongest sensory notes (Table III.9).

Table III.9. Odorants identified by GC-O in rPAF and mistelle incubated at 45ºC during 7 weeks and rPAF incubated at 75 ºC during 24 h in strict anoxic conditions.

RI polar	RI non polar	description	compound	rPAF7wT45	M7wT45	rPAF24hT75
1008		Solvent, cetone, strawberry	n.i. solvent	61	72	60
1143	798	Green, fresh grape, grass, tomato, vegetal	Z-3-hexenal	68	32	45
1302	973	Mushroom	1-octen-3-one	50	79	79
1373	981	Green, floral, olives, flower stem, geranium, sweet	(Z)-1,5-octadien-3-one	32	50	35
1392	856	Grass, green, flower stem, vegetal, rancid, soap, citric	Z-3-hexen-1-ol	55	48	25
1405		Soap, oil, rancid, coockies	E-2-hexen-1-ol	54	29	50
1439		Tobacco, vegetable, flower stem, earthy, dirty, rancid	E-2-octenal	53	43	35
1455	909	Baked potato	Methional	0	56	0
1457	1070	Boxwood, grapefruit, passion fruit, sweat	Dihydromyrcenol and/or linalool oxide	57	47	76
1507	1147	Wet paper, rancid, cucumber, green bug, coriander, cardboard	Z-2-nonenal	79	74	89
1537	1163	Plastic, cucumber, cardboard, rancid, wax	E-2-nonenal	56	53	50
1556	1095	Floral, roses, sweet	Linalool	31	45	53
1588	1156	Vegetal, plastic, rancid, cucumber, green, clhorine	(E,Z)-2,6-nonadienal	71	66	43
1705	1212	Oly, rancid, spicy, mushroom, yeasts	(E,E)-2,4-nonadienal	50	50	40
1749		Plasticine, fatty, gasoline, smoke, vegetable	TDN	58	0	32
1775	1230	sweet fruit, apple compoted, cucumber, green, flower stem	n.i 1775	45	50	32
1828	1393	Baked apple, stewed fruit, raspberry	β-damascenone	64	74	61
1852	1128	Grapefruit, citric, swear	3MH	98	79	94
1867	1087	Spicy, smoked, bacon, sweet	guaiacol	60	87	70
1923	1120	Roses, floral	β-phenylethanol	83	79	79
1952	1484	Spicy, violet, sweet, fruity, balsamic, toast, roses, caramel	β-ionone	58	45	35
1975		Cardboard, pool mat, solvent, bleach, saffron	n.i. 1975	35	69	38
2045	1078	Sugar cotton, cake, cupcake, sweet, spicy, caramel, sweet	furaneol	64	61	38
2095	1073	Animal, horse, horse stable, unpleasant	m/p-cresol	50	50	38
2146	1465	Citric, sweet, floral, leather	ethyl cinnamate	50	41	47
2173	1474	Sugar cotton, spicy, sweet, caramel, cabbage, toasted almond	γ-decalactone	58	52	84
2184	1352	Clove, spice, curry, pepper, cinnamon, dry herb	eugenol	71	72	38
2214	1099	toasted, spicy, pepper clove	sotolon	53	35	32
2260	1484	Coconut	Massoia lactone	48	48	50
2286		Spicy, clove, smoked, toast, bacon, pepper	2,6-dimethoxyphenol	83	83	67
2373	1451	Toast, citric, smoked, clove, dried fruit, spicy	E-isoeugenol	66	43	50
2595	1405	Vanilla, chocolate, smoke,	vanillin	80	69	83

Gas chromatographic data, olfactory description, chemical identity, modified frequency (MF) expressed as % for each compound.
RI, retention index on polar capillary column (DB-WAX), and non polar capillary column (DB-5); n.i., not identified.
M, mistelle; 7w, 7 weeks; T, tempranillo. 45, 45 ºC; 75, 75 ºC

Results revealed the presence of up to 32 different odorants at levels potentially relevant from the sensory point of view. Odorants present in the samples can be classified into several categories:

1. Lipid derivatives, that with 10 different odorants is the most numerous group and includes *Z*-3-hexenal, 1-octen-3-one, *Z*-1,5-octadien-3-one, *Z*-3-hexenol, *E*-2-octenal, *Z*-2-nonenal, *E*-2-nonenal, *E,Z*-2,6-nonadienal, *E,E*-2,4-nonadienal, γ-decalactone and massoia lactone.
2. Volatile phenols and vanillins, including guaiacol, cresols, eugenol, 2,6-dimethoxyphenol, *E*-isoeugenol and vanillin.
3. Norisoprenoids and terpenes, including linalool oxide (and/or dihydromyrcenol), linalool, TDN, β-damascenone and β-ionone.
4. Amino acid derivatives, including methional and sotolon.
5. Miscellaneous compounds, including β-phenylethanol, ethyl cinnamate, furaneol, 3-mercaptohexanol and three unidentified compounds.

As can be seen in Table III.9, there is a close proximity between the olfactometric profiles of the samples, since most of the odorants were present at not very different olfactometric scores. However, there are some differences which may explain some of the sensory specificities previously noted. Surely, the most remarkable is the complete absence of methional in the GC-O profile of the aged rPAFs, since this compound can be responsible for the cooked fruit and raisin character noted in aged mistelles (San Juan, Ferreira, Cacho, & Escudero, 2011). It can be assumed that this compound was formed by Strecker degradation of methionine present in the must. Since methionine was not retained in the SPE used for isolating PAFs, this explains the absence of methional in the aged rPAFs. Its presence in the aromatic profile of the mistelle can be considered a nuisance, since most methionine will be consumed in alcoholic fermentation.

Another odorant which was present at much higher GC-O score in mistelles was the unknown with RI 1975 with descriptors such as cardboard or bleach, which may also have some role on the lack of aromatic expression of aged mistelles.

A third remarkable difference is the non-detection of TDN in the GC-O profile of the mistelle (Table III.9). A look to Tables III.10 confirms that this compound was indeed present in the mistelle, but at a much smaller concentration level. A fourth remarkable difference is the lowest level of furaneol and *m*-cresol in rPAF hydrolyzed at 75 ºC (Table III.9), which is confirmed by the presence of lowest concentrations of these compounds in rPAF at 75 ºC (Tables III.10 and III.13).

Also remarkable is the much smaller olfactometric score obtained for 3MH in aged mistelle, which reached maxima score in aged rPAFs (Table III.9). The presence of 3MH at those olfactometric scores suggest that this powerful aroma compound is responsible for the fresh fruit character noted in the aged rPAFs. The presence of 3MH in both aged mistelles and rPAFs is surprising, since this compound is known to be produced by specific yeast-driven enzymatic hydrolysis of the glutathionylated and cysteinylated precursors. Our results, however, strongly suggest that it may be also produced by chemical degradation of the corresponding precursors.

Of the four families of aroma compounds found in aged rPAFs (Table III.9), lipid and amino acid derivatives will be most surely not relevant in wine, since aldehydes will be most likely reduced in fermentation to the corresponding alcohols (Perpète & Collin, 2000), and amino acids will be consumed by yeast. By contrast, volatile phenols, vanillins, terpenes and norisoprenoids will be just marginally affected by yeast metabolism, and may exert an impact on wine aroma (Loscos et al., 2007). These compounds were targeted by quantitative GC-MS. 3MH could not be quantified with the method used, which had a too high detection limit (1-2 µg/L) for this powerful aroma compound.

Table III.10. Average concentration (µg/L) of volatile compounds released after the accelerated hydrolysis at 45 ºC during 2, 4 and 7 weeks in anoxic conditions and in the presence of oxygen of: i) the reconstituted phenolic and aromatic fractions in synthetic wine (rPAF); ii) the mistelle from Tempranillo grapes; and iii) the reconstituted phenolic and aromatic fractions in synthetic wine and sugar (rsPAF).

		rPAF Tempranillo				Mistelle Tempranillo				rsPAF Tempranillo	
		2w	**4w**	**7w**	**7wO**	**2w**	**4w**	**7w**	**7wO**	**7w**	**7wO**
Norisoprenoids	α-Ionone	0.30 ± 0.02^{a}	0.32 ± 0.01^{a}	0.23 ± 0.01^{b}	0.68 ± 0.03	0.63 ± 0.02^{c}	0.75 ± 0.05^{a}	0.69 ± 0.06^{b}	0.91 ± 0.04	0.25 ± 0.01	0.78 ± 0.07
	β-Ionone	1.14 ± 0.09^{b}	1.32 ± 0.06^{a}	1.07 ± 0.01^{b}	2.64 ± 0.04	1.76 ± 0.06^{c}	2.37 ± 0.25^{a}	2.40 ± 0.16^{a}	2.99 ± 0.05	1.10 ± 0.02	3.21 ± 0.01
	β-Damascenone	14.9 ± 0.76	15.7 ± 0.69	15.5 ± 0.007	13.2 ± 0.49	16.9 ± 0.46	17.2 ± 1.25	16.4 ± 0.35	15.5 ± 0.05	15.8 ± 0.71	14.2 ± 0.14
	TDN	2.64 ± 0.14^{c}	19.1 ± 1.31^{b}	37.4 ± 2.09^{a}	49.0 ± 0.05	1.45 ± 0.01^{c}	5.74 ± 0.27^{b}	13.5 ± 1.08^{a}	15.1 ± 0.84	43.5 ± 1.48	63.0 ± 1.46
	Vitispirane A[1]	0.13 ± 0.01^{c}	0.65 ± 0.03^{b}	1.00 ± 0.04^{a}	0.82 ± 0.001	0.08 ± 0.01^{c}	0.28 ± 0.01^{b}	0.49 ± 0.02^{a}	0.45 ± 0.009	0.96 ± 0.009	0.92 ± 0.01
	Vitispirane B[1]	0.06 ± 0.01^{c}	0.33 ± 0.01^{b}	0.47 ± 0.01^{a}	0.36 ± 0.001	0.04 ± 0.02^{c}	0.14 ± 0.01^{b}	0.24 ± 0.01^{a}	0.22 ± 0.007	0.47 ± 0.02	0.42 ± 0.01
	Riesling Acetal[1]	0.12 ± 0.01^{b}	0.31 ± 0.01^{a}	0.30 ± 0.01^{a}	0.34 ± 0.001	0.06 ± 0.01^{c}	0.16 ± 0.01^{b}	0.25 ± 0.02^{a}	0.30 ± 0.02	0.21 ± 0.008	0.25 ± 0.01
Terpenoids	β-Citronellol	1.71 ± 0.16^{ab}	1.89 ± 0.16^{a}	1.43 ± 0.06^{b}	3.49 ± 0.12	1.57 ± 0.04^{b}	2.17 ± 0.09^{a}	2.17 ± 0.16^{a}	2.58 ± 0.17	1.54 ± 0.09	3.92 ± 0.23
	Geraniol	0.53 ± 0.06	0.65 ± 0.05	0.57 ± 0.04	0.80 ± 0.02	0.84 ± 0.005	0.79 ± 0.03	0.86 ± 0.03	1.00 ± 0.14	0.56 ± 0.09	0.75 ± 0.01
	Linalool	2.74 ± 0.01^{b}	2.26 ± 0.05^{c}	5.59 ± 0.12^{a}	3.22 ± 0.06	2.17 ± 0.10^{b}	2.61 ± 0.08^{b}	6.72 ± 0.26^{a}	2.88 ± 0.04	5.57 ± 0.16	3.55 ± 0.06
	α-Terpineol	16.0 ± 0.13^{b}	21.8 ± 0.46^{a}	8.69 ± 0.09^{c}	8.33 ± 0.73	2.52 ± 0.01^{c}	4.99 ± 0.05^{b}	7.38 ± 0.29^{a}	6.81 ± 0.35	13.8 ± 0.02	11.7 ± 0.43
Volatile phenols	Guaiacol	6.31 ± 0.06^{c}	11.6 ± 0.55^{b}	14.3 ± 0.09^{a}	19.9 ± 0.37	4.60 ± 0.01^{c}	8.18 ± 0.09^{b}	11.3 ± 0.27^{a}	12.1 ± 0.30	10.6 ± 0.01	12.9 ± 0.04
	Eugenol	0.67 ± 0.05^{c}	0.93 ± 0.06^{b}	1.28 ± 0.04^{a}	1.28 ± 0.09	0.85 ± 0.08	1.25 ± 0.43	1.24 ± 0.02	1.35 ± 0.10	1.25 ± 0.07	1.36 ± 0.02
	E-Isoeugenol	0.14 ± 0.01^{b}	0.17 ± 0.05^{b}	0.32 ± 0.01^{a}	0.08 ± 0.006	0.11 ± 0.001	0.17 ± 0.07	0.15 ± 0.002	0.15 ± 0.06	0.15 ± 0.02	0.09 ± 0.01
	Methoxyeugenol	1.68 ± 0.06^{b}	4.22 ± 1.17^{a}	5.91 ± 0.08^{a}	5.41 ± 0.98	1.73 ± 0.29^{b}	2.35 ± 0.29^{b}	3.47 ± 0.09^{a}	3.70 ± 0.18	7.78 ± 2.77	5.78 ± 0.02
	2.6-dimethoxyphenol	48.7 ± 2.75^{c}	107 ± 4.64^{b}	142 ± 4.92^{a}	203 ± 8.20	28.8 ± 1.36^{c}	65.7 ± 4.16^{b}	98.7 ± 0.01^{a}	116 ± 7.07	91.8 ± 1.78	121 ± 5.80
	m-Cresol	0.26 ± 0.01^{b}	0.54 ± 0.02^{a}	0.51 ± 0.02^{a}	0.80 ± 0.006	0.25 ± 0.01^{c}	0.33 ± 0.01^{b}	0.40 ± 0.01^{a}	0.58 ± 0.009	0.67 ± 0.02	1.01 ± 0.01
	o-Cresol	0.40 ± 0.01^{b}	0.45 ± 0.01^{a}	0.47 ± 0.02^{a}	0.50 ± 0.007	0.76 ± 0.01^{c}	0.77 ± 0.01^{b}	0.83 ± 0.01^{a}	0.83 ± 0.02	0.67 ± 0.27	0.50 ± 0.01
	4-Ethylguaiacol	< D.L^{b}	0.04 ± 0.01^{a}	0.06 ± 0.01^{a}	0.25 ± 0.19	0.06 ± 0.03	0.04 ± 0.01	0.07 ± 0.02	0.11 ± 0.02	0.35 ± 0.40	0.10 ± 0.03
	4-Vinylguaicol	71.2 ± 22.3	35.5 ± 13.3	27.7 ± 1.02	32.8 ± 0.25	39.5 ± 8.65	47.0 ± 22.3	46.9 ± 10.1	53.2 ± 10.3	47.5 ± 4.17	21.5 ± 7.78
	4-Vinylphenol	1280 ± 254	1060 ± 533	1225 ± 121	1152 ± 6.55	641 ± 99.2	725 ± 439	1361 ± 88.6	1076 ± 736	2561 ± 841	1154 ± 710
Vanillins	Acetovanillone	14.5 ± 0.48	17.1 ± 2.81	16.7 ± 0.68	32.3 ± 1.73	12.5 ± 1.27	12.1 ± 0.25	13.8 ± 0.13	33.6 ± 2.10	16.1 ± 0.03	23.4 ± 0.84
	Vanillin	34.8 ± 0.96	44.9 ± 4.45	41.3 ± 0.61	171 ± 0.62	30.9 ± 1.93	28.7 ± 0.70	35.5 ± 2.71	205 ± 14.3	46.5 ± 13.4	114 ± 1.02
Miscellaneous grroup	Furaneol[1]	0.20 ± 0.00^{a}	0.18 ± 0.01^{b}	0.13 ± 0.01^{c}	0.14 ± 0.007	0.13 ± 0.06	0.16 ± 0.007	0.16 ± 0.006	0.16 ± 0.01	0.14 ± 0.01	0.16 ± 0.01
	Ethyl cinnamate	0.14 ± 0.01^{c}	0.65 ± 0.03^{a}	0.25 ± 0.03^{b}	0.32 ± 0.02	1.20 ± 0.05	1.31 ± 0.06	1.35 ± 0.07	1.27 ± 0.05	0.29 ± 0.005	0.40 ± 0.004
	DMS	nd	nd	< D.L	nd	nd	nd	9.78 ± 0.00	nd	nd	nd

Different letters indicate significant differences according to Duncan's post-hoc test for the comparison of the time of incubation. [1], relative area; nd , no determined; < D.L, under detection limit. 2w, 2 weeks; 4w, 4 weeks; 7w, 7 weeks; O, incubated in the presence of oxygen.

Quantitative data given in Tables III.10 for Tempranillo, confirms the absence of DMS from aged rPAF and rsPAF, confirming that DMS precursors are not retained in the SPE procedure. DMS can explain the tomato and truffle notes specifically developed in aged mistelles (Table III.7 and III.8 for Tempranillo and Grenache, respectively) (Picard, Thibon, Redon, Darriet, de Revel, & Marchand, 2015; Segurel, Razungles, Riou, Salles, & Baumes, 2004).

Results also confirm that the formation of TDN strongly depends on the chemical context. TDN has been related to kerosene nuances (Simpson, 1978; Simpson & Miller, 1983; Winterhalter, 1991) and may explain the specific nuances noted in aged rsPAFs (Table III.7 and III.8 for Tempranillo and Grenache, respectively), since these samples are those accumulating highest levels of this compound (Table III.10 and III.11 for Tempranillo and Grenache, respectively). These results suggest that the presence of sugar promotes carotenoid degradation. However, there should be other factors influencing carotenoid degradation since minima levels of TDN were observed in aged mistelles, in spite of their high amount of sugars.

In fact, it is remarkable that in mistelles the aroma development seems to be slower. In all cases levels of TDN, vistispiranes, Riesling acetal and most volatile phenols and vanillin derivatives are present at smaller levels, while linalool and geraniol, are present at higher levels (Tables III.10 and III.11 for Tempranillo and Grenache, respectively). This can be best seen in Figure III.2, which shows the projection of samples on the plane formed by the two principal components of the PCA obtained with GC-MS data.

Table III.11. Average concentration (µg/L) of volatile compounds released after the accelerated hydrolysis at 45 ºC during 2, 4 and 7 weeks in anoxic conditions and in the presence of oxygen of: i) the reconstituted phenolic an aromatic fractions in synthetic wine (rPAF); ii) the mistelle from Grenache grapes; and iii) the reconstituted phenolic and aromatic fraction in synthetic wine and sugar (rsPAF).

		rPAF Grenache				Mistelle Grenache				rsPAF Grenache	
		2w	**4w**	**7w**	**7wO**	**2w**	**4w**	**7w**	**7wO**	**7w**	**7wO**
Norisoprenoids	α-Ionone	0.79 ± 0.12^{a}	0.96 ± 0.01^{a}	0.44 ± 0.07^{b}	0.52 ± 0.006	1.26 ± 0.05^{a}	1.06 ± 0.02^{b}	0.60 ± 0.03^{c}	0.67 ± 0.008	0.40 ± 0.09	0.36 ± 0.01
	β-Ionone	6.19 ± 0.18^{b}	6.88 ± 0.07^{a}	1.55 ± 0.04^{c}	1.65 ± 0.04	7.62 ± 0.01^{a}	6.69 ± 0.01^{b}	2.13 ± 0.12^{c}	2.13 ± 0.04	1.50 ± 0.07	1.28 ± 0.07
	β-Damascenone	17.3 ± 0.92	17.5 ± 0.87	15.1 ± 0.24	12.3 ± 0.08	16.2 ± 0.39	15.9 ± 0.51	15.7 ± 0.97	12.5 ± 0.32	14.9 ± 0.38	13.7 ± 0.42
	TDN	18.2 ± 0.20^{c}	25.1 ± 0.49^{b}	60.6 ± 1.12^{a}	51.7 ± 3.84	4.46 ± 0.11^{c}	11.5 ± 0.01^{b}	28.8 ± 2.06^{a}	45.8 ± 0.20	92.5 ± 7.31	78.6 ± 5.88
	Vitispirane A[1]	0.41 ± 0.007^{c}	0.54 ± 0.01^{b}	0.83 ± 0.02^{a}	0.75 ± 0.03	0.13 ± 0.01^{c}	0.27 ± 0.01^{b}	0.49 ± 0.02^{a}	0.51 ± 0.001	0.92 ± 0.06	0.79 ± 0.04
	Vitispirane B[1]	0.16 ± 0.001^{c}	0.22 ± 0.01^{b}	0.33 ± 0.01^{a}	0.29 ± 0.007	0.05 ± 0.01^{c}	0.10 ± 0.01^{b}	0.18 ± 0.01^{a}	0.20 ± 0.005	0.36 ± 0.008	0.30 ± 0.01
	Riesling Acetal[1]	0.51 ± 0.007^{b}	0.49 ± 0.02^{b}	0.57 ± 0.02^{a}	0.47 ± 0.02	0.19 ± 0.01^{c}	0.31 ± 0.01^{b}	0.45 ± 0.02^{a}	0.50 ± 0.003	0.35 ± 0.02	0.39 ± 0.02
Terpenoids	β-Citronellol	4.27 ± 0.14^{a}	4.81 ± 0.20^{a}	2.23 ± 0.20^{b}	3.71 ± 0.22	12.3 ± 0.51^{a}	10.3 ± 0.46^{b}	7.33 ± 0.13^{c}	5.18 ± 0.32	2.06 ± 0.07	2.33 ± 0.08
	Geraniol	3.03 ± 0.23^{a}	2.22 ± 0.02^{b}	0.42 ± 0.11^{c}	1.13 ± 0.004	9.39 ± 0.19^{a}	6.73 ± 0.03^{b}	3.58 ± 0.07^{c}	1.96 ± 0.07	0.43 ± 0.006	0.54 ± 0.03
	Linalool	11.1 ± 1.03^{a}	9.09 ± 0.11^{b}	1.88 ± 0.07^{c}	3.53 ± 0.002	24.1 ± 0.08^{a}	18.5 ± 0.14^{b}	9.59 ± 0.06^{c}	5.61 ± 0.006	1.28 ± 0.03	1.38 ± 0.02
	α-Terpineol	75.9 ± 0.18^{a}	67.6 ± 0.95^{b}	31.3 ± 0.04^{c}	42.2 ± 0.30	33.7 ± 0.02^{c}	50.3 ± 0.94^{b}	60.4 ± 0.67^{a}	50.0 ± 1.36	29.4 ± 0.37	30.2 ± 0.36
Volatile phenols	Guaiacol	4.97 ± 0.39^{c}	8.99 ± 0.31^{b}	12.8 ± 0.53^{a}	11.5 ± 0.35	3.79 ± 0.14^{c}	5.83 ± 0.02^{b}	7.04 ± 0.06^{a}	8.69 ± 0.23	8.27 ± 0.24	10.2 ± 0.01
	Eugenol	0.25 ± 0.01^{c}	0.30 ± 0.01^{b}	0.48 ± 0.03^{a}	0.30 ± 0.009	0.29 ± 0.01^{a}	0.30 ± 0.01^{a}	0.22 ± 0.01^{b}	0.32 ± 0.008	0.32 ± 0.03	0.36 ± 0.04
	E-Isoeugenol	0.26 ± 0.001^{c}	0.30 ± 0.01^{b}	0.35 ± 0.01^{a}	0.21 ± 0.05	0.25 ± 0.01^{b}	0.28 ± 0.03^{a}	0.25 ± 0.01^{b}	0.35 ± 0.01	0.19 ± 0.05	0.17 ± 0.06
	Methoxyeugenol	0.54 ± 0.02^{c}	6.65 ± 0.07^{b}	8.61 ± 0.16^{a}	1.23 ± 0.03	0.40 ± 0.01^{c}	0.71 ± 0.03^{b}	0.83 ± 0.01^{a}	1.78 ± 0.50	1.81 ± 0.12	1.56 ± 0.11
	2.6-dimethoxyphenol	29.1 ± 1.24^{c}	55.9 ± 2.16^{b}	87.2 ± 1.15^{a}	76.7 ± 1.03	19.6 ± 0.13^{c}	31.1 ± 0.49^{b}	41.9 ± 0.22^{a}	57.1 ± 0.60	52.9 ± 1.91	74.1 ± 1.90
	m-Cresol	0.87 ± 0.009^{c}	1.03 ± 0.01^{b}	2.44 ± 0.01^{a}	1.43 ± 0.01	0.42 ± 0.01^{b}	0.51 ± 0.01^{a}	0.52 ± 0.01^{a}	0.65 ± 0.003	1.91 ± 0.02	1.93 ± 0.01
	o-Cresol	0.49 ± 0.001^{b}	0.51 ± 0.01^{b}	0.58 ± 0.03^{a}	0.76 ± 0.01	0.81 ± 0.01^{b}	0.82 ± 0.01^{b}	0.91 ± 0.01^{a}	0.97 ± 0.03	0.59 ± 0.006	0.58 ± 0.01
	4-Ethylguaiacol	0.00 ± 0.00^{c}	0.56 ± 0.03^{b}	1.43 ± 0.05^{a}	0.08 ± 0.00	0.04 ± 0.01	0.05 ± 0.009	0.05 ± 0.006	0.21 ± 0.07	0.06 ± 0.006	0.00 ± 0.00
	4-Vinylguaicol	102 ± 50.4	59.0 ± 12.6	27.9 ± 2.18	68.0 ± 6.07	37.7 ± 3.19	51.8 ± 0.10	47.4 ± 6.09	85.3 ± 0.07	22.7 ± 2.49	50.6 ± 2.13
	4-Vinylphenol	328 ± 93.7	232 ± 56.5	207 ± 134	483 ± 35.7	144 ± 9.89^{b}	199 ± 4.80^{b}	333 ± 47.2^{a}	551 ± 19.9	237 ± 24.9	603 ± 45.6
Vanillins	Acetovanillone	22.4 ± 0.51	32.3 ± 9.41	34.9 ± 16.0	66.3 ± 2.46	21.2 ± 2.58	21.5 ± 0.50	19.0 ± 2.09	94.6 ± 2.1	23.7 ± 0.54	40.0 ± 2.28
	Vanillin	66.3 ± 1.61	91.5 ± 13.5	123 ± 20.0	404 ± 6.09	79.5 ± 1.65^{a}	76.1 ± 1.75^{a}	42.5 ± 4.03^{b}	415 ± 1.08	89.9 ± 0.18	425 ± 7.89
Miscellaneous group	Furaneol[1]	0.22 ± 0.004^{a}	0.21 ± 0.01^{b}	0.16 ± 0.01^{c}	0.17 ± 0.004	0.19 ± 0.01^{a}	0.20 ± 0.01^{a}	0.14 ± 0.01^{b}	0.13 ± 0.003	0.15 ± 0.002	0.17 ± 0.01
	Ethyl cinnamate	0.14 ± 0.01^{c}	0.24 ± 0.01^{b}	0.37 ± 0.02^{a}	0.51 ± 0.01	0.67 ± 0.01^{b}	0.72 ± 0.01^{b}	0.85 ± 0.03^{a}	0.75 ± 0.02	0.37 ± 0.02	0.41 ± 0.01

Different letters indicate significant differences according to Duncan's post-hoc test for the comparison of the time of incubation. [1], relative area; nd , no determined; < D.L, under detection limit. 2w, 2 weeks; 4w, 4 weeks; 7w, 7 weeks; O, incubated in the presence of oxygen.

As can be seen in Figure III.2, mistelles aged 2 weeks are at the bottom and right part of the group of samples from both varieties, Grenache in the upper and right part of the plot, and Tempranillo in the lower and left part. It is also most remarkable that in both varieties, 7-weeks aged mistelles lie close to the rPAFs aged just 2 weeks, and that rPAFs and rsPAFs aged 7 weeks are in the left part of the plane, confirming that these samples developed strongly aroma compounds derived from carotenoids and polyphenols, while contained minimal amounts of the unstable linalool and geraniol.

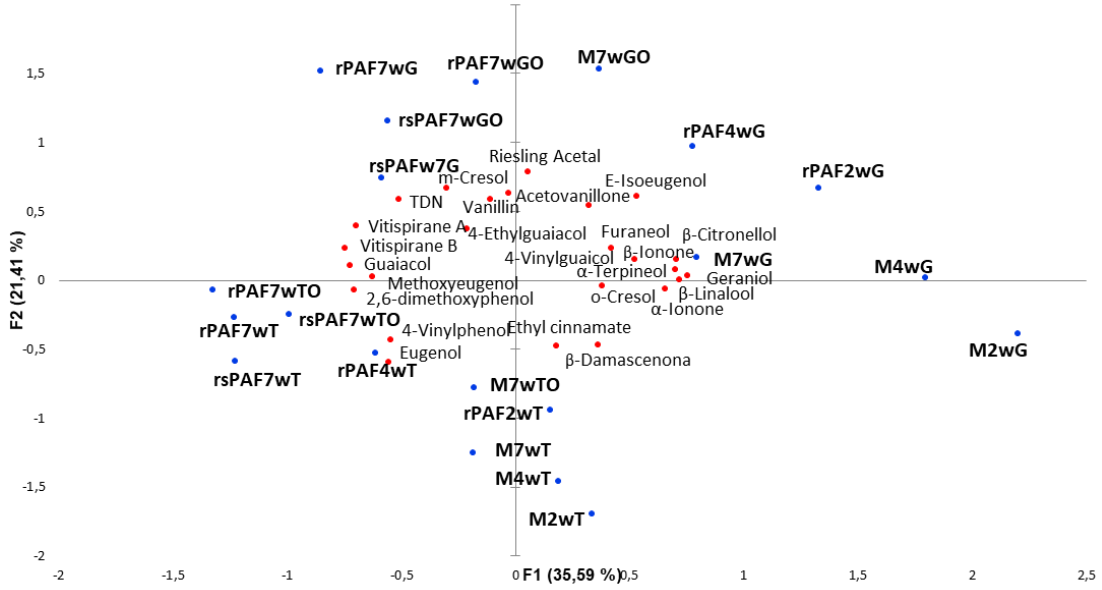

Figure III.2. Principal component analysis of rPAF, mistelle and rsPAF from Grenache and Tempranillo varieties incubated at 45 ºC during 2, 4 and 7 weeks in anoxic conditions as well as in the presence of oxygen. M, mistelle; T, Tempranillo; G, Grenache; 2w, 2 weeks; 4w, 4 weeks; 7w, 7 weeks; O, incubated in the presence of oxygen.

The plot confirms that levels of most compounds increase significantly ($P < 0.05$) with time, except terpenes and the two ionones in Grenanche, whose levels decreased significantly ($P < 0.05$) after 4 weeks of incubation (see Table III.11). The plot also reveals that both varieties had clearly different aroma profiles, richer in terpenols, β-ionone, vainillin and acetovanillone those of Grenache (Table III.11) and richer in volatile phenols those of Tempranillo (Table III.10), consistently with sensory nuances. For both varieties,

rPAF and rsPAF are plotted close together (Figure III.2), which indicates that the presence of sugar, quantitatively, does not produce substantial differences, except for the significantly ($P < 0.05$) higher concentration of TDN in rsPAFs (Tables III.10 and III.11 for Tempranillo and Grenache, respectively). Indirectly, this confirms that some compounds in the mistelle lost during the preparation of PAF are responsible for the slower aroma development. We have no clues about which compounds may exert such an effect.

Regarding the effect of oxygen, it is most remarkable that samples stored 7 weeks under air are in fact close to their corresponding anoxic pairs (except mistelles from Grenache) (Figure III.2), revealing that in spite of the strong aroma degradation noted in those samples, quantitatively changes in the volatile profiles represented in the figure were relatively modest. This confirms old reports revealing that aroma oxidative deterioration is mostly related to the formation of different aldehydes which were not quantified in the present work. Among compounds quantified at 7 weeks (Tables III.10 and III.11), oxidation increased systematically levels of acetovanillone and vanillin in both varieties, levels of TDN in Tempranillo and in mistelle from Grenache, levels of vinylphenols in Grenache and levels of ionones, guaiacol and 2,6-dimethyxyphenol in Tempranillo. Levels of β-damascenone were slightly but significantly reduced by oxidation. The highest impact of oxidation was noted on mistelles from Grenache, a variety that oxidizes more easily (Landrault, Poucheret, Ravel, Gasc, Cros, & Teissedre, 2001), whose aroma composition became in fact much closer to those of the aged PAFs, revealing that in this particular case oxidation accelerates the molecular degradation processes leading to the formation of aroma compounds derived from carotenoids and polyphenols.

It should be finally remarked that levels of some relevant aroma compounds such as β-damascenone, linalool, rose oxide, α-terpineol, guiaicol, eugenol, methoxyeugenol, 2,6-dimehtoxyphenol and vanillin derivates, formed after 7 weeks of anoxic aging are higher

than those observed in hydrolyzates obtained by previous methodologies (Hernández-Orte, Concejero, Astrain, Lacau, Cacho, & Ferreira, 2015), and that leaving aside β-damascenone, which is present at much increased levels, the concentrations of most compounds are between the ranges of occurrence observed in real wines (Ferreira, López, & Cacho, 2000; San Juan, Cacho, Ferreira, & Escudero, 2012).

As 7 weeks is a too long period for assessing grape quality, accelerated hydrolysis at higher temperatures were tried. The aroma development at 75 ºC in the rPAFs from Tempranillo previously used was monitored along 72 h, taking samples for sensory and GC-MS analysis at different times (3, 8, 14, 24, 38, 48, 60 and 72 h).

3.1.2.2. Accelerated hydrolysis at 75 ºC

Regarding sensory analysis, as can be seen in Table III.12, aroma development or rPAF from Tempranillo incubated at 75 ºC requires at least 24 h to reach high intensity. It was also obvious that aroma complexity reached an optimum, so that at higher hydrolysis times the aroma became simpler.

Table III.12. Sensory description of the samples obtained after the accelerated hydrolysis at 75 ºC of the phenolic an aromatic fractions from Tempranillo reconstituted in synthetic wine (rPAF) during 3, 8, 14, 24, 38, 48, 60 and 72 h in anoxic conditions.

	3h	8h	14h	24h	38h	48h	60h	72h
Aroma intensity	Low	Low	Medium	High	High	High	Medium	Medium
Sensory descriptors	Fruit, must, grape, black fruit	Fruit, must, grape, black fruit, tea and grape skin	Fruit, blueberry, sweet and smoky notes	Fresh fruit, blueberry, fruit in syrup, spicy, phenolic, olives and floral	Fresh fruit, fruit in syrup, spicy, plastic and grape skin	Fresh fruit, fruit in syrup and grape skin	Fruit, unspecific	Fruit, unspecific

Triangular tests revealed that samples obtained after 24, 38 or 48 h of hydrolysis were not significantly different between them ($P > 0.1$), while they were easily differentiated (significant at $P < 0.05$) from those aged 14 h or from those aged 60 h. Aromas developed

after 24 h were not much dissimilar to those observed at 45 ºC and their fruity nuances, recalled those observed in aged red wines (Escudero, Cacho, & Ferreira, 2000; San Juan et al., 2012).

Quantitative GC-MS results in Table III.13 confirm that levels of aroma compounds evolve with time in a similar way to that observed at 45 ºC.

All norisoprenoids except the ionones increased steadily with time. This same pattern is observed also for α-terpineol, guaiacol, eugenol, *E*-isoeugenol, methoxyeugenol, 2,6-dimethoxyeugenol, *m*-cresol, *o*-cresol, acetovanillone and vanillin. All these compounds are stable compounds and are degradation products of carotenoids, terpenoids or of phenols, respectively. In clear contrast, β-ionone, geraniol and linalool reached maxima levels before 24 hours, and then steadily decreased. β-citronellol, α-ionone, ethyl cinnamate and vinylphenol reached maximum levels after 24 hours.

Comparing the concentrations of aroma compounds after 7 weeks at 45 ºC (Table III.10) with that observed at 75 ºC (Table III.13), it can be appreciated that at 75 ºC levels of β-ionone, β-damascenone and geraniol after 24 h are slightly higher, while those of volatile phenols and vanillins are lower. In these last cases hydrolysis time have to be higher than 38 h to obtain similar levels. However, it can be also observed that TDN levels are much increased after 24 h. Levels in this sample (28 µg/L) are not far from those measured in the sample aged 7 weeks at 45 ºC (37 µg/L, Table III.10), while the sample hydrolyzed 38 hours at 75 ºC already contained more than 51 µg/L (Table III.13). It can be hypothesized that the poor aroma descriptors given to the rPAFs after 60 and 72 h of incubation at 75 ºC (Table III.12) are related to the huge levels of TDN they contained, even if the descriptors of this molecule were not specifically identified. The threshold for this molecule was first thought to be 20 µg/L (Simpson, 1978), but most recently, it has been corrected to 2 µg/L (Sacks, Gates, Ferry, Lavin, Kurtz, & Acree, 2012).

Table III.13. Average concentration (µg/L) of volatile compounds released after the accelerated hydrolysis at 75 ºC of the phenolic an aromatic fractions from Tempranillo grapes reconstituted in synthetic wine (rPAF) during 3, 8, 14, 24, 38, 48, 60 and 72 h in anoxic conditions.

		3h	8h	14h	24h	38h	48h	60h	72h
	Aroma intensity	Low	Low	Medium	High	High	High	Medium	Medium
Norisoprenoids	α-Ionone*	< D.L^{d}	0.12 ± 0.003^{c}	0.36 ± 0.025^{a}	0.37 ± 0.03^{a}	0.27 ± 0.04^{b}	0.39 ± 0.02^{a}	0.33 ± 0.01^{a}	0.36 ± 0.02^{a}
	β-Ionone*	0.50 ± 0.03^{d}	0.54 ± 0.05^{d}	1.63 ± 0.07^{a}	1.49 ± 0.07^{b}	0.93 ± 0.02^{c}	1.42 ± 0.01^{b}	1.03 ± 0.09^{c}	0.99 ± 0.008^{c}
	β-Damascenone*	10.1 ± 0.29^{d}	20.5 ± 0.25^{c}	22.0 ± 1.77^{bc}	22.5 ± 1.51^{bc}	24.1 ± 0.22^{b}	23.6 ± 0.62^{b}	28.2 ± 1.85^{a}	28.8 ± 0.17^{a}
	TDN*	0.46 ± 0.06^{g}	3.35 ± 0.11^{g}	14.5 ± 0.10^{f}	28.1 ± 1.13^{e}	51.7 ± 0.12^{d}	68.4 ± 0.98^{c}	97.6 ± 5.26^{b}	119 ± 1.29^{a}
	VitispiraneA[1]*	0.006 ± 0.00^{e}	0.10 ± 0.001^{e}	0.28 ± 0.007^{d}	0.60 ± 0.01^{c}	1.00 ± 0.02^{b}	0.98 ± 0.01^{b}	1.45 ± 0.14^{a}	1.50 ± 0.001^{a}
	VitispiraneB[1]*	0.003 ± 0.00^{g}	0.06 ± 0.001^{f}	0.17 ± 0.003^{e}	0.34 ± 0.003^{d}	0.56 ± 0.008^{c}	0.57 ± 0.006^{c}	0.81 ± 0.04^{b}	0.86 ± 0.007^{a}
	RieslingAcetal[1]*	0.008 ± 0.001^{f}	0.08 ± 0.002^{e}	0.19 ± 0.004^{d}	0.32 ± 0.02^{c}	0.41 ± 0.005^{b}	0.42 ± 0.005^{b}	0.50 ± 0.04^{a}	0.51 ± 0.006^{a}
Terpenoids	β-Citronellol*	0.62 ± 0.01^{f}	0.85 ± 0.02^{ef}	2.22 ± 0.01^{b}	2.50 ± 0.18^{a}	1.27 ± 0.01^{cd}	1.48 ± 0.13^{c}	1.17 ± 0.20^{cd}	1.05 ± 0.20^{de}
	Geraniol*	3.90 ± 0.38^{e}	5.61 ± 0.13^{a}	4.85 ± 0.28^{b}	4.68 ± 0.35^{bc}	4.31 ± 0.04^{cde}	4.30 ± 0.07^{cde}	4.56 ± 0.07^{bcd}	4.27 ± 0.17^{cde}
	Linalool*	1.38 ± 0.009^{e}	2.27 ± 0.006^{d}	3.92 ± 0.06^{a}	3.59 ± 0.10^{b}	3.27 ± 0.008^{c}	3.13 ± 0.08^{c}	3.15 ± 0.10^{c}	3.17 ± 0.07^{c}
	α-Terpineol*	1.36 ± 0.04^{f}	2.80 ± 0.13^{e}	6.18 ± 0.25^{d}	8.41 ± 0.07^{c}	10.3 ± 0.11^{b}	11.1 ± 0.08^{b}	12.3 ± 1.14^{a}	12.8 ± 0.65^{a}
Volatile phenols	Guaiacol*	1.19 ± 0.1^{h}	2.86 ± 0.01^{g}	5.45 ± 0.04^{f}	9.49 ± 1.10^{e}	14.0 ± 0.03^{d}	16.4 ± 0.42^{c}	22.4 ± 0.22^{b}	23.4 ± 0.04^{a}
	Eugenol*	0.46 ± 0.03^{e}	0.55 ± 0.02^{de}	0.68 ± 0.03^{d}	0.85 ± 0.14^{c}	0.98 ± 0.04^{c}	1.13 ± 0.09^{b}	1.56 ± 0.03^{a}	1.44 ± 0.03^{a}
	E-Isoeugenol*	0.17 ± 0.008^{f}	0.22 ± 0.01^{e}	0.22 ± 0.003^{e}	0.24 ± 0.01^{de}	0.26 ± 0.003^{d}	0.34 ± 0.02^{c}	0.39 ± 0.03^{b}	0.42 ± 0.008^{a}
	Methoxyeugenol*	2.21 ± 0.16^{e}	2.64 ± 0.25^{e}	2.90 ± 0.12^{de}	3.93 ± 0.42^{d}	4.99 ± 0.08^{c}	6.28 ± 1.07^{b}	7.91 ± 0.41^{a}	8.07 ± 0.09^{a}
	2,6-Dimethoxyphenol*	19.4 ± 0.29^{g}	39.3 ± 2.11^{f}	65.4 ± 0.95^{e}	106 ± 4.25^{d}	153 ± 2.82^{c}	181 ± 8.64^{b}	239 ± 20.9^{a}	251 ± 3.22^{a}
	m-Cresol*	0.13 ± 0.007^{d}	0.15 ± 0.01^{d}	0.24 ± 0.004^{c}	0.27 ± 0.009^{c}	0.33 ± 0.007^{b}	0.35 ± 0.008^{b}	0.44 ± 0.04^{a}	0.46 ± 0.007^{a}
	o-Cresol*	0.41 ± 0.02^{d}	0.42 ± 0.03^{d}	0.45 ± 0.002^{c}	0.47 ± 0.006^{bc}	0.52 ± 0.008^{b}	0.50 ± 0.01^{bc}	0.60 ± 0.03^{a}	0.59 ± 0.03^{a}
	4-Ethylguaiacol	0.07 ± 0.004	0.07 ± 0.006	0.07 ± 0.003	0.08 ± 0.01	0.07 ± 0.005	0.11 ± 0.06	0.08 ± 0.009	0.08 ± 0.007
	4-Vinylguaicol	15.3 ± 0.42	16.9 ± 0.37	20.1 ± 1.89	24.1 ± 3.15	19.8 ± 0.40	27.2 ± 10.4	25.7 ± 0.58	23.7 ± 0.25
	4-Vinylphenol*	59.7 ± 11.1^{c}	64.3 ± 6.58^{c}	141 ± 36.9^{a}	159 ± 33.9^{a}	81.1 ± 8.17^{c}	131 ± 0.00^{ab}	90.6 ± 5.02^{bc}	91.7 ± 6.48^{bc}
Vanillins	Acetovanillone*	11.3 ± 0.73^{c}	11.9 ± 0.16^{c}	11.8 ± 0.72^{c}	12.9 ± 0.57^{bc}	13.1 ± 0.17^{bc}	14.9 ± 2.24^{ab}	15.8 ± 1.34^{a}	16.4 ± 0.05^{a}
	Vanillin*	22.1 ± 1.18^{f}	23.7 ± 0.61^{ef}	27.9 ± 0.86^{de}	30.8 ± 0.18^{cd}	34.6 ± 0.66^{c}	35.6 ± 3.14^{bc}	41.2 ± 4.73^{a}	40.0 ± 0.87^{ab}
Miscellaneous group	Furaneol[1]	0.03 ± 0.0001	0.04 ± 0.001	0.04 ± 0.0008	0.04 ± 0.002	0.04 ± 0.001	0.04 ± 0.001	0.04 ± 0.002	0.04 ± 0.001
	Ethyl cinnamate*	0.11 ± 0.003^{c}	0.11 ± 0.02^{c}	0.22 ± 0.001^{ab}	0.23 ± 0.02^{a}	0.19 ± 0.005^{b}	0.25 ± 0.008^{a}	0.22 ± 0.03^{ab}	0.23 ± 0.01^{a}

Different letters indicate significant differences according to Duncan's post-hoc test. *, significative at 95% (P < 0.05); [1], relative area; < D.L, under detection limit.

Nevertheless, in order to facilitate comparison of data in Table III.10 and III.13, all rPAF from Tempranillo samples aged at 45 and 75 ºC were plotted together in the PCA plot shown Figure III.3.

The plot confirms that samples incubated 24, 38 or 48 hours at 75 ºC are chemically the ones more similar (or the least dissimilar) to the sample aged 7 weeks at 45 ºC. Since sensory notes after 24 hours were more intense and distinctive, likely because of the smaller presence of TDN and smoky volatile phenols, and relatively large levels of terpenols, β-ionone and β-damascenone, this hydrolysis time was retained as best compromise for the characterization of the aroma potential of red grapes used for making high quality wines intended for aging.

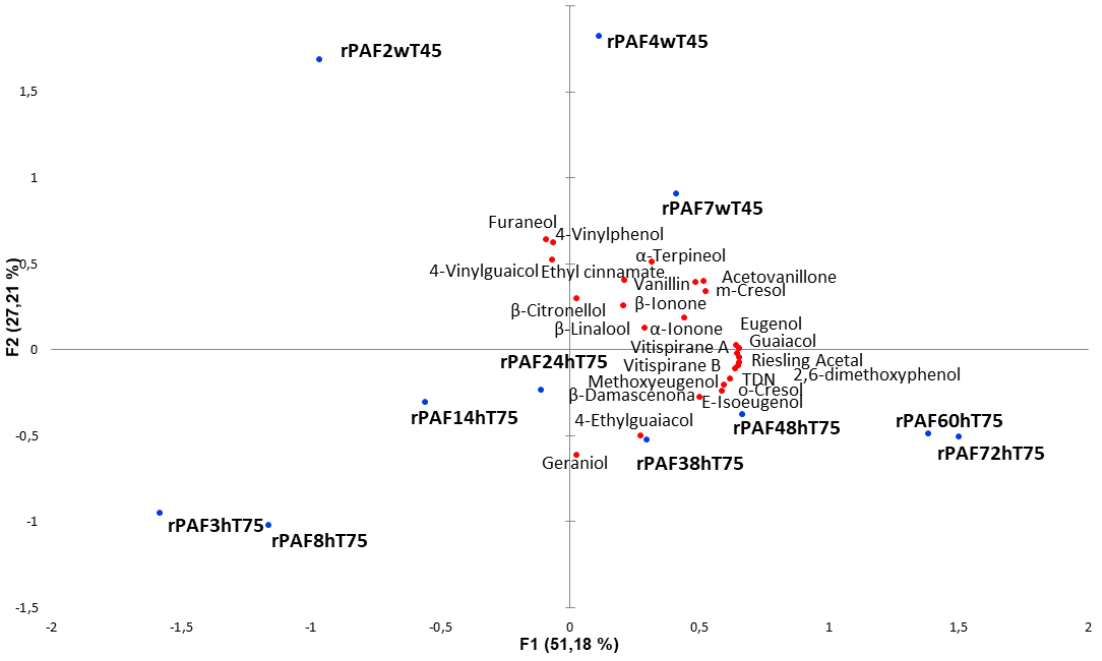

Figure III.3. Principal component analysis of rPAF from Tempranillo variety incubated at 45 ºC during 2, 4 and 7 weeks in anoxic conditions, and at 75 ºC during 3-72 h. T, Tempranillo; 2w, 2 weeks; 4w, 4 weeks; 7w, 7 weeks; 75, incubated at 75 ºC; and 45, incubated at 45 ºC.

Results in the tables, suggest that in the case of terpene-dependent varieties, or even for making wines not intended for aging, the optimal conditions of hydrolysis may turn to be different. It should be also acknowledged that what the present method provides is a general

strategy to develop and measure aroma molecules derived from different grape aroma precursors. This should be particularly useful for comparing grapes, for studying the effects of different agronomical or environmental conditions or as an aid for identifying new precursors. However, it is known that yeast metabolism (Oliveira & Ferreira, 2019) and different technological options can exert a large influence on varietal aroma profiles of aged wines (Clodoveo, Dipalmo, Rizzello, Corbo, & Crupi, 2016), which implies that for predicting wine aroma, the method will have to be validated using a complex design including fermentation and aging in cellar conditions.

3.2. Aroma potential of Grenache and Tempranillo grapes

Once the strategy was developed, it was used to characterize different lots of grapes (Table III.1). The phenolic and aromatic fractions (PAFs) extracted from 33 different lots of grapes from Grenache and Tempranillo were reconstituted in synthetic wine and further submitted to accelerated hydrolysis at 75 ºC for 24 h in strict anoxic conditions. Most samples developed strong aromatic nuances. The aroma developed by the different samples was characterized by sensory analysis, GC-O and GC-MS.

3.2.1. Sensory characterization

The first sensory study consisted of a sorting task aimed at grouping samples attending to their odor properties. Results of the sorting task are summarized in the dendrogram shown in Figure III.4. The labels (descriptors) most frequently used by the judges to describe the clusters created in the sorting task are also given. It can be first observed that the replicate samples introduced as controls (R_uGS4; and R_rTR1) are plotted together in the dendrogram, supporting the consistency of the panel. It can be also observed that the sensory task identified five different sensory categories split into two major groups (group A: clusters 1-2 and group B: clusters 3-5), each one containing samples predominantly from

a single variety. Thirteen out of the 16 samples belonging to clusters 1 and 2 are from Grenache, while 16 out of 19 in the other three clusters are from Tempranillo.

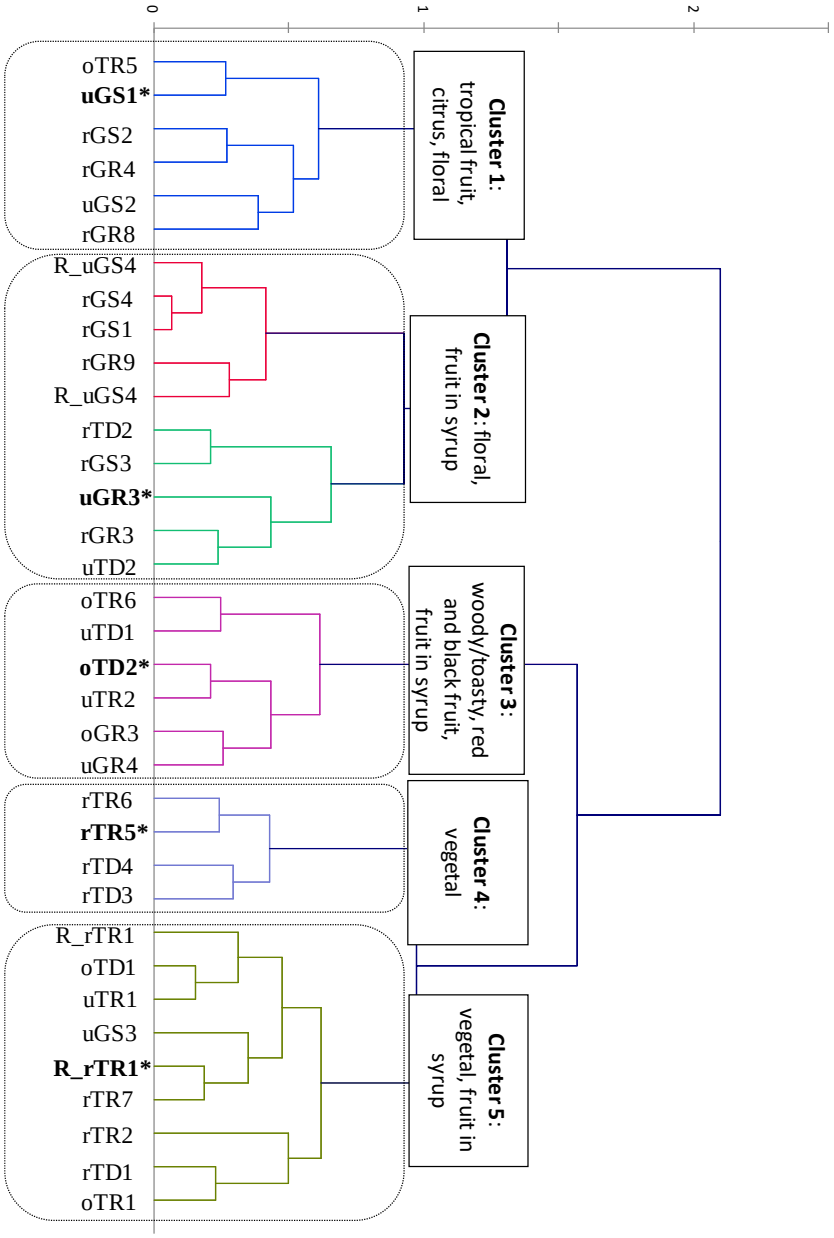

Figure III.4. Dendrogram showing the classes derived from the sorting task carried out on the 35 hydrolyzates obtained from 33 PAFs (plus two replicates, marked with R_). Samples in bold are those selected for further flash profiling. Codes: u, r or o, refers to underripe, ripe or overripe; T or G, refers to Tempranillo or Grenache; R, S or D, refers to Rioja, Somontano or Duero (geographical origin); the last number refers to the specific vineyard within the region.

The two clusters integrated in the main group A (cluster 1+2) were described as “tropical fruit/citrus” and “floral” for cluster 1 and as “floral” and “fruit in syrup” for cluster 2,

suggesting that “floral” is an attribute more specific of Grenache. For group B (clusters 3-5), containing mainly Tempranillo, three other sensory categories were identified. Cluster 3 was mainly described as “woody-toasty”, “red fruit” and “black fruit”, and “fruit in syrup”; cluster 4 as “vegetal”; and cluster 5 as “vegetal” and “fruit in syrup”. Remarkably, the cluster does not reveal any relevant effect of geographic precedence or of the degree of ripeness.

One sample per sensory category was selected as the most representative for each cluster (from cluster 1: uGS1; from cluster 2: uGR3; from cluster 3: oTD2; from cluster 4: rTR5; and from cluster 5: rTR1) for a deeper sensory characterization using flash profile. Results of this study are summarized in the GPA maps given in Figure III.5.

The two first components accumulate 35% and 29% of the original variance, respectively. A first observation from the distribution of samples observed in Figure III.5a is that the varietal distribution obtained in the previous sensory task, is not identified here. In fact, the two samples from Grenache are plotted in extreme positions in the first component. This apparent contradictory result should be attributed to the complementary nature of this second sensory task, which aims quantifying sensory descriptors in dissimilar samples, while the sorting task aims to classify samples. Nevertheless, most descriptors used in the sorting task in Figure III.4 were further cited in the flash profile (Figure III.5) and the sensory profiles obtained are relatively equivalent as will be seen.

In the task, eight descriptors emerged as the most relevant to describe the samples. In order of use: “alcoholic” (cited by 70% of panelists), “fruit in syrup” (63%), “vegetal” (50%), “kerosene” (40%), “tropical fruit/citrus” (40%), “woody/toasty” (29%), “red fruit” (29%) and floral (21%). Attributes differ attending to their ability to discriminate samples, as can be observed in the GPA planes shown in Figures III.5b, c and d.

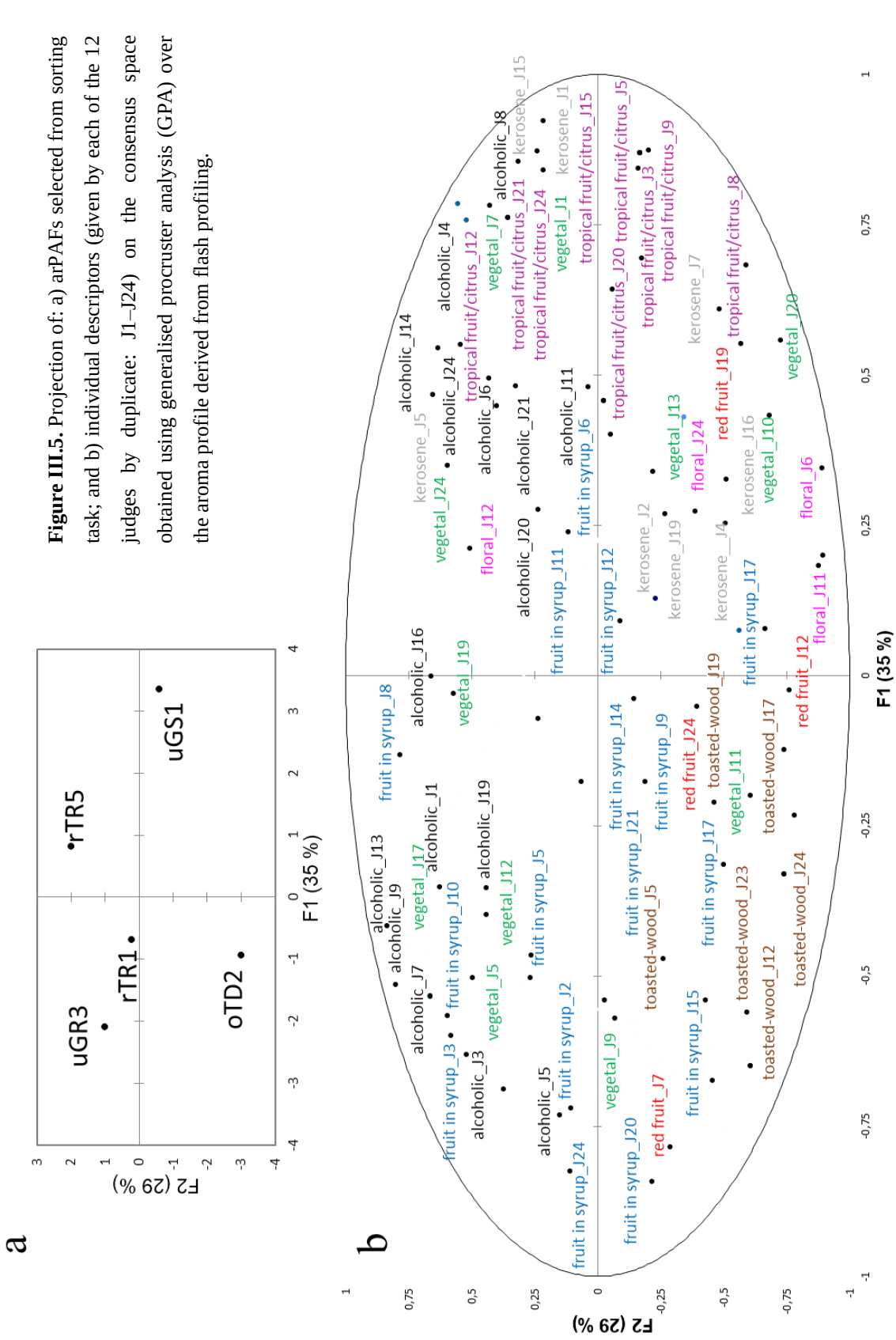

Figure III.5. Projection of: a) arPAFs selected from sorting task; and b) individual descriptors (given by each of the 12 judges by duplicate: J1–J24) on the consensus space obtained using generalised procruster analysis (GPA) over the aroma profile derived from flash profiling.

The most discriminant attributes are those occupying narrow areas of the plane, since specifically define one or two samples. By contrast, those more widely distributed in the plane are similarly used to define all the samples, indicating that represent common attributes. Attending to this criterion, attributes can be ranked into three categories: highly discriminant, discriminant and common. Highly discriminant attributes are characteristic of only one sample and occupy a quite narrow area of the plane. This is the case of "Tropical fruit/citrus" (right part) and "woody/toasty" (down left part) of Figure III.5b. Discriminant attributes are found in one of the halves of the plane, as can be observed in the cases of "alcoholic" (upper half), "floral" (right half), "kerosene" (right part) and "red fruit" (down half). The attribute "Fruit in syrup" is slightly less discriminant, since 73% of the times is found in the left half (Figure III.5b) while the attribute "vegetal" is not discriminant at all. As can be seen in Figure III.5b, it is evenly distributed within the plane, indicating that it is a common characteristic of all the samples.

The sample uGS1, which was representative of the first cluster (Figure III.4), is projected on the right part of the plot in Figure III.5a, indicating that it was described mainly with the terms "tropical fruit/citrus", and " kerosene", which are exclusive attributes for this sample, and as "floral", and "red fruit", which are attributes shared with other samples. The sample uGS1 is also the single one lacking the attribute "fruit in syrup", and scores very low in "alcoholic". This is mostly in agreement with results from the sorting task. The sample oTD2, the representative of the third cluster in Figure III.4, is identified as the second most different in this task. This sample is mainly described with terms such as toasty-woody (exclusive attribute), "red fruit" (shared with the previous one) and "fruit in syrup" (shared with all samples in the left plane). Samples rTR1, representative of cluster 5, and uGR3, representative of cluster 2 were mainly described as "alcoholic" and "fruit in syrup".

Finally, rTR5, representative of the cluster 4 was described with “alcoholic” and “vegetal” notes.

It is remarkable that the attribute “alcoholic” is present in the three samples which do not have specific sensory notes (uGR3, rTR1 and rTR5) (Figure III.5a and b). Since these wine models did not contain major fermentation volatiles, such as higher alcohols, this attribute was likely an exclusive characteristic of ethanol which was similarly present in all the samples. This suggests, that only some of the odorants present in samples uGS1and oTD2, likely also those ones responsible for their exclusive sensory characteristics, are able to mask the aroma (sweet, alcohol) and chemesthesic (pungent, harsh, hot) notes of alcohol. Furthermore, it can be hypothesized, that the “fruit in syrup” character is at least in part the result of the interaction between alcohol and odorants of fruity character, and that only the odorants specifically present in uGS1, likely the ones contributing to its exclusive “tropical fruit/citric” character, can mask. A similar observation was made when the addition of a small amount (1 ng/L) of a green odorant (4-methyl-4-mercapto-2-pentanone) to an aromatic reconstitution reproducing the aroma of a white wine from Macabeo changed the aroma from sweet, alcoholic, synthetic to fresh fruit (Escudero, Gogorza, Melus, Ortín, Cacgo, & Ferreira, 2004).

Therefore, from the sensory point of view, grapes from Tempranillo and Grenache contain aroma precursors able to develop a common vegetal character, general fruity characteristics at quite different levels of intensity and a differential set of sensory descriptors. Fruity notes likely become integrated with ethanol into the “fruit in syrup” aroma descriptor. The differential set of sensory descriptors, includes terms such as “tropical fruit/citric”, “kerosene”, “toasty-woody”, “floral”, and “red fruit”. Some of these sensory descriptors were at levels enough to mask the sensory characteristics of ethanol and are likely implied in the specific aromatic profiles of the varieties. Acknowledging the preliminary character

of this study, Grenache grapes seem to be able to specifically develop “tropical fruit/citric” and maybe also “floral” characteristics, while Tempranillo grapes seem to be able to develop a specific “woody-toasty” character.

3.2.2. GC-O analysis

In order to identify the odorants responsible for the distinctive descriptions between clusters, the 5 arPAFs studied by flash profiling were also submitted to GC-O. Data from the study are summarized in Table III.14, which shows the 27 different odor zones detected by the panel. Twenty-five odorants were identified as responsible for those odor zones with different levels of certainty. In 21 of the cases a single odorant seems to be responsible for the odor zone; in two others, marked with a 1 superscript in the table, there remains some doubts about the presence of additional odorants in the odor zone, since the odor descriptors of the identified odorants do not completely explain the odor descriptors given by the panel. In one of the odor zones, two odorants were identified. Additionally, no odorants could be identified in the odor zones with polar retention indexes at 1012, 1109 and 1779.

The 25 identified odorants can be classified attending to their biochemical origin into 5 different categories: lipid-derivatives with 11 members, phenol-derivatives (5 members), terpenes (4 members), norisoprenoids (2 members) and miscellaneous (3 members).

Within the lipid-derivatives category there are 7 unsaturated aldehydes, 2 unsaturated ketones and 2 lactones. Lipid derivatives are molecules with either 9 (six of them), 8 (two of them), 10 (two of them) or 6 (just one) carbon atoms. Within the phenol-derivatives category, there are 4 volatile phenols and ethyl cinnamate. Among terpenes, linalool, linalool oxide, dihydromyrcenol and α-terpineol were identified.

Table III.14. Summary of the GC-O experiment carried out on the five PAF-derived hydrolyzates selected as representative of each of the clusters found in the sensory sorting task. Retention indexes in polar (DB-Wax) and non polar (DB-5) stationary phases, odor description, identity and GC-O scores (modified frequency in %) ranked attending to the difference between the maxima and minima scores.

RI polar	RI non polar	description	Compound*	uGS1 cluster 1	uGR3 cluster 2	oTD2 cluster 3	rTR5 cluster 4	rTR1 Cluster 5	max-min
1859	1131	Grapefruit, tropical, guava, green	3-mercaptohexanol[b]	92..8	69.7	71.4	16.7	76.1	76.1
1464	1070	Grapefruit, citrus, floral, sweet	linalool oxide[a] + dihydromyrcenol[c]	67.7	21.5	25.5	0	25.5	67.7
1012		Solvent, ketone	n.i. 1012	9.6	28.9	58.9	73.6	63.6	64
1109		Strawberry, acid, caramel, strawberry-cream	n.i. 1109	50.9	28.9	33.3	16.7	0	50.9
1675	1049	Citrus, bitter almond, green, flower, nuts, cardboard	Phenylacetaldehyde[1b]	37.3	60.9	19.2	6.8	43	54.1
1381	986	Mushroom, blood, metal, iron	*Z*-1.5-octadien-3-one[b]	0	0	26.4	40.8	50	50
1958	1488	Floral, spicy, strawberry candy, rose	β-ionone[a]	26.4	41.9	10.8	0	21.5	41.9
1593	1159	Vegetable, green, cucumber, peas, flower	*E.Z*-2.6-nonadienal[a]	60.8	19.2	49.1	62.7	53.8	43.5
1562	1095	Floral, paint, herbal, citrus	linalool[a]	31.2	44.1	0	0	6.8	44.1
1779		Citrus, floral, grapefruit, fruity, sweet	n.i. 1779	32.3	48.1	16.7	6.8	6.8	41.3
2147		Floral, toasted, hand cream	ethyl cinnamate[a]	13.6	43.0	9.6	13.6	0	43
1873		Spices, clove, smoked, bacon	guaiacol[a]	38..2	66.7	74.5	41.9	62.7	36.3
1307	979	Mushroom, humidity	1-octen-3-one[a]	58.9	57.9	70.7	36.3	49.1	34.4
2007		Rubber, plastic, dust, earth	o-cresol[a]	38.5	23.6	23.6	15.2	50.5	35.3
2053	1058	Caramel, strawberry candy, sugar cotton	furaneol[a]	54.9	67.7	35.4	33.3	40.8	34.4
2020		Grilled meat, butter, cream, fried, rubber	y-nonalactone[1a]	31.2	45.6	9.6	20.4	13.6	36
2287	1359	Barbecue, fried corn, spicy, toasted	2.6-dimethoxyphenol[a]	19.2	31.2	41.9	9.6	11.8	32.3
1822	1332	Rancid, oily, toasted, spicy	*E.E*-2.4-decadienal[b]	66.7	58.9	58.9	40.8	45.1	25.9
2099	1077	Stable, horses, manure, animal pee, leather	m/ρ-cresol[a]	58.9	66.3	47.1	33.3	45.1	33
1835	1388	Apple compote, raspberry jam	β-damascenone[a]	88.2	88.2	90.5	65.4	82.5	25.1
1543	1165	Cucumber, fatty, rancid, carmine	*E*-2-nonenal[a]	68.0	78.2	69.7	66.3	56.1	22.1
1734	1192	Floral, sweet, anise, green, citrus	α-terpineol[a]	38.5	40.8	26.4	21.5	24.5	19.3
1621	1253	Rancid, paper, cucumber, plastic, mat	*Z*-2-decenal[b]	56.9	60.9	60.9	47.1	66.3	19.2
1710	1224	Fat, raw bread, wood, toasted, fried, wax	*E.E* -2.4-nonadienal[b]	52.7	43	50	40.8	43	11.9
2260	1484	Coconut, fruity, toasted, spicy, lactic	massoia lactone[a]	68.0	86	86	79.1	80.5	18
1147	800	Grass, stem, plant, green	*Z*-3-hexenal[a]	75.5	73.6	83.3	72.6	75.5	10.7
1513	1150	Rancid, paper, cardboard, fatty, cucumber	*Z*-2-nonenal[a]	91.3	89.8	92.2	87.4	95	7.6

n.i.. not identified.*Reliability of the identification. [a]retention indexes, odor and mass spectrometry equal to those of the pure standard; [b]as a but Mass Spectrum could not be properly recorded; cas a but data were obtained from literature (pure standard not available). 1 indicates that a second unidentified odorant may be also present within the odor zone

The two norisoprenoids are β-ionone and β-damascenone, and among the miscellaneous category, phenylacetaldehyde, 3-mercaptohexanol and furaneol were found. The former is an amino acid derivative, the second one is the product of the hydrolysis of different glutathionylated and cysteinylated precursors, and the third one is a sugar derivative.

Odorants in Table III.14 are ranked attending to the difference between the maxima and minima scores. This parameter, given in the last column, is an indication of the potential ability of an odorant to introduce sensory differences, so that most discriminant should be ranked first. Nevertheless, it should be noted that in those cases in which GC-O scores are close to saturation, such as *Z*-2-nonenal, this parameter can underestimate the discriminating ability of the odorant. In any case, attending to this criterion, the table reveals that the odorants potentially most discriminant between the five representative samples are poorly known compounds which in fact could not be quantified, two even identified, in the present study. Three out of the four most discriminant odorants are maxima in the sample representative of cluster 1 (Figure III.4) and, on the basis of their sensory descriptors, the two first odor zones in the table should be responsible for the specific tropical fruit and citrus character of samples in this cluster. The first odorant is 3-mercaptohexanol, which is an extremely powerful and well-known grape-derived odorant. Its presence, however, was not expected because the hydrolysis of the different precursors is assumed to occur exclusively via specific β-lyase activities of yeast (Roland, Schneider, Razungles, & Cavelier, 2011). It can be argued that it is an artifact formed by the relatively high temperatures at which the hydrolysis took place, but it was also found when the hydrolysis was carried out at 45 ºC (Alegre, Arias-Pérez, Hernández-Orte, & Ferreira, 2020) and in earlier studies, Darriet et al., (Darriet, Tominaga, Demole, & Dubourdieu, 1993) showed that it could be released by acid catalysis in the presence of ascorbic acid. On the other hand, it is known that its precursors can be present in Grenache at mg/L levels

(Concejero, Peña-Gallego, Fernández-Zurbano, Hernández-Orte, & Ferreira, 2014), so that less 0.1% cleavage would suffice for its detection. The odor zone eluting at RI 1464 also had a grapefruit and citrus character, and two odorants compatible with this odor were identified: linalool oxide and dihydromyrcenol. A third potentially discriminant odorant, the strawberry smelling compound eluting at 1109, was also maxima in this sample. Table III.14 also reveals the presence of two discriminant odorants maxima in the sample representative of cluster 4 with vegetal odor (Figure III.4) and scoring high also in the representative of cluster 5 with vegetal and fruit notes (Figure III.4). These two odorants are the unidentified solvent-smelling with RI 1012 and the mushroom-blood-metal smelling *Z*-1,5-octadien-3-one. This last compound has been recently shown to play a role in the perception of dry fig and geranium nuances in musts (Allamy, Darriet, & Pons, 2017). Both compounds may play a role in the perception of vegetal notes most clearly identified in clusters 4 and 5. Another discriminant odorant was identified as phenylacetaldehyde, and scored maxima in the sample representative of cluster 2 (floral) (Figure III.4). Other floral smelling odorants also scored high in this sample, such as linalool, ethyl cinnamate or β-ionone. On the other hand, many of the lipid derivatives, such as *E*,*E*-2,4-decadienal, *E*-2-nonenal, *Z*-2-decenal, *E*,*E*-2,4-nonadienal or *Z*-3-hexenal, have quite limited ranges of variability in the GCO scores (Table III.14), which suggests that these odorants derived from lipids are a common background in all samples contributing to vegetal notes.

3.2.3. Quantitative data

The 33 samples were also analyzed quantitatively by GC-MS. Targeted compounds included those found relevant in previous studies (Loscos et al., 2010; Oliveira & Ferreira, 2019) and belonged to six different chemical categories: norisoprenoids, terpenoids, lactones, volatile phenols, vanillin derivatives and miscellaneous. Unfortunately, some remarkable odorants identified by GC-O in Table III.14 could not be quantified, well

because of the low concentration at which they are found, well because they require specific analytical procedures involving chemical derivatization or selective isolation. Overall, 30 different aroma compounds could be quantified. Samples were organized according to the clusters observed in the sorting task, results from clusters 1 and 2 are given in Table III.15.

Table III.15. Concentration (µg/L) of volatile compounds released after the accelerated hydrolysis at 75 ºC during 24h in anoxic conditions of different PAFs grouped in the clusters 1 and 2 formed in the sorting task based on aroma properties.

	Cluster 1: Tropical fruit-citrus, floral						Cluster 2: Floral, fruit in syrup								
	rGR4	**oTR5**	**rGR8**	**uGS2**	**uGS1**	**rGS2**	**uGS4**	**rGS4**	**rGS1**	**rGR9**	**rTD2**	**rGS3**	**uGR3**	**rGR3**	**uTD2**
NORISOPRENOIDS															
β-ionone	1.15	1.11	1.15	1.24	1.21	1.12	1.45	1.51	1.46	1.60	1.45	1.64	1.65	1.66	1.65
α-ionone	0.38	0.42	0.40	0.41	0.41	0.39	0.42	0.46	0.46	0.44	0.44	0.48	0.49	0.43	0.43
β-damascenone	24.6	25.8	25.8	24.1	25.2	25.0	32.4	32.3	29.4	31.3	30.3	30.2	29.9	31.8	30.0
TDN	55.5	50.0	68.9	42.1	44.7	47.8	41.5	32.8	32.4	32.1	25.4	32.3	31.3	32.9	43.3
riesling acetal[1]	0.42	0.38	0.53	0.42	0.40	0.45	0.48	0.41	0.36	0.34	0.31	0.36	0.34	0.33	0.32
TERPENOIDS															
β-citronellol	1.84	1.73	1.78	1.87	1.89	1.83	2.19	2.07	1.93	1.95	1.79	2.13	2.09	2.25	1.79
geraniol	3.69	3.28	3.72	3.50	3.39	3.80	4.82	4.51	3.12	3.93	2.77	3.69	3.91	5.43	2.86
linalool	9.21	8.23	9.48	11.11	9.96	9.40	12.4	12.0	11.4	11.5	8.92	12.15	12.0	12.20	9.09
α-terpineol	31.1	26.4	33.5	29.8	29.2	32.1	34.2	31.8	25.5	26.6	19.7	28.6	28.0	30.9	19.9
nerol	0.91	0.90	1.05	0.91	0.96	0.94	1.33	1.24	0.97	1.19	0.76	1.15	1.21	1.34	0.92
1,8-cineole	1.30	1.27	1.29	1.35	1.30	1.30	1.33	1.34	1.32	1.26	1.25	1.27	1.25	1.21	1.14
r-limonene	10.6	10.1	9.89	14.0	13.4	10.9	21.9	22.3	21.2	22.8	18.1	23.5	25.5	25.3	24.7
linalool oxide	3.81	3.65	4.07	3.65	3.70	3.56	4.11	3.76	3.62	3.40	2.88	3.64	3.43	3.56	2.88
LACTONES															
massoia lactone	4.49	4.17	3.13	2.74	3.14	3.44	8.45	10.4	8.81	12.0	7.70	13.3	14.4	9.68	6.35
VOLATILE PHENOLS															
guaiacol	8.27	7.92	7.35	8.65	8.31	8.41	9.55	9.30	9.00	8.89	10.01	10.4	8.93	9.77	8.73
eugenol	0.26	0.28	0.24	0.27	0.24	0.28	0.33	0.28	0.25	0.32	0.35	0.28	0.30	0.40	0.37
E-isoeugenol	0.44	0.42	0.41	0.51	0.44	0.50	0.36	0.31	0.24	0.26	0.26	0.30	0.24	0.26	0.25
methoxyeugenol	1.57	1.88	1.44	1.40	1.37	1.44	1.67	1.70	2.10	1.63	3.14	1.94	1.57	1.62	2.09
2,6-dimethoxyphenol	66.7	66.2	61.6	64.4	66.8	63.3	79.4	76.2	74.3	75.2	85.4	81.7	73.8	80.9	80.1
m-cresol	0.49	0.45	0.46	0.49	0.46	0.49	0.47	0.47	0.42	0.40	0.39	0.46	0.41	0.42	0.34
o-cresol	0.61	0.58	0.53	0.59	0.62	0.60	0.53	0.54	0.52	0.53	0.52	0.52	0.58	0.58	0.57
4-ethylguaiacol	0.11	0.12	0.11	0.12	0.11	0.11	0.09	0.10	0.10	0.09	0.09	0.09	0.10	0.09	0.09
4-vinylguaicol	8.07	7.51	9.12	8.86	7.74	9.14	9.11	8.32	8.12	8.96	7.70	8.81	7.64	9.87	8.94
4-vinylphenol	112	119	112	79.2	93.2	103	90.0	89.9	102	86.1	126	97.3	75.0	62.3	93.5
VANILLIN DERIVATIVES															
acetovanillone	22.6	20.0	23.8	25.6	22.6	24.5	29.6	27.3	26.3	25.2	25.0	31.5	26.7	24.5	21.8
vanillin	96.8	81.3	86.8	99.0	93.6	98.6	108	102	100	98.8	89.6	115	96.3	90.8	83.0
syringaldehyde	180	176	177	179	180	175	288	277	270	254	245	292	236	211	236
MISCELLANEOUS GROUP															
furaneol	62.7	0.93	1.03	1.36	1.15	0.87	1.15	1.48	1.17	8.18	0.63	1.00	10.9	0.97	1.56
ethyl cinnamate	0.12	0.11	0.11	0.12	0.12	0.12	0.11	0.13	0.12	0.17	0.10	0.16	0.15	0.15	0.14
ethyl 2-hydroxy-4-methylpentanoate	0.08	0.06	0.05	0.05	0.05	0.05	0.04	0.05	0.05	0.05	0.05	0.05	0.04	0.05	0.04

[1]relative area

In the case of samples sorted in clusters 3 and 4, the quantitative data are summarized in Table III.16.

Table III.16. Concentration (µg/L) of volatile compounds released after the accelerated hydrolysis at 75 ºC during 24h in anoxic conditions of different PAFs organized in the clusters 3 and 4 formed in the sorting task based on aroma properties.

	Cluster 3: Toasted-woody, red fruit, black fruit, fruit in syrup						Cluster 4: Vegetal			
	oTR6	**oTD2**	**uTR2**	**uTD1**	**oGR3**	**uGR4**	**rTD4**	**rTD3**	**rTR5**	**rTR6**
NORISOPRENOIDS										
β-ionone	1.44	1.30	1.41	1.49	1.22	1.58	1.05	1.07	1.44	1.05
α-ionone	0.42	0.43	0.45	0.41	0.38	0.40	0.29	0.36	0.38	0.33
β-damascenone	17.5	20.9	19.6	22.9	23.2	22.6	10.9	11.2	13.0	9.68
TDN	18.9	24.3	16.8	14.7	26.4	19.3	15.5	14.9	14.2	12.6
riesling acetal[1]	0.23	0.24	0.19	0.19	0.22	0.26	0.15	0.15	0.14	0.14
TERPENOIDS										
β-citronellol	1.47	1.55	1.59	1.61	1.73	1.52	0.83	0.96	1.00	0.82
geraniol	2.10	2.15	2.19	2.11	3.30	3.41	0.98	0.95	1.01	1.06
linalool	6.57	5.83	7.22	6.17	8.54	9.91	5.96	5.72	5.74	4.68
α-terpineol	10.2	11.4	12.7	11.1	26.9	19.0	3.01	3.11	3.11	3.07
nerol	0.77	0.70	0.84	0.75	0.98	0.93	< D.L	< D.L	< D.L	< D.L
1.8-cineole	1.00	1.05	1.19	1.16	1.13	0.96	1.18	1.22	1.15	1.07
r-limonene	25.8	21.1	20.3	20.3	18.4	27.5	24.1	23.4	25.9	23.6
linalool oxide	1.74	1.83	1.74	1.53	2.99	1.97	1.58	1.36	1.35	1.34
LACTONES										
massoia lactone	6.50	4.10	3.86	4.60	2.92	6.01	3.13	3.14	3.39	3.85
VOLATILE PHENOLS										
guaiacol	12.1	10.0	9.99	9.38	11.6	8.48	12.3	10.7	10.1	12.0
eugenol	0.63	0.60	0.53	0.60	0.41	0.42	0.81	0.72	0.70	0.70
e-isoeugenol	0.39	0.33	0.59	0.43	0.33	0.36	0.58	0.53	0.48	0.51
methoxyeugenol	3.73	3.48	3.23	4.92	2.15	2.06	5.49	4.63	4.27	4.02
2.6-dimethoxyphenol	112	99.9	91.8	96.8	95.4	88.7	130	116	118	123
m-cresol	0.24	0.23	0.24	0.26	0.29	0.28	0.13	0.13	0.13	0.13
o-cresol	0.45	0.41	0.45	0.43	0.42	0.46	0.32	0.36	0.32	0.30
4-ethylguaiacol	0.09	0.09	0.09	0.09	0.09	0.09	0.11	0.08	0.07	0.10
4-vinylguaicol	10.1	9.56	9.78	9.62	11.0	9.60	7.60	5.75	5.85	5.49
4-vinylphenol	329	370	241	261	163	174	204	176	185	203
VANILLIN DERIVATIVES										
acetovanillone	20.4	17.3	18.5	17.2	23.8	22.9	15.1	13.9	13.3	13.9
vanillin	81.6	62.9	72.6	67.0	88.4	86.6	47.0	41.8	43.8	49.3
syringaldehyde	221	166	166	172	243	233	53.9	50.0	60.0	94.4
MISCELLANEOUS GROUP										
furaneol	1.06	0.86	1.55	1.09	1.46	0.79	< D.L	< D.L	84.35	< D.L
ethyl cinnamate	0.09	0.09	0.08	0.08	0.13	0.08	0.19	0.18	0.19	0.18
ethyl 2-hydroxy-4-methylpentanoate	0.05	0.04	0.04	0.05	0.04	0.05	0.05	0.05	0.12	0.06

[1]relative area

In Table III.17 it can be observed the concentration of the compounds released in the samples belonging to cluster 5 in the sorting task.

Table III.17. Concentration (µg/L) of volatile compounds released after the accelerated hydrolysis at 75 ºC during 24h in anoxic conditions of different PAFs grouped in the cluster 5 formed in the sorting task.

	Cluster 5: Vegetal, fruit in syrup							
	oTD1	**rTR1**	**oTR1**	**uGS3**	**uTR1**	**rTD1**	**rTR2**	**rTR7**
NORISOPRENOIDS								
β-ionone	1.66	1.47	1.43	1.52	1.48	1.57	1.69	1.64
α-ionone	0.51	0.49	0.46	0.45	0.50	0.47	0.48	0.47
β-damascenone	17.0	17.0	16.5	17.4	15.1	19.1	17.9	20.6
TDN	7.52	9.58	13.5	13.3	13.4	11.4	14.7	11.1
riesling acetal[1]	0.13	0.17	0.19	0.19	0.18	0.16	0.18	0.15
TERPENOIDS								
β-citronellol	1.08	1.19	1.27	1.20	1.10	1.13	1.04	1.03
geraniol	0.98	1.08	1.26	1.21	1.27	1.00	1.05	1.18
linalool	5.68	6.71	7.22	7.53	5.70	5.97	5.71	5.66
α-terpineol	2.78	5.36	6.32	9.43	5.55	5.03	5.03	5.02
nerol	< D.L	< D.L	< D.L	< D.L	< D.L	< D.L	< D.L	< D.L
1.8-cineole	0.97	1.10	1.16	1.16	1.04	0.93	0.91	0.88
r-limonene	37.2	25.7	24.1	23.9	25.7	29.3	30.8	30.8
linalool oxide	0.90	1.45	1.52	1.78	1.52	1.32	1.49	1.30
LACTONES								
massoia lactone	4.33	5.21	3.63	3.15	3.68	3.15	3.83	3.32
VOLATILE PHENOLS								
guaiacol	7.81	9.76	11.2	8.18	8.10	10.29	9.58	8.04
eugenol	0.70	0.61	0.59	0.53	0.63	0.62	0.51	0.56
e-isoeugenol	0.69	1.13	0.93	0.68	0.82	0.65	0.77	0.62
methoxyeugenol	8.64	5.58	4.53	3.10	3.63	4.43	3.52	3.50
2.6-dimethoxyphenol	80.1	86.9	98.1	83.6	86.8	91.5	90.3	91.1
m-cresol	0.17	0.20	0.20	0.21	0.17	0.19	0.17	0.17
o-cresol	0.44	0.48	0.42	0.43	0.40	0.46	0.42	0.44
4-ethylguaiacol	0.08	0.09	0.09	0.07	0.11	0.09	0.10	0.09
4-vinylguaicol	6.50	7.33	7.17	6.84	6.95	7.07	6.66	5.42
4-vinylphenol	225	211	153	145	185	192	199	186
VANILLIN DERIVATIVES								
acetovanillone	20.3	19.8	18.9	19.6	15.4	16.8	14.6	14.1
vanillin	49.3	67.9	64.1	68.2	57.0	51.8	57.0	44.5
syringaldehyde	107	129	135	139	103	115	104	102
MISCELLANEOUS GROUP								
furaneol	< D.L	1.90	< D.L	25.09	< D.L	< D.L	< D.L	< D.L
ethyl cinnamate	0.06	0.06	0.06	0.05	0.05	0.05	0.06	0.05
ethyl 2-hydroxy-4-methylpentanoate	0.04	0.04	0.04	0.05	0.03	0.03	0.04	0.03

[1]relative area

Quantitative data were processed by one-way ANOVA considering as factors the sensory cluster, grape variety, geographical precedence and degree of ripeness. The most influential factor was the sensory cluster for which all aroma compounds except furaneol, varied significantly with differences in many cases of large magnitude, as can be seen in Table III.18.

Table III.18. Average (±standard deviation) concentrations of compounds (µg/L) found in hydrolysates reconstituted PAFs. Data are segregated attending to the sensory clusters identified by sorting task. F quotients found in the corresponding one-way ANOVAs. The two last columns of the table Sensory thresholds and potential sensory discrimination abilities are also given as the ratios OAVmax/OAVmin with the condition OAV>1 and between brackets with the condition OAV > 0.2.

		Sensory Cluster						Sensory relevance	
		cluster 1	**cluster 2**	**cluster 3**	**cluster 4**	**cluster 5**	**F**	**Sensory threshold**	**OAVmax/ OAVmin**
Norisoprenoids	β-ionone	1.16±0.05^{a}	1.57±0.09^{c}	1.41±0.13^{b}	1.15±0.19^{a}	1.56±0.10^{c}	20.6*	0.09[1]	1.6
	α-ionone	0.40±0.02^{b}	0.45±0.02^{c}	0.41±0.02^{b}	0.34±0.04^{a}	0.48±0.02^{d}	27.0*	2.6[1]	0
	β -Damascenone	25.1±0.07^{d}	30.8±1.10^{e}	21.1±2.20^{c}	11.2±1.40^{a}	17.6±1.70^{b}	153.5*	0.05[1]	3.3
	TDN	51.6±9.60^{d}	33.8±5.40^{c}	20.1±4.40^{b}	14.3±1.30^{ab}	11.8±2.40^{a}	56.7*	2[2]	9.2
	Riesling Acetal[x]	0.43±0.05^{d}	0.36±0.05^{c}	0.22±0.03^{b}	0.14±0.00^{a}	0.17±0.02^{a}	67.1*	n.a.	n.a.
Terpenes	β -citronellol	1.83±0.06^{d}	2.02±0.17^{e}	1.58±0.09^{c}	0.90±0.09^{a}	1.13±0.08^{b}	111.0*	100[1]	0
	Geraniol	3.56±0.20^{c}	3.90±0.90^{c}	2.54±0.63^{d}	1.00±0.05^{a}	1.13±0.12^{a}	38.2*	20[1]	0 (1.4)
	Linalool	9.56±0.95^{c}	11.3±1.30^{d}	7.37±1.60^{b}	5.52±0.58^{a}	6.27±0.77^{ab}	30.8*	6[3]	2.1(2.7)
	α -terpineol	30.3±2.50^{c}	27.2±5.00^{c}	15.2±6.60^{b}	3.08±0.05^{b}	5.56±1.90^{a}	57.7*	250[1]	0
	Nerol	0.94±0.06^{b}	1.12±0.20^{c}	0.83±0.11^{b}	0.00±0.00^{a}	0.00±0.00^{a}	139.4*	300[4]	0
	1.8-cineole	1.30±0.03^{c}	1.26±0.06^{c}	1.08±0.09^{b}	1.15±0.06^{b}	1.02±0.11^{a}	16.2*	1.1[5]	1.2(1.5)
	R-limonene	11.5±1.80^{a}	22.8±2.30^{b}	22.2±3.60^{b}	24.3±1.10^{b}	28.5±4.50^{c}	26.3*	15[6]	2.5(3.8)
	Linalool oxide	3.74±0.18^{c}	3.48±0.40^{c}	1.96±0.52^{b}	1.41±0.11^{a}	1.41±0.25^{a}	72.3*	n.a.	n.a.
Lactones	Massoia lactone	3.52±0.67^{a}	10.1±2.60^{b}	4.67±1.40^{a}	3.38±0.34^{a}	3.79±0.70^{a}	25.6*	10[7]	1.4(5.2)
Volatile phenols	Guaiacol	8.15±0.46^{a}	9.39±0.57^{bc}	10.3±1.40^{cd}	11.3±1.00^{d}	9.12±1.30^{ab}	7.3*	9.5[1]	1.3(1.7)
	Eugenol	0.26±0.02^{a}	0.32±0.05^{a}	0.53±0.09^{b}	0.73±0.05^{c}	0.59±0.06^{b}	63.5*	6[1]	0
	E-isoeugenol	0.45±0.04^{a}	0.28±0.04^{a}	0.40±0.10^{b}	0.53±0.04^{b}	0.79±0.17^{c}	28.8*	6[1]	0
	Methoxyeugenol	1.52±0.19^{a}	1.94±0.49^{a}	3.26±1.10^{b}	4.60±0.64^{bc}	4.62±1.80^{c}	12.0*	1200[1]	0
	2.6-dimethoxyphenol	64.8±2.10^{a}	78.6±3.90^{b}	97.5±8.20^{d}	121±4.70^{e}	88.6±5.50^{c}	82.1*	570[1]	0 (1.1)
	m-cresol	0.47±0.02^{e}	0.42±0.04^{d}	0.26±0.02^{c}	0.13±0.00^{a}	0.18±0.02^{b}	183.7*	68[1]	0
	o-cresol	0.59±0.03^{d}	0.54±0.02^{c}	0.44±0.02^{b}	0.33±0.02^{a}	0.44±0.02^{b}	95.3*	31[1]	0
	4-ethylguaiacol	0.11±0.01^{b}	0.09±0.01^{a}	0.09±0.00^{a}	0.09±0.02^{a}	0.09±0.01^{a}	7.9*	33[1]	0
	4-vinylguaicol	8.40±0.72^{b}	8.61±0.72^{b}	9.95±0.56^{c}	6.17±0.96^{a}	6.74±0.60^{a}	27.2*	40[1]	0 (1.4)
	4-vinylphenol	103±14.0^{a}	91.4±18.0^{a}	257±82.0^{c}	192±14.0^{b}	187±27.0^{b}	20.7*	180[1]	2.1(6.0)
Vanillins	Acetovanillone	23.2±1.90^{c}	26.4±2.80^{d}	20.0±2.90^{b}	14.1±0.77^{a}	17.5±2.50^{b}	24.3*	1000[1]	0
	Vanillin	92.7±7.20^{d}	98.2±9.80^{d}	76.6±11.0^{c}	45.5±3.30^{a}	57.5±8.70^{b}	40.9*	995[1]	0
	Syringaldehyde	178±2.00^{c}	257±27.0^{d}	200±36.0^{c}	64.6±20.0^{a}	117±15.0^{b}	63.9*	50000[1]	0
Miscellaneous	Furaneol	11.3±25.0	3.00±3.80	1.14±0.31	21.1±42.0	3.37±8.80	1.0	5[1]	17 (84)
	Ethyl cinnamate	0.12±0.00^{c}	0.14±0.02^{d}	0.09±0.02^{b}	0.18±0.01^{e}	0.05±0.00^{a}	63.0*	1.1[1]	0
	Ethyl 2-hydroxy-4-methylpentanoate	0.06±0.01^{bc}	0.05±0.01^{ab}	0.04±0.00^{ab}	0.07±0.03^{c}	0.04±0.01^{a}	4.8#	51	0

[x]relative area; < D.L. under detection limit; *Significant at P<0.0005; #Significant at P<0.05; Different letters indicate significant differences between sensory clusters according to Fischer post-hoc test; n.a.: not available; G, Grenache; T, Tempranillo; [1]San Juan et al., 2012; [2]Sack et al., 2012; [3]Buttery, Teranishi, Ling, & Turnbaugh, 1990; [4]López et al., 2002; [5]Herve, Price, & Burns, 2003; [6]Zalacain, Marín, Alonso, & Salinas, 2007; [7]Pons, Allamy, Lavigne, Dubordieu, & Darriet, 2017; [8]Lytra, Tempere, de Revel, & Barbe, 2012.

These observations are further supported by the PCA carried out with quantitative data, as can be seen in Figure III.6. Samples are distributed in the plane following exactly the same five clusters identified in the sorting task. This similarity between the sensory and chemical spaces is quite infrequent in wine flavor chemistry, and suggests that the sensory classes identified in the sorting task, are the consequence of quite specific profiles of volatiles. Since some aroma relevant molecules detected in the GC-O experiment have not been quantified, it seems that those profiles of volatiles reflect the existence of specific metabolic patterns. Additionally, and comparing to the difficulties found in wine to correlate sensory and chemical spaces, it can be hypothesized that major fermentation volatiles largely complicate and distort the relationship between the chemical and the sensory spaces.

In order to facilitate the discussion of results and, in particular, in order to focus the discussion on the odorants most relevant from the sensory point of view, the two last columns in Table III.18 contain the odor thresholds of the quantified odorants and the ratios OAVmax/OAVmin. Such ratios are indicative of the potentiality of the odorant to introduce sensory differences within the pool of samples. If such ratios are calculated including only those (odour activity values) OAVs>1 (strict criterion), the odorants potentially responsible for higher sensory variability are β-damascenone, TDN, linalool, limonene, furaneol and 4-vinylphenol, whose ratios are higher than 2. If the ratios are calculated including all those OAVs>0.2 (conservative criterion), then massoia lactone also shows a high discriminating potential reaching a 5.2 ratio. Odorants with some ability (ratio <2 but >1.3) to introduce sensory differences attending to these ratios are also β-ionone, geraniol, 1,8-cineole, guaiacol and 4-vinylguaiacol. The highest ratios measured for furaneol are due to spurious very large concentration values registered in some individual samples. This is the most polar and difficult to extract compound in the list, so that such extreme behavior could be attributed to limitations of the analytical method.

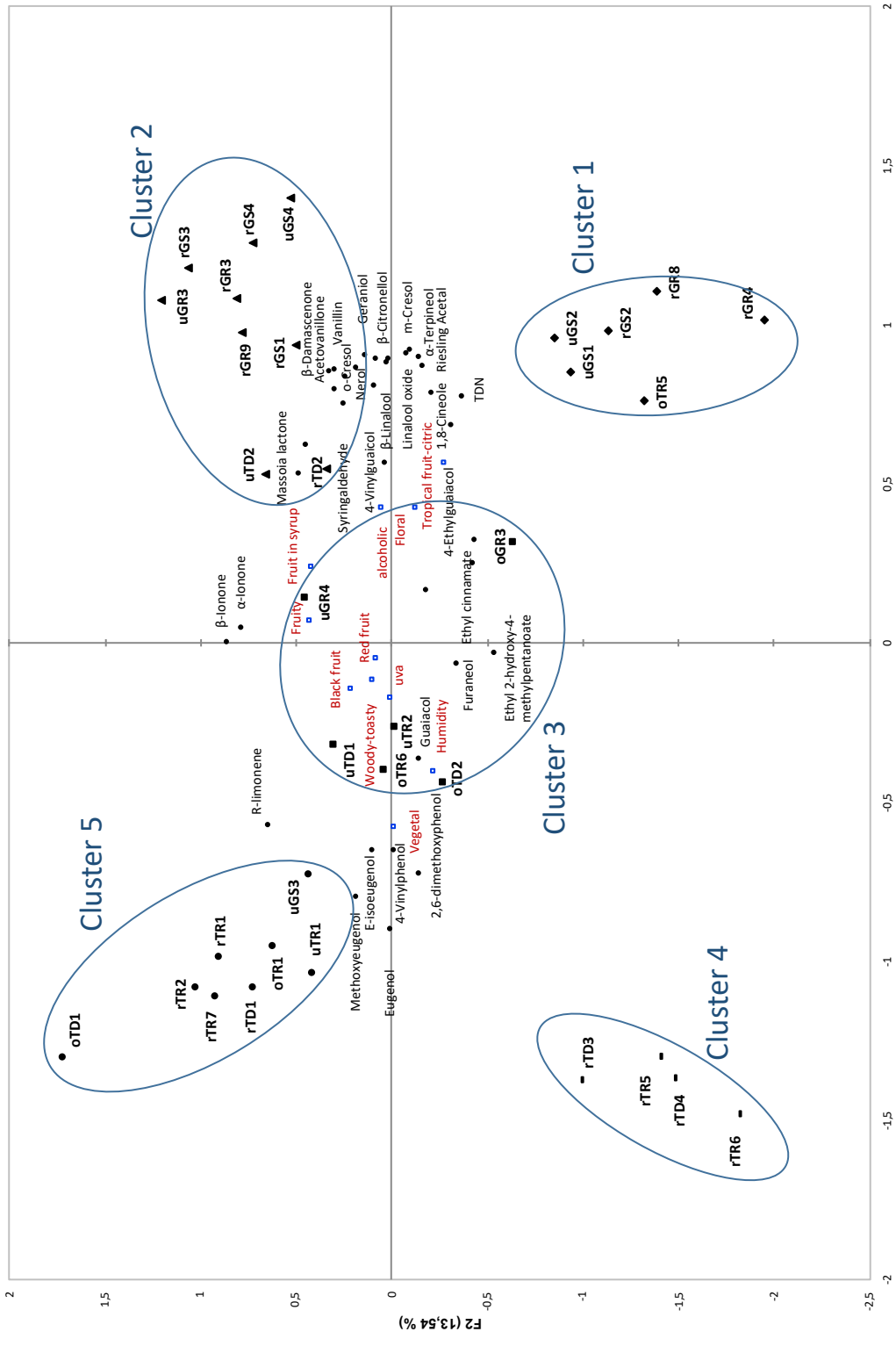

Figure III.6. Projection of samples and variables in the PCA plane obtained from exclusively GC-MS quantitative data. Sensory variables are projected as illustrative variables but did not take part in the analysis. The superimposed circles delimit the clusters identified in the sensory sorting task shown in Figure III.4.

The plot in Figure III.6 basically states that Grenache samples are found at the far-right part of the plane, split into two major groups, one at the North (coinciding with cluster 2 in Figure III.4) and a second at the South (cluster 1 in Figure III.4), two other samples (uGR4 and uGR3) more centered and a single odd sample (uGS3) in the left part of the plane. Samples from Tempranillo are all of them but three (uTD2, rTD2 and oTR5), at the left part of the plane, split into three groups corresponding to the clusters 3, 4 and 5 identified in the sorting task (Figure III.4).

Then, considering Figure III.6 and data in Table III.18, it can be said that samples from Grenache are richest in norisoprenoids (except ionones), terpenoids (except limonene) and vanillin derivatives, while those of Tempranillo are richest in most volatile phenols. This has to be relevant from the sensory point of view, first because differences affect to relatively large number of compounds having similar aroma properties (terpenols, vanillins, volatile phenols) whose sensory effects will be cooperative; second because some of the components have high OAVmax/OAVmin ratios, in particular β-damascenone, TDN, linalool and massoia lactone, which are maxima in Grenache and 4-vinylphenol which is maxima in Tempranillo.

Going into more detail with the help of Table III.18, the two Grenache clusters clearly differ because cluster 2 contains highest levels of β-ionone, β-damascenone, linalool, limonene (second highest) and of massoia lactone, while cluster 1 contains highest levels of TDN. The high contents of TDN in Grenache has been recently observed (Oliveira & Ferreira, 2019). These compositional differences explain the floral and fruit in syrup character of samples in cluster 2, and the specific kerosene attribute of samples in cluster 1 (Figure III.5), but cannot explain the tropical fruit and citrus character of samples in cluster 1 Figure III.4. Attending to the olfactometric study in Table III.14, these should be attributed to 3-mercaptohexanol, linalool oxide and dihydromyrcenol which were not quantified.

Among the Tempranillo clusters, cluster 4, is characterized by its minima contents in most aroma compounds (Table III.18). It contains highest levels of guaiacol, eugenol, and 2,6-dimethoxyphenol, but only the former is barely above threshold. This would explain that samples in this cluster were characterized only by vegetal and alcoholic notes, which are the general background notes, as was seen in Figure III.5. Samples in cluster 5 also have close to minima contents in most aroma components, but have higher levels than those of cluster 4 in α and β-ionones, and in β-damascenone (Table III.18). This, together with the presence of *Z*-1,5-octadien-3-one could explain their fruit in syrup character, in addition to the vegetal and alcoholic notes (Figure III.4 and Figure III.5).

Finally, samples in cluster 3 have an intermediate composition to those of clusters 2 and 5. They have higher levels of volatile phenols, particularly of vinylphenols, and smaller levels of terpenes, vanillin derivatives, massoia lactone and β-damascenone than those of samples in cluster 2 (Table III.18). They also have, except for most volatile phenols, higher levels of aroma compounds than samples in cluster 5. The higher levels of volatile phenols would explain the woody/toasty character of samples in cluster 3 (Figure III.4). Attending to previous results (San Juan et al., 2011), it can be hypothesized that the red fruit character and the lack of alcoholic character would be the consequence of a smaller fruit in syrup character, because of the smaller levels of massoia lactone, β-damascenone than samples in cluster 2 and smaller levels of Z-1,5-octadien-3-one and higher levels of fruity odorants than samples in cluster 5 (Table III.18).

The second most influential factor was grape variety, for which 24 out of the 30 aroma compounds varied significantly, in some cases also with large differences, as can be also seen in Table III.19.

Table III.19. Average (±standard deviation) concentrations of compounds (expressed in μg L^{-1}) found in hydrolysates reconstituted PAFs. Data are segregated attending to the grape variety. F quotients found in the corresponding one-way ANOVAs. Different letters indicate significant differences between sensory clusters according to Fischer post-hoc test.

	Grenache	Tempranillo	F
NORISOPRENOIDS			
β-ionone	1.46±0.18	1.37±0.22	0.0
α-ionone	0.43±0.04	0.43±0.06	0.0
β -Damascenone	25.8±5.97[b]	19.6±6.12[a]	20.5*
TDN	30.3±11.5[a]	23.9±17.9[a]	17.8*
Riesling Acetal[1]	0.32±0.11[b]	0.24±0.11[a]	34.6*
TERPENES			
β -citronellol	1.79±0.40[b]	1.36±0.36[a]	32.9*
Geraniol	3.34±1.32[b]	1.93±0.98[a]	49.2*
Linalool	10.1±2.26[b]	6.88±1.49[a]	73.5*
α -terpineol	24.0±1.09[b]	11.9±10.0[a]	66.6*
Nerol	0.88±0.48[b]	0.42±0.44[a]	28.9*
1.8-cineole	1.22±0.13[b]	1.12±0.13[a]	17.1*
R-limonene	21.7±5.35[a]	22.6±7.21[a]	6.1#
Linalool oxide	3.06±0.95[b]	2.03±0.97[a]	48.2*
LACTONES			
Massoia lactone	7.00±4.22[b]	4.39±1.28[a]	7.0#
VOLATILE PHENOLS			
Guaiacol	9.32±0.96	9.63±1.58	4.1
Eugenol	0.38±0.14[a]	0.54±0.17[b]	41.8*
E-isoeugenol	0.40±0.14[a]	0.56±0.24[b]	8.9#
Methoxyeugenol	2.16±0.99[a]	3.84±1.72[b]	34.8*
2.6-dimethoxyphenol	80.9±13.9[a]	92.2±18.6[a]	19.3*
m-cresol	0.37±0.12[b]	0.25±0.12[a]	39.1*
o-cresol	0.51±0.08[b]	0.45±0.09[a]	18.8*
4-ethylguaiacol	0.09±0.01	0.10±0.01	0.9
4-vinylguaicol	8.46±1.31[a]	7.74±1.50[a]	7.3#
4-vinylphenol	116±43.4[a]	195±74.3[b]	26.0*
VANILLIN DERIVATIVES			
Acetovanillone	24.0±4.66[b]	18.5±3.51[a]	48.0*
Vanillin	89.4±19.9[b]	66.6±17.0[a]	51.8*
Syringaldehyde	210±68.02[b]	145 ±57.3[a]	20.7*
MISCELLANEOUS GROUP			
Furaneol	9.33±21.8	4.07±14.6	0.2
Ethyl cinnamate	0.12±0.04	0.10±0.05	2.3
Ethyl 2-hydroxy-4-methylpentanoate	0.05±0.02	0.05±0.01	0.0

*Significant at P<0.0005; #Significant at P<0.05; [1]relative area

By contrast, the factor with smallest influence in the dataset was the degree of ripeness (Table III.20), for which only 4-vinylphenol reached significance.

Table III.20. Average (±standard deviation) concentrations of compounds (expressed in µg L^{-1}) found in hydrolysates reconstituted PAFs. Data are segregated attending to the ripeness state. F quotients found in the corresponding one-way ANOVAs. Different letters indicate significant differences between sensory clusters according to Fischer post-hoc test.

	Underripe	Ripe	Overripe	F
NORISOPRENOIDS				
β-ionone	1.47±0.15	1.40±0.24	1.36±0.19	0.6
α-ionone	0.43±0.03	0.42±0.06	0.40±0.04	0.4
β -Damascenone	23.9±5.67	22.4±8.02	20.2±3.79	0.6
TDN	28.1±13.9	27.3±17.2	23.4±14.7	0.2
Riesling Acetal[1]	0.30±0.11	0.28±0.13	0.23±0.08	0.6
TERPENES				
β -citronellol	1.68±0.35	1.50±0.51	1.47±0.26	0.7
Geraniol	2.87±1.16	2.53±1.55	2.18±0.96	0.5
Linalool	9.11±2.38	8.37±2.74	7.01±1.20	1.4
α -terpineol	19.9±9.99	17.2±13.1	14.0±10.3	0.5
Nerol	0.78±0.45	0.56±0.56	0.56±0.44	0.7
1.8-cineole	1.19±0.13	1.18±0.02	1.09±0.11	1.0
R-limonene	21.7±4.82	22.2±6.49	22.8±8.96	0.5
Linalool oxide	2.63±1.03	2.56±1.15	2.10±1.01	0.5
LACTONES				
Massoia lactone	5.63±3.55	6.00±3.52	2.27±1.20	0.6
VOLATILE PHENOLS				
Guaiacol	8.83±0.63	9.66±1.31	10.1±1.88	2.2
Eugenol	0.42±0.14	0.46±0.20	0.53±0.16	0.7
E-isoeugenol	0.47±0.18	0.49±0.23	0.51±0.25	0.1
Methoxyeugenol	2.51±1.17	3.06±1.45	4.06±2.45	1.8
2.6-dimethoxyphenol	81.2±10.5	88.8±20.6	92.0±16.3	0.9
m-cresol	0.33±0.12	0.31±0.15	0.26±0.10	0.5
o-cresol	0.51±0.08	0.47±0.10	0.45±0.06	0.7
4-ethylguaiacol	0.09±0.01	0.09±0.01	0.93±0.01	0.0
4-vinylguaicol	8.51±1.12	7.60±1.40	8.65±1.83	1.9
4-vinylphenol	144±69.0[a]	144±51.3[a]	226±102[b]	3.6*
VANILLIN DERIVATIVES				
Acetovanillone	22.0±4.46	20.7±5.83	20.1±2.17	0.3
Vanillin	83.2±16.6	75.4±25.6	71.3±14.9	0.7
Syringaldehyde	193±54.8	164±82.8	175±51.0	0.5
MISCELLANEOUS GROUP				
Furaneol	4.46±7.88	9.66±24.4	0.72±0.59	0.6
Ethyl cinnamate	0.10±0.03	0.13±0.05	0.09±0.28	2.2
Ethyl 2-hydroxy-4-methylpentanoate	0.04±0.005	0.54±0.02	0.04±0.008	1.7

*Significant at P<0.05

The factor geographical origin, had a small but significant influence on the levels of 16 aroma compounds (Table III.21). Nevertheless, the real influence of this factor cannot be well assessed since the experiment was not adequately balanced, but results in any case suggest that its influence is much smaller than that of the variety.

Table III.21. Average (±standard deviation) concentrations of compounds (expressed in µg L^{-1}) found in hydrolysates reconstituted PAFs. Data are segregated attending to the origin. F quotients found in the corresponding one-way ANOVAs. Different letters indicate significant differences between sensory clusters according to Fischer post-hoc test.

	Rioja	Somontano	Duero	F
NORISOPRENOIDS				
β-ionone	1.42±0.21	1.39±0.18	1.41±0.24	0.0
α-ionone	0.43±0.05	0.43±0.03	0.42±0.07	0.3
β -Damascenone	21.3±6.40^{ab}	26.9±5.08^{b}	20.3±7.42^{a}	2.7
TDN	25.9±17.4^{ab}	35.9±10.9^{b}	19.6±11.3^{a}	2.5
Riesling Acetal[1]	0.26±0.11^{a}	0.38±0.08^{b}	0.20±0.08^{a}	6.9*
TERPENES				
β -citronellol	1.49±0.43^{a}	1.89±0.30^{b}	1.34±0.35^{a}	4.3*
Geraniol	2.52±1.39^{ab}	3.51±1.09^{b}	1.73±0.84^{a}	4.3*
Linalool	8.02±2.38^{a}	10.7±1.67^{b}	6.67±1.45^{a}	8.3*
α -terpineol	16.4±11.7^{a}	27.6±7.78^{b}	9.50±7.24^{a}	6.8*
Nerol	0.59±0.53	0.94±0.41	0.39±0.42	2.7
1.8-cineole	1.13±1.33^{a}	1.30±0.06^{b}	1.11±0.12^{a}	6.9*
R-limonene	22.5±6.62	18.9±5.20	24.8±6.05	1.9
Linalool oxide	2.37±1.06^{a}	3.48±0.71^{b}	1.78±0.73^{a}	7.2*
LACTONES				
Massoia lactone	5.53±3.35	6.67±4.07	4.56±1.66	0.9
VOLATILE PHENOLS				
Guaiacol	9.54±1.50	8.97±0.75	9.91±1.35	1.0
E-isoeugenol	0.53±0.25	0.42±0.14	0.46±0.17	0.7
Methoxyeugenol	2.93±1.28^{a}	1.84±0.57^{a}	4.60±1.96^{b}	8.4*
2.6-dimethoxyphenol	88.6±17.5^{b}	73.7±7.97^{a}	97.1±16.9^{b}	4.6*
m-cresol	0.28±0.12^{a}	0.43±0.09^{b}	0.23±0.09^{a}	7.3*
o-cresol	0.47±0.09^{a}	0.55±0.06^{b}	0.44±0.08^{a}	3.9*
4-ethylguaiacol	0.09±0.11	0.10±0.02	0.09±0.01	0.9
4-vinylguaicol	8.03±1.71	8.37±0.79	7.84±1.42	0.3
4-vinylphenol	164±66.9^{b}	100±19.6^{a}	206±84.9^{b}	5.5*
VANILLIN DERIVATIVES				
Acetovanillone	19.9±4.38^{a}	25.9±3.77^{b}	18.4±3.67^{a}	7.8*
Vanillin	74.3±18.8^{a}	98.1±13.8^{b}	61.6±17.4^{a}	9.2*
Syringaldehyde	166±60.8^{ab}	225±62.3^{b}	143±74.7^{a}	3.5*
MISCELLANEOUS GROUP				
Furaneol	10.3±24.3	4.16±8.46	0.52±0.61	0.9
Ethyl cinnamate	0.11±0.05	0.12±0.03	0.11±0.05	0.1
Ethyl 2-hydroxy-4-methylpentanoate	0.05±0.02	0.05±0.004	0.04±0.008	0.6

*Significant at P<0.05

4. CONCLUSIONS

Most grape aroma precursors and polyphenols present in 750 mL of mistelle can be isolated by SPE on a partially dealcoholized mistelle (2-3% (v/v) ethanol) through 10 g Sep Pak-C18, and further elution with 100 mL of ethanol. In these PAFs, amino acids, metal cations and DMS precursors are not retained. In addition, the reconstitution of PAFs in model wine and further aging in strict anoxia during 7 weeks at 45 ºC or during 24 h at 75 ºC lead to the development of strong aromas reminding some odor nuances observed in aged red wines. By contrast, mistelles aged similarly develop strong caramel and raisin-like aromas likely associated to Strecker aldehydes. The addition of sugars to PAFs, induced the formation of kerosene notes attributed to TDN. The presence of oxygen, even if does not induce strong changes in the volatile profiles, causes a strong distortion of the sensory profiles.

The 32 odorants identified by GC-Olfactometry in aged rPAFs are similar between samples aged at 45 and 75 ºC and belong to 4 major categories (lipid derivatives; volatile phenols and vanillins, and norisoprenoids and terpenes) and a 5th miscellaneous group which surprisingly includes 3-mercaptohexanol. Moreover, GC-MS and GC-O profiles of rPAFs aged 7 weeks at 45 ºC are also relatively similar to those obtained after 24 h at 75 ºC, which suggests that this fast hydrolysis of rPAFs can be a promising tool for the study of the aroma potential of winemaking grapes.

Hydrolyzates obtained from PAFs extracted from 33 lots of grapes from Tempranillo and Garnacha have aromas classified into five different sensory categories with a common vegetal background character. Grenache-related categories may have specific tropical fruit/citric, kerosene and floral characteristics, while Tempranillo-related may develop

specific toasty-woody and red fruit sensory notes. Specific sensory notes seem to mask alcoholic and fruit in syrup aroma descriptors which would be also common.

The GC-O profiling of representative samples revealed that 3-mercaptohexanol, linalool oxide and dihydromyrcenol, two unidentified odorants, phenylacetaldehyde and *Z*-1,5-octadien-3-one are potentially the most discriminant odorants of the data set. A large group of powerful lipid-derivatives including 7 unsaturated aldehydes, 2 unsaturated ketones and 2 lactones, having 9 (6 of them), 10, 8 (2 of them each) or 6 (just 1) carbon atoms, may be responsible for the vegetal background and have also implications in the fruit in syrup perception. Other identified odorants were 4 volatile phenols, ethyl cinnamate, β-ionone and β-damascenone, linalool and α-terpineol and furaneol.

The PCA derived from quantitative data (30 odorants, including only 12 out of the 27 detected by GC-O) showed a clustering perfectly matching the one found by sensory analysis, which suggests the existence of 5 specific metabolomic profiles behind the 5 specific sensory profiles. Quantitative data confirm that Grenache is richest in norisoprenoids (except ionones), terpenoids (except limonene) and vanillin derivatives, while those of Tempranillo are richest in most volatile phenols.

The integration of all data suggests that 3-mercaptohexanol, maybe together with linalool oxide and dihydromyrcenol, would be responsible for the tropical fruit/citrus character, that TDN is responsible for kerosene notes and that volatile phenols, notably guaiacol and 4-vinylphenol, would be responsible for the woody/toasty character. It is also suggested that β-damascenone and massoia lactone, likely with *Z*-1,5-octadien-3-one would be main contributors to fruit in syrup and alcoholic notes and would mask red fruit character.

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FINAL CONCLUSIONS

Five *non-Saccharomyces* yeasts for Verdejo musts and five for Tempranillo were able to generate wines with high quality aromatic profiles. Of these yeasts, 2 for each variety were able to generate similar aromatic profiles by fermenting musts from different terroirs and vintages. The analysis of the musts allowed us to observe that there were differences between musts, which indicated that these *non-Saccharomyces* yeasts generated similar aromatic profiles independently of the precursors present in the starting musts.

In addition, the co-inoculation with *Saccharomyces cerevisiae* did not generate differences in the aromatic profile obtained with the yeasts used with Verdejo variety, unlike those of the Tempranillo variety in which the aromatic profile was determined by the *S. cerevisiae* yeast.

The amino acid profile had an effect on the metabolism of polyfunctional mercaptans precursors, with a greater release of 4MMP and 3MH in wines with the profile of Chardonnay variety. The cysteinylated precursor of 4MMP and the glutathionylated precursor of 3MH were preferably consumed by yeasts. Yeasts could use cysteinylated and glutathionylated precursors as a source of GSH and/or cysteine. Moreover, the addition of cysteine at 20 mg/L generated an overexpression of the IRC7 and CYS3 genes that encodes for a enzymes with β and γ-lyase activities, respectively. This addition also led to an upregulation of the gene OPT1, which encode for a GSH transporter. The overexpression of these genes results in a release of higher concentrations of polyfunctional mercaptans.

Most grape glycosidic precursors and polyphenol present in grapes could be isolated by SPE from dealcoholized mistelle using cartridges of 10 g of C18. The reconstitution of the PAF in synthetic wine followed by aging in anoxia for 7 weeks at 45 °C or for 24 h at 75

°C led to the development of strong aromas reminiscent of some odor nuances observed in aged red wines. In addition, GC-MS and GC-O analyses of rPAF incubated for 7week at 45 °C were also relatively similar to those obtained after 24 h at 75 °C, suggesting that this rapid hydrolysis of rPAFs may be a promising tool for the study of the aroma potential of winemaking grapes.

The characterization of the 33 samples allowed to identify that different aromatic profiles found in Grenache and Tempranillo grapes are due to eight aldehydes, β-damascenone and massoia lactone that provide a common "vegetal" and "fruit in syrup" background, and linalool, linalool oxide, 3MH, furaneol, guaiacol and metoxieugenol that seem to be responsible for the distinctive aromatic nuances. Grenache was rich in norisoprenoids, terpenoles and vanillin derivatives and Tempranillo in volatile phenols.

CONCLUSIONES FINALES

Cinco levaduras *no-Saccharomyces* para mostos de la variedad Verdejo y cinco para Tempranillo fueron capaces de generar vinos con perfiles aromáticos de alta calidad. De estas levaduras, 2 para cada variedad generaron perfiles aromáticos similares fermentando mostos de diferentes terroirs y añadas. La cuantificación de los precursores presentes en los mostos nos permitió observar que había diferencias entre los mostos, lo que indicó que estas levaduras *no-Saccharomyces* generaron perfiles aromáticos similares independientemente de los precursores presentes en los mostos iniciales.

Además, la coinoculación con *Saccharomyces cerevisiae* no generó diferencias en el perfil aromático obtenido con las levaduras utilizadas para la variedad Verdejo, al contrario que las de la variedad Tempranillo en la que el perfil aromático fue determinado por la levadura *S. cerevisiae.*

El perfil de aminoácidos tuvo un efecto sobre el metabolismo de los precursores de mercaptanos polifuncionales, con una mayor concentración de 4MMP y 3MH en vinos con el perfil de la variedad Chardonnay. La levadura consumió preferentemente el precursor cisteínico de 4MMP y el precursor glutatiónico de 3MH. Además, las levaduras podrían usar los precursores cisteínicos y glutatiónicos como fuentes de GSH y/o cisteína. La adición de 20 mg/L de cisteína generó una sobreexpresión de los genes IRC7 y CYS3 que codifica enzimas con actividades de β y γ-liasa, respectivamente. Esta adición también condujo a una regulación positiva del gen OPT1, que codifica para un transportador GSH. La sobreexpresión de estos genes da como resultado mayor liberación de los mercaptanos polifuncionales.

Final conclusions

La mayoría de los precursores glicosídicos y polifenoles presentes en las uvas podrían aislarse por SPE de mistelas desalcoholizadas usando cartuchos C18 de 10 g. La reconstitución de las fracciones fenólicas y aromáticas (PAF) en vino sintético seguido del envejecimiento en anoxia durante 7 semanas a 45 °C o durante 24h a 75 °C condujo al desarrollo de aromas fuertes que recuerdan a algunos matices de olor observados en vinos tintos envejecidos. Además, los análisis GC-MS y GC-O de rPAF incubada durante 7 semanas a 45 ° C también fueron relativamente similares a los obtenidos después de 24 h a 75 °C, lo que sugiere que esta rápida hidrólisis de rPAF puede ser una herramienta prometedora para el estudio del potencial aromático de las uvas de vinificación.

La caracterización de las 33 muestras permitió identificar que los diferentes perfiles aromáticos encontrados en las uvas de Garnacha y Tempranillo se deben a ocho aldehídos, β-damascenona y massoia lactona que proporcionan un fondo común con notas "vegetal" y "fruta compotada", y linalol, óxido de linalol, 3MH, furaneol, guaiacol y metoxieugenol que parecen ser responsables de los matices aromáticos distintivos. La garnacha fue rica en norisoprenoides, terpenoles y derivados de vainillina y el Tempranillo en fenoles volátiles.