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Tesis Doctoral

INNOVATIVE ANALYTICAL PLATFORMS FOR SCREENING THE ABILITY OF MICRO-ORGANISMS TO PRODUCE HIGH IMPACT AROMA COMPOUNDS IN FERMENTATIVE PROCESSES

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- I'm sure it's a kind of secret message from the past… Let's crack the code! -

Universidad Zaragoza

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Laboratorio de Análisis de Aroma y Enología

Marie Denat

Innovative analytical platforms for screening the ability of micro-organisms to produce high impact aroma compounds in fermentative processes

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PhD THESIS
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Supervisors:

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Laboratorio de Análisis de Aroma y Enología

Innovative analytical platforms for screening the ability of micro-organisms to produce high impact aroma compounds in fermentative processes

Dissertation presented for the degree of

Philosophy Doctor in Analytical Chemistry

Marie Denat

Supervisors

Dr. Vicente Ferreira González

Dr. Amparo Querol Simón

2022

LABORATORIO DE ANÁLISIS DEL AROMA Y ENOLOGÍA

AROMAGENESIS

Departamento de
Química Analítica
Universidad Zaragoza

D. VICENTE FERREIRA GONZÁLEZ, Catedrático del Departamento de Química Analítica de la Universidad de Zaragoza y Dña. AMPARO QUEROL SIMÓN, Profesora de Investigación del Consejo Superior de Investigaciones Científicas (CSIC) en el Instituto de Agroquímica y Tecnología de Alimentos (IATA),

CERTIFICAN

Que la presente memoria, titulada "*Innovative analytical platforms for screening the ability of micro-organisms to produce high impact aroma compounds in fermentative processes*", correspondiente al Proyecto de Tesis aprobado por el Departamento de Química Analítica y presentada para optar al grado de Doctor, ha sido realizada bajo nuestra dirección por *Dña. Marie Denat*, autorizando su presentación para proseguir los trámites oportunos y proceder a su calificación por el tribunal correspondiente.

Fdo. Dra. Amparo Querol Simón

Fdo. Dr. Vicente Ferreira González

« On ne cesse de devenir, chaque jour de notre existence »
Marie Robert

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Presentation

This project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement number 764364. It was part of the innovative training network called *Aromagenesis* (`www.aromagenesis.eu`), gathering 14 PhD students from 10 research groups, both from academia and industry, in 7 European Union countries, investigating biochemistry and genetics of flavor production in yeasts used in wine and beer fermentations. This project aims to generate new yeast strains with improved flavor profiles and to develop novel approaches to expand flavor profiles through co-fermentation with different yeast and bacterial species.

This thesis was hosted at the Laboratorio de Análisis del Aroma y Enología, Zaragoza (LAAE) and performed in close collaboration with the Instituto de Agroquímica y Tecnología de Alimentos, Valencia (IATA) and Lallemand Bio S.L. (Barcelona, Spain), from 2018 to 2022. Two one-months secondments were completed in March 2019 at IATA and October 2019 in Lallemand Bio experimental winery (Logroño, Spain), and shorter secondments were also done at the Instituto de Ciencias de la Vid y el Vino, Logroño (ICVV).

This work was focused on the evaluation of the impact of the fermenting yeast on wine aroma profile and in particular *Saccharomyces cerevisiae*. It has been carried out in close collaboration with the PhD student Dolores Pérez, affiliated to Lallemand and IATA, studying the impact of non-conventional yeasts and new yeast hybrids on wine aroma profile.

After a brief summary in Spanish, the present thesis is divided into the following parts:

- **Chapter 1: Introduction,** in which the actual context about wine aroma is presented. Its constitution is detailed, the origin of each component including the different metabolic pathways in *S. cerevisiae* involved in their formation and modulation, and the analytical strategies mostly used to quantify them. This part is closed by a series of hypothesis that will be tested along this thesis and the main objectives.

- **Chapter 2: Materials and Methods,** which presents the common methodologies used during this thesis, separated into microbiology, metabolomics, analytical chemistry, sensory and statistics. The experimental designs and specific techniques are detailed in each chapter.

– **Experimental Chapters:** in which the experimental works carried out during this thesis and the discussion of the results are presented. A short summary of the experimental work carried out in each chapter is given below:

Chapter 3: The effects of *S. cerevisiae* strains carrying alcoholic fermentation on the fermentative and varietal aroma profiles of young and aged Tempranillo wines

In this chapter, 10 strains were used to fermented a semi-synthetic must supplemented with polyfunctional mercaptans (PFMs) precursors and with a phenolic and aroma precursors fraction (PAF) extracted from Tempranillo. After fermentation, samples were submitted to a process of accelerated aging at 50 ºC in anoxia during 5 weeks. The major fermentative and varietal volatile metabolites were analysed by gas chromatography (GC) and GC-mass spectrometry (MS), including higher alcohols and their acetates, linear and branched fatty acids and their ethyl esters, carbonyl compounds, lactones, terpenes, norisoprenoids, cinnamates, vanillin derivatives and volatile phenols. Analyses were carried out in young wines and after aging. The volatile fraction lost during fermentation was also recovered, odorants of this fraction were identified by gas chromatography-olfactometry (GC-O) and quantified by GC and GC-MS.

Chapter 4. Chemo-sensory impact of *S. cerevisiae* strains on the aroma profiles of Tempranillo red wines throughout accelerated aging

In this chapter, a real must of Tempranillo was fermented with 2 strains selected from the results of the previous chapter. The same accelerated aging was performed and the same volatiles were quantified. Sensory evaluations were also carried out, in order to determine whether the samples were different and eventually to correlate volatile profile with the differences observed.

Chapter 5. Influence of *S. cerevisiae* yeasts on the aromatic longevity of non-sulfite added white wines

In this chapter, 3 strains were used to fermented a semi-synthetic must supplemented with PFMs precursors and Albariño PAF. After fermentation, samples were submitted to accelerated aging in anoxia at two temperatures; at 50 ºC up to 8 weeks and at 75 ºC up to 96 hours. The same volatiles previously quantified were also determined, plus free and total Strecker aldehydes, PFMs and free and total sulfur dioxide. Analyses were performed in the recently fermented wines and at 4 different times during aging at each temperature.

Chapter 6. Influence of *Saccharomyces* wine strains on the aroma precursor fraction during fermentation. A preliminary metabolomic approximation.

In this chapter, four different yeast strains were selected attending to their differential abilities to modulate aroma volatiles derived from specific precursors, such as PFMs, norisoprenoids, terpenes, volatile phenols, cinnamates and vanillin derivatives, as determined in previous chapters and in the work of the PhD student Dolores Pérez. A must obtained combining 6 varieties (Tempranillo, Garnacha, Riesling, Chardonnay, Gewürztraminer and Macabeo) was fermented with the 4 different strains to obtain young wines which were aged in anoxia at 75 ºC 12, 24 and 96 hours. The same volatiles quantified in the first chapter plus PFMs were determined in the recently fermented and aged wines. In parallel, the precursor fractions from the must and young wines were characterized using UPLC-QTOF-MS untarget analysis.

– **Conclusions**

– **Annexes**

The results from the three first chapters have resulted in the following publications so far:

Denat, M., Pérez, D., Heras, J. M., Querol, A. & Ferreira, V. (2021). The effects of *Saccharomyces cerevisiae* strains carrying alcoholic fermentation on the fermentative and varietal aroma profiles of young and aged Tempranillo wines. Food Chemistry: X, 9 (March 2020), 100116. https://doi.org/10.1016/j.fochx.2021.100116

Denat, M., Pérez, D., Heras, J. M., Sáenz-Navajas, M. P. & Ferreira, V. (2021). Impact of two yeast strains on Tempranillo red wine aroma profiles throughout accelerated ageing. OENO One, 55(4), 181–195. https://doi.org/10.20870/oeno-one.2021.55.4.4732

Denat, M., Ontañón, I., Querol, A. & Ferreira, V. (2022). The diverse effects of yeast on the aroma of non-sulfite added white wines throughout aging. LWT, 113111. https://doi.org/10.1016/j.lwt.2022.113111

Additionally, the work carried out in collaboration with Dolores Pérez, using the methods and data treatment developed in this thesis, have produced the following papers:

Pérez, D., Denat, M., Minebois, R., Heras, J. M., Guillamón, J. M., Ferreira, V. & Querol, A. (2022). Modulation of aroma and chemical composition of Albariño semi-synthetic wines by non-wine *Saccharomyces* yeasts and bottle aging. Food Microbiology, 104(January). https://doi.org/10.1016/j.fm.2022.103981

Pérez, D., Denat, M., Heras, J. M., Guillamón, J. M., Ferreira, V. & Querol, A. (2022). Effect of non-wine *Saccharomyces* yeasts and bottle aging on the release and generation of aromas in semi-synthetic Tempranillo wines. International Journal of Food Microbiology, 365, 109554. https://doi.org/10.1016/j.ijfoodmicro.2022.109554

Pérez, D., Denat, M., Pérez-Través Laura, Heras, J. M., Guillamón, J. M., Ferreira, V. & Querol, A. Generation of intra- and interspecific *Saccharomyces* hybrids with improved oenological and aromatic properties. Microbial Biotechnilogy, (submitted).

Presentación

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El trabajo persigue evaluar el impacto de la levadura a cargo de la fermentación, y en particular de *Saccharomyces cerevisiae*, sobre el perfil aromático del vino con un énfasis particular en el desarrollo del aroma varietal y en la longevidad del vino.

La tesis consta de una introducción en la que se presenta el estado del conocimiento actual del aroma del vino, detallando el origen de cada componente, sus rutas metabólicas en *S. cerevisiae* y las estrategias analíticas más comunes para cuantificarlos, de un capítulo de metodología general y de cuatro capítulos experimentales independientes.

En el primer y tercer capítulos se presentan los resultados obtenidos con mostos semi-sintéticos suplementados con una fracción fenólica y aromática (FFA) extraída de uvas de Tempranillo o Albariño, y con precursores de mercaptanos polifuncionales de síntesis. Después de fermentar, los vinos sufrieron un proceso de envejecimiento anóxico a 50 y/o 75 grados. En el primer trabajo se analizó todo el sistema, incluyendo los volátiles perdidos por evaporación en la fermentación, se trabajó con 10 cepas *Saccharomyce*s independientes y se analizaron un amplio conjunto de aromas varietales y fermentativos. En el tercer capítulo, se trabajó solo con tres cepas de levadura, pero se amplió el número de compuestos aromáticos analizados, incluyéndose aldehídos de Strecker y mercaptanos polifuncionales, además de la práctica totalidad de aromas fermentativos y varietales, ya cuantificados anteriormente, y también se realizó un estudio más exhaustivo de la evolución del aroma durante el envejecimiento a 2 temperaturas.

El segundo capítulo experimental es un corolario del primero en el que un mosto de tempranillo se fermentó con dos de las levaduras más diferentes del primer trabajo. En este caso, además de la composición aromática, cuyos datos se transformaron mediante el concepto de vectores aromáticos y se procesaron empleando diversos conceptos psicofísicos, se midieron las características sensoriales de las muestras empleando diversas estrategias de análisis sensorial, con el fin de verificar el impacto de la levadura sobre las propiedades sensoriales del vino todo a lo largo de su envejecimiento.

En el cuarto capítulo experimental, se seleccionaron 4 cepas de levaduras mostrando máximas diferencias en su capacidad de modular el aroma varietal. Con ellas, se fermentó un mosto obtenido con una mezcla de 6 variedades de uva diferentes (Tempranillo, Garnacha, Riesling, Chardonnay, Gewürztraminer and Macabeo) con el fin de producir la máxima variabilidad posible sobre el pool de precursores aromáticos del aroma varietal. La composición del aroma de los vinos producidos fue extensivamente estudiada a lo largo del envejecimiento. En paralelo, la fracción de precursores del mosto y del vino recién fermentados fueron caracterizadas mediante análisis no dirigido en UPLC-QTOF-MS. El tratamiento de los datos buscó señalar los componentes detectados por UPLC-MS que pudieran estar relacionados con la variabilidad observada en el aroma varietal con el fin de señalar posibles precursores aromáticos y de comprender el mecanismo de acción de la levadura sobre los mismos.

La conclusión fundamental de la presente tesis es que la fermentación, y en particular la levadura que la lleva a cabo, no sólo determina la composición del aroma fermentativo, sino que determina la evolución a lo largo del tiempo del aroma varietal y condiciona el desarrollo de aromas oxidativos, afectando por tanto la calidad sensorial y longevidad del vino. Esto lo realiza de diferentes maneras:

1. Actuando sobre el aroma varietal primario y sobre su evolución a lo largo del envejecimiento
    (a) Acelerando la hidrólisis de los precursores, adelantando la formación del aroma, pero sin alterar la cantidad final de aroma formada:
        – De forma directa (vía actividad enzimática, por ejemplo, para la β-damascenona o geraniol)
        – De forma indirecta (acidulando el pH, TDN)
    (b) Metabolizando el precursor en otra molécula, por tanto, disminuyendo la cantidad final de aroma formado (TDN, massoia lactone, guaiacol)
    (c) Transformando componentes del mosto en precursores aromáticos, por tanto, aumentando la cantidad final de aroma formado (vainillina, metoxieugenol, oxido de rosa, *beta*-citronellol)
    (d) Formando de novo el aroma varietal (linalol y geraniol)
    (e) Formando especies reactivas que destruyen el aroma varietal:
        – Vinilfenoles reactivos hacia los mercaptanos polifuncionales
        – SO_2, reactivo hacia la β-damascenona
2. Actuando sobre los aldehídos de Strecker de al menos, dos maneras diferentes:

(a) Produciendo cantidades diferentes de los mismos durante la fermentación

(b) Produciendo el medio reactivo necesario (aminoácidos residuales + dicarbonilos) para su acumulación en anoxia

3. Produciendo aromas fermentativos precursores de aromas relevantes en el vino envejecido (ácidos ramificados precursores de ésteres etílicos frutales, ácido leucidico y cinámico)

Finalmente, el estudio metabolómico preliminar que completa esta tesis, ha permitido confirmar que la fermentación afecta de una manera muy intensa a los componentes de la uva, con la posible excepción de los glicósidos de moléculas aromáticas, y que el envejecimiento lleva también asociado un cambio profundo en las moléculas procedentes de la uva, incluyendo en esta ocasión los precursores de aromas varietales. Se han anotado además un conjunto importante de señales UPLC-MS potencialmente implicadas en la generación del aroma varietal y en su transformación por la levadura.

Index

Chapter 1

Introduction

1.1 Wine sensory properties and wine aroma vectors

The number of molecules which can be part of the volatile fraction of wines is very large, exceeding most likely several thousands. However, it has been suggested that the different aroma nuances of wines are due to around 80 volatile compounds, that can be found in wines at concentrations peri- or above threshold, in the range 10^{-9} to 10^{2} g/L (Culleré et al., 2019). One of the actual challenges of wine science is to determine whether it is possible to predict wine aroma from its chemical composition.

1.1.1 Olfaction process

Wine flavor is constituted by the combined and integrated action of aroma, taste and mouthfeeling (touch and chemesthesis). The largest part of the qualitative characteristics of flavor is caused by odor. Odorous compounds are detected by the receptors of the olfactory epithelium, situated in the nasal cavity. As described by (Firestein, 2001), most odor compounds are recognized by more than one olfactory receptor, and those receptors can also detect combinations of odors; so that human nose can perceive and differentiate several thousands of compounds and endless different odors.

In addition, the sensory perception of odor and flavor involves the brain limbic system, associated with emotion and memory (Swiegers et al., 2005b).

1.1.2 OTs, OAVs and their limitations

The minima concentration of a volatile necessary for a sensory detection is called odour threshold (OT). OT are commonly used to explain the chemical bases of aroma perception. The ratio volatile concentration/OT is known as odor activity value (OAV). OAV should be understood as concentrations in odor scale, but they are not measurements of odor perception.

The relationship between odor intensity (I) and the intensity of the stimulant -the odorant- (C_i) is odorant-specific and it is known as psychophysical plot. As explained in Ferreira et al. (2021a), those plots have a sigmoid structure for which the first half can be approximated by the Stevens's law ($I = k_i C_i n_i$) where k_i and n_i are odorant-specific coefficients, and C_i can be replaced by the OAV of the odorant. However, as n and k are only known for a few volatiles, many researchers naturally use directly the OAV of the odorants as a direct measurement of their importance in a food product, and tend to believe that those odorants with OAV < 1 are irrelevant in the odor properties of such a product. However, such view has several limitations.

First, OT are relatively imprecise, since they depend on several factors including the high inter-personal variability, which depends on intrinsic and extrinsic factors such as genetic heritage, previous training to sensory analysis, mood and experimental conditions (Tempere et al., 2011). Moreover, OT are by definition obtained in the absence of any other odorant, except perhaps ethanol, while in normal olfaction the detection takes place in the presence of many other odorants, some of which could affect not only to the sensitivity of the chemoreceptors, but also to the ulterior processing of the olfactory signals (Ferreira et al., 2021a). Indeed, it has been described that some compounds can have sensory relevance even at subthreshold levels by synergic interactions (Escudero et al., 2004). Because of that, it has been proposed that only aroma chemicals present at concentrations one order

of magnitude below their corresponding OT, can be safely considered irrelevant to wine aroma, OAV < 0.1 (Ferreira et al., 2021a).

1.1.3 Aroma vectors

In order to palliate these limitations and understand wine aroma in a more integrated way, the concepts of wine buffer and aroma vectors have been developed (Ferreira, 2010). The aroma buffer is constituted by 27 compounds generally present at concentrations well above threshold (ethanol, diacetyl, acetaldehyde; **Fusel alcohols:** isobutanol, isoamyl alcohols, hexanol, β-phenylethanol, methionol; **organic acids:** acetic acid, butyric acid, hexanoic acid, octanoic acid, decanoic acid; **isoacids:** isobutyric acid, 2-methylbutyric acid, isovaleric acid; **organic acid ethyl esters:** ethyl acetate, ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate; **Fusel alcohol acetates:** isobutyl acetate, isoamyl acetate, β-phenylethyl acetate; **isoacids ethyl esters:** ethyl isobutyrate, ethyl 2-methylbutyrate, ethyl isovalerate). Minor changes in the composition of this mixture, do not affect their sensory descriptors, fruity and alcoholic (Escudero et al., 2004).

The most outstanding property of this mixture is its ability to suppress many aroma nuances, ability which seems to be mostly caused by isobutyl and isoamyl alcohols (De-la Fuente-Blanco et al., 2016). The buffer can be broken by some specific odorants or by groups of similar odorants acting in a concerted way. It is precisely this ability of our olfactory system to provide an integral odor signal for groups of odorants of similar aroma, what makes it possible to define aroma vectors.

A compound or group of compounds able to break the buffer, transmitting a specific sensory note is called aroma vector. In wine, 93 aroma compounds have been described and classified into 43 aroma vectors, belonging to 12 sensory categories, according to their similarity in terms of aroma and chemical structure (Ferreira et al., 2021a), leading to a considerable simplification of the representation of wine aroma perception. They are presented in the Table 1.1.3.

Table 1.1: Main compounds involved into wine aroma profile, their OT in μg/L, specific descriptors, the aroma vector related and their generic descriptor.

generic descriptor	**aroma vector**	**compound**	**OT (μg/L)**	**specific descriptor**
acetic	acetic acid	acetic acid	300000[1]	acetic, vinegar
	ethyl acetate	ethyl acetate	12300[2]	glue
alcoholic, solvent	higher alcohols	isobutanol isoamyl alcohol β-phenylethanol	40000[3] 30000[3] 14000[4]	harsh, spirit, solvent
yeasty, oxidized	acetaldehyde	acetaldehyde	500[3]	green apple, oxidised
	isoaldehydes	isobutyraldehyde 2-methylbutanal 3-methylbutanal	6[5] 16[5] 4.6[5]	malty, yeasty
	methional	methional	0.5[6]	potato, oxidised, overripe
	phenylacetaldehyde	phenylacetaldehyde	1[5]	honey, oxidised
lactic, acid	fatty acids	butyric acid hexanoic acid octanoic acid decanoic acid	173[4] 420[4] 500[4] 1000[4]	cheesy, soapy
	branched acids	isobutyric acid isovaleric acid	50[7] 33[4]	cheesy, sweaty
	diacetyl	diacetyl	100[3]	buttery, milky
reduced	VSC	H_2S MeSH EtSH	1.1[8] 1.8[8] 1.1[9]	rotten egg, cooked cabbage
	acetates	isobutyl acetate	1605[1]	banana

		isoamyl acetate	30[3]	
	ethyl ester	ethyl propanoate	5500[10]	fruits, apple, strawberry
		ethyl butyrate	125[10]	
		ethyl hexanoate	62[10]	
		ethyl octanoate	580[11]	
		ethyl decanoate	200[4]	
		ethyl isobutyrate	15[4]	
		ethyl 2-methylbutyrate	18[4]	
		ethyl isovalerate	3[4]	
		ethyl 4-methylvalerate	10[12]	
		ethyl cyclohexanoate	0.03[10]	
		ethyl leucate	900[13]	
	lactones	γ-octalactone	238[14]	peachy
		γ-nonalactone	30[15]	
		δ-decalactone	386[4]	
	damascenone	β-damascenone	0.05[3]	baked apple, dry plum
	furaneol	furaneol	5[1]	strawberry, cotton candy
		homofuranel	125[1]	
	massoia lactone	massoia lactone	11[16]	overripe, dried, cooked fruits
flowery	terpenes	linalool	25.2[4]	jasmine, muscat, orange blossom
		geraniol	30[3]	
		nerol	300[17]	
		citronellol	40[17]	
		dihydromyrcenol	79*	
		α-terpineol	250[4]	
		linalool oxides	> 3600[18]	
	ionones	α-ionone	2.6[11]	violets, berry

		β-ionone	0.09[4]	
	cinnamates	trans-ethyl cinnamate ethyl dihydrocinnamate	1.1[4] 1.6[4]	sweet, balsamic
	phenylethyl acetate	β-phenylethyl acetate	250[3]	floral, rose, sweet
	rose oxide	(+/-)-cis/trans-rose oxide	0.5-160[19]	rose, litchi
freshness, citric, green	MH	MH	0.06[20]	grapefruit
	MHA	MHA	0.004[20]	passion fruit
	MP	MP	0.0008[20]	box tree
	piperitone and menthane lactones	piperitone minlactones menthofuran	70[21] 0.00012[22] 0.052[22]	minty, fresh
	cineole	1,8-cineole	1.1[23]	eucalyptus
spice, woody	ethylphenols	4-ethylphenol 4-ethylguaiacol m-cresol o-cresol	35[10] 33[4] 68[24] 31[11]	spice, leather
	methoxyphenols	eugenol guaiacol trans-isoeugenol methoxyeugenol 4-propylguaiacol syringol	6[4] 9.5[4] 6[5] 1200[5] 10[10] 570[25]	clove, smoky
	vinylphenols	4-vinylphenol 4-vinylguaiacol	180[26] 40[3]	medicinal

	vanillins	vanillin acetovanillone methyl vanillate ethyl vanillate	995[5] 1000[5] 990[25] 3000[25]	vanilla, nutmeg
	TDN	TDN	2[27]	kerosene
	rotundone	rotundone	0.016[28]	pepper
	whiskylactones	cis/trans-whiskylactone	790/67[29]	oaky, peachy
	DMS	DMS	25[9]	black truffle, black olive
empyreumatic	fermentative thiols	BM FFT	0.0003[30] 0.0004[31]	toasted, smoke coffee, roasted
cooked fruits, prune, figs	sotolon	sotolon	15[27][32]	licorice, curry
	ketones	3-methyl- 2,4-nonanedione	0.016[38][33]	anise, hay, prune
		(Z)-1,5-octadien-3-one	0.09[36][34]	geranium

*calculated in LAAE, in 12 % (v/v) ethanol/water, 5 g/L tartaric acid, pH 3.5; [**1**] from (Ferreira et al., 2002), in 10 % (v/v) ethanol/water, pH 3.2; [**2**] from (Escudero et al., 2004), in 10 % (v/v) ethanol/water, 5 g/L tartaric acid, pH 3.2; [**3**] from (Guth, 1997a), in 10 % (v/v) ethanol/water; [**4**] from (Ferreira et al., 2000), in 11 % (v/v) ethanol/water, 7 g/L glycerol, 5 g/L tartaric acid, pH 3.4; [**5**] from (Culleré et al., 2007), in 10 % (v/v) ethanol/water, 5 g/L tartaric acid, pH 3.2; [**6**] from (Escudero et al., 2000), in 11 % (v/v) ethanol/water, 5 g/L tartaric acid, 7 g/L glycerin, pH 3.4; [**7**] from (van Gemert, 2003), in water; [**8**] from (Siebert et al., 2010), in wine; [**9**] from (Goniak and Noble, 1987), in white wine; [**10**] from (San Juan et al., 2012), in 10 % (v/v) ethanol/water, 5 g/L tartaric acid, pH 3.2; [**11**] from (Etievant, 1991), in 12 % (v/v) ethanol/water, except for t-whiskylactone in 30 % (v/v) ethanol, and t-2-hexenol in beer; [**12**] from (Takeoka et al., 1995), in water; [**13**] from (Falcao et al., 2012), in 12 % (v/v) ethanol/water, 4 g/L tartaric acid, pH 3.5; [**14**] from (Cooke et al., 2009), in red wine, 12.8 % (v/v) ethanol, pH 3.5, SO_2 levels 117 mg/L total and 21 mg/L free; [**15**] from (Nakamura et al., 1988), in 10 % (v/v) dealcoholized and dearomatized wine/ethanol; [**16**] from (Pons et al., 2017), in 12 % (v/v) ethanol/water, 5 g/L tartaric acid, pH 3.5; [**17**] from (Ohloff, 1978), in water; [**18**] from (Ribéreau-Gayon et al., 1975), in sweet water; [**19**] from (Yamamoto et al., 2002), in water; [**20**] from (Tominaga et al., 1998a), in 12 % (v/v) ethanol/water, 5 g/L tartaric acid, pH 3.5; [**21**] from (Pons et al., 2016), in red wine; [**22**] from (Picard et al., 2017), in 12 % (v/v) ethanol/water; [**23**] from (Poitou et al., 2017), in red wine; [**24**] from (Culleré et al., 2004), in 11 % (v/v) ethanol/water, 7 g/L glycerol, 5 g/L tartaric acid, pH 3.4; [**25**] from (López et al., 2002),

in 10 % (v/v) ethanol/water, pH 3.2; **[26]** from (Boidron et al., 1988), in 12 % (v/v) ethanol/water, 8 g/L glycerol and different salts; **[27]** from (Sacks et al., 2012), in 10 % (v/v) ethanol/water, 1 % (m/v) tartaric acid; **[28]** from (Wood et al., 2008), in red wine; **[29]** from (Otsuka et al., 1974), in 30 % (v/v) ethanol/water; **[30]** from (Tominaga et al., 2003b), in hydroalcoholic solution; **[31]** from (Tominaga et al., 2000), in 12 % (v/v) ethanol/water, 8 g/L glycerol and different salts; **[32]** from (Martin et al., 1992), in white wine; **[33]** from (Pons et al., 2008), in 12 % (v/v) ethanol/water, 5 g/L tartaric acid, pH 3.5; **[34]** from (Allamy et al., 2017), in synthetic wine.

1.2 Analytical strategies for the quantification of wine aroma volatiles

Aroma vectors are composed by chemical compounds with different analytical properties and present at quite different concentrations, which implies the existence of quite different levels of analytical difficulty. In any case, and as aforementioned, methods detection limits should allow to quantify at least one order of magnitude below OT.

Wine odorants can be classified attending to the difficulty with which they can be determined.

1.2.1 Easy-to-analyze volatiles

Those volatiles are usually present at concentrations above 1 μg/L and have a relatively low reactivity and polarity.

Quantitatively, the most abundant wine volatiles are by-products of fermentation usually present at levels superior to several hundreds or thousands of μg/L, including acetic acid, ethyl acetate, higher alcohols and their acetates, branched fatty acids, linear short chain fatty acids and their ethyl esters, acetaldehyde and diacetyl. All of them are easily analyzed by GC-FID (Ortega et al., 2001), apart from the latter two, for which an approximate amount will be obtained.

Other compounds present at inferior concentrations, but still superior or around the μg/L are, for example, ethyl esters of branched acids, ethyl cinnamates, terpenes (rose oxide, linalool, geraniol), norisoprenoids (β-damascenone, ionones, TDN), lactones, vanillins and volatile phenols (ethylphenols, methoxyphenols and vinylphenols). Those volatiles can be very easily determined using simple isolation strategies, including SPE, followed by a simple GC separation coupled with an MS detector (López et al., 2002).

1.2.2 Aldehydes, volatile sulfur compounds, sulfur dioxide

Those compounds form complexes and are usually present between the μg-mg/L.

Sulfur compounds and aldehydes are present in both free and bonded forms in wines. While free forms can be directly analyzed via GC-MS or, in the case of volatile sulfur compounds (DMS, EtSH, MeSH, H_2S), by means of a specific detector such as SCD (Ontañón et al., 2019) or pFPD (Franco-Luesma and Ferreira, 2014); a previous step is required to liberate those complexes for the analysis of bonded forms, usually known as total forms. This can be achieved by heating the acidulated sample, as for sulfur dioxide (Carrascon et al., 2017), by a combination of dilution, temperature and adding a complexing agent (Cl^-), as for H_2S and mercaptans, or by adding higher levels of derivatization agent and using stronger reaction conditions, as for Strecker aldehydes (acetaldehyde, isoaldehydes, methional, phenylacetaldehyde). In the latter case, particular attention is necessary regarding contaminations, since some aldehydes are also present in air and water (Culleré et al., 2004).

1.2.3 Highly polar compounds

Volatiles such as furaneol (2,5-dimethyl-4-hydroxy-3(2H)-furanone), homofuraneol (2-ethyl-4-hydroxy-5-methyl-3 (2H)-furanone) and sotolon (4,5-dimethyl-3-hydroxy-2(5H)-furanone) are very polar compounds present at few μg/L, whose analysis requires quite demanding extraction procedures and highly inert chromatographic phases. A procedure uses SPE with an extra cleaning step consisting in washing the non-polar compounds with non-polar solvent. Extracts can then be analyzed by GC-MS (Ferreira et al., 2003; San Juan et al., 2012).

1.2.4 Ultra-trace compounds

Other volatiles are present at levels inferior to the μg/L and require highly selective and sensitive analytical strategies.

PFMs are powerful compounds with a very low odor threshold, around the ng/L. Other compounds in this category are alkylmethoxypyrazines (Wen et al., 2018), rotundone (Culleré et al., 2016), piperitone (Picard et al., 2016) and other limonene derivatives (Picard et al., 2017). The least polar aroma compounds (all of them except polyfunctional mercaptans) are extracted via stir bar sorptive extraction (SBSE), and the extract is further thermally desorbed in a bi-dimensional GC-MS. In the case of PFMs, a previous derivatization is required (Mateo-Vivaracho et al., 2010).

1.3 Scheme of the main metabolic pathways leading to the production of volatile compounds involved in wine aroma

Most volatile compounds taking part in the different aroma vectors are affected by the yeast in charge of alcoholic fermentation. The most widely used in wine industry is *Saccharomyces cerevisiae*, which is also the most widely studied micro-organism in particular about its sensory impact on wine (Tempère et al., 2018).

Fermentation is an anaerobic energy-producing process in which C_6-sugars are bio-transformed into ethanol and carbon dioxide. Firstly, during glycolysis, C_6-sugars are converted into pyruvate, generating energy under the form of ATP at the expense of NADH accumulation, the reduced form of the NAD^+ co-enzyme. As reviewed by Pronk et al. (1996), pyruvate is then metabolized following several routes. In the dominant, it is converted into (1) ethanol, allowing the re-oxidation of NADH. A second option is its transformation into (2) acetyl-CoA, which is further introduced into the TCA cycle and further converted into lipids and sterols. It can be also transformed into (3) α-acetolactate, which is further involved into the synthesis of amino acids.

A comprehensive vision of wine aroma genesis was proposed in the Figures 1.1 and 1.2. The genesis of the main volatiles involved in the aroma vectors was

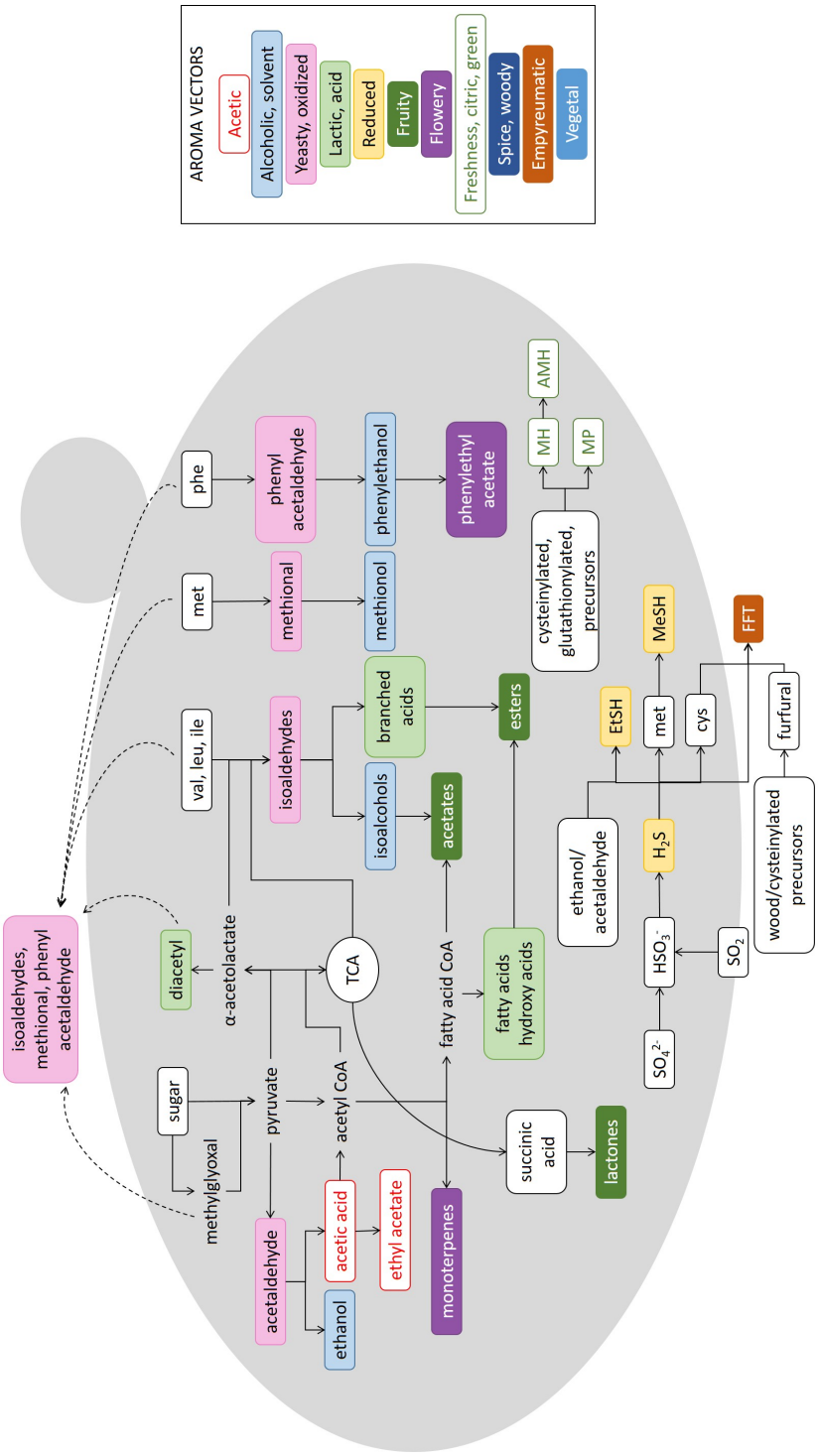

Figure 1.1: Secondary metabolism of *S. cerevisiae* during alcoholic fermentation leading to the liberation of the main volatile metabolites involved into the perception of wine aroma. Dotted lines are processes that hypothetically happen during wine aging. Adapted from Swiegers et al. (2005a).

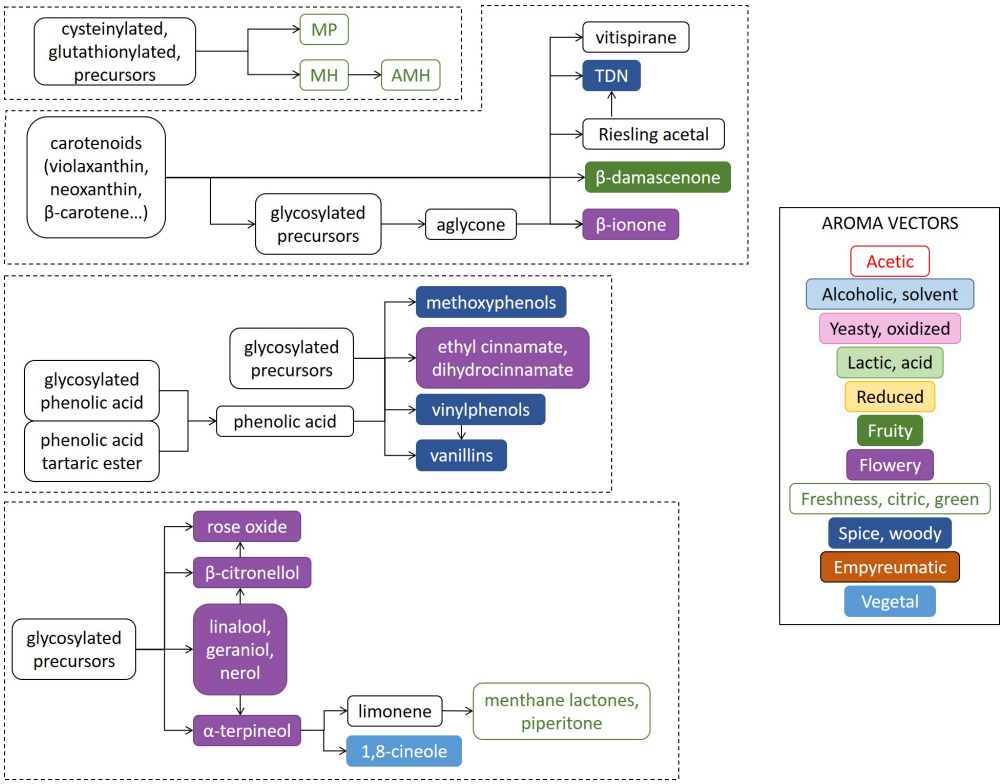

Figure 1.2: Genesis of the main volatiles involved into wine aroma by processes naturally occurring during vinification and/or wine aging, and in which *S. cerevisiae* yeasts have a demonstrated or supposed effect.

represented, and their sensory contribution was indicated by the colored rectangles. Figure 1.1 represents the main metabolic pathways and intracellular processes leading to the formation fermentative volatiles by *S. cerevisiae*; while Figure 1.2 represents the processes involved in the formation of varietal and other volatiles, either during vinification or throughout aging. While in these cases mostly chemical processes, such as acid hydrolysis, or diverse molecular rearrangements take place, yeast plays o may play also an impact on their modulation.

Volatile compounds are generally separated into two major categories: varietal and fermentative compounds. While fermentative compounds derive from yeast primary metabolism; varietal aroma compounds derive from specific precursors produced by grapes. A third category of aging bouquet (Simpson, 1979) is usually added for the compounds requiring aging time to form and accumulate. However, and as it can be seen in the Figures 1.1 and 1.2, the frontiers between fermentative and varietal and between varietal and aging bouquet are often unclear since many volatiles can have a multiple origin. The impact of yeast on varietal and particularly on aging-bouquet compounds is mostly unknown.

In the following parts, the different metabolic pathways leading to the formation of wine aroma, will be detailed. In the first part, we will describe those best known, including studies explaining their evolution in wines. In the second part, those for which the effect of the yeast is not fully understood will be mentioned.

1.4 Aroma vectors affected by the primary metabolism of *S. cerevisiae*

1.4.1 Higher alcohols

Both 2 and 3-methylbutanol are usually quantified together and known as isoamyl alcohol. Together with isobutanol, and with a minor sensory relevance, β-phenylethanol and methionol are higher alcohols, also called Fusel alcohols. These

compounds are present in all wines. Isoamyl alcohol is found at concentrations well above 100 mg/L, way above its detection threshold (Culleré et al., 2019), while isobutanol can be found at levels close to 50 mg/L in some wines. They are involved into the alcoholic, solvent aroma vector (Ferreira et al., 2021a) and can exert a strong aroma suppression effect (De-la Fuente-Blanco et al., 2016).

These compounds are produced by yeast via the Ehrlich pathway from the amino acids valine, leucine, isoleucine, phenylalanine and methionine (Ehrlich, 1907). Amino acids are firstly transaminated into α-keto acids, decarboxylated into aldehydes and they are further reduced into alcohols or, alternatively, oxidized into acids, depending on the redox state of the cell. Each step is enzymatically catalyzed and several genes encoding for these enzymes have been described (Hazelwood et al., 2008). Their concentration is usually unaffected by wine aging (Marais and Pool, 1980).

1.4.2 Acetates from higher alcohols

The yeast can transform higher alcohols produced through the Ehrlich pathway in acetates, via an intracellular esterification enzymatically catalyzed by acyltransferases, requiring acetyl-CoA (Nordström, 1962). The genes encoding for such enzymes have been identified and their organization and expression have also been characterized (reviewed by Mason and Dufour (2000)). The quantity formed in wine depends on the availability of the alcohol precursors (Yoshimoto et al., 2002) and on the activity of the enzymes involved in their synthesis and hydrolysis, which is highly strain dependent (Peddie, 1990). They are especially relevant for young wine aroma since during wine aging, these compounds hydrolyze and their concentration drastically decrease (Marais and Pool, 1980).

Isobutyl and isoamyl acetates can be found in wines at concentrations around 0.1 to 10 mg/L, sometimes largely exceeding their odor thresholds. At low levels, these two compounds are integrated within the fruity aroma vector, while at concentrations above 2 mg/L, they confer to the wine a banana odor nuance.

β-phenylethyl acetate, which has a strong floral, honey character, can contribute to floral notes when present at levels above 0.5 mg/L (Lilly et al., 2000).

Due to their relatively high abundance in young wines and their pleasant notes, the aroma impact of higher alcohol acetates has been the subject of intensive research from the second half of the XXth century in fermented beverages such as wine (Ferreira et al., 1995; Ramey and Ough, 1980) and beer (Engan, 1972). More recently and thanks to the improvement of non-GMO techniques, such as hybridization or adaptive evolution, engineered wine yeast have emerged with a more powerful flavor profile, for example, by favoring higher alcohols and their acetates (Rollero et al., 2016). However, the flavors produced by those strains, can be in many instances too simple for high-quality table wines.

1.4.3 Lineal fatty acids and their ethyl esters

Lineal fatty acids are usually present in wines at concentrations around 1 mg/L, above their olfaction threshold. While isolated, they are described as cheesy and soapy; their role in wine aroma perception is unclear because their main contribution is through perceptual interactions with other aroma compounds (Ferreira et al., 2021b).

Short chain (C2-C4) and medium chain (C6-C12) fatty acids are derived from acetyl-CoA formation by yeasts. They are intermediates of long-chain fatty acids, themselves precursors for the synthesis of lipids formed to build the yeast plasma membrane (Mbuyane et al., 2021). Their formation involves first, the transformation of acetyl-CoA into malonyl-CoA via acetyl-CoA carboxylase, and the further repetitive condensation of acetyl-CoA (or propionyl-CoA for odd numbered fatty acids) and malonyl-CoA by fatty acid synthetases (Lambrechts and Pretorius, 2000).

Since fatty acids are inhibitors of fermentation, *S. cerevisiae* yeasts are able to form ethyl esters as a detoxification mechanism, and most likely also as semiochemicals to communicate with other species. They are formed by the enzymatic condensation between fatty acid-CoA and ethanol (Ramey and Ough,

1980).

Various studies have been dedicated to the study of the aromatic impact of esters on wine aroma, (Engan, 1972) already suggested the existence of an additive sensory effect on the fruity perception, which was further supported by Ferreira et al. (1995). This hypothesis has been studied in more detail by several authors. Lytra et al. (2013) demonstrated that even sub or peri-threshold esters can contribute to the fruity aroma in model solutions. More recently, De-la Fuente-Blanco et al. (2020) demonstrated that all ethyl esters integrate within a single fruity vector in complex wines, and have further corroborated the relevant role played by sub- and peri-threshold esters on the intensity, but not the on quality, of the fruity vector.

While fatty acids concentration are usually not affected during wine aging (Marais and Pool, 1980), the evolution of esters mainly depends on the esterification equilibrium (San Juan et al., 2012).

1.5 Influence of the yeast on varietal aroma vectors

Varietal compounds are derived from grape specific precursors. These are non-volatile molecules yielding volatiles by enzymatic transformations, among others glycosidases or lyases, and/or acid catalyzed hydrolysis and, eventually, by a series of further spontaneous chemical rearrangements (Ferreira and Lopez, 2019).

1.5.1 Aroma derived from cysteinylated and glutathionylated precursors

PFMs are derived from cysteinylated and glutathionylated precursors. 3-mercaptohexanol (MH) and 4-methyl-4-mercaptopentan-2-one (MP) are present in grapes linked to cysteine (Tominaga et al., 1998b), glutathione (Des Gachons et al., 2002), cysteine-glycine (Capone et al., 2011) or γ-glutamyl-cysteine (Bonnaffoux et al., 2017). There is no direct correlation between the content of

precursors in grapes, which are in the μg/L-mg/L range and the volatiles liberated in wine, whose concentrations are only few ng/L for MP or up to few μg/L for MH in the case of Sauvignon Blanc for example (Concejero et al., 2014; Mateo-Vivaracho et al., 2010).

Yeasts are able to liberate only a small fraction of precursor through carbon-sulfur β-lyase activity during fermentation; while the metabolization of the other part of the precursors is still unknown (Bonnaffoux et al., 2017). This activity for which several genes have been identified has a great intra-specific variability (Belda et al., 2016). Interestingly, one of the genes (IRC7) may have been favored during the domestication process since it resulted to be predominant in the wine strains and is absent from the wild ones (Ruiz et al., 2021). Additionally, MHA is derived from the esterification of MH via alcohol acetyltransferase and is formed up to several tens of ng/L (Swiegers and Pretorius, 2007).

MH, MHA and MP have very low olfaction thresholds; 60, 4 and 0.4 ng/L, respectively. They are characterized by box tree and blackcurrant notes for MP, grapefruit for MH and passion fruit for MHA. They all globally participate to the perception of freshness, green and citric notes in wines (Mateo-Vivaracho et al., 2010).

Due to the interesting sensory properties of these compounds, their modulation by yeasts have been extensively studied and yeasts with increased ability to release PFMs have been investigated. For example, yeasts liberating higher amounts of MP thanks to a particularly efficient β-lyase activity have been selected (Belda et al., 2016). Other authors have enhanced MH production via the overexpression of STR3 gene encoding a cystathionine β-lyase (Holt et al., 2012). First studies were directed towards increased levels of the fruitier MHA at the expense of MH. This was achieved via ATF1 overexpression, which encodes the enzymes responsible for the production of acetates (Swiegers et al., 2006).

However, recent research carried out in our laboratory, has demonstrated that MH and MP can be also released by spontaneous hydrolysis during accelerated

anoxic aging of wine or of polyphenolic and aromatic fractions extracted from grapes (Alegre et al., 2020b; Denat et al., 2022). This discovery may imply that the fraction of odorless cysteinyl and glutathionyl precursors have an active role on the lifespan of MP and MH during wine aging. From this point of view, preserving a large pool of the precursor fraction in fermentation seems to be a relevant requisite for producing wines able to keep their freshness and black fruit character for long periods.

1.5.2 Aroma derived from glycosidic precursors

Another major family of precursors are glycosylated compounds. Glycosylation is a process by which plants store and transform volatile compounds, presumably at the end of each biosynthesis pathway. Glycosylation increases the stability of labile molecules, increases solubility in the intracellular aqueous media, and decreases toxicity (Winterhalter and Skouroumounis, 1997). This pool constitutes a reserve of aroma. Its composition is highly dependent on the cultivar.

Grapes qualified as "aromatic" such as Gewurztraminer, Muscat and some of their derivatives, such as Traminette or Torrontés riojano, contain large amounts of linalool, cis-rose-oxide, geraniol and other terpenols, in free and under the form of glycosidic precursors. By contrast, some grapes have a rather neutral aroma, and are qualified as "neutral", such as Monastrell, Tempranillo or Grenache (Ferreira and Lopez, 2019). These grapes contain low amounts of terpenols, but can contain a relevant pool of other aroma glycosidic precursors such as norisoprenoid or benzenoid types.

Glycosidic precursors are formed by a sugar unit linked to an aglycone which will lead to the volatile after liberation via yeast glycosidases. The sugar unit can be constituted by a monosaccharide or a disaccharide. In the latter case, the action of a sugar-specific enzyme is required before the proper liberation of the aglycone via β-glucosidase (Gunata et al., 1988).

Contrary to the grape glycosidases, yeast glycosidases are not inhibited by the presence of sugar but have low tolerance to ethanol and acid pH, and some of them

have an aglycone-specificity (Winterhalter and Skouroumounis, 1997). This implies that at the end of the fermentation, there will be a significant part of the pool of aroma glycosidic precursors remaining intact. Different strategies are commonly used in wine industry in order to increase their hydrolysis and concomitant liberation of more aroma compounds. For example, exogenous enzymes or non-*Saccharomyces* yeasts can be added at pre-fermentative stages, or mixed fermentations combining *Saccharomyces* and non-*Saccharomyces* can be tried, since some of these yeasts possess glycosidases with a higher tolerance to ethanol and acid pHs (Belda et al., 2016).

Many different volatiles can be found glycosylated in grapes. Chemical families include lactones, aliphatic alcohol derivatives, terpenes, sesquiterpenoids, norisoprenoids and benzenoid compounds (Caffrey et al., 2020; Ferreira and Lopez, 2019; Wirth et al., 2001).

Terpenol glycosides were the first volatiles precursors identified at the end of the XX$^{\text{th}}$ century (Ribéreau-Gayon et al., 1975). Geraniol and linalool are two of the most sensorily relevant volatiles liberated from glycosides. These compounds are present in most wines at sub or peri-threshold concentration, and in some particular wines can reach levels above the thresholds (25 and 30 μg/L, for linalool and geraniol respectively). Other less aromatic terpenols, such as nerol and β-citronellol are also found at relatively similar levels. These compounds have a demonstrated implication in the flowery notes of many wines (Ferreira et al., 2021a). Several different glycosidic precursors of these compounds have been identified (Hjelmeland and Ebeler, 2015).

Within the benzenoid family, there are different classes of relevant odor volatiles. Volatile phenols include a series of carnation, smoke or clove-like odorants, such as guaiacol, eugenol, isoeugenol and 2,6-dimethoxyphenol; two other vinylphenols, 4-vinylguaiacol and 4-vinylphenol, with carnation notes for the first and with phenolic and chemical notes for the second. Vanillin derivatives includes vanillin, acetovanillone, syringaldehyde, ethyl and methyl vanillate, have odors remaining of vanillin and nutmeg. In most cases, levels of those aroma compounds are low

or moderate, contributing to different spicy, phenolic and even woody notes of wines (Ferreira et al., 2021a). However, high levels of vinylphenols are considered detrimental to wine quality (Chatonnet et al., 1993). Those high levels are most often produced not by the corresponding glycosidic precursors, but from the direct decarboxylation of the phenolic acid precursors.

In the actual context of climate change and the recrudescence of bushfires near vineyards, many wines have been affected by smoky-taint, an off-odor caused by excessive amounts of guaiacol, 4-methylguaiacol and syringol originated from increasing β-D-glucopyranoside precursors in grapes (Ristic et al., 2017). In this case, wines were characterized by smoky, ashy and medicinal notes. Other aroma compounds derived from grape glycosidic precursors within the benzenoid family are ethyl cinnamate and ethyl dihydrocinnamate, herein referred as "cinnamates". These compounds were firstly identified in Pinot noir, and were erroneously proposed as key odorants of the wines made with this variety (Moio and Etievant, 1995). Nevertheless, these two powerful aroma compounds are involved in flowery notes of neutral wines at the few μg/L levels at which they can be found (Culleré et al., 2019). Their modulation by the yeast has not been studied.

Finally, wine also contain little amounts of several lactones, such as δ-, γ-octa, nona or decalactone, generally present in wines at concentrations of few to several tens of μg/L. These peachy and coconut smelling compounds, at the very low levels at which they are usually found can contribute, however, to sweet and floral notes of wines if associated to other compounds such as cinnamates or vanillins (Loscos et al., 2007). The existence of glycosidic precursors has not been explicitly demonstrated, although the presence of γ-nonalactone in hydrolysates from the precursor fraction has been reported (Loscos et al., 2009).

1.5.3 Other enzymatic activities: modulation of volatile phenols, lactones, terpenoids, norisoprenoids and sesquiterpenoids

In some cases, the frontier between fermentative and varietal aroma is not clear, because of a number of reasons. One obvious reason is the *de novo* production by the yeast of an aroma compound also produced by the grape. In other cases, there is a specific precursor of grape origin, but it has to be transformed by yeast to produce the volatile. And in some cases, the compounds have clear fermentative origin, but the compositional profile of the grape must has a deep influence on the profile of volatiles produced.

1.5.3.1 *De novo* synthesis: lactones, terpenes, sesquiterpenoids

This can be the case for γ and δ-lactones. Several formation pathways of these compounds in yeast have been hypothesized, from the intramolecular cyclisation of unsaturated fatty acids (Wanikawa et al., 2000), or glutamic acid or derivatives, such as succinic acid (Muller et al., 1973).

It can be also the case of some terpenes (Carrau et al., 2005) and sesquiterpenes (Camesasca et al., 2018), since these compounds can be secondarily synthetized by *S. cerevisia* yeasts under certain conditions via the mevalonate pathway (MVA) for sterol biosynthesis, and particularly in the case of biological aging (Morales et al., 2020). The impact of sesquiterpenoids, such as nerolidol or farnesol, on wine aroma is unclear, but they have many biological activities such as quorum-sensing (Rodrigues and Černáková, 2020).

While the mechanisms involved into their evolution during aging have been studied in Valpolicella wine (Slaghenaufi and Ugliano, 2018), there is not much information about the impact of the yeast on their modulation. Their *de novo* synthesis in wine has already been observed by *S. cerevisiae* (Gamero et al., 2011).

In grapes, this pathway also lead to the formation of α-guaiene, further transformed by chemical (Huang et al., 2014) or enzymatic (Takase et al., 2016)

oxidation into the potent aroma molecule rotundone. Although the mechanisms used by yeasts to modulate this volatile have not been properly elucidated, it has been demonstrated that its concentration may be greatly reduced by fermentation with the cryotolerant *S. uvarum* (Geffroy et al., 2017). This compound has an olfaction threshold of around several ng/L (Wood et al., 2008) and characteristic pepper notes perceptible in some Syrah and Duras grapes. It could, however, be considered a taint at certain high concentrations (Geffroy et al., 2018; Williamson et al., 2012).

1.5.3.2 Other enzymatic activities

Volatile phenols and vanillins also can have from multiple origins. They are formed from hydroxycinnamic acids. These compounds are present in grapes in free form, as esters with tartaric acid (Lorrain et al., 2013) or as glycosidic precursors (Ferreira and Lopez, 2019). Esterified acids are previously released via yeasts cinnamoyl esterase activities (Smit et al., 2003). Coumaric and ferulic acids from grape are then decarboxylated into the volatile compounds 4-vinylphenol and 4-vinylguaiacol via the yeast cinnamate decarboxylase (Chatonnet et al., 1992). These compounds, known as vinylphenols can be consecutively reduced into ethylphenols via vinylphenol reductase, enzyme particularly active in *Brettanomyces/Dekkera* spoilage yeast (Benito-Vazquez et al., 2021), or oxidized to vanillin as observed in orange juice (Naim et al., 1993). Vanillin may also arise directly from ferulic acid (Peleg et al., 1992).

Cinnamic acid can also be esterified by the yeast, yielding ethyl cinnamate and 2,3-dihydrocinnamate.

1.5.3.3 Other enzymatic activities involved in long-term modulation

Several authors have proposed the existence of many other enzymatic transformations, apart from the cleavage of glycosidic bonds, such as reduction, oxidation, acetylation, hydroxylation, to explain the differences observed in the

modulation of terpenes such as rose oxide (Koslitz et al., 2008), β-citronellol (Gramatica et al., 1982; Slaghenaufi et al., 2020), but also of norisoprenoids such as TDN, Riesling acetal, vitispirane (Grebneva et al., 2019; Sponholz and Hühn, 1997) and β-damascenone (Lloyd et al., 2011).

Rose oxide is an aroma-powerful terpene identified in the aromatic variety Gewürtztraminer (Guth, 1997b). It may arise from multiple precursors such as 3,7-dimethyl octa-2,5-dien-1,7-diol which, through enzymatic reduction and cyclization yields rose oxide (Koslitz et al., 2008). Among its 4 isomers, the (-)-cis form is the most potent sensorily, with an olfaction threshold below the μg/L which confers to the wine characteristic litchi and rose-like notes (Yamamoto et al., 2002).

In the case of norisoprenoids, the major aglycones liberated through β-glucosidase activity are 3-hydroxy-β-damascone, dihydro-β-ionone, 3-oxo-α-ionol and vomifoliol. They require further acid-catalyzed transformations to yield the odorant. As reviewed by Mendes-Pinto (2009), they come from carotenoid breakdown. Their formation requires a first step of cleavage by 9,10,(9',10')-carotenoid cleavage dioxygenase (CDD) present in grapes, leading to primary cleavage products. One of these is β-ionone (Eugster et al., 1991). The other products will be further transformed into non-aromatic, possibly glycosylated forms, which can be transformed into β-damascenone via acid catalyzed rearrangements and hydrolysis.

To date, their modulation by yeast has been observed but the mechanisms involved are not fully understood (Oliveira and Ferreira, 2019). Since CCDs are not naturally occurring in *S. cerevisiae*, some recent research has tried the development of bioengineered strains or enzymes optimized for the initial cleavage (López et al., 2020).

Regarding the aromatic impact of these compounds, it seems to be, in the cases of β-ionone and β-damascenone, very dependent on the matrix (Tomasino and Bolman, 2021). Both components have very low detection thresholds, inferior to 100 ng/L. Their concentrations in wine can reach several hundreds of ng/L in the case of

β-ionone, and several μg/L in that of β-damascenone. With its characteristic violet aroma, β-ionone is involved into the perception of flowery notes in wines. On its part, β-damascenone is a known enhancer of fruity notes (Ferreira et al., 2002; Pineau et al., 2007), which at higher levels imparts overripe fruits notes (Ferreira et al., 2021a).

1.6 Influence of yeast on wine aroma longevity

Wine longevity is assured, firstly, by the continuous presence of positive aroma compounds throughout aging and, secondly, by the non-accumulation of negative aroma compounds. Yeasts can have a direct impact on both of these two points through a series of processes. First, yeast can accelerate the hydrolysis of precursors, which can be translated into higher levels of aroma compound in young wines, but smaller levels in aged wines. Second, yeast can modify chemically the precursor, affecting to the neat yield of aroma compound, positively or negatively. Third, yeast can also produce increased levels of fermentative compounds which are relevant aroma precursors, as is the case of branched acids, precursors of their fruity ethyl esters. A similar case with a negative outcome is the different levels of amino acid precursors of Strecker aldehydes remaining after fermentation. Fourth, yeast can also leave fermentation by-products, such as α-dicarbonyls, able to induce the Strecker degradation of those amino acids.

In the following section, and as it will be demonstrated in the experimental parts of this thesis, some of the most important actions of yeast on wine longevity will be highlighted.

1.6.1 Evolution of positive aroma throughout aging

The evolution with time of positive aroma compounds depends on several factors. First, on the stability and reactivity of the odorants. Second, on the amounts of precursors able to form the aroma compound. Third, on the complexity of

the reactions leading to the formation of the odorant from the precursors and; forth, on the possible action of the yeast in charge of fermentation on those aroma precursors. The different combinations of these factors explain the different accumulation profiles observed during aging. The most important and examples of these types of evolution are briefly commented.

1.6.1.1 Evolutions with marked and early maxima: linalool and geraniol

This type of evolution is prototypical of labile aroma compounds with a limited pool of precursors able to yield the aroma compound by simple and relatively fast chemical reactions. Linalool and geraniol, follow nearly always this type of evolution, since they are formed by direct liberation from specific precursors and are labile at wine pH, so that these compounds will be always found at higher levels in young or at least in not too-aged wines (Ribéreau-Gayon et al., 1975).

1.6.1.2 Evolutions reaching a plateau: β-damascenone

This type of pattern is followed by stable or relatively stable aroma compounds with a limited pool of precursors. A prototypical example is that of β-damascenone. Since the formation from the precursors is more complex than that of terpenols, the plateau is reached after one or two years of aging, depending on the amounts and complexity of the pool of precursors (Alegre et al., 2020b; Oliveira and Ferreira, 2019). The stability of β-damascenone will also be affected by the use of SO_2. Relatively high levels of this compound cause the decomposition of β-damascenone (Daniel et al., 2004).

1.6.1.3 Evolutions with continuous increases

This type of pattern is the most common, and is followed by stable or relatively stable aroma compounds with an abundant pool of precursors usually requiring a complex set of chemical transformations to form the aroma compounds. The fruity ethyl esters of branched acids, norisoprenoids such as TDN

(1,1,6-trimethyl-1,2-dihydronaphthalene), DMS (dimethylsulfide), different terpene derivatives and benzenoids, FFT (2-furfurylthiol) usually follow this pattern, as will be briefly commented later.

TDN - Although not demonstrated, a pathway involving the yeast reductase activity of a grape specific precursor was recently proposed (Grebneva et al., 2019). Also, the existence of several precursors has been demonstrated (Versini et al., 1996), as well as their numerous intermediaries, including vitispiranes (Winterhalter, 1991) and Riesling acetal (Daniel et al., 2009).

Sensorily, the case of TDN is double-edged. This compound was firstly detected in old Riesling wines as responsible for the typical kerosene notes of some aged wines of this variety (Simpson and Miller, 1983). With a detection threshold of 2 μg/L, it can be responsible for the perception of spicy notes, but it may also impart unpleasant notes at levels above 60-80 μg/L (Ziegler et al., 2019). It is a rather ubiquitous compound, which is present in many other varieties, such as Garnacha (Oliveira and Ferreira, 2019) or Tempranillo (Alegre et al., 2020a), and which can cause aging off-odors in wines made with highly insolated grapes.

DMS - It can be formed during fermentation by enzymatic reduction of dimethyl sulfoxide, but it is mainly formed during aging by the slow hydrolysis of the specific precursor, S-methylmethionine (Loscos et al., 2008). Under limited yeast-assimilable nitrogen, yeast can metabolize nearly completely this precursor. Anyway, DMS increases during anoxic aging in nearly all wines (Franco-Luesma and Ferreira, 2016), so that it does not come with surprise that it has been found an essential part of the aging bouquet of Bordeaux wines (Picard et al., 2015).

Individually described with truffle, olive and asparagus notes, its impact on wine aroma is very dependent on the matrix composition. It can play the role of aroma enhancer on the black fruits notes of wines, but can also be detrimental to wine quality by increasing the negative maturation bouquet (Ferreira et al., 2021a).

Oxidation and degradation terpene derivatives - Linalool furan and pyran oxides and α-terpineol and will follow a similar trend since they are derived from the acid catalyzed rearrangements of the labile linalool, geraniol and nerol (Ribéreau-Gayon et al., 1975). These compounds are less relevant from a sensory point of view with olfaction thresholds superior to several hundreds of μg/L. However, they could also participate to the perception of flowery notes in wines (Versini et al., 1994).

Dihydromyrcenol could also be included into this category, although its accumulation pattern is unknown. This oxygenated monoterpene was detected in wines (Petronilho et al., 2021) and grapes (Alegre et al., 2020b), suggesting in the latter study its possible implication into the perception of citric, fruity notes.

Limonene derivatives - They could also be included in this category although their accumulation pattern is unknown. Firstly identified in Bordeaux aged wines (Picard et al., 2016; Pons et al., 2016) piperitone (p-menth-1-en-3-one) and other p-menthane lactones (mintlactone, isomintlactone and menthofuranlactone) are very potent odorants formed from limonene. They have been found at several hundreds of ng/L and are individually described as minty. They all contribute to the perception of freshness in aged wines (Picard et al., 2017). Limonene is part of the monoterpene biosynthesis in grapes and is formed by enzymatic dehydration of α-terpineol (Marais and J. Marais, 1983). While detected in grapes at around 10 to 50 μg/L (Nasi et al., 2008), its concentration in wines drops below the μg/L (Lisanti et al., 2021), far below threshold (34 μg/L in water pH 3.8 (Averbeck and Schieberle, 2009)). The influence of the yeast on its liberation is mostly unknown.

The 1,8-cineole could also be included in this category, although its origin is most likely exogenous such as *Eucalyptus* sp. trees near the vineyard (Poitou et al., 2017). However, a formation pathway from α-terpineol or limonene has been proposed (Fariña et al., 2005). It has been detected in wines not exposed to eucalyptus trees at concentrations inferior to 3 μg/L; described as minty, its contribution to the perception of green notes has been demonstrated and particularly via an additive

effect with 3-isobutyl-2-methoxypyrazine (Poitou et al., 2017).

Aged-related thiols FFT and BM - FFT has multiple origins: on the one hand, it is formed in wooden barrels by direct chemical reaction between furfural and the H_2S formed during fermentation (Blanchard et al., 2001), so that maxima levels will be observed when the fermentation is carried out directly within a new toasted barrel rich in furfural. On the other hand, a fermentative origin has been suggested in Baijiu. In this case, the formation would take place by the enzymatic cleavage of the conjugate cysteine-furfural, which would form spontaneously (Zha et al., 2017). The genes encoding for a carbon-sulfur lyase have been identified, yielding FFT from the cleavage of the cysteine-furfural conjugate (Zha et al., 2018). While H_2S is a natural by-product of alcoholic fermentation (Swiegers and Pretorius, 2007), furfural mainly comes from toasted wood. However, it has also been found in free and bonded form to cysteine in beer (Bustillo Trueba et al., 2021) and in little amounts in Merlot grapes and wine (Ferreira et al., 2018).

FFT was firstly identified in aged Champagne (Tominaga et al., 2003a,b) together with BM, whose origin and accumulation pattern are mostly unknown. FFT and BM amounts are present in wine at levels below 0.4 μg/L (Mateo-Vivaracho et al., 2010). Both are powerful aroma compounds with olfaction thresholds below the ng/L and described with burnt and coffee notes.

Fruity ethyl esters from branched acids and hydroxyacids - During wine aging, branched acids derived from Ehrlich pathway are slowly esterified. The corresponding esters are found in wines at levels above thresholds, around several tens of μg/L (Díaz-Maroto et al., 2005). In particular, ethyl isobutyrate, ethyl isovalerate and ethyl 2-methylbutyrate are involved into the fruity perception of aged red wines (San Juan et al., 2012).

Hydroxyacids also belong to the ester aroma vector and although their accumulation pattern is not fully known, they could be included into this category. In particular, ethyl leucate (ethyl 2-hydroxy-4-methylpentanoate) can be present

in wines at concentrations around several hundreds of μg/L and is responsible for the fruity notes of some aged wines (Campo et al., 2006; Falcao et al., 2012). Its formation pathway has not been fully elucidated, however it has been hypothesized that its direct precursor 2-hydroxy-4-methylpentanoic acid may arise from lipid metabolism degradation (Marullo et al., 2021), while some bacteria are able to yield this acid from leucine (Butel et al., 1995). It has been observed that the amount of this acid precursor follows a decreasing tendency during aging (Gracia-Moreno et al., 2015). While the effect of the yeast on the ethyl ester of branched acids has been demonstrated (Gammacurta et al., 2014), their impact on hydroxyacids is mostly unknown.

1.6.1.4 Complex evolutions

Varietal PFMs - The evolutions with aging of MH, MHA and MP are very complex because these molecules are quite reactive. On the one hand they are relatively strong nucleophiles (Nikolantonaki and Waterhouse, 2012; Nikolantonaki et al., 2012; Romanet et al., 2019) reactive towards the different wine electrophiles and, on the other hand, they are also very sensible towards oxidation (Hofmann et al., 1996). Furthermore, as previously discussed, yeast exerts a decisive influence on the levels of free forms of these compounds in wine and has also a powerful influence on the levels of their precursors remaining after fermentation.

The persistence in wine of these compounds, which is essential for the conservation of freshness and tropical fruits notes in aged wines (Piano et al., 2014; Picard et al., 2015), will then be assured as long as: (1) it contains low levels of highly electrophilic species to which they could react, (2) wine does not oxidize, and (3) the levels of precursors still available after fermentation remain high. Their persistence during wine aging can be improved with the addition of antioxidants such as phenolic acids (Lambropoulos and Roussis, 2007), glutathione (Piano et al., 2014), SO_2 or ascorbic acid (Nikolantonaki et al., 2014).

Vinylphenols - In opposition to the latter case, vinylphenols are electrophiles, and are reactive toward wine nucleophiles, such as anthocyanins, with which they form stable pigments, as reviewed elsewhere (De Freitas and Mateus, 2011). The effect of the yeast on these molecules has been the subject of intensive research as a way to modulate wine color (Božič et al., 2020; Morata et al., 2019), and its evolution during aging. Their transformation by *Dekkera/Brettanomyces* spoilage yeast into the odorous ethylphenols is also of microbiological interest, since represents a persistent problem (Benito et al., 2009).

The evolution during aging of vinylphenols will therefore depend on many factors, such as the amount formed by the yeast during fermentation, the amount of glycosylated precursors remaining after fermentation, and the content in nucleophilic species of the wine. High levels of vinylphenols may be detrimental because they will react with PFMs and because, if present in excess, their contribution to wine aroma is objectionable.

1.6.2 Evolution during aging of aroma compounds involved in off-odors and premature aging

Some lipid derivatives (massoia lactone, 3-methyl-2,4-nonadione; γ-nona and -decalactones), together with the amino acid derivatives, sotolon and, particularly, Strecker aldehydes (phenylacetaldehyde and methional) can be responsible for premature aging characteristics if present in high amounts (Mislata et al., 2020). On the other hand, H_2S and other small mercaptans can be involved in reductive off-odors.

1.6.2.1 Premature aging

Massoia lactone - Massoia lactone (5,6-dihydro-6-pentyl-2H-pyran-2-one) is likely formed by internal esterification of the δ-hydroxyacid precursor, which explains its presence in hydrolysates from fractions containing precursors (Alegre et al., 2020b). Present in wine at around several μg/L (Qian et al., 2020), it is responsible

for the overripe notes of dried and cooked fruits (Allamy et al., 2018). Levels of massoia lactone decrease in fermentation since it is reduced by the yeast (Pons et al., 2017).

Other ketones: 3-methyl-2,4-nonadione and (Z)-1,5-octadien-3-one - The β-diketone 3-methyl-2,4-nonadione was detected by GC-O in Spanish and French aged wines (Ferreira et al., 2009; Pons et al., 2008). Some furanoid fatty acids have been identified as precursors of this molecule in soy bean oil (Guth and Grosch, 1991). A recent study determined that yeast reductase can transform 2-methyl-2,4-nonadione into 2-hydroxy-3-methylnonan-4-one during alcoholic fermentation, and the reverse reaction occur spontaneously during wine aging, yielding 3-methyl-2,4-nonadione (Peterson et al., 2020). This molecule has a very low OT of just 16 ng/L, and in isolation it was described with anise and hay notes. Its aromatic contribution to wine aroma, however, seems to be very dependent on its concentration. When diluted at around the μg/L, it could contribute to the prune aroma nuances of prematurely aged wines, and particularly in presence of β-damascenone and γ-nonalactone (Dubourdieu et al., 2013; Pons et al., 2008). Interestingly, 3-methyl-2,4-nonadione has been found to be the best agonist for the human olfactory receptor OR1A1, generating the most important signal among 190 key food odorants tested. And, most surprisingly, the other 400 olfactory receptors tested did not respond to this volatile. Such specificity could denote a potential biological relevance for this particular odorant (Geithe et al., 2017). OR1A1 also responded to γ-nonalactone, confirming its potential role in the perception of premature aging notes.

(Z)-1,5-octadien-3-one has been identified in red musts and it could also be involved into the perception of prune notes in wines (Allamy et al., 2017). Its origin in grapes and wine is unknown, although it has been hypothesized that α-linolenic acid could be its precursor. This powerful aroma compound has a threshold of 90 ng/L and is described in isolation with geranium notes. At sub-threshold levels it may confer to the wine prune and dried fig notes (Allamy et al., 2017) and at

supra-threshold levels it imparts geranium and green notes and may suppress the fresh character of wine (Alegre et al., 2020b; Arias-Pérez et al., 2021).

Sotolon - With its typical curry note, sotolon (3-hydroxy-4,5-dimethyl-2(5H)-furanone) is characteristic of oxidized-style and botrytized wines. It has been demonstrated that sotolon was mainly formed during aging, from the aldol condensation of 2-ketobutyric acid with acetaldehyde (Pons et al., 2010). Interestingly, this reaction only yielded sotolon if the amount of acetaldehyde was superior to 0.5 mg/L. 2-Ketobutyric acid can originate from threonine degradation by *S. cerevisiae* yeasts via Ehrlich pathway, or from the oxidative degradation of ascorbic acid, present in small amounts in grapes and usually added as antioxidant in wine after bottling. In the same study, the capacity of *S. cerevisiae* to modulate 2-ketobutyric acid during alcoholic fermentation was demonstrated; however, its impact on the formation of sotolon during wine aging have not been studied.

Strecker aldehydes - Strecker aldehydes have a most dominant effect on wine characteristics. On the one hand, all of them, when present at little amounts (tens of μg/L), introduce typical oxidation characteristics, leading to a clear quality loss (Marrufo-Curtido et al., 2021). On the other hand, phenylacetaldehyde can suppress fruity aroma in red wines (San-Juan et al., 2011), may participate in floral notes at low levels, and at medium to high levels produces typical oxidation-related honey notes.

Strecker aldehydes can be in free or bonded forms, since they form α-hydroxyalkylsulfonic acids, which are stable and reversible adducts between an aldehyde and sulfur dioxide (Baert et al., 2012; De Azevedo et al., 2007). They can also form Schiff bases by weaker associations with the amine groups of amino acids and proteins (Baert et al., 2012) or even with the sulfhydryl (-SH) group of cysteine (Baert et al., 2015). Only free forms are odor-active, and bonded forms act like a reservoir that can be liberated later on.

These compounds are key intermediates in the Ehrlich pathway, although it is generally thought that they are completely reduced or oxidized, so that the remaining amounts of these compounds after fermentation are negligible. However, significant amounts of residual aldehydes have been found in beer (Perpète and Collin, 2000; Saison et al., 2010) and in synthetic wine (de Oliveira, 2019), proving that in some conditions, yeasts are unable to reduce them to the alcohol or to oxidized them into the corresponding acid. It has been suggested that the presence of SO_2, prevents their transformation by a quenching effect (Perpète and Collin, 2000).

During wine aging, Strecker aldehydes can can be formed through the reaction between the amino acid precursor and different α-dicarbonyls via Strecker degradation (Rizzi, 2006, 2008). Since both reactants, amino acids and α-dicarbonyls such as diacetyl or glyoxal, are normal by-products of all fermentations, their formation during normal wine aging cannot be discarded, even in non-oxidative conditions. The most relevant wine α-dicarbonyls formed during fermentation are diacetyl, glyoxal and methylglyoxal. In all cases, the modulation by the yeast has been demonstrated, which suggests a possible indirect sensory impact by participating to the Strecker degradation.

Diacetyl is described as buttery, yeasty, nutty and toasty. While desirable at low concentrations, it is considered as an off-flavor at levels above 5 mg/L. However, its contribution to wine aroma seems very dependent on the type of wine (Bartowsky et al., 2002; Martineau et al., 1995). It arises from α-acetolactate, formed by yeast from pyruvate via the action of the enzyme aceto-hydroxy acid synthase, is excreted from the cell and spontaneously decarboxylated into diacetyl (Pronk et al., 1996). Diacetyl can then be reduced by *S. cerevisiae* to acetoin and 2,3-butanediol via acetoin and butanediol dehydrogenases respectively (Bartowsky and Henschke, 2004). It is thought that the majority of diacetyl is metabolized by reduction to acetoin and 2,3-butanediol in order to decrease its toxicity (Martineau et al., 1995). However, *S. cerevisiae* yeasts can release high quantities of α-acetolactate,

considered as a potential source of diacetyl during aging in presence of oxygen (Ochando et al., 2018).

Glyoxal is an intermediary between pyruvate and lactic acid in presence of glutathione (Cooper, 1984). The activity of lactate dehydrogenase responsible for this conversion has been found to be limited in *S. cerevisiae* (Dequin and Barre, 1994).

1.6.2.2 Reduction off-odors

Hydrogen sulfide (H_2S), methanethiol (MeSH) and ethanethiol (EtSH) are powerful aroma compounds with olfaction threshold around the μg/L. They are responsible for the reductive aroma in wine, characterized by rotten eggs and cooked cabbage notes. While the production pathway of H_2S have been the subject of intensive research, less information is available regarding the other two. As reviewed in (Swiegers and Pretorius, 2007) H_2S is produced from sulfate via the sulfate assimilatory reduction pathway, leading to the synthesis of sulfur amino acids cysteine and methionine. It can then combine chemically or enzymatically with ethanol or acetaldehyde to form EtSH. MeSH arises from methionine degradation and its production would involve a S-lyase enzymatic activity and/or a non-enzymatic process. Their modulation by several fermenting strains have recently been investigated (Jimenez-Lorenzo et al., 2021), including H_2S liberation during fermentation (De Guidi et al., 2021). The overproduction of hydrogen sulfide is one of the principal challenges of oenology, since it is the main cause of the so-called reductive problems or reductive off-odors. An in-dept discussion of this complex and quite specific topic is out of the scope of the present review.

1.7 Final remarks

As shown in the introduction, it is evident that the effects of yeast on wine aroma go far beyond the synthesis of fermentative aroma volatiles. Yeast is able to affect in different ways also to grape-derived precursors, and hence will affect not only the aroma of young wines, but also to the evolution with time of wine aroma, since many of these precursors play relevant roles on the development of wine aroma throughout aging. Evidences also show that some yeast by-products are reactive molecules which can have an ulterior role on wine aroma formation or degradation. While the direct effect of yeast on wine aroma has been the subject of many researchers, there is a clear lack of studies trying to assess the long-term effects of yeast on wine aroma and wine longevity.

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1.8 Hypothesis and objectives

The influence of yeasts on wine aroma manifests not only on the existence of specific aroma profiles of fermentative aroma compounds, but extends throughout the whole wine shelf life, affecting also aroma compounds derived from grape specific precursors, and potentially affecting wine aroma longevity by a number of direct and indirect processes. The main objective of this PhD thesis is to assess the impact of the yeast on wine aroma profile and on its evolution during wine aging. To reach this objective the following operational objectives have been addressed:

1. Evaluate the impact of 10 *S. cerevisiae* yeast strains on the fermentative and varietal aroma of Tempranillo wine during and after fermentation, and after a period of accelerated aging.

2. Evaluate the impact of 2 *S. cerevisiae* strains on the sensory characteristics of Tempranillo wines, to assess whether those strain-related sensory characteristics are consistently kept during aging, and to elucidate the chemical changes in aroma composition potentially responsible for those aroma sensory properties.

3. Assess the differences introduced by 3 *S. cerevisiae* yeast strains in the development of varietal aroma throughout aging and wine longevity, paying particular attention to Strecker aldehydes and PFMs.

4. Evaluate the impact of 4 *Saccharomyces* yeasts on the aroma precursors fraction during fermentation and its evolution during aging.

These objectives are complementary to some of those of the PhD Thesis of Dolores Pérez, specifically working on non-oenological yeasts, and together will try to expand the knowledge about the possible roles played by fermentation microorganism on wine aroma and wine shelf-life.

Chapter 2

Materials and Methods

In this part, the general analytical, sensory and statistical methods used in the Thesis are described. The experimental design and specific aspects of each experiment are described in the corresponding chapters.

2.1 Reagents and standards

2.1.1 Solvents

Ultrapure water was purified in a Milli-Q system from Millipore (USA). DCM, n-hexane, ethanol and methanol for gas chromatography were supplied by Fisher Scientific (Loughborough, UK). Methanol, acetonitrile and formic acid for liquid chromatography-mass spectrometry were purchased from Merck (Darmstadt, Germany) and Fisher Scientific (Loughborough, UK).

2.1.2 Reagents and standards

Reagents and standards were supplied by Panreac (Barcelona, Spain), Sigma-Aldrich (Steinheim, Germany), Fluka (Madrid, Spain), TCI (Tokyo, Japan), Scharlau (Barcelona, Spain), VWR Chemical (Llinars del Vallés, Spain), Eptes Sarl (Vevey, Switzerland), Merck (Darmstadt, Germany), ChemLab (Zedelgem, Belgium), Akras (Biedermannsdorf, Austria), Roowin (Riom, France), Synchem UG&Co

(Felsberg, Germany), Lancaster (Eastgate, UK), Oxford Chemicals (Hartlepool, UK), Polyscience (Hirschberg an der Bergstrasse, Germany), Firmenich (Genève, Switzerland), Chemservice (Worms, Germany), Alfa Aesar (Kandel, Germany).

2.1.3 Materials

PTFE and nylon filters (0,22 μm) were supplied by Branchia Labbox (Barcelona, Spain) and Micron Analitica S.A. (Madrid, Spain) respectively. Isolute ENV+ and LiChrolut EN resins were supplied by Biotage (Uppsala, Sweden) and Merck (Darmstadt, Germany). Bond Elut ENV and Sep Pak C18 SPE cartridges were supplied by Varian (Walnut Creek, USA) and Waters (Dublin, Ireland). AnaeroGen^{TM} oxygen scavengers were provided by ThermoFisher (Waltham, USA).

2.2 Vinification

2.2.1 Grapes PAF

Due to the multiplicity and complexity of aroma precursors in grapes, it is essential to use a natural extract from grapes (Ferreira and Lopez, 2019). In the recent years, several strategies have been considered to predict the aromatic potential of grapes. One of the most recent strategies has been developed in the laboratory (Alegre et al., 2020) and consists in the preparation of mistelles to solubilize in the ethanolic must grape polyphenols and aroma precursors, and in the further extraction of the polyphenolic and aroma precursors (PAF) by solid phase extraction. The extract can be reconstituted and subjected to accelerated aging in anoxic conditions to liberate the aroma from the precursors and evaluate winemaking grapes potential. In the present case, PAFs were used in the chapters 1 and 3, but they were further reconstituted with acidified water and sugar to produce the semi-synthetic must for fermentation.

2.2.1.1 Mistelles preparation

As detailed in Alegre et al. (2020), after destemming, ethanol at 15 % (w/w) and 0.05 g per kg of must of sodium metabisulfite (7681-57-4, Merck, purity 97 %) were added to the crushed grapes. The mixture was left in maceration at 8 ºC during 7 days for red grapes, and only 5 hours for white grapes. The mistelles were then pressed, left in decantation for one week at 5 ºC, filtered and stored at 5 ºC in the dark. The mistelles preparation from winemaking grapes was carried out at the ICVV (Logroño, Spain).

2.2.1.2 PAF extraction

PAF were extracted from grape mistelles. Mistelles were firstly centrifuged at 10 °C, 4500 rpm for 20 min, dealcoholized and passed through a 10 g Sep Pack-C18 SPE cartridge previously conditioned with 44 mL of methanol, 44 mL of Milli-Q water containing 2 % of ethanol. Resin was washed with 88 mL of Milli-Q water at pH 3.5 and dried under vacuum. The compounds retained were then eluted with 100 mL of ethanol. PAF was stored at -20 ºC, in the dark, in hermetically closed vials without headspace.

2.2.2 Synthetic must preparation

2.2.2.1 Basic nutrients

Synthetic must preparation was adapted from Hernandez-Orte et al. (2006). It contained sugars (105 g/L glucose, 105 g/L fructose), organic acids (4 g/L tartaric acid, 3 g/L malic acid, 0.3 g/L citric acid), salts (2 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 0.15 g/L $CaCl_2$), vitamins (0.3 g/L myo-inositol, 1 mg/L thiamine, 1 mg/L nicotinic acid, 1 mg/L pyridoxine, 1 mg/L pantothenic acid, 0.04 mg/L biotin, 1 mg/L p-aminobenzoic acid, 0.2 mg/L riboflavin, 0.2 mg/L folic acid), trace elements (4.7 mg/L $MnCl_2$, 2 mg/L $ZnCl_2$, 1 mg/L H_3BO_3, 0.54 mg/L $CuCl_2$, 1.29 mg/L KIO_3, 0.49 mg/L $Co(NO_3)_2$, 0.19 mg/L $NaMoO_4$), and anaerobic factors (0.05 % (v/v)

Tween 80, 15 mg/L ergosterol).

Nitrogen content was adjusted by mixing 220 mg/L of $(NH_4)_2HPO_4$ and a mixture of amino acids containing 44.4 mg/L GABA, 58.5 mg/L alanine, 14.3 tyrosine, 17.7 mg/L valine, 14.4 mg/L isoleucine, 13.4 mg/L leucine, 86.5 mg/L aspartate, 85.6 mg/L glutamate, 60.1 mg/L serine, 6.5 mg/L glycine, 137.4 mg/L histidine, 72.3 mg/L threonine, 673.1 mg/L arginine, 302.3 mg/L proline, 25.2 mg/L methionine, 7.5 mg/L phenylalanine, 13.7 mg/L lysine, 177.3 mg/L glutamine (Hernández-Orte et al., 2002). After pH adjustment to 3.5 with NaOH, synthetic must was sterilized by filtration (0.45 μm) inside a vertical laminar flow chamber (PV-100, Tesltar S.A., Barcelona, Spain).

2.2.2.2 Addition of specific aroma precursors

Two types of precursors were added to the must: synthetic precursors of polyfunctional mercaptans synthesized by Roowin (Riom, France, purity $\geq$ 95 %); and natural precursors extracted from grapes PAF. Glutathionylated and cysteinylated precursors of MH and MP were added from a Milli-Q water solution sterilized by filtration (0.45 μm) and stored at -20 ^{o}C. Final concentrations in must were 0.1 mg/L Cys-MH, 0.05 mg/L Cys-MP, 1 mg/L Glu-MH, 0.05 mg/L Glu-MP.

PAF was firstly dealcoholized by evaporation until dryness using a Rotavapor, resuspended in sterile distilled water and added to the sterile synthetic must at 10 % (v/v).

2.2.3 Fermentation monitoring and control

2.2.3.1 Microbiological aspects

The commercial *S. cerevisiae* active dry yeast were from Lallemand Bio S.L. (Barcelona, Spain), they were rehydrated in ten times their weight of sterile water at 37 ^{o}C during 30 min and added to the must at 30 g/hL. Once open, they were hermetically closed and kept at 4 ^{o}C.

The strains stored in glycerol were firstly pre-cultured into GPY broth overnight

(2 % glucose, 0.5 % peptone, 0.5 % yeast extract) at 25 ºC. After quick centrifugation, GPY broth was discarded, colonies were resuspended in sterile distilled water and inoculated into synthetic must at 10^6 cells/mL.

During fermentation, yeast growth was monitored using, either flow cytometry following the methodology described by Tilloy et al. (2014), or by plating the appropriate dilution of fermenting must on a solid medium adapted for yeast growth such as YPD (1 % yeast extract, 2 % peptone, 2 % glucose) and incubated at 25 ºC for 2 days (Rollero et al., 2018), or by the measurement of the optical density at 600 nm after the appropriate dilution. In the latter case, 10^6 cells/mL are equivalent to an absorbance of 0.1 (Su et al., 2019).

2.2.3.2 Fermentation monitoring and end of fermentation

Fermentations were monitored either by daily weighing, or by quantification of glucose and fructose (methods described below).

At the end of fermentation, i.e. when the weight loss was inferior to 0.1 g between two consecutive days, wines were centrifuged at 10 °C for 15 min and stored at 4 ºC up to their analysis or further accelerated aging.

2.2.3.3 Oenological parameters

Several oenological parameters were measured in the recently fermented wines using either global, or specific methods. Glucose, fructose, ethanol, glycerol and organic acids were measured by UHPLC as described in (Su et al., 2019). After centrifugation and dilution in distilled water (3 times), samples were filtered through 0.22 mm nylon membranes and injected into a UHPLC Ultimate 3000, (ThermoFisher) equipped with refraction index UV–visible detectors. The column was a HyperREZ XP Carbohydrate H+ 8 mm (ThermoFisher), the mobile phase was 1.5 mM H_2SO_4 with a flux of 0.6 mL/min. Concentrations were calculated by response factors calculated in the analysis of synthetic calibrated solutions. The specific methods are described below.

– *Ethanol:*

Pure 1-butanol (71-36-3, Sigma-Aldrich, purity 99.8 %) was used as IS. A 125 μL volume of wine was spiked with 4 μL of SI and brought to 10 mL volume with MilliQ. A 0.5 μL volume was manually injected in Split mode in a GC8000 Fisons (today ThermoFisher) gas chromatograph equipped with a split/splitless injector and a flame ionization detector (FID). The column was a DB-WAXetr (30 m x 0.53 mm i.d., 2 μm film thickness) from J&W Scientific (Folsom, USA). The oven temperature was kept at 70 ^{o}C during 3 min. Quantification was done by interpolating the SI-normalized peak area in the straight lines built by the repeated analysis of calibrated solutions.

– *Sugars:*

Reducing sugars were determined by oxidation with divalent copper. Glucose and fructose were analyzed either enzymatically using a Y15 Biosystems auto-analyzer (Barcelona, Spain), or with a commercial enzymatic kit for D-glucose/D-fructose (Cat. No. 10139106035, R-Biopharm AG) using a UV-vis spectrophotometer UV-1700 Pharma Spec from Shimadzu (Kyoto, Japan).

– *Acidity:*

pH was determined using a pH-meter MicropH 2000 from Crison (Barcelona, Spain), total acidity by titration with NaOH and volatile acidity using the Garcia-Tena method. Such method is based in a fractional distillation of wine and in the further acidimetry of the fractions collected.

– *Sulphur dioxide:*

Free and total SO_2 were analyzed following the procedure validated by Carrascon et al. (2017) with some modifications. Ethyl methyl sulfide (624-89-5, Sigma-Aldrich, purity 96 %) was used as IS. For both determinations, 4.5 mL of the sample were placed in a 10 mL-vial and spiked

with the IS in a free-O_2 chamber from Jacomex (Dagneux, France). For free SO_2, 0.5 mL of ortho-phosphoric acid (7664-38-2, Fluka, purity $\geq$ 85 % in water) was added just before the analysis, with a syringe through the septum. Samples were then incubated 15 min at 40 ºC and 400 μL of headspace were injected in split mode (split ratio 20:1). For total SO_2, 0.5 mL of ortho-phosphoric acid (85 %) was added with a syringe through the septum and the sample was pre-incubated at 100 ºC during 20 min in order to cleave the sulfur dioxide adducts. The sample was then incubated at 80 ºC for 15 min and 200 μL of headspace were injected in split mode (split ratio 100:1).

The chromatographic system used for free and total SO_2 analysis was described in Ontañón et al. (2019) and consists on an Agilent 7890B gas chromatograph with a selective detector SCD 8355 from Agilent Technologies (Santa Clara, USA). The capillary column was a SPB-1 SULFUR (30 m $\times$ 0.32 mm i.d., 4 μm film thickness) from Supelco (Bellefonte PA, USA) preceded by a (3 m $\times$ 0.32 mm i.d.) pre-column, of fused silica with a polar deactivation. The injection was made into a MMI injector equipped with an ultra-inert liner of 4 mm i.d. from Agilent. The auto-sampler was a Combi-PAL from CTC Analytics (Zwingen, Switzerland) with a static headspace unit. After the injection, the syringe was purged with nitrogen for 5 min. The oven temperature was set at 35 ºC for 3 min and then raised of 10 ºC/min up to 45 ºC, and of 50 ºC/min up to 140 ºC and hold for 1 min. Helium was the carrier gas at 2 mL/min. The chromatographic analysis lasts 7 min. Base and burner temperatures were 280 ºC and 800 ºC, respectively. Air was used as oxidizer for the detector at a 50 mL/min.

The area of the sulfur dioxide peak was normalized by that of the IS and converted into concentration value by means of a response factor obtained by the analysis of a spiked synthetic wine containing 5 g/L tartaric acid, 10 g/L glycerol, 1.5 %, (w/w) 1,2-propanediol, 12 % (v/v) ethanol and pH adjusted at 3.5 and known amounts of SO_2 (5 and 10 mg/L of free and total respectively).

2.2.4 Accelerated anoxic aging

An important limitation when studying wine evolution and longevity is the long time required for observing changes at room temperature. Because of that, accelerated aging strategies have been traditionally used. A high temperature exposure in the absence of oxygen was already proposed 60 years ago (Singleton, 1962). Here, the methodology recently developed by Vela et al. (2017) has been used. It consists in aging samples at 50 ºC in strict anoxia, including sample preparation. Five weeks of aging through this process is roughly equivalent to one year of bottle aging.

In the present work, wines were conditioned into a free-O_2 chamber. Samples were placed either into 18 mL-glass tubes, or in 720 mL-glass containers with metallic screw caps and bagged in two high density plastic bags containing oxygen scavengers AnaeroGen^{TM}. Samples were then incubated at 50 ºC or 75 ºC several weeks or hours, respectively. At the end of aging, samples were stored in anoxia at 4 ºC up to their analysis.

2.3 Untarget analysis of aroma precursors

2.3.1 Extraction

Aroma precursors were extracted as described by Alegre et al. (2020) with some minor modifications and rescaled for 10 mL of sample. These modifications were effectuated and optimized in collaboration with the PhD student Elayma Sánchez Acevedo, and Dr. Ignacio Ontañón Alonso, both from LAAE. After dealcoholization and addition of 2 mg/L of phenyl β-D-glucopyranoside (1464-44-4, Sigma-Aldrich, purity 95 %) as IS, the samples were passed through a 500 mg Sep Pak C18 cartridge previously conditioned with 2.2 mL of methanol and 2.2 mL of MilliQ water. After washing with 4.4 mL of acidic MilliQ water at pH 3.5, the resin was dried under vacuum and eluted with 5 mL of methanol (LC-MS grade). The extracts were then evaporated to dryness under nitrogen flow, resuspended into 250 μL of methanol (LC-MS grade) and filtered through 0.22 μm polytetrafluoroethylene

(PTFE) membranes.

2.3.2 Samples and QC

One quality control (QC) pool was prepared mixing 10 μL of each of the 30 extracts. At the beginning of the batch, one blank of methanol was injected followed by 5 QCs and the samples in randomized order. QC was re-injected every six injections and at the end of the batch (Arapitsas and Mattivi, 2018).

2.3.3 UHPLC-QTOF-MS analysis

The untargeted analysis was based on the method described by Flamini et al. (2014) with some modifications. It was carried out with the help of María Savirón Sánchez and Jesús Orduna Catalán from ICMA (University of Zaragoza, Spain). Seven μL of each extract were injected and analyzed by a UHPLC Elute coupled with an electrospray ionization (ESI), interface to TIMS-QTOF MS from Bruker Daltonics (Billerica, USA). The column was a C18 (100 x 2.1 mm, 1.7 μm) from Waters (Milford, USA), maintained at 35 ^{o}C. Solvent A was water and B was acetonitrile both with 0.1 % of formic acid (LC-MS grade). The total run time was 18.7 min, the flow was 0.3 mL/min. The gradient elution program was (in % of B): 0 min, 5 %; 8.70 min, 45 %; 11.30 min, 65 % and 13.40, 90 %.Samples were analyzed in both positive and negative modes, the capillary voltage was set to 4500 and 3000 V respectively. The drying gas flow was set to 8 L/min at 200 ^{o}C, the nebulizer was set to 4.0 bar. The scan range was set to m/z 50–1300 for the MS and MS/MS mode. In all cases, acquisition cycles began by high resolution MS, and re-fragmentation, with a collision energy of 30 eV, of the most intense ions to obtain MS/MS spectra. If necessary, injections in MS/MS were eventually repeated in a narrow isolation to ensure ion selectivity.

2.3.4 Specific statistic treatment

As described in Ontañón et al. (2020), raw files of the QCs were compared during the sequence using Bruker Compass DataAnalysis (v4.2) and data treatment was performed using MetaboScape (v7.0.1), both from Bruker Daltonics. Alignment was performed in default mode, peak picking was performed with a minima intensity threshold of 1000, from 0.3 to 15 min of the total 18 min of acquisition. A PCA was firstly built with all the samples, including QC in order to check for their proper clustering. QCs were then excluded and one-way ANOVA analysis was performed on the dataset. Significantly affected buckets (p-value < 0.05) were selected. For negative buckets, those presenting a formic acid adduct were filtered since it is the predominant ion formed for glycosides (Caffrey et al., 2020).

2.4 Wine aroma profiling

All the compounds analyzed and standards used are presented into Tables A.1-A.4 in Annex A.

2.4.1 Major aroma compounds

Major compounds analysis - including some carbonyl compounds, higher alcohols and their acetates, volatile fatty acids and their ethyl esters - was performed as described in Ortega et al. (2001). The details related to the standards used, the analytes retention time and their detection limits are resumed in the Table A.1 in Annex A.

Three mL of the sample previously spiked with the IS solution (2-octanol, 4-methyl-2-pentanol, ethyl heptanoate and heptanoic acid, 30 mg/L of each) were diluted into 7 mL of Milli-Q water, added with 4.1 g of ammonium sulfate (7783-20-2, Sigma-Aldrich, purity ≥ 99 %), and extracted with 0.25 mL of DCM after 90 min of horizontal agitation. After centrifugation at 2500 rpm for 10 min, the organic phase was recovered with a syringe.

The gas-chromatograph was a Varian CP-3800 (Palo Alto, USA). The column was a ZB-WAX (30 m x 0.32 mm i.d. with 0.5 mm film thickness) from Phenomenex (Torrance, USA) preceded by an uncoated pre-column (2 m x 0.53 mm i.d.). The oven temperature program was initially set at 40 ºC for 5 min, then raised at 3 ºC/min to 200 ºC. Carrier gas was hydrogen at a 3 ml/min flow. The injection of 3 μL of sample was performed in split mode with a 30 ml/min split flow. Detection was made by FID. The areas of the peaks of the analytes were normalized by those of the corresponding IS and converted into concentration by means of a response factor obtained by the analysis of a calibrated synthetic wine prepared with tartaric acid (4 g/L), ethanol (13 %, v/v), glycerin (10 g/L), quinine (7 g/L), arabic gum (70 g/L), tannic acid (100 g/L) and pH adjusted at 3.5.

2.4.2 Trace aroma compounds

Trace compounds include minor branched chain esters, terpenes, norisoprenoids, vanillin derivatives, cinnamates, lactones and volatile phenols. The details related to the standards used, analytes retention time, *m/z* ratios and their detection limits are summarized in the Table A in Annex A.

The SPE was carried out as described by López et al. (2002): 15 mL of sample spiked with IS (2-octanol, 3-octanone and 3,4-dimethylphenol, 250 μg/L each) were loaded into a 65 mg LiChrolut EN (or a 70 mg Isolute ENV+) SPE cartridge previously conditioned with 2 mL of DCM, 2 mL of methanol and 2 mL of hydro-alcoholic solution (12 %, v/v). The resin was then washed with an aqueous solution of methanol (30 %, v/v) containing 1 % (w/v) of sodium bicarbonate (w/v), dried under vacuum and retained analytes were finally eluted with 0.6 mL (or 0.8 mL in the case of Isolute ENV+) of DCM containing 5 % (v/v) of methanol.

The GC-MS analysis was performed as described in Oliveira and Ferreira (2019) with some modifications. The chromatographic system was a QP2010 gas chromatograph equipped with a quadrupole mass spectrometer detector from Shimadzu. The column was a DB-WAXetr (30 m x 0.25 mm i.d., 0.5 μm film

thickness) from Agilent, preceded by a medium-polar uncoated pre-column (3 m x 0.25 mm i.d.). The carrier gas was helium at 1.26 mL/min. The temperature program of the chromatographic oven initially started at 40 ºC during 5 min, raised at 1 ºC/min to 65 ºC then at 2 ºC/min to 220 ºC and hold for 50 min. A split/splitless SPL injector was used at a temperature of 250 ºC. The injection of 2 μL of sample was carried out in splitless mode using a pressure pulse to ensure a column flow of 4.50 mL/min during 1.5 min. The ion source and interface were kept at 220 ºC and 230 ºC respectively. The mass analyzer was set in SIM mode and the *m/z* ratios list is available in the table in the Table A in Annex A.

The area of the analytes peaks was normalized by the area of the IS and was converted into concentration value by means of a response factor which was obtained by the analysis of a calibrated synthetic wine prepared with tartaric acid (4 g/L), ethanol (13 %, v/v), glycerin (10 g/L), quinine (7 g/L), arabic gum (70 g/L), tannic acid (100 g/L) and pH adjusted at 3.5.

2.4.3 Polyfunctional mercaptans

Five polyfunctional mercaptans were analyzed: MH, MP, MHA, FFT and BM using two different methodologies. The details related to the standards used, the analytes retention time, *m/z* ratios and their detection limits are summarized in the Table A.3 in Annex A.

2.4.3.1 Quantification by GC-GC-MS

This analysis is based on that described in Mateo-Vivaracho et al. (2010) with some modifications. These modifications were effectuated and optimized by the PhD student Oscar Castejón Musulén, and Dr. Ignacio Ontañón Alonso, from LAAE.

Ethylenediaminetetraacetic acid disodium salt 2-hydrate (EDTA, 6381-92-6, purity 99 %), L-cysteine hydrochloride hydrate (345909-32-2, purity 99 %) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 6674-22-2, purity 98 %) were from Sigma-Aldrich. 2,3,4,5,6-Pentafluorobenzyl bromide (PFBBr, 1765-40-8,

purity $\geq$ 98 %) and o-methylhydroxylamine hydrochloride (593-56-6, purity 98 %) were from Fluka.

Fifteen mL of samples were firstly added with EDTA (5 g/L) and L-cysteine chlorhydrate (0.1 M). The deuterated analytes, used as IS (MH-d_5 at 700 ppt in wine, MHA-d_5 at 200 ppt, MP-d_{10} at 100 ppt, FFT-d_2 at 70 ppt, BM-d_5 at 40 ppt) were spiked and pure O-methylhydroxylamine was added to form the oximes of MP. This oximation was performed at 55 ^{o}C during 45 min. Six mL of the sample were then loaded into a 50 mg BondElut-ENV SPE cartridge previously conditioned with 1 mL of DCM, 1 mL of methanol and 1 mL of hydro-alcoholic solution at 12 % (v/v). The cartridge was then washed with 4 mL of a 40 % (v/v) methanol/water solution in phosphate buffer (0.2 M) at pH 7.7 and, after this, with 1 mL of Milli-Q water. Analytes retained in the resin were derivatized by adding 1 mL of a DBU (6.7 %, v/v) and 50 μL of PFBBr (2 g/L in hexane) solutions, and letting the imbibed cartridge for 20 min at room temperature. The excess of reagent was removed by the addition of 100 μL of thioglycerol (2 g/L) in DBU at 6.7 % (v/v), and allowed to react for 20 min at room temperature. The resin was rinsed with 4 mL of a 40 % (v/v) of a methanol/water solution with phosphoric acid (0.2 M) and with 1 mL of Milli-Q water and dried under vacuum. Derivatized analytes were eluted with 1.6 mL of methanol. Finally, 200 μL of the extract were diluted in 1.8 mL of Milli-Q water and extracted by SBSE using previously conditioned PDMS Twister® from Gerstel (Müllheim an der Ruhr, Germany). Stirbars were then dried under nitrogen flow at 50 ^{o}C.

The stirbar was desorbed using thermal desorption unit (TDU) and a cryo-cooled injection system (CIS 4) with a programmable temperature vaporization (PTV) inlet equipped with a MPS auto-sampler both from Gerstel. The TDU temperature was programmed started at 30 °C during 0.2 min, temperature raised at 120 °C/min to 300 °C and held for 5 min. The transfer line of the TDU was kept at 250 °C. The initial temperature of the CIS was set at -80 °C using liquid nitrogen. The CIS was then heated to 250 °C at a rate of 12 °C/s and held for 30 min to inject the trapped

compound into the capillary columns in solvent vent mode.

The chromatographic system was an Agilent 7890A gas chromatograph equipped with a Deans switch device from Agilent allowing the selective transfer of heart cuts from the first column to the second. The first column was a DB-5ms (15 m x 0.25 mm i.d., 0.25 μm film thickness) from J&W Scientific connected to a FID and the Deans switch. An uncoated, deactivated column (6.7 m x 0.18 mm i.d.) from Agilent was used as a restrictor between the FID detector and the Deans switch. The second column was a DB-WAXetr (30 m x 0.25 mm i.d., 0.5 μm film thickness) from Agilent, connected to an Agilent 5975C MS. The carrier gas was helium at 36 psi in the first column and 31 psi in the second one. The temperature program of the chromatographic oven initially started at 40 ºC during 4 min, temperature raised at 25 ºC/min to 180 ºC then at 10 ºC/min to 220 ºC then at 4 ºC to 250 ºC and hold for 20 min. The FID was kept at 280 °C and operated with 40 mL/min hydrogen and 450 mL/min air. The pressure in the MS was kept constantly at 31 psi. A quadrupole mass detector was operated in SIM mode with electron ionization. The temperature of the ion source was set at 230 °C and the transfer line was kept at 240 °C. The mass analyzer was set in SIM mode and the m/z ratios list is available in the Table A.3 in Annex A.

2.4.3.2 Quantification by UHPLC-MS/MS

This analysis is based on the method described by Vichi et al. (2015) with some modifications. These modifications were effectuated and optimized by the Dr. Alexis Marsol and Dr. Ignacio Ontañón Alonso, both from LAAE.

It consists in the extraction and derivatization of the polyfunctional mercaptans by selenium-containing reagent, 2-phenyl-1,2-benzisoselenazol-3(2H)-one (Ebselen, 60940-34-3, TCI, purity > 98 %) under anoxic conditions. After addition of the deuterated internal standards, 10 mL of samples are introduced into the anoxic chamber, Ebselen is added (0.1 mM in DCM) and the mix is 1-min vortex agitated at room temperature. Out of the anoxic chamber, the samples are centrifuged

(4500 rpm, 15 min) and 1 mL of the organic phase is recovered and dried under nitrogen flow. It is resuspended into 100 μL of methanol (LC-MS grade) and filtered through 0.22 μm PTFE membranes.

Seven μL of extracts were analyzed through LC-QqQ with an Intensity Solo C18-2 column (100 x 2.1 mm and 2 μm particle) from Bruker Daltonics, maintained at 40 ^{o}C. Solvent A was water and B was methanol, both with ammonium formate (10 mM). Flow was set at 0.5 mL/min, the analysis lasted 17 min and the gradient of B was: 0 min, 60 %; 2 min, 60 %; 10 min, 78 %; 10.1 min, 100 %; 15.1 min, 100 %; 15.2 min, 60 %; 17 min, 60 %. The parameters of the source were the following: Spray Voltage Positive: 4500.0 V; Spray Amp Positive: 100.0 μA; Cone Pressure: 25.0 PSI; Probe Pressure: 25.0 PSI; Nebulizer Pressure: 60.0 PSI; Spray Voltage Negative: 4000.0 V; Spray Amp Negative: 100.0 μA; Cone Temperature: 250.0 ^{o}C; Probe Temperature: 450.0 ^{o}C; Exhaust On: true. Two transitions were selected for each analyte in multiple reaction monitoring (MRM) in positive mode (Table A.3).

In all cases, the area of the analytes peaks was normalized by the area of the IS and was converted into concentration value by means of a response factor which was obtained by the analysis of a spiked mix of samples with a known quantity of analytes.

2.4.4 Strecker aldehydes

Strecker aldehydes were quantified following the methods described and validated in Culleré et al. (2004) and Ferreira et al. (2006) with some modifications. These modifications were effectuated and optimized by the PhD student Oscar Castejón Musulén, and Dr. Ignacio Ontañón Alonso, both from LAAE.

O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (86356-73-2, Fluka, purity 99 %) was used as derivatization reagent. The details related to the standards used, the analytes retention time, m/z ratios and their detection limits are resumed in the Table A.4 in Annex A.

For the analysis of total forms, samples were first introduced into the anoxic

chamber, where 12 mL aliquots were spiked with the IS solution (2-methylpentanal, 3-methylpentanal, deuterated methional and deuterated phenylacetaldehyde, 200 μg/L each) and sealed. The sealed vials were then taken out and incubated at 50 ^{o}C for 6 hours to ensure equilibration. After this, 360 μL of a 10 g/L PFBHA solution were added and the reaction allowed to develop at 35 ^{o}C for 12 hours. After this, 10 mL of sample were then percolated through 1 mL SPE cartridges packed with 30 mg of LiChrolut-EN resins. The cartridges were then washed with 10 mL of a solution containing 60 % (v/v) MeOH and 1 % (w/w) $NaHCO_3$, then dried and finally eluted with 1.2 mL of hexane.

For the analysis of free forms, 5 mL of the sample was passed through a 100 mg LiChrolut-EN SPE cartridge previously conditioned with 2 mL of hexane containing 10 % (v/v) of diethyl ether, 2 mL of methanol, 2 mL of hydro-alcoholic solution at 12 % (v/v). The cartridge was further rinsed with 1 mL of Milli-Q water, 5 mL of an aqueous solution containing 1 % (m/v) of sodium bicarbonate and 1 mL of Milli-Q water. Carbonyls retained in the cartridge were directly derivatized by passing 1 mL of an aqueous solution of PFBHA (5 g/L in Milli-Q water), and letting the cartridge imbibe the reagent for 15 min at room temperature. Excess of reagent was removed with 5 mL of a 0.05 M sulfuric acid solution and 1 mL of Milli-Q water. After drying under a flow of nitrogen, derivatized analytes were eluted with 1 mL of hexane containing 10 % (v/v) of diethyl ether. The extract was spiked with the IS (2,3,6-trichloroanisole, 30 ppm) and dried with sodium sulfate.

For both free and total aldehydes, the chromatographic system was a QP2010 gas chromatograph equipped with a quadrupole mass spectrometer detector from Shimadzu. The column was a DB-WAXetr (30 m x 0.25 mm i.d., 0.5 μm film thickness) from Agilent, preceded by a (2 m x 0.25 mm i.d.) medium-polar uncoated pre-column. The carrier gas was He at 1 mL/min. The chromatographic oven was held at 40 ^{o}C for 4 min, then raised to 250 ^{o}C at 10 ^{o}C/min, and held for 10 min. A SPL injector (split/splitless) was used at a temperature of 250 ^{o}C. The chromatographic analysis lasted 35 min. For both extracts, 3 μL were injected in

splitless mode, with a pulse pressure of 40 psi for 1.50 min. The temperatures of the ion source and the interface were set at 220 ºC and 230 ºC respectively. The mass analyzer was set in SIM and the complete list of m/z ratios are shown in in the Table A.4.

Concentrations were obtained by using response factors calculated by the analysis of table wines spiked with known amounts of analytes.

2.5 Sensory related methods

2.5.1 Gas Chromatography-Olfactometry

Sniffings were carried out in a Thermo 8000 series GC equipped with a FID and a ODO-1 sniffing port from SGE (Ringwood, Australia) connected by a flow splitter to the column exit. The column was a DB-WAX (30 m x 0.32 mm i.d., 0.5 μm film thickness) from J&W Scientific. Carrier gas was hydrogen at 3 mL/min. Injection volume was 1 μL in splitless mode, with a splitless time of 1 min. Injector and detector temperatures were both fixed at 250 ºC. The program of the chromatographic oven was initially set at 40 ºC for 5 min, then raised at 4 ºC/min up to 100 ºC, at 6 ºC/min up to 136 ºC, and at 3 ºC/min up to 220 ºC, and was held for 10 min. A panel constituted of 4 to 6 members, with extensive experience with GC-O, carried out the sniffings (Ferreira et al., 2003). Each judge evaluated the extract once in two time segments of 20 min to avoid fatigue; one session per day. The panelists were asked to measure the intensity of each odor using a 0-3 intensity scale, 0.5 of increment allowed: -0- not detected; -1- weak, hardly recognizable odor; -2- clear but no intense odor; -3- intense odor.

Data treatment was performed following the procedure of modified frequencies (MF) (Dravnieks, 1985): $MF(\%) = \sqrt{F(\%).I(\%)}$, where F is the detection frequency of an aromatic attribute in percentage and I is the average intensity of the maximum intensity in percentage.

The odorants were identified by comparison of their descriptors, chromatographic

retention index (RI) in DB-WAX and DB-5 columns and MS spectra with those of pure reference compounds.

2.5.2 Sensory analysis

2.5.2.1 Experimental conditions

The samples were encoded with random 3-digit numbers and presented in normalized (German Institute for Normalization, DIN) dark wine glasses from Sensus (Schott Zwiesel, Germany) covered with petri dishes and served at room temperature in individual booths. Samples were presented in a randomized order, different for each participant and exclusively evaluated orthonasally. Sensory evaluations were carried out in a ventilated and air-conditioned tasting room at around 20 °C under ambient light. Participants were not informed about the nature of the samples and were not paid for their participation. Sensory analyses were performed by judges with extensive experience in wine sensory analysis and belonging to LAAE (Zaragoza, Spain) and ICVV (Logroño, Spain).

2.5.2.2 Nonverbal characterization: sorting task

Participants were asked to smell the samples and group them according to their aroma similarities. Once the sorting was achieved, panelists were asked to describe the groups using 1 to 3 attributes. The instructions for the sorting task in Spanish are available in the Figure A.1 in Annex A.

Data obtained in the sorting task were summarized in a matrix (sample x sample) obtained by summing the number of times a pair of samples was sorted in the same group. This similarity matrix was submitted to MDS. Then, HCA was calculated on all the dimensions derived from MDS, considering Euclidean distances, Ward's method and automatic truncation. The terms generated for the group description were filtered, eliminating hedonic descriptors, and were submitted to lemmatization and categorization. These processes consist in an arrangement of the terms according to their root (lemmatization) and semantic similarities (categorization). It was

performed individually by 3 experienced researchers from LAAE. The final list of terms was obtained by triangulation of the 3 lists generated independently (Abric, 2005). Only descriptors with a citation frequency superior to 20 % were considered.

2.5.2.3 Descriptive analysis: flash profile

Flash profile (FP) consisted in 3 parts of 30 min each held the same day and separated by at least one hour. In the first part, participants were asked to smell the samples orthonasally and generate discriminant descriptors, without number restriction. Then, all the descriptors were gathered and grouped into categories by 3 experienced experimenters independently. Final list of descriptors was obtained by consensus. In the second part, panelists were trained with commercial aroma references prepared in ethanol 15 % (v/v) by LAAE. Panelists were asked to associate the references to the descriptors. Judges able to correctly associate 80 % of the references were qualified. In the third part, qualified panelists evaluated the samples together with one sample in duplicate to evaluate the repeatability of the panel. All the samples were presented simultaneously and panelists were asked to rank the samples for each attribute of the final list on a 10 cm graduated scale (1 cm intervals), from 0 (low intensity) to 10 (high intensity). The instructions for the flash profile in Spanish are available in the Figure A.2 in Annex A.

Generalized procrustes analysis (GPA) was performed on individual matrices (sample x attributes) built by entering the product ranking for each panelist. HCA was calculated on the coordinates obtained.

2.5.2.4 Olfaction threshold determination

OT was determined following the standard practice recommendations (ASTM, 2008), in 12 % (v/v) water/ethanol, 5 g/L tartaric acid, pH 3.5. Instructions in Spanish are in the Figure A.3 in Annex A.

2.6 Statistical analysis

2.6.1 Odor Activity Value

In order to facilitate data treatment, concentration values were, in some cases, transformed into OAV, calculated as the ratio of the concentration for a given compounds and its corresponding OT. When concentration was below detection limit (DL), it was replaced by the DL itself and divided by the corresponding OT. However, this method is limited since it is known that, even at concentration below threshold, an odor can be perceived as the result of perceptual synergism (Atanasova et al., 2005). In order to palliate this issue, compounds displaying similar aroma can be gathered into aroma vectors.

2.6.2 Statistics

Statistical analysis was performed using R software (v3.5.0, Boston, USA) via Jupyter Notebook (v6.3.0) programming environment hosted by Anaconda Navigator (v2.0.4). One, two and three-way analysis of variance (ANOVA) and Tukeys's honestly significance difference (HSD) test were performed with the functions *anova* from *car* package (v3.0.2) and *HSD.test* from *agricolae* package (v1.3.3) from *stat* package (v3.6.2). Principal Component Analysis (PCA) were performed and plotted using *factoextra* package (v1.0.5). Heatmap was built using *heatmap* function.

Statistical analyses related to sensory data treatment were performed using XLSTAT from Addinsoft (version 2020.1.3, New York, USA).

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Chapter 3

The effects of 10 *Saccharomyces cerevisiae* strains carrying alcoholic fermentation on the fermentative and varietal aroma profiles of young and aged Tempranillo wines

3.1 Introduction

Wine aroma is its most outstanding sensory property and is essential for its quality and differentiation (Charters and Pettigrew, 2007). Although the number of volatile molecules that can be a part of the volatile fraction of wines is very large, exceeding most likely several thousands, it has been suggested that 70 different odor chemicals are those playing major roles on the aromatic properties of wines (Ferreira et al., 2021).

Quantitatively, the most abundant wine odorants are alcoholic fermentation by-products, particularly higher alcohols, ethyl esters and acetates, some carbonyls and acids. By contrast, many other relevant odorants derive from grape specific precursors and can be present at very limited concentrations, within the ng/L range in the case of polyfunctional mercaptans, few hundreds of ng/L in the case of β-ionone, around the few μg/L in the case of β-damascenone or below 0.2 mg/L in the cases of terpenols, volatile phenols and vanillin derivatives (Ferreira and Lopez, 2019; Ruiz et al., 2019).

Some of these compounds are directly formed or liberated during alcoholic fermentation. This is the case of most fermentation by-products derived from yeast amino acid and fatty acid metabolisms. Significant amounts of these compounds are formed from the early stages of alcoholic fermentation, so that these compounds made up the aroma fraction lost by evaporation during fermentation (Gómez-Plaza et al., 1993; Guerrini et al., 2016). Some other compounds, such as the monoterpenoids geraniol and linalool, are directly liberated from grape-specific glycosylated precursors during fermentation by the action of yeast β-glucosidases (Gunata et al., 1988).

On the contrary, the formation and accumulation of some other volatiles requires aging time. In fact, a quantitatively relevant group of aroma compounds and aroma precursors are subjected to several slow chemical reactions such as acid hydrolysis, esterification or intra-molecular rearrangements that will greatly affect wine aroma profile. This is the case of most norisoprenoids and vanillin derivatives, such as

β-damascenone and TDN or vanillin and acetovanillone, whose levels increase during aging as the consequence of different transformations of grape carotenoid metabolites (Winterhalter and Gök, 2013) or of grape glycosylated precursors (Ferreira and Lopez, 2019). Notably, it has been recently observed that the yeast genera plays a major role in the modulation of TDN concentration in wine after some time of aging (Oliveira and Ferreira, 2019). A third group of odorants suffering changes during aging are labile molecules, such as linalool or geraniol, which degrade during aging to form α-terpineol, nerol or 1,8-cineole (Waterhouse et al., 2016). Other groups of odorants deeply affected by aging are fruity esters and acetates. The acetates of higher alcohols are quickly hydrolyzed and their levels soon fade away. On the contrary, the ethyl esters of branched acids - isobutyric, 2-methylbutyric and isovaleric acids - and of other minor acids, slowly and steadily increase by esterification of their precursor acids with ethanol (Díaz-Maroto et al., 2005). The existence of all these processes makes that aging time should then be considered as an important factor to assess the role of yeasts on wine aroma modulation.

Saccharomyces cerevisiae is the micro-organism most widely studied regarding its sensory impact on wine (Tempère et al., 2018). However, most previous studies have dealt with the short-term impact of this yeast on both fermentative and varietal aroma profiles (Gamero et al., 2011; Gammacurta et al., 2017; Molina et al., 2009), neglecting aging effects. This aspect will be specially addressed in the present work whose objective is to evaluate the impact of 10 *S. cerevisiae* yeast strains on the fermentative and varietal aroma of Tempranillo wine during and after fermentation, and after a period of accelerated aging.

3.2 Materials and methods

3.2.1 Wine elaboration

All the methods and compositions are detailed in the Chapter 2, Materials and Methods.

3.2.1.1 Synthetic must preparation

A semi-synthetic must was prepared and added with a phenolic and aroma precursor fraction (PAF) extracted from Tempranillo grapes. Synthetic glutathionylated and cysteinilated precursors of MH and MP were also added.

3.2.1.2 Yeast strains

Ten *S. cerevisiae* strains (Lallemand Bio SL, Madrid, Spain) conditioned as active dry yeasts were used: Lalvin ICV D254™ (D254), Lalvin Clos™ (CLOS), Uvaferm HPS™ (HPS), Enoferm BDX™ (BDX), Lalvin Rhône 2056® (RHONE), Lalvin ICV D80™ (D80), Lalvin 71B™ (71B), Lalvin Persy™ (PERSY), Lalvin ICV OKAY™ (OKAY), IONYS wf™ (IONYS). They were rehydrated and 10^6 living cells/mL were inoculated in each fermenter. Cell viability and vitality were monitored by flow cytometry.

3.2.1.3 Fermentation monitoring

Fermentations were carried out at 25 ºC, under agitation at 150 rpm using a magnetic stirrer. Carbon dioxide release was monitored by weighing. The main chemical fermentative parameters were analysed at the beginning and at the end of fermentation, including sugars, acids and alcohols by UHPLC.

3.2.1.4 Fermentation system

Fermentations were carried out in triplicates, in 100 mL-glass fermenters containing 50 mL of synthetic must so that headspace represented 50 % of the total volume. Fermenters were tightly closed with a perforated silicone cork in which an airlock (Micromalta S.L., Madrid, Spain) was inserted. Needles and 5 mL-syringes were inserted into the silicon cork to allow sampling without opening the fermenter.

3.2.1.5 End of fermentation and accelerated aging

At the end of fermentation, samples were centrifuged and conditioned for accelerated anoxic aging into 18 mL-glass tubes and incubated at 50 ºC for 5 weeks.

3.2.1.6 Experimental design

Fermentations were performed in triplicates and were repeated with CLOS and IONYS in must without PAF addition, whose volume was replaced by sterile distilled water. Unfermented controls of synthetic must with and without PAF were also included in duplicates. The experimental procedure is summarized in the Figure 3.1.

3.2.2 Analysis of young and aged wines

Major metabolites of alcoholic fermentation (higher alcohols and their acetates, volatile fatty acids and their ethyl esters, branched fatty acids and their ethyl esters, acetoin, diacetyl, and acetaldehyde), usually present in wines at levels above 0.2 mg/L, were analysed by the GC-FID.

Minor aroma compounds present in wine at levels around 0.1-200 μg/L (branched ethyl esters, terpenes, norisoprenoids, vanillin derivatives, volatile phenols) were analysed by GC-MS.

3.2.3 Analysis of the aroma evaporated during fermentation

A pre-purified standard SPE cartridge filled with 160 mg of LiChrolut EN resin was lodged into the airlock of the fermenters to trap volatiles emitted during the fermentation. When this finished, the cartridge was removed, dried under vacuum and eluted with 1.6 mL of dichloromethane containing 5 % (v/v) of methanol.

For GC-O, a single extract was prepared by mixing 100 μL of each one of the 40 extracts obtained from the 40 different fermentations. The extract was carefully concentrated by evaporation of the solvent under nitrogen flow until a 0.2 mL final volume and injected into the GC-O system.

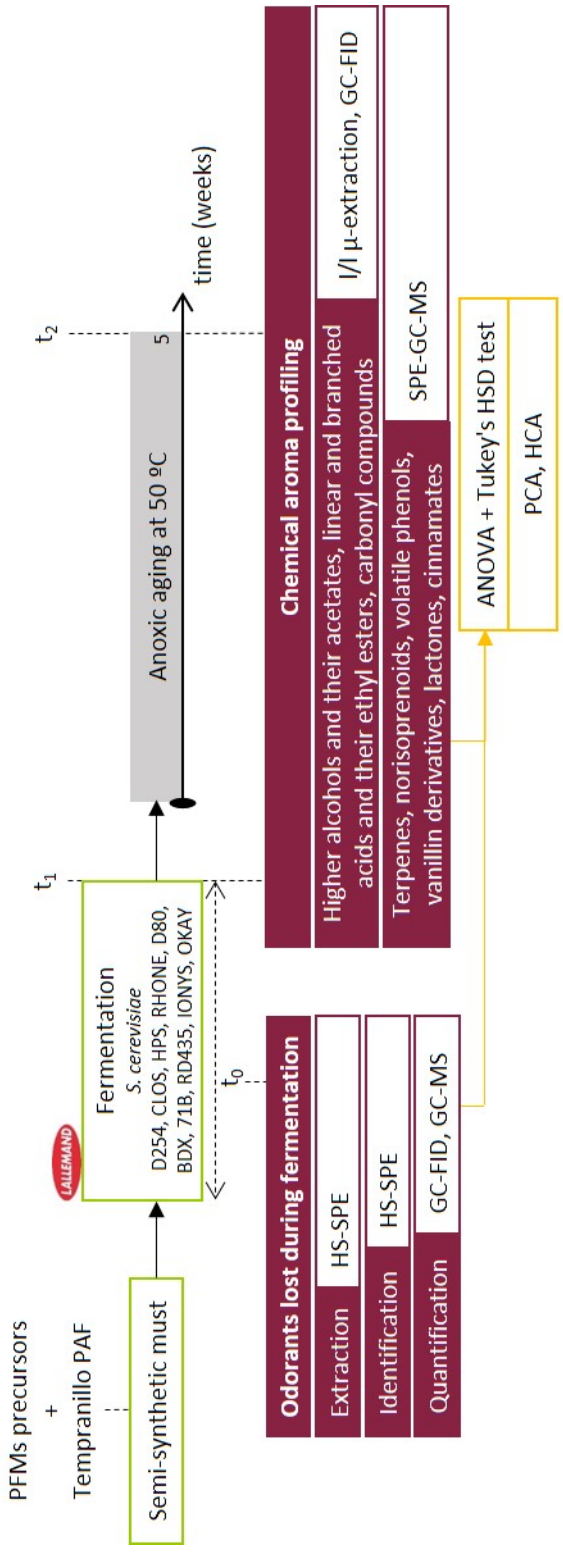


Figure 3.1: Experimental procedure - A semi-synthetic must of Tempranillo was fermented with 10 *S. cerevisiae* yeast strains and wines were submitted to accelerated aging at 50 ºC during 5 weeks. The aroma profile of the wines were analysed.

Table 3.1: List of the additional compounds quantified by GC-MS in this study. Quantification m/z ratio are provided, the first one was used for quantification.

compound	CAS	m/z
isobutyraldehyde	78-84-2	57, 55, 71
2-methylbutanal	96-17-3	57, 58
3-methylbutanal	590-86-3	58, 57
propyl acetate	109-60-4	61, 73
isopropyl acetate	108-21-4	61, 59
isobutyl acetate	110-19-0	73, 86
ethyl isobutyrate	97-62-1	71, 116
2-methylbutyric acid	116-53-0	74, 57
3-methylbutyric acid	503-74-2	60, 87

For quantification of the odorants present in the extracts, these were spiked with the internal standards, concentrated by evaporation under nitrogen up to 0.2 mL and analysed. Major fermentative and minor compounds were directly quantified by GC-FID and GC-MS analysis respectively, as described in the part 2 Materials and Methods. Some additional odorants were added according to the results of the GC-O analysis. Those compounds are presented in the Table 3.1 and were analysed in GC-MS as follow: in a Shimadzu QP2010 (Quioto, Japan) equipped with a DB-WAXetr (30 m x 0.25 nm, 0.5 μm film thickness) from Agilent, preceded by an uncoated pre-column (3 m x 0.25 mm). Carrier gas was He at 1.26 mL/min. Injection volume was 1 μL in split mode, with a split ratio of 1/30. Injector temperature was 250 ºC. Chromatographic oven temperature was initially at 30 ºC for 1 min, then raised at 1 ºC/min to 35 ºC, held for 1 min, then at 1 ºC/min to 40 ºC, at 15 ºC/min to 55 ºC, held for 5 min, at 15 ºC/min to 72 ºC, held for 5 min, at 15 ºC/min to 150 ºC, held for 15 min. The Ion source was kept at 220 ºC and the interface at 230 ºC. The mass analyser was set in single ion monitoring mode. The list of the compounds quantified, including their m/z ratios is available in Table 3.1.

3.2.4 Statistical analysis

Significance of the factors yeast, PAF and aging were determined by one, two and three-way analysis of variance (ANOVA) and Tuckey's HSD test was performed on one-way ANOVA results. PCA and hierarchical clustering were also performed.

3.3 Results

3.3.1 Odorants lost during fermentation

In order to assess the type and amounts of odorants lost during fermentation, a small trap was installed in the fermenters. A GC-O screening procedure was carried out on an extract obtained by mixing small aliquots of all the extracts obtained in

Table 3.2: Identification of odorants purged out during fermentations and trapped in LiChrolut EN cartridges placed before the Muller valves of the fermenters. The GC-O experiment was carried out on an extract made by mixing the eluates of the different traps. Retention indexes (RI) in DB-WAX and DB-5 columns, identifications, olfactometric scores (MF %) and odor descriptors are detailed. Compounds are marked by letters according to the reliability of their identification (see legend below).

RI_{DB-WAX}	RI_{DB-5}	compound*	MF	odor description
1220	< 900	isoamyl alcohol^{A}	87	cheese, rancid
940	< 900	3-methylbutanal^{A}	78	cheese, rancid
935	< 900	isopropyl acetate^{A}	62	fruity, strawberry
975	< 900	ethyl isobutyrate^{A}	62	fruity, strawberry
2097		cresol^{B}	55	phenolic, leather
1929	1115	β-phenylethanol^{A}	53	floral, rose
1039	< 900	ethyl butyrate^{A}	46	fruity, strawberry
1241	988	ethyl hexanoate^{A}	45	fruity
1513	1133	Z-2-nonenal^{B}	38	rancid, cucumber
1128	< 900	isoamyl acetate^{A}	37	banana
1441	1193	ethyl octanoate^{B}	35	mushroom, plastic, humidity
1544	1153	E-2-nonenal^{B}	29	fat, rancid
1621	1391	ethyl decanoate$A$	29	soap
1696	< 900	2 and 3-methylbutyric acid^{A}	26	sweat, rancid
1842		guaiacol^{B}	26	smoky, burn
966	< 900	ethyl propanoate^{A}	24	fruity
1425		1-nonen-3-one^{C}	24	mushroom, undergrowth
1468		decanal^{C}	24	grass, floral
1953		Z-whisky lactone^{C}	24	spicy
2169		2-phenoxyethanol^{C}	24	rancid, carton
2245		4-vinylguaiacol^{C}	24	spicy
2214		sotolon^{C}	22	spicy

*__A__: identification conclusive, experimental RIs in two columns, odor and MS corresponded to the one obtained with the pure chemical standard; __B__: identity highly likely, one of the previous criteria (two RIs, odor, MS) failed; __C__: tentative identification based on RI on a single column, odor and previous literature.

the experiment. Results are summarized in the Table 3.2.

Overall, twenty-two odorants were detected with GC-O scores above 20 %. Eleven out of the twenty-two odorants were identified at maxima level of confidence. In five other cases, some of the identity criteria could not be completely fulfilled because of different reasons, such as excessively low levels to get a good MS spectrum (Z and E-2-nonenals), co-elution in the non-polar column (cresol and guaiacol) or discrepancy in the odor (ethyl octanoate). The identification of the six less intense odorants was based only on the coincidence of the odor and retention index of the odorant in the polar column with those of the standard, and should be considered tentative.

The most intense odorants were mainly by-products of alcoholic fermentation: isoamyl alcohol and 2-phenylethanol, 3-methylbutanal, and a numerous group of esters (isopropyl and isoamyl acetates, and the ethyl esters of isobutyric, butyric, hexanoic, octanoic and decanoic acids). Apart from these, cresol and Z and E-2-nonenals were also between the twelve most intense. The origin of cresol is not clear, but it could be a breakdown product of the polyphenols present in fermentation media, while nonenals are known derivatives of the auto-oxidation of grape fatty acids (Ferreira et al., 1997). Results, therefore, confirm that the most relevant odorants purged out during fermentation are by-products derived from yeast metabolism or grape fatty acid auto-oxidation and not varietal aroma compounds released from specific aroma precursors in grape, such as terpenols, norisoprenoids or PFMs.

3.3.2 Quantitative assessment of the volatiles lost during fermentation

The amounts of eighteen odorants trapped in the cartridges installed in the PAF-containing fermenters are summarized in Table 3.3. In general, levels were low and in some cases were affected by a high imprecision. The total mass of volatiles trapped in the cartridges ranged from around 1 mg (D80) to around 2 mg (71B).

Table 3.3: Amounts in μg of volatile compounds purged out during fermentation and trapped in LiChrolut EN cartridges placed before the Muller valves of the fermenters containing 50 mL of synthetic must. The first column gives the significance of the factor yeast on the levels of volatiles found in the fermentations performed with PAF addition (p-values in bold are inferior to 0.05). n.d. indicates that the compound was not detected (below detection limits). - indicates that data was not available.

	p-value	MUST	CLOS	IONYS	71B	BDX	D254	D80	HPS	OKAY	PERSY	RHONE
acetates												
propyl acetate	**6.24E-10**	0.10 ± 0.02	0.6 ± 0.2	4.1 ± 0.3	0.9 ± 0.2	0.49 ± 0.06	0.6 ± 0.2	0.5 ± 0.2	0.9 ± 0.2	2.1 ± 0.4	3.7 ± 0.8	0.9 ± 0.2
isopropyl acetate	**5.80E-05**	1 ± 7	135 ± 11	320 ± 43	158 ± 34	135 ± 33	130 ± 17	129 ± 54	162 ± 43	199 ± 18	250 ± 33	171 ± 23
isobutyl acetate	**4.47E-02**	n.d.	0.7 ± 0.1	6 ± 2	2.0 ± 0.7	1.8 ± 0.5	1.2 ± 0.4	1 ± 1	2.2 ± 0.6	1.4 ± 0.4	3.1 ± 0.1	1.1 ± 0.5
isoamyl acetate	**4.70E-04**	15 ± 4	6 ± 3	104 ± 18	23 ± 17	-	17 ± 11	8 ± 15	18.2 ± 8.1	29 ± 5	-	14 ± 31
acids												
3-methylbutyric acid	1.14E-01	n.d.	3 ± 1	4 ± 3	4 ± 2	2.4 ± 0.2	4 ± 1	1.8 ± 0.4	5.1 ± 2.0	2.7 ± 3.0	3 ± 2	4 ± 1
2-methylbutyric acid	7.92E-02	0.1 ± 0.1	0.4 ± 0.2	0.8 ± 0.7	0.9 ± 0.6	0.59 ± 0.09	0.9 ± 0.4	0.41 ± 0.09	1.1 ± 0.5	1.4 ± 0.8	0.6 ± 0.6	1.0 ± 0.3
alcohols												
isobutanol	**4.69E-02**	74 ± 20	130 ± 24	129 ± 8	128 ± 4	-	176 ± 30	142 ± 112	184 ± 31	127 ± 54	-	120 ± 16
isoamyl alcohol	1.57E-01	387 ± 13	420 ± 107	488 ± 58	484 ± 226	-	497 ± 163	325 ± 318	507 ± 125	664 ± 116	-	364 ± 110
carbonyls												
acetaldehyde	**3.07E-03**	5 ± 2	3 ± 2	3.6 ± 0.6	14 ± 10	-	3.3 ± 0.8	4 ± 1	4 ± 1	4 ± 2	-	5 ± 2
isobutyraldehyde	6.39E-02	n.d.	904 ± 482	684 ± 124	1291 ± 528	957 ± 464	548 ± 268	392 ± 210	692 ± 367	493 ± 267	644 ± 243	534 ± 113
2-methylbutanal	5.85E-02	n.d.	1.4 ± 0.2	2.2 ± 0.7	3 ± 1	1.1 ± 0.5	1 ± 1	0.7 ± 0.1	1.2 ± 0.8	2.4 ± 0.8	1.7 ± 0.1	0.9 ± 0.9
3-methylbutanal	2.05E-01	n.d.	3.5 ± 0.9	7 ± 1	12 ± 4	4.2 ± 2.3	3 ± 4	3. 0 ± 0.3	4 ± 3	10 ± 4	6.5 ± 0.3	4 ± 4
esters												
ethyl propanoate	**5.28E-04**	0.1 ± 0.2	6.5 ± 0.6	38 ± 18	5 ± 1	4.9 ± 0.3	6 ± 7	5 ± 2	7 ± 2	7.5 ± 0.5	10 ± 3	6 ± 2
ethyl butyrate	**2.95E-02**	0.2 ± 0.1	0.10 ± 0.03	0.86 ± 0.05	0.19 ± 0.01	-	0.23 ± 0.04	0.1 ± 0.1	0.3 ± 0.1	0.20 ± 0.03	-	0.2 ± 0.1
ethyl isobutyrate	**3.07E-03**	n.d.	0.3 ± 0.2	0.30 ± 0.05	0.32 ± 0.01	0.34 ± 0.15	0.5 ± 0.2	0.21 ± 0.09	0.5 ± 0.1	0.32 ± 0.06	0.38 ± 0.02	0.4 ± 0.1
ethyl hexanoate	3.09E-01	2.7 ± 0.8	4 ± 2	7.0 ± 0.6	6 ± 5	-	5.9 ± 2.3	3 ± 1	4 ± 2	6.7 ± 0.9	-	3 ± 5
ethyl octanoate	4.89E-01	10 ± 5	7 ± 4	11 ± 2	11 ± 8	-	11 ± 4	5 ± 1	7 ± 3	14 ± 4	-	6 ± 9
ethyl decanoate	**1.23E-04**	0.32 ± 0.05	2.2 ± 0.7	8 ± 2	4 ± 2	-	4 ± 1	2.9 ± 0.9	3.6 ± 0.9	6.3 ± 0.1	-	5 ± 1

The major volatile was isobutyraldehyde, which accounts for more than 50 % of the total of volatiles trapped. Four other odorants, isopropyl acetate, isoamyl acetate, isobutanol, and isoamyl alcohol can be also found at levels above 100 μg. The levels of some volatiles released were significantly related to the yeast strain, although in most cases differences were not very high. Major differences correspond to the strain IONYS, whose cartridges contained highest levels of acetates and some ethyl esters, followed by the strain 71B, with maxima levels of aldehydes.

As expected, volatiles purged during fermentation are mostly non-polar aroma compounds such as esters, acetates or carbonyls. Only two acids and two alcohols were found amongst the quantifiable volatile compounds. In these cases, the amounts evaporated corresponded to very small fractions of the volatiles produced. In the particular case of isoamyl alcohol, the 664 μg found in the cartridge of OKAY strain correspond to 13 mg/L in the 50 mL of liquid. Considering that the recently fermented wine contained around 190 mg/L of this compound (Table B.1 in Annex B), it can be estimated that less than 5 % of the total amount of isoamyl alcohol produced was evaporated. On the opposite side, isobutyraldehyde was found in the cartridge from the strain 71B at 1.29 mg, which amounts to 26 mg/L in the 50-mL volume, while reported levels of this compound in wine are well below 0.1 mg/L (Culleré et al., 2007). This suggests that isobutyraldehyde is a major fermentation volatile which is nearly completely ($>$ 99 %) lost by evaporation. Similar considerations applied to isopropyl and isoamyl acetates and ethyl propanoate indicate that more than 70 % of these aromas are lost by evaporation. Levels purged out of higher esters, such as ethyl butyrate, isobutyrate, hexanoate, octanoate and decanoate, and of higher aldehydes, such as 2 and 3-methylbutanal, were far more modest. Yet, they represent significant fractions of the total formed. In the cases of ethyl hexanoate and decanoate, for instance, the fractions lost are 68 and 35 % of the total amounts formed, respectively.

In any case, results reveal that the carbon dioxide released during fermentation carried out large amounts of isobutyraldehyde, isopropyl acetate, ethyl propanoate

and isoamyl acetate, which in fact are mostly lost in this period; and also significant amounts in absolute but not in relative terms, of isobutanol and isoamyl alcohol. Some other esters, such as ethyl butyrate, hexanoate, octanoate and decanoate were produced at much smaller levels but yet, were significantly lost by evaporation.

3.3.3 Major fermentative aroma compounds

Twenty-six major fermentation volatiles were detected at concentrations superior to detection limits in young wines (Table B.1 in Annex B). In this case, 19 out of the 26 quantified volatiles were significantly related to the yeast strain. The most different profiles of volatiles were obtained in wines fermented with IONYS, which produced maxima levels of most ethyl esters and acetates, and also of isovaleric acid, acetoin and γ-butyrolactone, and minima levels of acetic and decanoic acids. Other strains showing specific profiles of volatiles were 71B, BDX, D80, and PERSY. 71B produced maxima levels of methionol, butanol, hexanol and minima levels of most ethyl esters. BDX produced maxima levels of isobutanol and isoamyl alcohol. D80 produced maxima levels of acetic, isobutyric and decanoic acids. Finally, PERSY produced maxima levels of ethyl octanoate, octanoic acids, ethyl lactate and minima levels of isoamyl alcohol and isobutanol.

In general, differences introduced by the strains were of little to moderate magnitude. Levels of isoamyl alcohol ranged from 160 to 310 mg/L, a factor 2; those of isobutanol from 20 to 60 mg/L, a factor 3. These differences are, however, large enough to have sensory significance (De-la Fuente-Blanco et al., 2017). Differences in the levels of acetic acid were much higher and amounted to a factor 20, from just 30 mg/L (IONYS) to 600 mg/L (RHONE). However, leaving aside IONYS, differences become more modest, ranging from 340 to 600 mg/L, less than a factor 2. Similar ranges of variation were observed for hexanoic, octanoic, decanoic and isovaleric acids, while levels of isobutyric acid ranged a factor close to 4. Levels of esters were, in general, very low as a possible consequence of their strong evaporation, and in some cases, they were not even detected. Leaving aside IONYS, their ranges

of variation were not large.

3.3.4 Trace aroma compounds: varietal or fermentative origin?

The complete data sets with the concentrations of up to 34 trace aroma components in recently fermented and in aged wines, in the different controls introduced in the study, and the results of the different ANOVA studies carried out on the data are compiled in the Tables B.1-B.5 in Annex B.

Regarding the varietal or fermentative origin of the aroma compounds, the study of the controls including or not the PAF material extracted from the grapes and those others including or not fermentation (all compiled in Table B.2 and statistical analysis in Table B.3 in Annex B), reveals that some aroma compounds cannot be unequivocally classified into fermentative or varietal. Rather, there are several intermediate categories, as the answer to the following three simple questions asked to each aroma compound, reveals;

1. Is the aroma compound present in fermented controls not containing grape PAF?

2. Is it present in unfermented controls containing grape PAF?

3. Is it at significantly larger amounts in fermented samples containing grape PAF than in the corresponding controls not containing grape PAF?

A positive answer to the 1st question implies an unequivocal fermentative origin; the compound can be formed by yeasts from the basic list of nutrients supplied. On the contrary, a negative answer indicates that the formation of the compound requires the presence of grape components. The answers to the two following questions will indicate whether the compound is present in the grape PAF as specific precursor (positive answer to 2) and whether yeast is required for its formation (positive answer to 3). Attending to the answers, five different origin-related categories emerged, as schematized in the Figure 3.2.

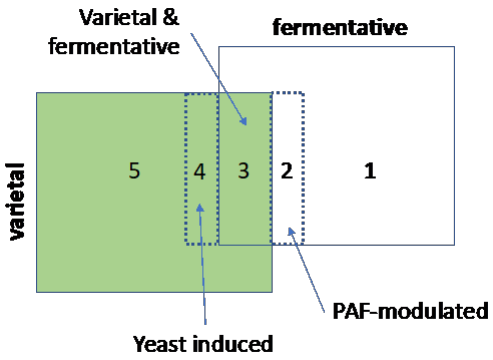


Figure 3.2: The five origin-related categories in which aroma compounds of the experiment should be classified.

1. Pure fermentative compounds (answer YNN) are those which were present in fermented samples not containing PAF and whose levels were not influenced by the presence of PAF. Compounds in this category were isobutyl acetate, ethyl isovalerate, ethyl 2-methylbutyrate and δ-decalactone.

2. PAF-modulated fermentative compounds (answer YNY) are those aroma compounds formed by yeast, but whose levels are significantly influenced by the presence of PAF. Compounds in this category were β-phenylethyl acetate, ethyl isovalerate, ethyl leucate, γ-octalactone, β-citronellol, geraniol, nerol.

3. Fermentative and varietal aroma compounds (answers YYY and YYN), are those aroma compounds which can be formed by yeast from basic nutrients and which can be also found in unfermented controls containing PAF. Linalool and its oxide belong to this category.

4. Yeast-induced varietal aroma compounds (answer NNY), are those aroma compounds found exclusively in fermented PAF. β-ionone, ethyl dihydrocinnamate belong to this category.

5. Varietal aroma compounds (answer NYY), are those compounds found exclusively in samples containing grape PAF. Most compounds belong to this category (massoia lactone, β-damascenone, TDN, vitispirane, Riesling acetal, vanillin, acetovanillone, syringaldehyde, syringol, guaiacol, 4-ethyguaiacol, 4-ethylphenol, 4-vinylguaiacol, 4-vinylphenol, eugenol, methoxyeugenol, trans-isoeugenol, 4-propylguaiacol).

It should be noted that, directly or indirectly, levels of all compounds were influenced by the existence of fermentation, which suggests, as it will be further seen in the next sections, that yeast is going to play a relevant role on nearly the complete wine aroma profile.

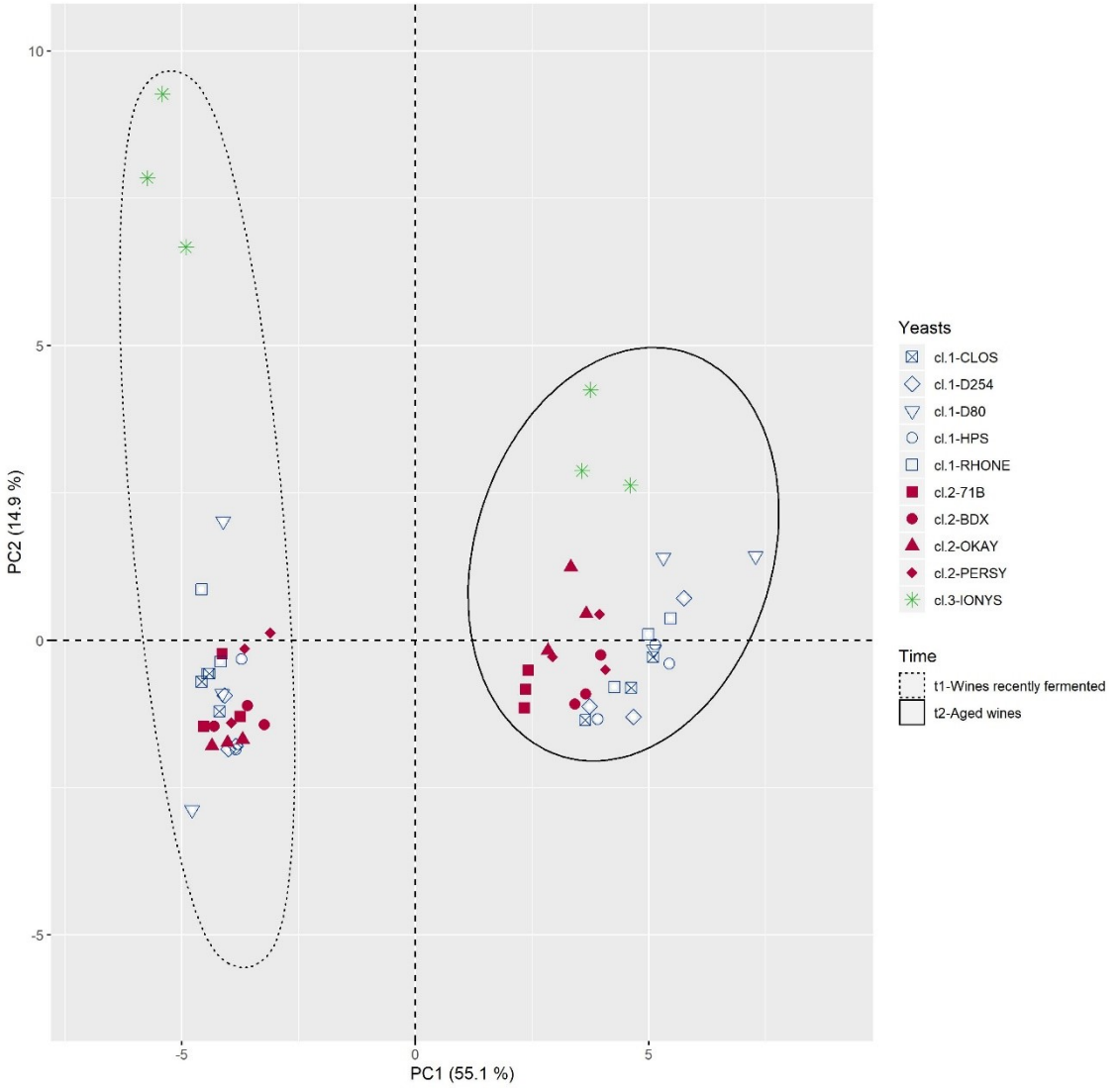


Figure 3.3: PCA calculated from the concentrations of trace compounds in the samples with PAF fermented by 10 *S. cerevisiae* yeasts, analyzed after fermentation and after accelerated aging. Clusters mentioned in the legend are the one resulting from the heatmap built from aged wines volatiles concentrations (Figure 3.4). Yeasts represented with an empty blue icon belong to the cluster 1 (cl.1), full red icon belong to the cluster 2 (cl.2) and the green asterisk represents IONYS, belonging to the cluster 3 (cl.3).

3.3.5 Yeast strain and aging: global overview

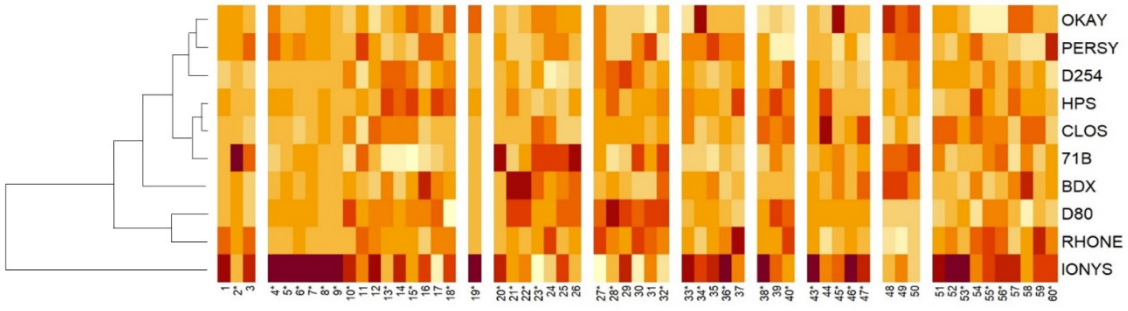
Both factors, yeast strain and aging have a strong influence on the trace aroma composition of wines, although aging is the dominant factor. Such dominance is most evident in the PCA plane given in Figure 3.3, which shows the projection of the sixty samples (10 yeast x 2 times of analysis x 3 replicates) in the two first dimensions. The first component (55.1 % of the original variance) separates samples attending to age, with young samples on the left, and aged samples on the right. The variable loadings (not shown) reveals that most compounds, including norisoprenoids, esters, volatile phenols, vanillin derivatives, increase during aging and that only terpenes (except for linalool oxide), massoia lactone and β-phenylethyl acetate decrease during aging.

The plot reveals two other important characteristics. First, that samples fermented with IONYS are very well separated from the others before and after aging. Young samples because of their highest levels in β-phenylethyl acetate, linalool and geraniol, and aged samples because of their highest levels of isobutyl acetate, γ-octalactone and δ-decalactone. The second remarkable characteristic, is that leaving aside IONYS, the yeast strain is an active grouping factor in trace aroma compounds only after aging.

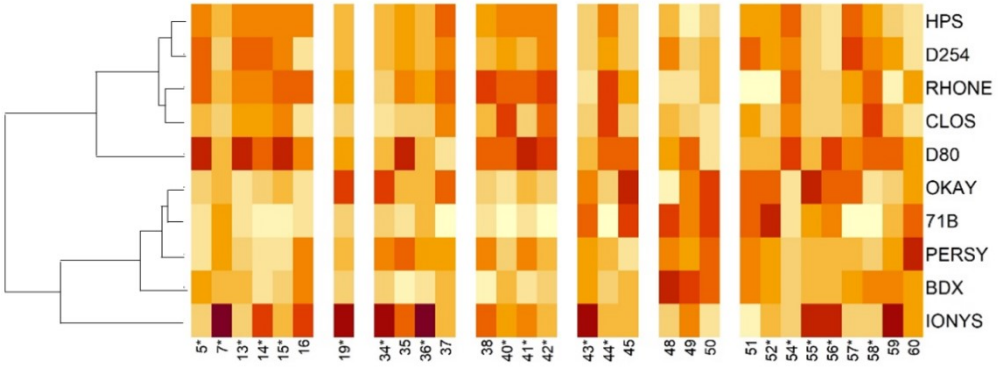
3.3.6 Effects of yeast strain on wine aroma

In order to analyze the influence of yeast taking into consideration the dominance of the aging time, two different heatmaps were generated with the aroma compounds quantified. The first one is given in 3.4a and includes data from major and trace aroma compounds measured in young wines. The second one can be seen in Figure 3.4b and includes only trace aroma compounds in aged wines. The results of the hierarchical clustering are also displayed on the left part of each plot.

Figure 3.4a confirms the singularity of IONYS in young wines and the apparently low diversity existent between the other strains. After aging, however, a much clear structure emerges as can be seen in Figure 3.4b, where yeast strains can



(a)



(b)

	carbonyls	17	ethyl lactate	32	decanoic acid	47	nerol
1	acetaldehyde	18	diethyl succinate		**lactones**		**vanillin derivatives**
2	diacetyl		**cinnamates**	33	γ-butyrolactone	48	vanillin
3	acetoin	19	ethyl dihydrocinnamate	34	γ -octalactone	49	acetovanillone
	esters		**alcohols**	35	γ -nonalactone	50	syringaldehyde
4	ethyl acetate	20	butanol	36	δ-decalactone		**volatile phenols**
5	isobutyl acetate	21	isobutanol	37	massoia lactone	51	syringol
6	isoamyl acetate	22	isoamyl alcohol		**norisoprenoids**	52	guaiacol
7	β-phenylethyl acetate	23	hexanol	38	β-damascenone	53	4-ethylguaiacol
8	ethyl propanoate	24	benzyl alcohol	39	β-ionone	54	4-ethylphenol
9	ethyl butyrate	25	β-phenylethanol	40	TDN	55	4-vinylguaiacol
10	ethyl hexanoate	26	methionol	41	Riesling acetal	56	4-vinylphenol
11	ethyl octanoate		**acids**	42	vitispirane	57	eugenol
12	ethyl decanoate	27	acetic acid		**terpenes**	58	methoxyeugenol
13	ethyl isobutyrate	28	isobutyric acid	43	linalool	59	trans-isoeugenol
14	ethyl 2-methylbutyrate	29	isovaleric acid	44	linalool oxide	60	4-propylguaiacol
15	ethyl isovalerate	30	hexanoic acid	45	β-citronellol		
16	ethyl leucate	31	octanoic acid	46	geraniol		

(c)

Figure 3.4: Heatmap from normalized concentrations of the volatiles compounds quantified in young (a) and aged (b) wines. Compounds from 1 to 60 are defined in the Table (c), * indicates that the compound was significantly affected by the yeast (p-value < 0.05).

be classified into three different clusters. Cluster 1 is integrated by a quite homogeneous group formed by D254, HPS, RHONE and CLOS and with more dissimilarity, by D80. The second cluster is formed by PERSY, OKAY, 71B and with more dissimilarity, BDX. And finally, IONYS is the single component of the most different cluster 3. It can also be seen that yeasts in cluster 1 produced higher levels of pure fermentative compounds such as isobutyl acetate, ethyl isobutyrate, 2-methylbutyrate and isovalerate and released higher levels of norisoprenoids (TDN, vitispirane and Riesling acetal). Cluster 2 released in general smaller levels of volatiles, except of some volatile phenols, such as vanillin and guaiacol.

3.3.7 Modulation of varietal aroma

The effect of yeast strain on those genuine varietal aroma compounds naturally present in unfermented controls containing grape PAF can be assessed with the help of the plots given in Figure 3.5. The plots compare levels of aroma compounds found in fermented aged samples with those obtained in the unfermented aged controls. These representations facilitate the identification of the general role of fermentation on the fate of these varietal aroma compounds and also of the specific role played by the yeast strain. Compounds can be classified into three different categories depending on the effect of fermentation.

1st category: Positive effect of fermentation, including five aroma compounds whose levels in fermented samples were much above those found in the unfermented controls, suggesting that some of the specific precursors of these aroma compounds could be formed by the action of yeasts. Compounds in this category are acetovanillone, β-ionone (this last not shown in Figure 3.4, since it was only found in young samples), ethyl dihydrocinnamate, 4-propylguaiacol, 4-ethylguaiacol, trans-isoeugenol and eugenol (these 5 last are not represented in Figure 3.4 since they were not detected in the unfermented must). Only in the case of eugenol the effect of yeast was significant. Levels of acetovanillone in the aged fermented

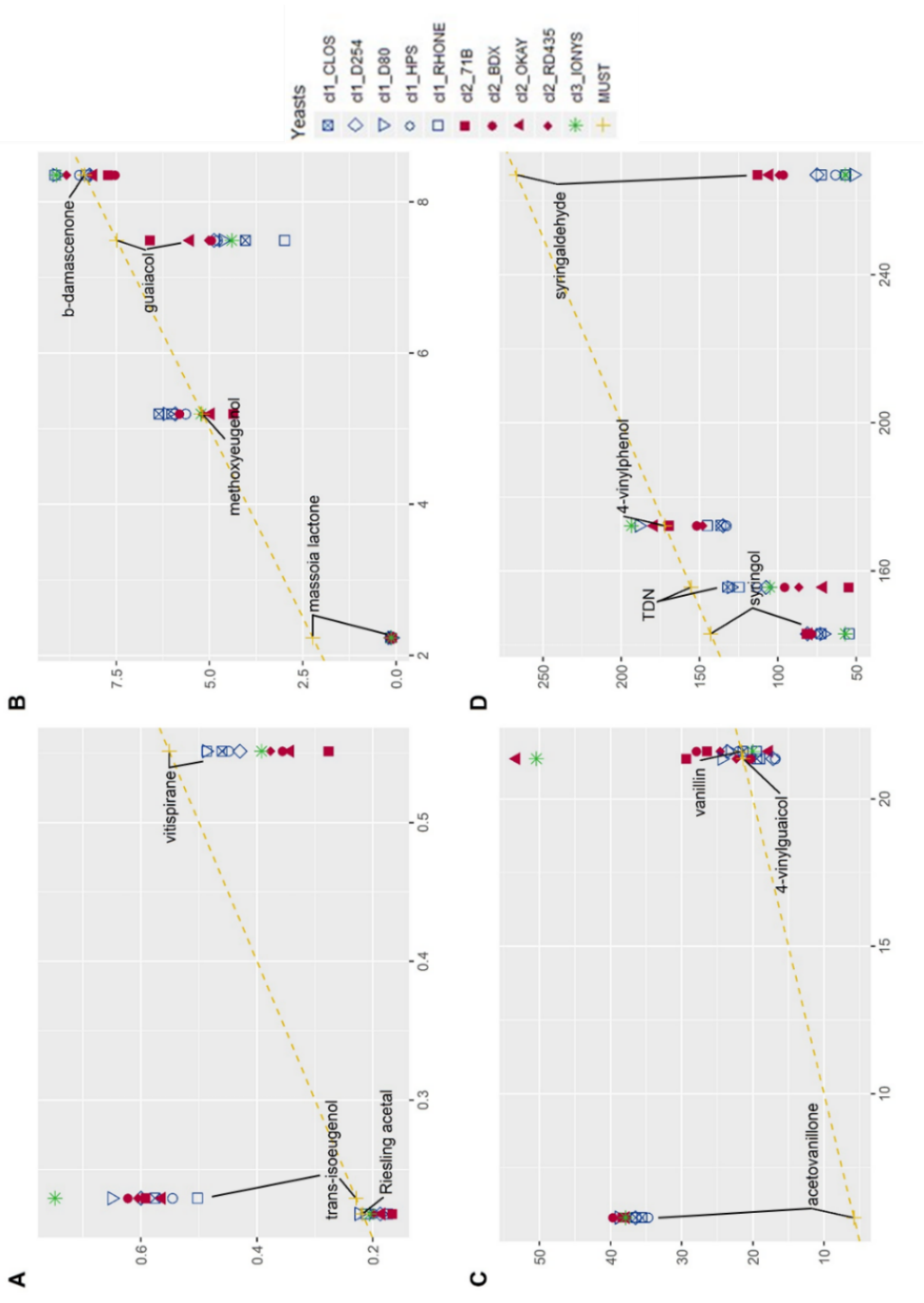


Figure 3.5: Compounds concentrations in samples compared to its levels in the unfermented musts in μg/L. Yeasts belonging to the cluster 1 are represented in blue, from cluster 2 in red and cluster 3 in green. Unfermented must is represented in yellow. Each yeast strain is represented by a different shape.

samples are 10 times higher than those found in the unfermented control, however, no difference was observed between the strains.

2nd category: No effect of fermentation, effect of strain. Includes compounds having in common that, in average, levels of fermented samples are not dissimilar to those of the unfermented controls. There are however strong differences attending to the differential effect introduced by yeast. In the cases of Riesling acetal (Figure 3.5A), and of methoxyeugenol and β-damascenone (Figure 3.5B), there is no effect of the yeast, which suggests, that at least for Tempranillo, the levels of these varietal aroma compounds cannot be modulated by yeast.

The case of β-damascenone, which is an aroma enhancer (Pineau et al., 2007) and modules the ripeness-character of fruity perception (San-Juan et al., 2011), deserves special mention. Levels of this odorant in recently fermented samples were above those measured in the corresponding unfermented controls (Tables B.4-B.5 in Annex B) and were significantly influenced by the yeast strain. Samples fermented with IONYS showed levels up to 4 times higher than those found in those made with OKAY. This suggests that yeasts cannot change the long-term level of this aroma compound but can accelerate its formation. The second subcategory includes vanillin (Figure 3.5C), methoxyeugenol (Figure 3.5B), and 4-vinylphenol (Figure 3.5D), for which the yeast strain exerts a moderate and significant influence, so that differences of around a 50 % between the minimum and the maximum are observed. 4-Vinylguaiacol can be also classified within this subcategory but with two strains, IONYS and OKAY, showing a clear outlier over-productive character.

3rd category: Negative effect of fermentation. It includes aroma compounds whose levels in fermented samples were below those found in the unfermented controls. There are strong differences between compounds attending to the effect played by the strain of yeast. Massoia lactone (Figure 3.5B) is a particular case whose levels drop to nearly zero in all the fermented samples with no difference between strains. Massoia lactone is an important marker of over-ripeness and

contributor to prune aroma, and its levels are known to decrease during fermentation (Pons et al., 2017). Our results reveals that such decrease intensifies during aging, which suggests that fermentation reduces also the precursors. It would be of interest to see whether such reduction is equally effective in grapes containing higher levels of precursors of this molecule.

Compounds in the category for which the yeast strain introduced significant differences were, from less to more intense, syringol, vitispirane, syringaldehyde, guaiacol and TDN. It is apparent that the specific precursors of these compounds are metabolized differently by yeasts. This can have a strong technological relevance since guaiacol and TDN can take part in relevant odor faults, and suggests that selected strains are a potentially effective remedial tool.

Guaiacol is an aroma compound contributing to the characteristic toasty-woody notes of Tempranillo, but it can be a serious off-odor developed with time in wines made with grapes exposed to smoke (Ristic et al., 2017). Results in Figure 3.5 reveal that yeasts within the cluster 1 seem to metabolize the precursor at higher levels. In the case of RHONE, levels of guaiacol were reduced by almost a factor three comparing with the unfermented control. Powerful reductions linked to specific yeast strains can be also observed for TDN, known responsible for kerosene notes developed in aged Riesling wines and with a 2 μg/L detection threshold (Sacks et al., 2012) will surely contribute to unpleasant notes in aged red wines. The Figure 3.5 reveals that yeasts in cluster 2, notably 71B can reduce levels by a factor 3 with respect to the control or D80. A similar yeast-induced and vitispirane-independent decrease of TDN levels has been recently observed for non-*Saccharomyces* yeast (Oliveira and Ferreira, 2019) but to the best of our knowledge, it has not been observed for *Saccharomyces* strains. This ability can have a notable sensory importance, since levels of TDN are expected to increase due to climate change (Winterhalter and Gök, 2013).

Many varietal aroma compounds in Figure 3.5 derive from ferulic acid and its glycosides (vanillin, acetovanillone, isoeugenol, eugenol and 4-vinylguaiacol). The

higher levels of these aroma molecules measured in aged fermented samples suggest that yeast transforms ferulic acid glycosides into the corresponding aroma glycosides and that those transformations are strain specific. Aged wines made with BDX have maxima contents of vanillin and acetovanillone, those made with IONYS have maxima contents of eugenol while those made with OKAY have minima levels of vanillin and maxima levels of 4-vinylguaiacol. Some of those specificities seem to be shared by strains in the same cluster. Yeasts in cluster 2 show higher levels of vanillin, acetovanillone and 4-vinylguaiacol than those in cluster 1. 4-Vinylguaiacol and 4-vinylphenol deserve a specific comment, since their levels are extremely dependent of the yeast strain in unaged samples, with factors around 10 between the minimum and maximum concentrations. Because of their reactivity differences between maxima and minima shrink to factors 3 (case of 4-vinylguaiacol) and 1.7 (case of 4-vinylphenol). In both cases, maxima levels were observed for IONYS, and minima levels for BDX and HPS.

3.3.8 Modulation of other relevant aroma molecules

Linalool and geraniol are the two most important terpenols of wine. In the present case, these two molecules were hardly detected in the unfermented controls, suggesting that the grape material did not have much precursors. The two compounds were found in the controls not containing grape extract, so that yeasts were able to form weak, or moderate in the case of IONYS, amounts of these molecules. Fermented unaged samples contained both molecules at the expected concentration ranges of Tempranillo (< 6 μg/L) except for the samples fermented by IONYS, whose levels were above 20 μg/L, which suggests that this quite unique yeast strain will produce young wines with markedly different characters. During aging, levels of these compounds and the other terpenes decreased (< 1 μg/L), while those of linalool oxide, its oxidation product, increased.

Ethyl leucate or ethyl 2-hydroxy-4-methylvalerate, is a remarkable aroma compound identified in aged wines (Campo et al., 2006) and suggested to be key in

the specific blackberry aroma of Bordeaux red wines (Falcao et al., 2012). Results from this paper have shown that maxima levels are found in aged PAF-containing fermented samples and that the yeast strain exerts a significant influence, with levels found in IONYS, BDX, D80, PERSY and RHONE twice those found in 71B or OKAY (Table B.4 in Annex B).

Ethyl esters of branched acids are the most important fruity esters in aged red wines, contributing concertedly to fruity aroma (De-la Fuente-Blanco et al., 2020) and are slowly formed by esterification of the corresponding acids synthesized during fermentation through Ehrlich pathway. The influence of yeasts becomes most obvious after aging. Minima levels are found in 71B, PERSY (cluster 2), and maxima in D80 and D254 (cluster 1), with differences as large as factors between 4 and 6.5.

Isoamyl and β-phenylethyl acetates are relevant in the aroma of young wines, since their levels quickly fade by hydrolysis of the esters. The influence of yeasts in their levels is overwhelming. In the case of β-phenylethyl acetate, levels in young wines (Table B.4 in Annex B) range from 0.1 mg/L (cluster 1) to 1.5 mg/L (cluster 3), with levels in samples from cluster 2 between 0.18 and 0.29 mg/L. Similar results were already observed for the isoamyl acetate and isopropyl acetates evaporated during fermentation (Table 3.3).

3.4 Conclusion

Volatiles lost by evaporation during fermentation are mostly fermentative compounds and not grape-related aroma compounds. Quantitatively, vapors are majorly composed of 2-methylpropanal, isopropyl acetate, isoamyl acetate, ethyl propanoate, isobutanol and isoamyl alcohol. While the fraction of alcohols lost is very low, that of the aldehydes and esters can be well above 90 % of the total volatile produced.

The strong impact exerted by the yeast strains on wine aroma composition becomes in many cases only evident after aging, since levels of ethyl esters of

branched acids, of most grape-related aroma compounds and of many minor yeasts-derived aroma compounds mostly increase during aging. The 10 strains can be classified into three clusters showing marked differences in fermentative and varietal aroma profiles.

The boundaries between fermentative and varietal aroma compounds are in many cases blurred. First, the study has confirmed a fermentative origin for linalool and geraniol, found at high levels in samples fermented by one of the strains. Second, the presence of polyphenolic and aromatic fractions from grape exerts a strong influence on yeast metabolism and, third, the strains of yeast not only hydrolyze glycosidic precursors, but metabolize quite differently the precursors of relevant aroma compounds, such as phenolic acids and norisoprenoids. These characteristics have interesting practical consequences on the potential of yeasts to control the wine aroma profile and, most remarkably, some wine aging attributes. Results have shown that the rates of accumulation of β-damascenone are strain-related, and that some strains may be specifically used to mitigate relevant aging-related off odors, such as those related to guaiacol, massoia lactone or TDN.

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Chapter 4

Impact of two yeast strains on Tempranillo red wine aroma profiles throughout accelerated aging

4.1 Introduction

The use of commercial *Saccharomyces cerevisiae* strains in wineries to carry out alcoholic fermentation is very common nowadays. Apart from facilitating fermentation monitoring and control, it also permits the modulation of wine styles, particularly through modifications of its aroma profile (Pretorius and Bauer, 2002). Although the number of volatile molecules which can be a part of the volatile fraction of wines is very large, it has been suggested that around 70 odor chemicals are those playing major roles on the aromatic properties of wines (Ferreira et al., 2021). Most of these compounds can be efficiently modulated by *S. cerevisiae* yeasts either because they are fermentation by-products or because even being derived from grape specific precursors, yeast exerts a role in their release (Swiegers et al., 2005).

The most abundant volatile compounds that take part in the fermentative aroma profile of wines are higher alcohols, acids and ethyl and acetate esters. These compounds are derived from the transformation of basic nutrients of must through amino acid and fatty acid metabolisms of yeasts. The modulation of the levels of these compounds and, particularly, of the ethyl esters of short and branched acids by the strain in charge of fermentation leads to sensory differences. A higher production of these compounds is supposed to improve the fruity characteristics of wines (Molina et al., 2009).

On the other hand, wine varietal aroma profile is composed of volatiles present at lower concentrations and derived from grape specific precursors. For example, ethyl cinnamates, vanillin derivatives, terpenes and lactones participate in the perception of floral notes in wines, even at sub and peri-thresholds levels (Loscos et al., 2007). Other relevant grape-derived aroma compounds are nor-isoprenoids such as β-damascenone, β-ionone or TDN.

Aging is responsible for important changes in wine aroma profile because of a series of spontaneous chemical changes such as acid hydrolysis, chemical rearrangements of unstable molecules or esterification processes (Waterhouse et al., 2016). Levels of some fermentative compounds, such as higher alcohols or fatty

acids, are usually little affected during bottle aging, while levels of others can be greatly modified, particularly those of ethyl esters of branched short chain fatty acids and the acetates of higher alcohols. The latter strongly decrease, while the former slowly increase during aging (Antalick et al., 2014; Cassino et al., 2019; Díaz-Maroto et al., 2005; Makhotkina and Kilmartin, 2012; Marais and Pool, 1980; Ramey and Ough, 1980). Concerning varietal aroma profile, changes in the profile of terpenoids, sesquiterpenes and norisoprenoids have been reported (Simpson and Miller, 1983). Wine aging is crucial for the apparition and accumulation of some varietal compounds such as TDN (1,1,6-trimethyl-1,2-dihydronaphthalene), responsible for the kerosene off-odor (Rapp et al., 1985; Slaghenaufi and Ugliano, 2018).

However, few studies have been dedicated to study the sensory impact of yeasts after some time of aging. Among those, King et al. (2011) evidenced a strong sensory impact of seven *S. cerevisiae* yeasts on the aroma of Sauvignon blanc. Changes were still evident after three years of bottle aging. Unfortunately, these types of studies require long periods of time, which represents an important inconvenient. Accelerated aging strategies have consequently been employed, being thermal treatment among the most commonly used (Francis et al., 1994; Simpson, 1978; Singleton et al., 1964; Slaghenaufi and Ugliano, 2018). More recently, accelerated aging was applied in total absence of oxygen, including sample preparation in an anoxic chamber. An aging period of five weeks at 50 ^{o}C in strict anoxia would be roughly equivalent to one year of bottle aging at room temperature (Vela et al., 2017). Recently, this accelerated aging has been applied to demonstrate that the fermentative and varietal aroma profile of wines can be efficiently modulated by sequential inoculation of strains of *Pichia*, *Torulaspora* or *Lachancea* followed by *Saccharomyces* (Oliveira and Ferreira, 2019). This was also the case in the previous chapter comparing several *S. cerevisiae* strains. In both cases, aging was crucial for the appreciation of some of these changes.

In the previous chapter, the capacity to produce volatiles of 10 *S. cerevisiae*

strains was screened using a semi-synthetic must of Tempranillo supplemented with natural aroma precursors and polyphenols extracted from Tempranillo grapes. Yeasts became separated into three different clusters according to their production of volatiles. The strains IONYS wf™ and Lalvin ICV D254™ belonged to two different clusters. Globally, the latter produced medium quantities of most volatile compounds and higher levels of ethyl esters of branched acids (ethyl isobutyrate and isovalerate); while IONYS was characterized by a maxima production of most volatiles, particularly of lineal ethyl esters (ethyl propanoate, butyrate, hexanoate, decanoate), acetates from higher alcohols (isobutyl, isoamyl, β-phenylethyl acetates), lactones (γ-butyro, γ-octa, γ-nona and δ-decalactone), volatile phenols (guaiacol, 4-vinylguaiacol), terpenes (geraniol, linalool), ethyl leucate, dihydrocinnamate, and a very low production of acetic acid.

In this context, the objectives of the present work are to evaluate the impact of these two strains on the sensory characteristics of Tempranillo wines, to assess whether those strain-related sensory characteristics are consistently kept during aging, and to elucidate the chemical changes in aroma composition potentially responsible for those aroma sensory properties.

4.2 Materials and methods

All the methods are detailed in the Chapter 2, Materials and Methods. The experimental procedure is schematized on the Figure 4.1.

4.2.1 Wine elaboration

4.2.1.1 Must preparation

Fermentations were carried out in Lallemand Bio experimental winery (Logroño, Spain). Twenty kilograms of Tempranillo grapes (D.O. Ca Rioja, 2019 vintage) were harvested, destemmed and manually crushed. Potassium metabisulfite was added at 3 g/hL and yeast assimilable nitrogen (YAN) was supplemented by the

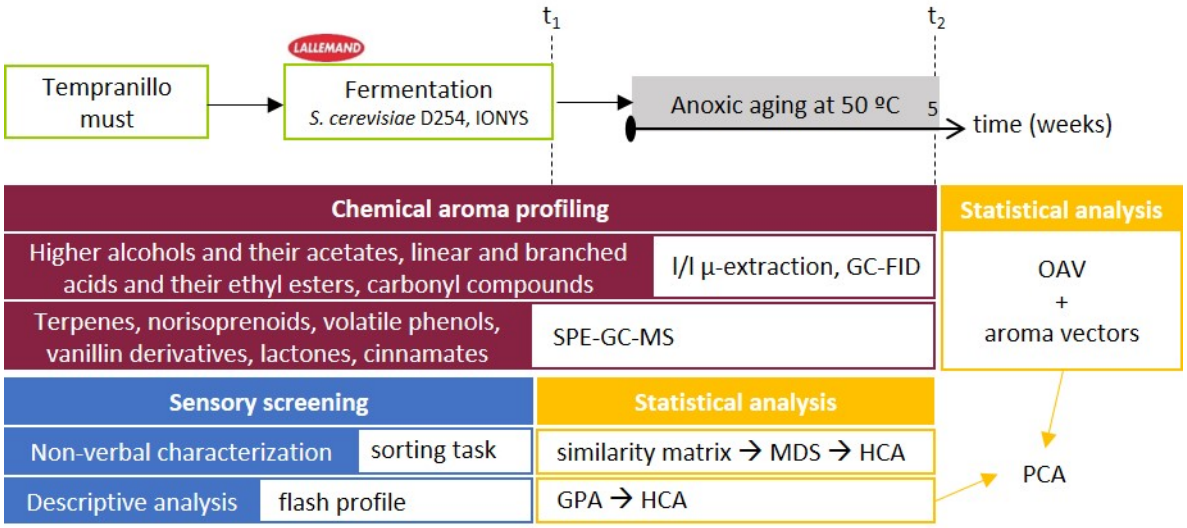


Figure 4.1: Experimental procedure - A must from Tempranillo grapes was fermented with 2 *S. cerevisiae* yeast strain. Wines were submitted to anoxic accelerated aging for 5 weeks at 50 ºC. Wines aroma profiles were analyzed via volatiles quantifications and sensory analysis.

addition of 160 mg N/L of Nutrient Vit™ from Lallemand S.L. (Barcelona, Spain). The must was divided into three-kilogram batches and homogeneously distributed into 5-L fermenter jars.

4.2.1.2 Yeast strains

The two *S. cerevisiae* were active dry yeasts, IONYS wf™ (IONYS) and Lalvin ICV D254™ (D254) from Lallemand Bio. Both were rehydrated with GO-FERM PROTECT™ (30 g/hL), which is a stimulant and protector agent and added to the must. During the fermentation, viable yeasts were counted every two days by plating diluted fermentation media on YPD agar plates, which were further incubated at 30 ºC for 48 hours.

4.2.1.3 Vinification, wine aging and samples

Fermentations were performed in triplicates at 19-22 ºC. At the end of the alcoholic fermentation (reducing sugars $<$ 1 g/L), potassium metabisulfite, Bactiless™ (Lallemand Bio) and Gecoll Supra® (Laffort, Bordeaux, France) were added at 40 mg/L of total SO_2, 20 g/hL and 40 mL/hL, respectively. Wines were decanted for 48 hours. Before bottling, wines were further supplemented with potassium metabisulfite (15 mg/L of total SO_2). A volume of approximately 3 L of wine was recovered of each replicate. Then, a 1.4 L of this volume was divided into two 720-mL glass containers which were submitted to a process of accelerated aging during 5 weeks at 50 ºC. Samples were vertically maintained into the incubator oven so that wine was not in contact with the screw cap and was separated by approximately 20 mL of Argon headspace.

A total of 12 wine samples were generated: six young wines recently fermented (t1) with two yeast strains (I: IONYS, D: D254) in triplicate (I-t1a, I-t1b, I-t1c; D-t1a, D-t1b, D-t1c) and the corresponding six wines after accelerated aging (I-t2a, I-t2b, I-t2c; D-t2a, D-t2b, D-t2c). Each sample manipulation was carried out in strict anoxia. After opening, samples were transferred and kept at 4 ºC in 750,

500 and 350-mL green glass bottles closed with vacuum wine stopper from Vacu Vin (Castellón de la Plana, Spain). These samples were chemically and sensory characterized with the methods described in the following sections.

4.2.1.4 Conventional oenological analysis

Conventional oenological parameters of grape juice and of recently fermented wines were analyzed using the following methodologies: glucose and fructose, YAN, free ammonia nitrogen (FAN), lactic and malic acid and acetaldehyde were analyzed by enzymatic kits using a Y15 Biosystems auto-analyzer (Barcelona, Spain). Total and volatile acidities were determined by potentiometric titration. Free and total sulfur dioxide were analyzed by colorimetry and alcohol content was analyzed by NIR spectrometry. Fermentations were monitored by daily measurements of glucose and fructose (International Organisation of Vine and Wine, 2019).

4.2.2 Chemical characterization of volatile composition

Major volatile compounds (higher alcohols, acetates and ethyl esters, volatile fatty acids – in concentrations from 10 to 200 mg/L) were analysed by GC-FID and trace volatile compounds (acetate and ethyl esters, vanillin derivatives, volatile phenols, terpenes, norisoprenoids and lactones – in concentrations from 0.1 to 1000 μg/L) by GC-MS.

4.2.3 Sensory analysis

4.2.3.1 Experimental conditions

The twelve samples were encoded with random 3-digit numbers. Ten-mL samples were presented to each judge.

Wines were evaluated following two different sensory strategies during three sessions held on two different days in two different weeks. Session 1 was devoted to nonverbal characterization (i.e., sorting task); sessions 2 (recently fermented wines) and 3 (wines after accelerated aging) were devoted to descriptive analysis by flash

profile methodology. Sensory analyses were performed by judges with extensive experience in wine sensory analysis and belonging to the laboratory LAAE and ICVV. Sorting task was carried out by twenty judges (13 women and 7 men, from 22 to 55 years, in average 34 ± 9 years) without previous training, and flash profile by fourteen judges (8 women and 6 men, from 22 to 64 years, in average 36 ± 12 years) previously trained to identify and score the specific attributes of the present study using odor references. Sensory tests, including training, are detailed in the following parts.

4.2.3.2 Non-verbal characterization: sorting

The twelve samples (the three biological replicates of the two recently fermented and of the two aged wines) were presented simultaneously together with a duplicated control consisting of two samples of a commercial red wine (Tempranillo, 2018). Participants were asked, during a 20-min session, to smell the fourteen samples and to group them according to their aroma similarities; the number of groups formed should be between 2 and 13, both inclusive. Once the sorting was achieved, panelists were asked to describe the groups using 1 to 3 attributes. Panel repeatability was assessed by verifying that the two replicates of control wines were grouped together.

4.2.3.3 Descriptive analysis: flash profile (FP)

The twelve samples were evaluated separately in two different days. In the first one, the six recently fermented samples, and in the second one, the six aged samples. Each of the two groups of samples were described by FP, which consisted in three 30 min sub-sessions each, held the same day and separated by at least one hour. In the first sub-session, participants were asked to smell the samples orthonasally and generate discriminant descriptors, without any restriction in the number used. Then, all descriptors were gathered and grouped in categories by three experienced experimenters independently. The final list of descriptors was obtained by consensus (see Table 4.1).

Table 4.1: List of descriptors generated through flash profile for wines recently fermented (A) and aged wines (B).

A	B
acetic	dried fruits
alcoholic	fruits in syrup
black fruits	fruity
dried fruits	green
fruits in syrup	lactic
leather	leather
red fruits	metallic
solvent	rubber, plastic
vegetal	spicy
white fruits	white fruits

In the second sub-session, panelists were trained with commercial aroma references prepared in ethanol 15 % v/v by LAAE. Panelists were asked to associate the references to the descriptors. Judges able to correctly associate 80 % of the references were qualified. In the third sub-session, qualified panelists evaluated the six samples object of study (one replicate of each sample) together with one sample in duplicate to evaluate panel repeatability. The seven samples were presented simultaneously and panelists were asked to rank the samples for each attribute of the final list on a 10 cm graduated scale (1 cm intervals), from 0 (low intensity) to 10 (high intensity).

4.2.4 Statistical analysis

4.2.4.1 Volatile compounds quantifications: data transformation

For the purpose of understanding the potential sensory effects linked to aging or to the strain of yeast, concentrations were firstly transformed in OAVs by dividing concentration by odor threshold. Aroma compounds were then arranged into aroma vectors. Aroma vectors are groups of aroma compounds sharing chemical and sensory characteristics, whose sensory action is known to be additive (Ferreira et al., 2018). As a first rough approximation of the intensity of any vector in a given sample, the OAVs of the aroma compounds within the vector were summed. As OAVs are simply concentrations normalized by the threshold (available in Table 1.1.3 in the Chapter 1, Introduction), they cannot be used to predict the relative importance of a given odorant or group of odorants in a mixture, since that will ultimately depend on the particular psychophysical functions, which are not known, and on the existence of perceptual interactions between aroma vectors, most of which are also poorly known. However, they can provide a rough estimation of the number of different primary odors present in the mixture at detectable levels, and a reasonable estimation of their relative intensity between samples.

Aroma vector composition is detailed in the Table 4.2. Terpenes were separated into two groups. Terpenes 1 includes the members of this family found at higher

Table 4.2: Composition of aroma vectors, their generic and specific aroma descriptors in isolation (Ferreira et al., 2021). Compounds in grey indicate that they were not detected.

generic descriptor	**aroma vector**	**compound**	**specific descriptor**
acetic	acetic acid	acetic acid	acetic, vinegar
alcoholic, solvent	ethyl acetate	ethyl acetate	glue, ethyl acetate
	higher alcohols	β-phenylethanol isoamyl alcohol isobutanol benzyl alcohol 1-butanol cis-3-hexenol 1-hexanol methionol	harsh, spirit, solvent
flowery	cinnamates	ethyl dihydrocinnamate trans-ethyl cinnamate	sweet, balsamic
	ionones	β-ionone α-ionone	violets, berry
	β-phenylethyl acetate	β-phenylethyl acetate	floral, rose, sweet
	rose oxide	(+)-cis/trans-rose oxide	rose, litchi
	terpenes 1	β-citronellol geraniol linalool nerol 1,8-cineole R-limonene	jasmine, muscat, orange blossom
	terpenes 2	α-terpineol cis/trans-linalool oxide	jasmine, muscat, orange blossom
fruity	acetates	isoamyl acetate isobutyl acetate hexyl acetate	banana
	β-damascenone	β-damascenone	baked apple, dry plum
	ethyl esters	ethyl 2-methylbutyrate ethyl butyrate ethyl hexanoate ethyl isobutyrate ethyl isovalerate ethyl D/L-leucate ethyl octanoate ethyl propanoate diethyl succinate ethyl lactate ethyl 4-methylvalerate ethyl cyclohexanoate ethyl decanoate	fruity, apple, strawberry
	furaneol	furaneol	strawberry, sugary
	lactones	γ-nonalactone massoia lactone γ-butyrolactone	peachy

		δ-decalactone γ-octalactone trans/cis-whiskylactone	
lactic, acid	branched acids	isobutyric acid isovaleric acid	cheese, sweaty
	diacetyl	diacetyl	buttery, milky, yogurt
	linear fatty acids	decanoic acid hexanoic acid octanoic acid butyric acid	cheese, soapy
spice, woody	methoxyphenols	4-ethylguaiacol 4-vinylguaicol 4-vinylphenol guaiacol m-cresol methoxyeugenol o-cresol trans-isoeugenol eugenol 4-propylguaiacol syringol 4-ethylphenol	clove, smoky
	TDN	TDN	kerosene
	vanillins	acetovanillone vanillin syringaldehyde	vanilla, nutmeg
yeasty, oxidized	acetaldehyde	acetaldehyde	green apple, oxidized

levels in young wines, while terpenes 2, includes those ones found at higher levels in aged samples. Some compounds presenting specific chemo-sensory characteristics were not grouped and appear individually. It is the case of acetaldehyde, acetoin, diacetyl, acetic acid, ethyl acetate, β-phenylethyl acetate, β-damascenone, furaneol, rose oxide and TDN. For each aroma vector, OAVs of individual compounds were summed (summed-OAV). Aroma vectors with summed-OAV (or OAV in the case of aroma vector composed of an individual compound) inferior to 0.2 were not considered, since they were probably non-perceptible from a sensory point a view. This arbitrary value has been fixed according to previous studies (San Juan et al., 2012). That was the case for furaneol (not detected), acetoin, rose oxide, vanillin and terpenes 2. For each group of samples (young and aged), a PCA was generated with the OAVs of the retained aroma vectors as active variables. The potential sensory difference introduced by the strain of yeast in each aroma vector was assessed calculating the ratio between maximum and minimum average OAVs (average of 3 replicates).

4.2.4.2 Analysis of variance

The effects of the factors yeast (IONYS and D254) and aging (young and aged wines) were evaluated using one-way and two-way analysis of variance (ANOVA).

4.2.4.3 Sensory data treatment

Sorting task and flash profile data treatment are detailed in the Materials and methods part.

4.2.4.4 Correlation between chemical and sensory data

In order to investigate the correlations between the levels of volatile compounds and sensory scores obtained in the flash profile, two PCAs were generated, one for each group of recently fermented or aged samples. All the aroma vectors were considered as active variables. The scores (sample ranks) obtained in flash profile

of the descriptors with frequencies of citation superior to 20 %, were introduced in the PCA plots as supplementary variables. Spearman correlation coefficients were also calculated. RV coefficients were calculated to evaluate the degree of similarity between chemical and sensory spaces generated through PCA (chemical variables) and GPA (sensory descriptive variables), respectively.

4.3 Results and discussion

Fermentations of Tempranillo must were carried out with two *S. cerevisiae* yeast strains. In both cases, fermentations lasted 9 days. Maxima yeast population was reached after 3 days of fermentation at 13.3 ± 5.8 x 10^7 CFU/mL for D254 and at 4.6 ± 2.0 x 10^7 CFU/mL for IONYS, not significantly different.

The chemical parameters of the original must and of the recently fermented wines are presented in Table 4.3. As can be seen, the wine fermented by D254 contained a slightly but significantly higher amounts of residual sugars than that fermented by IONYS. Nevertheless, the low levels measured suggest that alcoholic fermentation was properly performed. No other significant difference was found, except for volatile acidity, whose level is three times smaller for IONYS (0.15 g/L) than for D254 (0.52 g/L). The production of low levels of acetic acid by IONYS in comparison with other commercial yeasts has already been reported in previous studies (Pérez et al., 2018).

4.3.1 Volatile composition and aroma vectors

Major and trace volatile compounds were measured after fermentation in the two strains and after anoxic accelerated aging (5 weeks at 50 ^{o}C). Overall, 64 compounds were detected at concentrations above detection limits (data presented as average ± standard deviation is available in supplementary material, Table C.1 in Annex C).

Overall, the aroma compounds measured were compiled into 17 aroma vectors

Table 4.3: Conventional oenological parameters of must and wines after alcoholic fermentation carried out by the yeast strains D254 and IONYS. Significance of the factor yeast is indicated by * (pvalue < 0.05).- indicates that data is not available.

	must	**IONYS**	**D254**
alcohol (% v/v)	-	12.5 ± 0.4	13.0 ± 0.1
glucose and fructose (g/l)*	203 ± 6	0.6 ± 0.1	1.0 ± 0.2
free SO_2 (mg/l)	-	4 ± 2	4 ± 2
total SO_2 (mg/l)	-	13 ± 4	13 ± 4
pH (20 °c)	3.33 ± 0.02	3.43 ± 0.08	3.48 ± 0.01
total acidity (g/l)	4.3 ± 0.1	7.2 ± 0.1	7.3 ± 0.2
volatile acidity (g acetic/l)*	-	0.15 ± 0.01	0.52 ± 0.02
L-malic acid	2.0 ± 0.1	1.81 ± 0.09	1.74 ± 0.09
lactic acid	-	0.08 ± 0.01	0.09 ± 0.01
acetaldehyde (g/l)	-	54 ± 10	32 ± 16

with combined OAVs above 0.5 in at least one of the samples. Seven of them are mono-component, and the rest are formed by mixtures of odorants of similar odors. The most complex aroma vectors are the ethyl ester aroma vector, integrated by 13 ethyl esters (De-la Fuente-Blanco et al., 2020) and the methoxyphenols vector, which integrates 10 odorants, most of them at sub-threshold levels. Table C.2 in Annex C gives the combined OAVs of the 17 aroma vectors, together with results from the two-way ANOVA to evaluate the effect of yeast, aging and their interaction on those aroma vectors. The exact composition of each vector and the complete ANOVA results are available in the Tables 4.2 and C.3 respectively.

4.3.2 The effect of accelerated aging on aroma vectors

Twelve out the 17 aroma vectors resulted significant for the aging effect. These are marked with a T in the Table C.3 and are diacetyl, acetic acid, ethyl acetate, acetates, β-phenylethyl acetate, cinnamates, ionones, terpenes 1, ethyl esters, lactones, methoxyphenols and TDN. Interestingly, 2 vectors (ethyl acetate and TDN) showed a significant interaction of yeast and aging factors (marked with a *), suggesting that the effect of aging on these vectors was dependent on the yeast that fermented the original wine. Results are also represented as boxplots in Figure 4.2.

Aging was not significant for acetaldehyde, higher alcohols, linear and branched fatty acids and β-damascenone, mostly in accordance with previous reports (Cassino et al., 2019; Makhotkina and Kilmartin, 2012; Marais and Pool, 1980).

Regarding fermentative compounds, levels of diacetyl decreased a factor 2 in average during aging, likely because of its reactivity towards amino acids (Bueno et al., 2018; Pripis-Nicolau et al., 2000) and polyphenols (Blanco-Vega et al., 2011). On the contrary, levels of acetic acid increased significantly around 200 mg/L for both strains. The cause of these increases is not clear. Aging took place under strict anoxia and at 50 ^{o}C, and other oxidation or microbial spoilage markers, such as acetaldehyde or fatty and branched acids, respectively, did not show any increase,

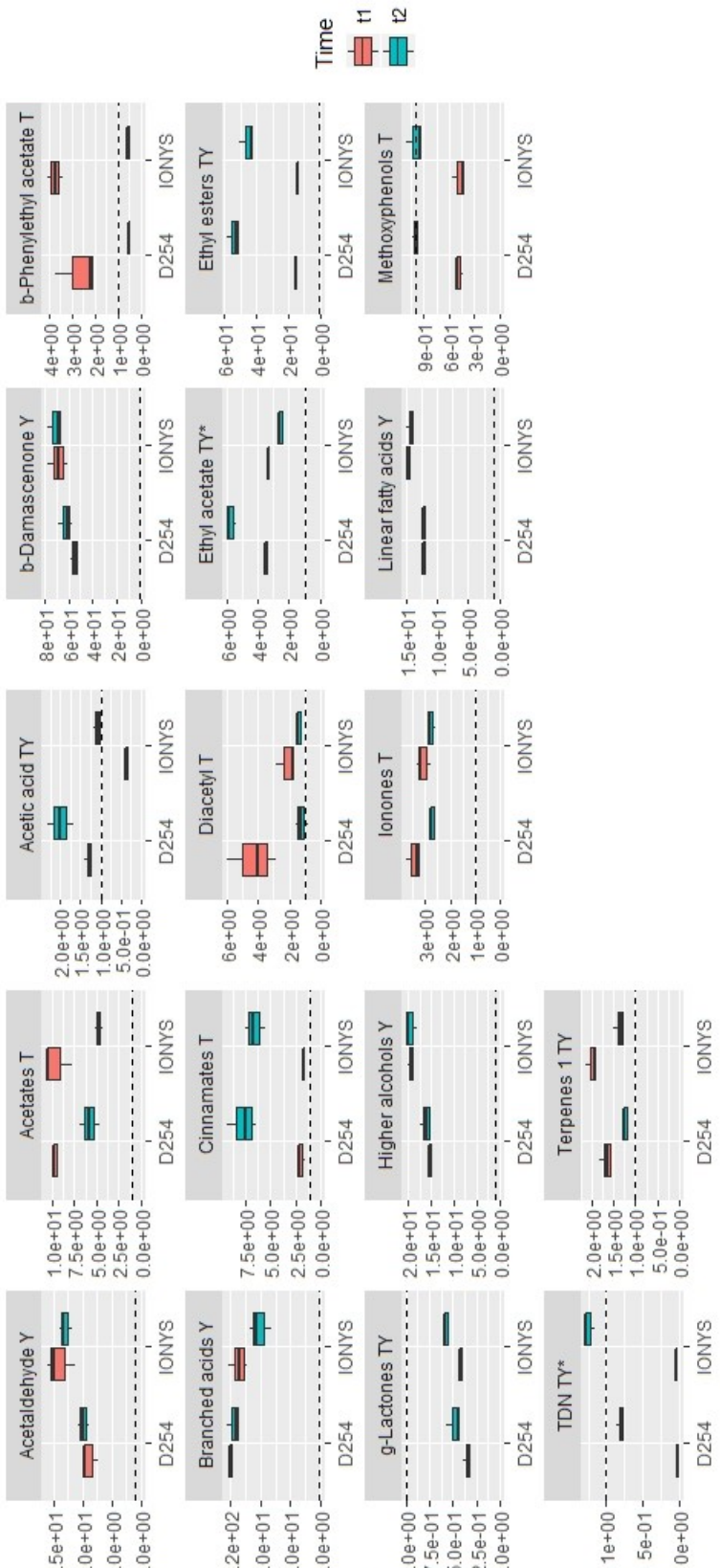


Figure 4.2: Boxplots representing the combined OAVs of each aroma vector for the four different samples produced in this study. t1, wines recently fermented (in red); t2, wines after accelerated ageing (in blue). Significance of the factors yeast, ageing time and their interaction are indicated by Y, T and * respectively. The dotted horizontal line is set at y = 1.

which suggests that the increase is not due to oxidation of microbial spoilage. The origin could be the hydrolysis of acetates, but the decreases with aging of isoamyl and β-phenylethyl acetates are too low to justify the observed increase in acetic acid, which may suggest the existence of an acetate not quantified in the present experiment. On the other hand, the ethyl esters aroma vector strongly increased (factor > 3) during aging. The increase is nearly entirely attributed to the slow esterification with ethanol of branched acids, 2-methylbutyric, isobutyric and isovaleric acids (Table C.1), to yield the corresponding aroma-powerful ethyl esters whose concentration increases by a factor 4. Ethyl esters of short chain fatty acids (ethyl propanoate, ethyl butyrate), esters of organic acids (ethyl succinate, lactate) and ethyl leucate also increased around factor 1.5; while esters of long chain fatty acids (ethyl octanoate and hexanoate) slightly decreased. Similar evolutions have already been observed during bottle aging (Cassino et al., 2019; Díaz-Maroto et al., 2005; Marais and Pool, 1980).

Leaving aside β-damascenone, varietal aroma vectors deeply change during aging. Ionones and terpenes in the first group, which are labile aroma compounds, decreased, while ethyl cinnamates, lactones, methoxyphenols and TDN, increase, mostly in agreement with previous reports (Denat et al., 2021; Loscos et al., 2010; Oliveira and Ferreira, 2019). The vector called cinnamates is formed by the ethyl ester of cinnamic acid - ethyl cinnamate - and by ethyl dihydrocinnamate. It is interesting to note that these compounds have a characteristic sweet and flowery note, whose increase counterbalances the loss of the floral terpenols, particularly of the two most powerful; geraniol and linalool, whose levels strongly decrease during aging. The increase of TDN with its kerosene note may have some sensory relevance, as well as the increase of the vector formed by methoxyphenols, with spicy and toasted notes. As aforementioned, this complex vector is composed by 10 odorants at subthreshold levels (Figure 4.2) and its increase is due to strong increases with aging of 4-vinylguaiacol, 4-vinylphenol, guaiacol and methoxyeugenol (4-allyl-2,6-dimethoxyphenol), likely as the consequence of the

hydrolysis of glycosidic precursors (Ferreira and Lopez, 2019).

In summary, aging deeply changes the aroma profiles of wine in our accelerated aging conditions. The most powerful effects are the strong decrease in the characteristic fruity and flowery notes of acetates and terpenes, which are replaced by the subtler flowery notes of ethyl cinnamates, by the fruity notes of ethyl esters, and by the spicy, toasty and empyreumatic character developed with time as the consequence of the increases in methoxyphenols and TDN.

4.3.3 The effect of accelerated aging on sensory properties: sorting task

In the non-verbal classification, control duplicate samples were clustered close together, confirming that the classification task was solid. The 14 samples (the 12 wines plus the 2 controls) led to 3 main clusters as can be seen in Figure 4.3. It can be observed that samples were clearly differentiated according to aging time, which was the dominant factor on the sensory characteristics of the samples. Aged wines were grouped in cluster 1 and were mainly characterized by the descriptors "lactic", "solvent", "dried fruits" and "fresh fruits". The effect of yeast strain was not clearly recognized in this set of wines, suggesting that age was a too-strong dominant or salient factor. It is known that in these types of tasks, panelists tend to sort samples according to the most salient differences (Moussaoui and Varela, 2010). On the contrary, in recently fermented wines the effect of strain is secondary, but it can be observed, since the wines are classified attending to the yeast in two subclusters within cluster 2, with a mismatch in Dt1c, and were described as "fresh fruits". Cluster 3 includes the mismatched replicate of D254 and the duplicates of the commercial young wine and is mainly described as "vegetal" and "spicy". Both series of samples were further analyzed separately in order to evaluate more precisely the effect of strain.

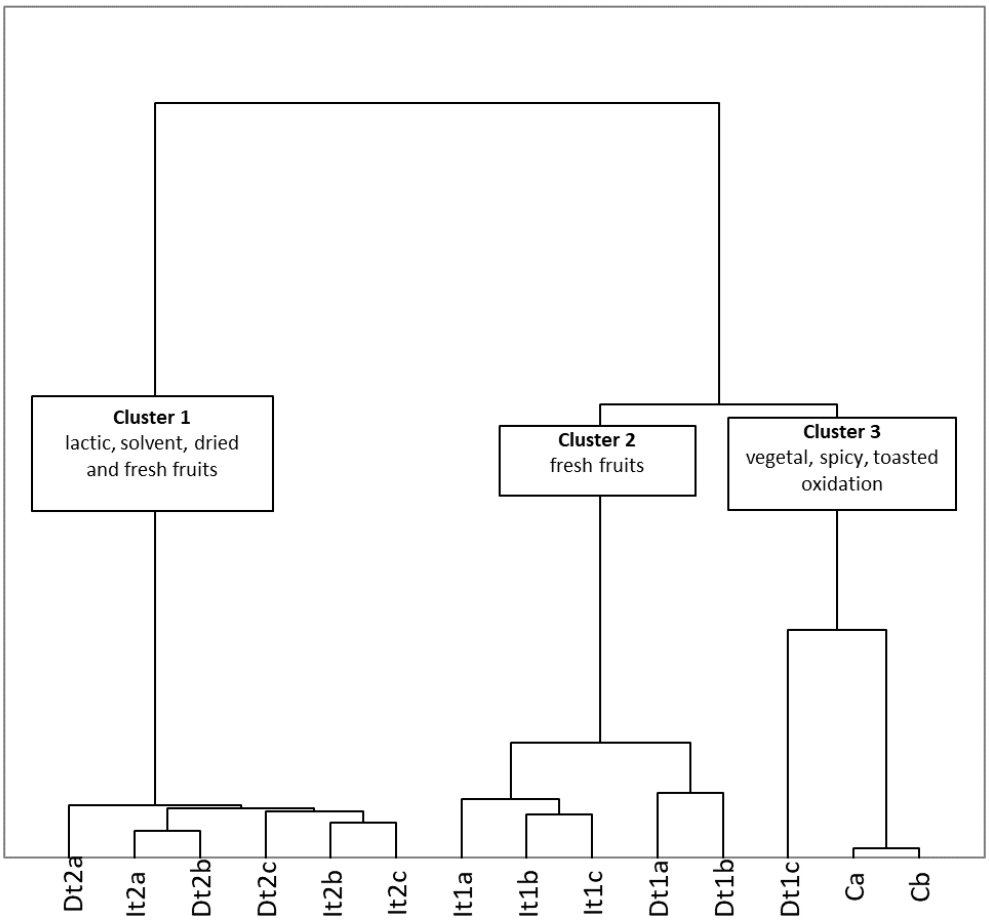


Figure 4.3: Dendrogram obtained from the data generated with the sorting task. Samples are encoded as follows: I, IONYS; D, D254; t1, wines recently fermented; t2, wines after accelerated aging; C, commercial wine; a-b-c, replicates.

4.3.4 Effect of yeasts on aroma vectors

As can be seen in Table C.2, the strain of yeast exerted a significant effect on 11 out of the 17 aroma vectors; seven out of 10 fermentative vectors and 4 out of 7 varietal vectors were significantly affected. Diacetyl and the 2 acetate vectors were the only ones not affected among fermentative compounds, while cinnamates, ionones and methoxyphenols were the varietals not significantly affected by the yeast strain. Detailed results of the ANOVA study are given in Table C.3. The relative weight of each one of the aroma vectors on the differentiation between strains can be seen with the help of Figure 4.4. That figure shows, for young and aged wines, the ratios between the highest average-OAV and the lowest average-OAV for each pair of samples fermented with the two strains. The most noticeable observation is that most differences are of little magnitude.

In fact, such ratio is significant and above 1.5 only for acetic acid and acetaldehyde in young wines (diacetyl was too variable), while only acetic acid, ethyl acetate and TDN were above this value in aged wines. It should be noted, however, that these most discriminant aromatic vectors are not likely to have a major sensory impact given its actual levels in the present set of wines (see Table C.1). The higher levels of acetic acid measured in D254 samples (Table C.1), will surely make the fruity character of this sample decrease, as it has been previously reported for levels above 0.5 g/L (San Juan et al., 2012), but levels are not enough to perceive the specific acetic acid character.

The effects of acetaldehyde, present at 4.5 and 7 mg/L will be most likely significant but subtle, as recently suggested (Arias-Pérez et al., 2021). As for ethyl acetate, its maximum levels are below 70 mg/L, while it has been reported that its nail polish remover notes only becomes perceptible above 80 mg/L (Plata et al., 2003). Finally, TDN levels are above reported thresholds in white wine, but yet, are very low and it can be anticipated that effects in red wine will be less obvious. The other seven aroma vectors introducing significant differences differ by factors smaller than 1.5, as can be seen in Figure 4.4, which suggests that the sensory effects

introduced by yeast are subtle and are the consequence of little variations in many aroma vectors.

4.3.5 Effect of yeasts on sensory properties: descriptive analysis

Results from the descriptive analysis were processed by GPA and HCA, as summarized in Figure 4.5. The clusters found in the HCA are within the dotted circles which, as can be seen, contain samples made with a single strain. This confirms that in both young and aged wines, the sensory effects introduced by the strains were clearly recognized. As the ANOVA study revealed, young wines fermented by D254 were characterized by black fruits, while those fermented by IONYS were characterized by white fruits. Aged samples made with D254 were characterized by fresh fruits and fruity, while those made with IONYS are described by the terms "lactic", "fruits in syrup" and "white fruits". As observed in the sorting task, the replicates Dt1c and Dt2a were more dissimilar. The relationships between the chemical and sensory spaces are summarized in the PCA plots provided in Figures 4.6a and 4.6a, for recently fermented and aged samples, respectively.

The first relevant observation is that in both plots there are some descriptors which are in areas without aroma vectors. This is particularly obvious in plot 4.6b for the descriptors green, metallic and lactic, but also in plot 4.6a for the descriptor vegetal. This result is not surprising and has a double origin. First, it has been shown that some vegetal character seems to be part of any wine aroma reconstitution not having any marked sensory descriptor (Ferreira et al., 2016) and, second; vegetal, green and metallic characters may be also related to the presence of up to 15 aldehydes (unsaturated, saturated and Strecker aldehydes) at sub and peri-threshold levels which were not quantified here (Arias-Pérez et al., 2021).

A second relevant observation is that the two sensory descriptors projected on the center of both plots, alcoholic in 4.6a and dried fruits in 4.6b, have been also reported to be common characters to all wine-like aroma reconstitutions (Alegre

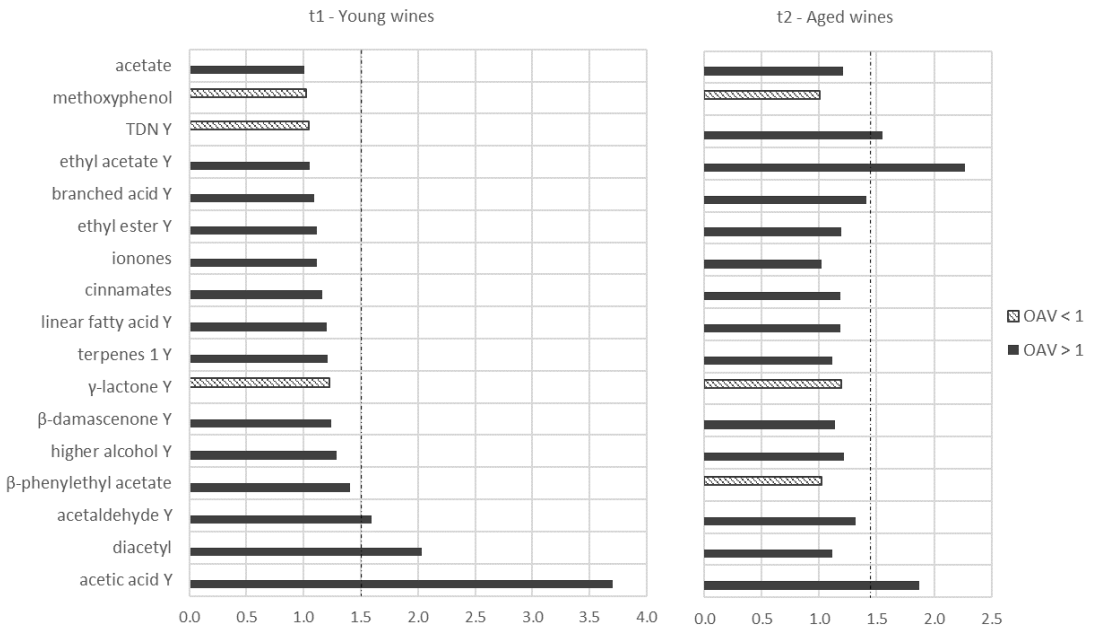


Figure 4.4: Representation of the ratio OAVmax/OAVmin for wines recently fermented (on the left) and aged wines (on the right). Aroma vectors with OAV > 1 are coloured in black and the ones with OAV < 1 are hatched. The dotted line placed at 1.5 was placed to highlight the most discriminant vectors.

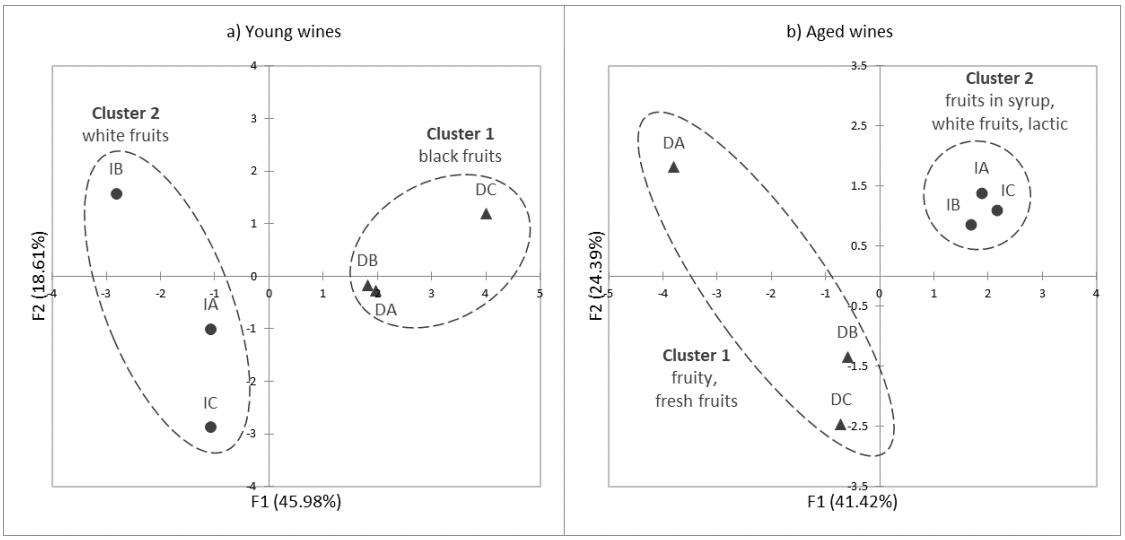
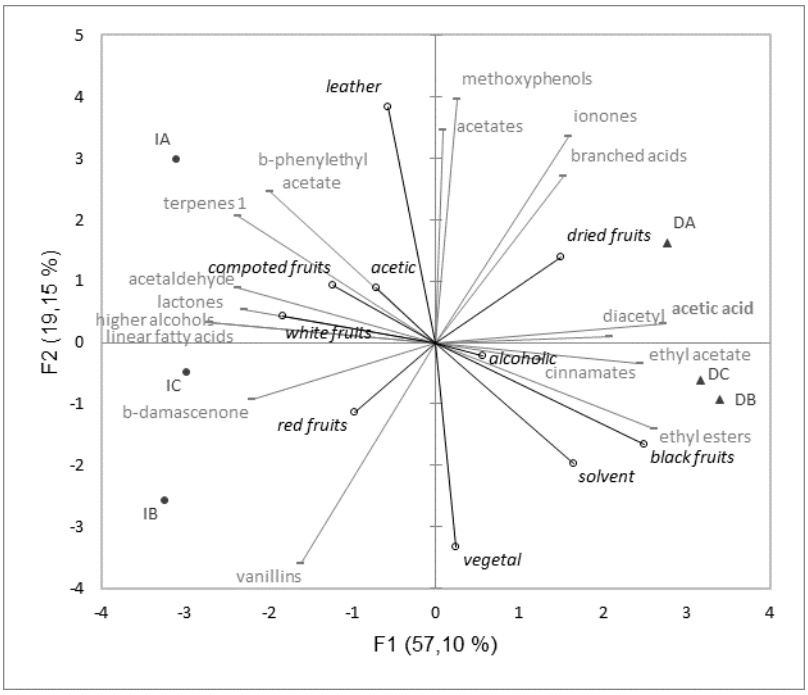
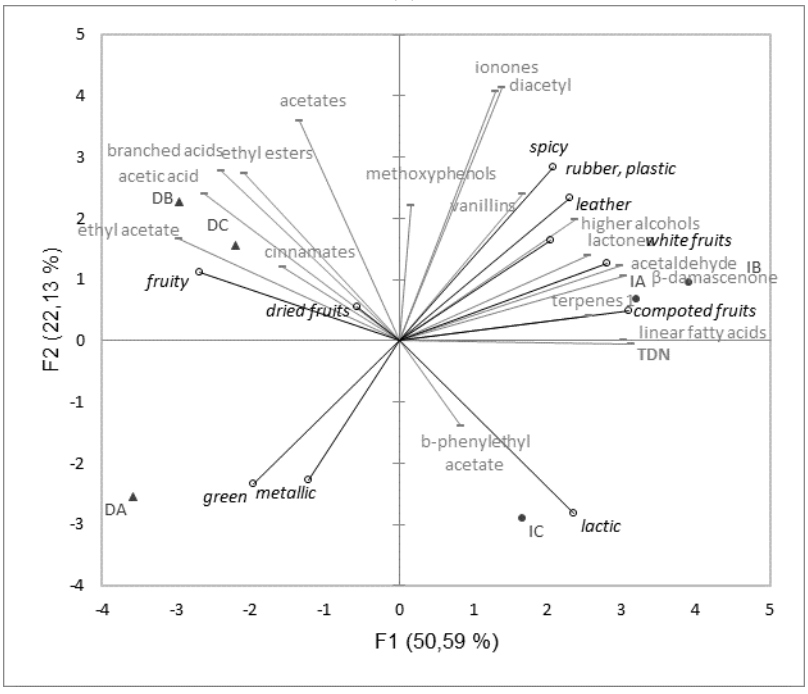


Figure 4.5: Graphical representation of the two first dimensions obtained by GPA from flash profile. On the left, samples recently fermented; on the right, samples after accelerated aging. Dotted circles indicate the clusters generated in the HCA study and the associated descriptors. Samples are codified as follows: I, IONYS; D, D254; A-B-C, replicates.



(a)



(b)

Figure 4.6: PCA of the young (a) and aged (b) wines are represented. Aroma vectors, as principal variables are coloured in grey. Descriptors, as supplementary variables, are represented in italic black.

et al., 2020; Ferreira et al., 2016). This implies that they are not discriminant which explains their position in the plots.

Third, in both plots, the terms black fruit (4.6a) and fruity (4.6b), are at the opposite side of compote and white fruits and, in both cases the former terms are related to ethyl esters, acetic acid, cinnamates and ethyl acetate, and the latter terms are related to linear fatty acids, β-damascenone, acetaldehyde, higher alcohols and lactones. The similarity between both representations strongly suggests that these ratios are key determinants of the type of fruity descriptors perceived in these Tempranillo wines. It is noteworthy that this seems to happen even if the composition of the fruity vector completely changes as a result of aging.

Finally, in both representations it can be seen that the leather character could be related to the presence of methoxyphenols.

4.4 Conclusion

Both, aging time and the yeast strain used in fermentation, introduce deep changes in the chemical and sensory aroma profiles of Tempranillo red wines. Aging affected to 12 and yeast strain to 11 out of the 17 aroma vectors in which the odorants of the wines were classified. From the sensory point of view, aging was clearly dominant, since judges used age as the first criterion to classify samples. This is because during aging and in our conditions of accelerated aging, the characteristic fruity and flowery notes of acetates and terpenols, were replaced by the less explicit fruity and sweet-flowery notes of ethyl esters and of cinnamates, respectively. Levels of methoxyphenols and TDN also increased. Nevertheless, the effects of the yeast strain were evident and were consistently identified through aging, both from the sensory and chemical points of view. Chemically, wines made with D254 contained consistently higher levels of ethyl esters, acetic acid, cinnamates and ethyl acetate and lower levels of linear fatty acids, β-damascenone, acetaldehyde, higher alcohols and lactones than those made with IONYS. The first profile was related to black and fresh fruit notes, while the second to white and compote fruits.

This highlights that, by introducing quantitatively small but systematic changes in many aroma vectors, both fermentative and varietal, the yeast strain can consistently modulate wine aroma throughout its shelf life.

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Chapter 5

The diverse effects of yeast on the aroma of non-sulfite added white wines throughout aging

5.1 Introduction

While the general white wine market is expected to grow at slow pace, there is an increasing demand for premium products with attractive characteristics, aging potential and free from added sulfites (Fact.MR, 2017). One obvious ways to seek for such a goal is by using selected strains, not only to ensure a reliable and controlled fermentation process, but also to optimize the release and/or formation of varietal aroma and, if possible, to guarantee and even increase wine longevity (Swiegers and Pretorius, 2005).

The ability of yeast strains to modulate fermentative aroma profiles is well-known. Those compounds are alcoholic fermentation by-products such as acetic acid, hydrogen sulfide, ethyl acetate, ethyl esters of fatty acids, higher alcohols and their acetates, usually present at concentrations above 0.2 mg/L (Swiegers et al., 2005). Selected strains modulating some of these compounds have been commercially available for several years now. For instance, for higher production of the acetates of higher alcohols (Rollero et al., 2016), or of ethyl esters (Swiegers et al., 2006), or of smaller amounts of acetic acid (Tilloy et al., 2014). There is still an active research for strains minimizing the formation of hydrogen sulfide (Agarbati et al., 2020) or of ethanol and higher alcohols (Zheng et al., 2020). The ability to increase or optimize aroma varietal characteristics is also highly demanded and has been the subject of intensive research (Gamero et al., 2011; Lambrechts and Pretorius, 2000; Loscos et al., 2007). An obvious target, given their strong and dominant aromatic characteristics, is the overproduction of varietal polyfunctional mercaptans (PFMs). Numerous researchers have identified yeast strains able to produce higher levels of these compounds from the same pool of precursors (Roland et al., 2011; Swiegers and Pretorius, 2007) and particularly, for being able to transform 3-mercaptohexanol (MH) in the more aroma-explicit 3-mercaptohexyl acetate (MHA) (Swiegers et al., 2009).

The action of yeasts on aromas present in grapes as glycosidic precursors has also been the subject of much research (Bisotto et al., 2015; Ugliano et al.,

2007). Here, the identification of good candidates is far more complex because of a series or reasons, including the lack of so-clear target aroma compounds, the complexity of the precursors fraction and, particularly, because of the relevance of aging in the release or the decay of some aroma compounds (Ferreira and Lopez, 2019). For instance, premature hydrolysis of linalool and geraniol glycosidic precursors will enhance wine aroma in the short place but it will inevitably reduce wine aging potential, since these compounds are extremely short-lived at wine pH (Williams et al., 1980). Additionally, recent research has also demonstrated that yeasts can modulate some aroma molecules formed only after long periods of aging such guaiacol (Denat et al., 2021a; Oliveira and Ferreira, 2019), or TDN (1,1,6-trimethyl-1,2-dihydronaphthalene), most likely via the specific action of reductases on the precursors (Grebneva et al., 2019).

The contribution of yeasts to wine longevity is yet poorly known, not only because of the limited number of studies including a long aging perspective, but because wine aroma longevity itself is mostly related to three major factors which, to the best of our knowledge, have never been studied together. These factors are (1) the accumulation of Strecker aldehydes, (2) the survival during aging of PFMs and (3) the formation during aging of fruity ethyl esters by esterification with ethanol of branched acids. Leaving aside this last group of compounds which constitutes the backbone of the fruity perception in aged wines and for which the impact of yeast strain is already known (Gammacurta et al., 2014), there is very little, if any, information about the effects of yeast on the long-term aging of the other aroma compounds.

Strecker aldehydes have a most dominant effect on wine characteristics. If present at little amounts (tens of μg/L), they will introduce typical oxidation characteristics, leading to a clear quality loss in table wines (Marrufo-Curtido et al., 2021). Although Strecker aldehydes are related to wine oxidation, they could be also formed via some pathways unrelated to oxidation. First, they are part of the Ehrlich pathway, which is essential to produce fermentative aroma compounds (Hazelwood et al., 2008). In

fact, we have recently demonstrated that isobutyraldehyde (2-methylpropanal) is a major component of the volatile fraction evaporated during fermentation (Denat et al., 2021a). Additionally, Strecker aldehydes can be formed through the reaction between the amino acid precursor and different α-dicarbonyls (de Revel et al., 2000; Rizzi, 2006, 2008), some of which are normal by-products of all fermentations. Therefore, the formation of Strecker aldehydes during non-oxidative wine aging cannot be discarded. The effect of yeast on these two potential pathways for the formation of Strecker aldehydes is not known.

Regarding the survival of PFMs during aging, a previous report already alerted that the influence of yeast extended throughout wine aging (King et al., 2011). Furthermore, there are increasing evidences provided by metabolomic studies, suggesting that wine longevity is strongly related to the presence of sulfur-containing compounds in wine (Romanet et al., 2019), mostly proteins and peptides (Romanet et al., 2021), which may have an antioxidant capacity comparable to those of phenolic compounds. It seems apparent that those sulfured compounds can protect PFMs from their irreversible reaction with wine quinones, both by forming disulfides (Nikolantonaki et al., 2012), and by competitive reaction (Nikolantonaki et al., 2014). The possible impact of yeast on the stability of these labile compounds with aging is not known.

Because of all these reasons, our main goal in this paper is to assess the differences introduced by the yeast strain in the development of varietal aroma throughout aging and wine longevity, paying particular attention to Strecker aldehydes and PFMs. The impact of three *S. cerevisiae* strains will be studied on the complete chemical aroma profiles derived from the fermentation of semi-synthetic grape must containing phenolics and aroma precursors extracted from Albariño grapes, plus cysteinyl and glutathionyl aroma precursors, throughout their accelerated anoxic aging.

5.2 Materials and methods

All the methods and compositions are detailed in the Chapter 2, Materials and Methods.

5.2.1 Vinification

5.2.1.1 Semi-synthetic must preparation

A semi-synthetic must was prepared and added with a Phenolic and Aroma precursor Fraction (PAF) extracted from Albariño grapes. Synthetic glutathionylated and cysteinylated precursors of MH and MHA were also added.

5.2.1.2 Yeast strains

The three *S. cerevisiae* yeast strains were Lalvin QA23^{TM}, Lalvin Sauvy^{TM} and Affinity ECA5^{TM} active dry yeasts from Lallemand Bio. They were rehydrated and added at 30 g/hL to the must. Yeast cell viability was monitored by plating the appropriate dilution of fermenting must on YPD solid media (2 % glucose, 2 % agar, 0.5 % peptone, 0.5 % yeast extract).

5.2.1.3 Fermentation system and monitoring

Fermentations were carried out in triplicates, 0.8 L of must was placed in 1-L Pyrex bottles closed with Muller valves. Sterile semi-synthetic must without yeast inoculation was also submitted to the same preparation process. Constant agitation was set at 200 rpm. Temperature was maintained at 18-22 ^{o}C. Fermentations were monitored by daily weighing.

5.2.1.4 End of fermentation and accelerated aging

At the end of fermentation, when the weight loss between two consecutive days was smaller than 0.1 g, wines were decanted during 24 hours at 4 ^{o}C and then centrifuged at 10 ^{o}C, 4500 rpm during 10 minutes. Wines were bottled in 0.75-L green glass

bottles, closed with a wine stopper (Vacu Vin, Spain) after displacement of air with a nitrogen flux.

Wines were conditioned and submitted to accelerated anoxic aging at 50 ºC during 1, 2, 5 and 8 weeks, and 75 ºC during 12, 24, 48 and 96 hours.

5.2.1.5 Conventional enological analysis

Oenological parameters were measured in the recently fermented wines including reducing sugars, ethanol, pH, volatile and total acidity, free and total sulfur dioxide.

5.2.2 Experimental design

As represented in the Figure 5.1, five sampling times were considered in this study: t0 - recently fermented wines, t1 - wines after 1 week of accelerated anoxic aging at 50 ºC or 12 h at 75 ºC; t2 - wines after 2 weeks at 50 ºC or 24 h at 75 ºC; t3 - wines after 5 weeks at 50 ºC or 48 h at 75 ºC; t4 - wines after 8 weeks at 50 ºC or 96 h at 75 ºC. The yeast strains were codified as follow: QA23, SAUVY and ECA5. Samples were stored at 4 ºC after conditioning in anoxia, and extracts at -20 ºC.

In the first part of the chapter, the role of yeast on the modulation of wine volatiles throughout aging will be analysed and discussed using the data obtained through accelerated aging at 50 ºC. The relationships between the two aging temperatures will be discussed in a second part.

5.2.3 Wine aroma analysis

5.2.3.1 Major compounds analysis

Major metabolites of alcoholic fermentation (higher alcohols and their acetates, volatile fatty acids and their ethyl esters, branched fatty acids and their ethyl esters, acetoin, diacetyl, and acetaldehyde), usually present in wines at levels above 0.2 mg/L, were analysed by GC-FID.

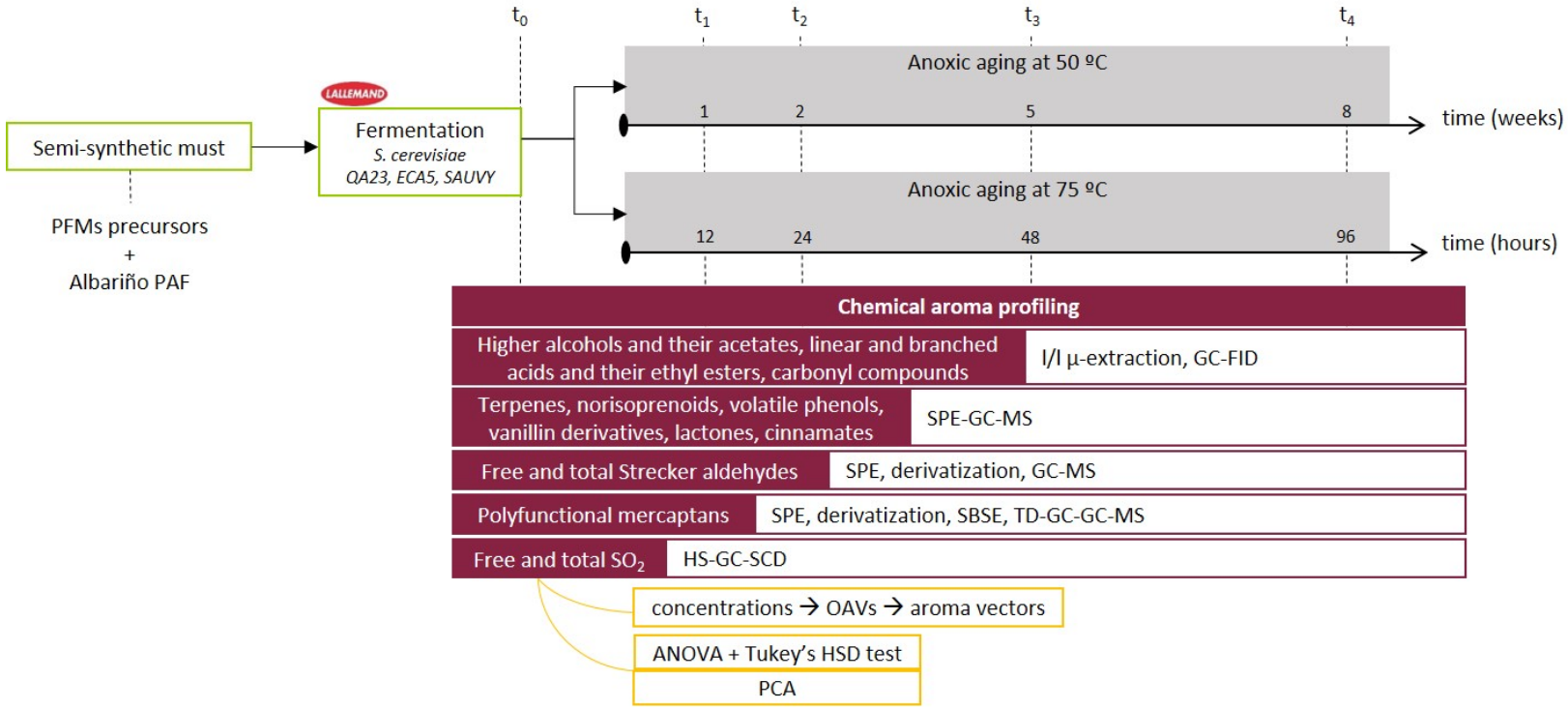


Figure 5.1: Experimental procedure - A semi-synthetic must of Albariño was fermented with 3 *S. cerevisiae* yeast strains. Wines were submitted to anoxic accelerated aging at 50 and 75 ºC. Wine aroma profiles were analysed via volatiles quantifications.

5.2.3.2 Trace compounds analysis

Minor aroma compounds present in wine at levels around 0.1-200 μg/L (branched ethyl esters, terpenes, norisoprenoids, vanillin derivatives, volatile phenols) were analysed by GC-MS.

5.2.3.3 Strecker aldehydes analysis

The analysis of free and total Strecker aldehydes (isobutyraldehyde, 2 and 3-methylbutanal, methional and phenylacetaldehyde) were carried out by GC-MS.

5.2.3.4 PFMs analysis

PFMs, including MH, MMP, MHA and FFT, were analysed by GC-GC-MS,

5.2.4 Statistical analysis

5.2.4.1 Effect of the factors yeast and aging: data set obtained at 50 ^{o}C

The significance of the factors yeast and aging time were determined via 2-way ANOVA on the data (volatiles concentration) collected after aging at 50 ^{o}C. One-way ANOVA and Tukey HSD test were also performed at each of the 5 sampling points in order to determine the specific differences between the yeasts. A PCA was also carried out.

5.2.4.2 Assessing potential sensory relevance

Concentrations were normalized by their olfaction threshold to yield Odor Activity Values (OAVs). If a compound was not detected, its concentration was replaced by its detection limit in the corresponding analytical method. Aroma compounds were then gathered into 26 aroma vectors, whose composition is detailed in the Table D.1 in Annex D, and the OTs are available in the Table 1.1.3 (Chapter 1, Introduction). Compounds presenting specific chemo-sensory characteristics were considered individually: acetic acid, ethyl acetate, diacetyl, acetaldehyde,

methional, phenylacetaldehyde, β-phenylethyl acetate, (+)-cis/trans-rose oxide, β-damascenone, MH, MP, MHA and TDN. The odor intensity scores of these 13 cases were estimated as the square root of their OAVs. The other 13 aroma vectors were composed of several volatiles sharing chemical and sensory characteristics, whose sensory action is known to be additive (Ferreira et al., 2021b). These were higher alcohols, acids, isoaldehydes, cinnamates, ionones, terpenes, esters, acetates, lactones, vanillins, ethylphenols, vinylphenols and methoxyphenols. In these cases, their odor intensity scores were estimated as the square root of the sum of the OAVs of all compounds in the vector. Additionally, some more generic aroma vectors were created by grouping together aroma vectors with relatively similar aroma (spicy, fresh, fruity, flowery, yeasty, lactic, alcoholic and acetic) as indicated in Table D.1. Odor intensity scores were equally estimated as the square root of the sum of the OAVs of all compounds in the vector.

A discriminant ratio was also calculated for each aroma vector and generic descriptor by calculating the ratio between the maxima and the minima odor intensity score within the 3 yeasts. In the cases in which the minima OAV was inferior to 1, the discriminant ratio was simply the maxima odor intensity score.

5.2.4.3 Correlations between accelerated aging at 50 and 75 ºC

Pearson correlation coefficients (R) and their significance were calculated.

5.3 Results and discussion

5.3.1 Effects of yeast on aroma composition and evolution at 50 ºC

Overall, 86 different aroma compounds have been successfully quantified using five different GC methods in samples fermented with three commercial yeasts and aged at 50 ºC for 5 different times. The complete set of results is given in the Tables D.2 to D.6 and the ANOVA results in the Table D.7 in Annex D. Both yeast and

aging time exerted a deep and strong effect on aroma composition. A 2-way ANOVA analysis revealed that levels of more than 50 aroma compounds were significantly affected by yeast and more than 60 were affected by time. In addition, in 33 cases the interaction yeast x time was significant (Table D.7). The combined effects of yeast and time can be visualized in the PCA plot shown in Figure 5.2.

In order to simplify the variable biplot, only the compounds significantly affected by yeasts and/or aging time were conserved (Table D.7). Since total and free Strecker aldehydes amounts were very close, only the total concentrations were conserved and total SO_2 was also preferred to the free concentrations, close to the detection limit. It can be appreciated that in this particular case and in clear contrast to a previous work (Denat et al., 2021a), the effect of yeast is evident and approximately equivalent throughout the whole wine shelf-life. This is quite surprising since, as the variable loading plot shows, very few compounds, including higher alcohols, linear fatty acids and their ethyl esters, and γ-octalactone, remain approximately constant throughout wine shelf-life. Labile terpenes, vinylphenols, acetates and polyfunctional mercaptans decrease during aging, while a complex amalgam of many compounds including stable terpenols, ethyl esters of branched acids, carotenoid breakdown products or the aglycones of some glycosides, increase during aging. This implies that yeast is able to introduce equivalent but distinct differences throughout aging, so that yeast-strain markers as well as yeast-strain related sensory properties will change with aging. In the following sections, these changing differences will be analyzed, with a particular emphasis on those not previously described (Denat et al., 2021a; Oliveira and Ferreira, 2019) and/or with a more likely impact on wine sensory properties.

5.3.1.1 Aroma compounds related to the Ehrlich pathway

The Ehrlich pathway is a net of metabolic routes related to the amino acid catabolism of yeast and is one of the most important sources of aromatically relevant secondary metabolites of *S. cerevisiae* yeasts (Hazelwood et al., 2008). Seventeen

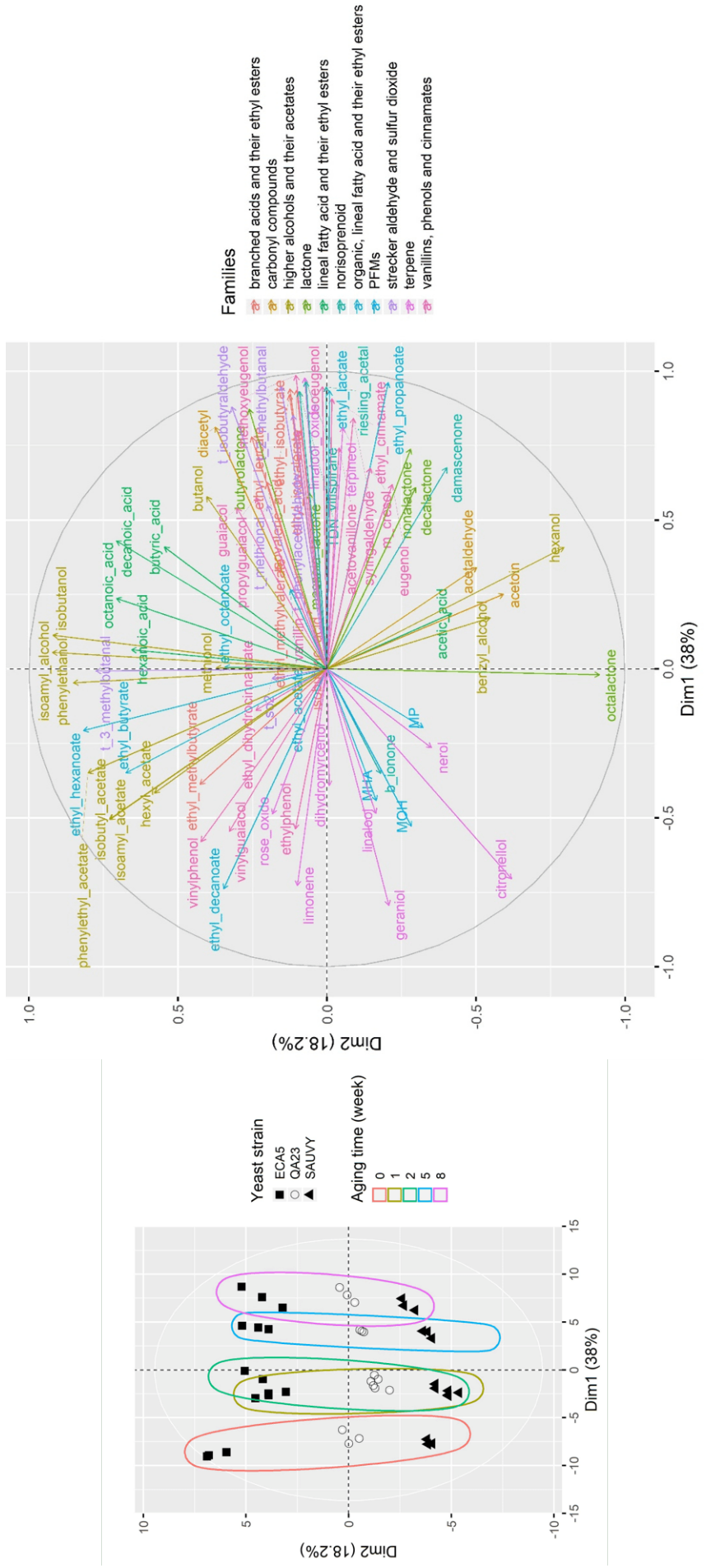


Figure 5.2: PCA obtained with the complete dataset of volatiles found in samples recently fermented by three *S. cerevisiae* strains and their evolution during aging at 50 ºC.

of the aroma compounds quantified in the present work belong to this group, including isobutyl, isoamyl and β-phenyl alcohols and their acetates, isobutyric, 2 and 3-methylbutyric acids, and their ethyl esters, methionol and Strecker aldehydes. Levels of all of them, except of isovaleric acid, were significantly related to the yeast. The evolution with time of these compounds is very diverse and complex, contributing to the extended and changing influence of yeast during aging. This can be visualized in Figure 3a for aroma compounds specifically related to the catabolism of leucine and isoleucine.

The figure reveals that among freshly fermented samples, those fermented with ECA5 contain highest levels of isoamyl alcohol and of its acetate, slightly higher levels of isovaleric acid and of 3-methylbutanal, but there were no differences in levels of ethyl isovalerate and 2-methylbutanal, whose levels immediately after fermentation were very low. However, as aging progresses, levels of isoamyl acetate decrease by hydrolysis, so that this difference becomes secondary in aged samples but it is replaced by the increasing levels of ethyl isovalerate and 3-methylbutanal, which accumulate at higher rates in samples fermented with ECA5.

In the cases of isoamyl alcohol, isovaleric acid and 3-methylbutanal, levels and differences remain approximately stable during aging. It should be noted that this pattern of changes may not be generalizable to other situations. While it is likely that levels of isoamyl alcohol, isovaleric acid and even 3- and 2-methylbutanals formed during fermentation, are somehow correlated, levels of isoamyl acetate are known to be dependent on the acetyltransferase activity of yeast and on the down-regulation of the genes involved in sterol biosynthesis, making acetyl-CoA more available for acetate synthesis (Rollero et al., 2016).

5.3.1.2 Strecker aldehydes

Strecker aldehydes are seldom determined in freshly fermented wines. In fact, the fermentative formation of Strecker aldehydes via the Ehrlich pathway is thought to be marginal, since it is assumed that the aldehyde is just an

intermediate which is quickly reduced or oxidized to the corresponding alcohol or acid. However, results presented here, together with results from a previous work (Denat et al., 2021a) in which high amounts of isobutyraldehyde were found in the volatile fraction evaporated from fermenting media (ca. 0.3-1.2 mg/L), demonstrate that little levels of Strecker aldehydes are already formed during fermentation. In the present case levels formed were 3.2-3.5, 9-20, 4.9-8.8, 2-2.3 and 1.3-3.0 μg/L of 2-methylbutanal, 3-methylbutanal, isobutyraldehyde, methional and phenylacetaldehyde, respectively. Levels of 3-methylbutanal, isobutyraldehyde and phenylacetaldehyde were strain-dependent.

Most remarkably and confirming previous unpublished results from Oliveira PhD thesis (de Oliveira, 2019), the fermentative formation of Strecker aldehydes is highly influenced by the SO_2 produced by yeast. This can be deduced from the fact that the ratios aldehyde/alcohol and aldehyde/acid measured in unaged recently fermented samples are positively and significantly correlated to measured levels of total SO_2 in these samples. In particular, for the ratios 2 and 3-methylbutanal/isoamyl alcohol, $R = 0.93$ (significant at p-value < 0.01), for isobutyraldehyde/isobutanol, $R = 0.78$ (significant at p-value < 0.05), for methional/methionol, $R = 0.88$ (significant at p-value < 0.05) and for isobutyraldehyde/isobutyric acid, $R = 0.81$ (significant at p-value < 0.05). Slopes were in all cases positive, which suggests that higher levels of intracellular SO_2 prevents a fraction of the Strecker aldehyde produced within the Ehrlich pathway from being enzymatically reduced or oxidized by the corresponding dehydrogenases.

This was time ago observed in the cold fermentation for the production of alcohol-free beer (Perpète and Collin, 2000). The level of aldehyde in wine immediately after fermentation is therefore related to both, the level of higher alcohol produced, which is a measure of the activity of Ehrlich pathway in that particular strain, and to the intracellular level of SO_2. Since both are genetically determined, those levels depend primarily on the yeast strain.

However, the most relevant contributions to the formation of Strecker

aldehydes are the strong increases with aging of 2-methylbutanal (Figure 5.3) and isobutyraldehyde (Figure 5.4), the moderate increase of phenylacetaldehyde and the modest but significant increase of methional, also shown in the Figure 5.4

These sensory-relevant increases may be attributed to the Strecker degradation of isoleucine, valine, phenylalanine and methionine, respectively, since aging took place in strict anoxia. The anoxic conditions are validated by the stable levels of acetaldehyde (Tables D.2-D.6, in Annex D) and of 3-methylbutanal, and make it possible to discard the possibility of a formation via oxidation of the alcohol or of the α-keto acid. This implies that the α-dicarbonyl carrying out the Strecker degradation should be already present in the fermenting media. Unfortunately, in the present work only diacetyl was quantified and no other major wine dicarbonyls, such as glyoxal or methyl glyoxal, which seem to be more reactive. The ability of methylglyoxal to carry out the Strecker degradation of amino acids in wine model solutions at 80 ^{o}C has been recently demonstrated (Monforte et al., 2020). As amino acids remaining after fermentation were not analyzed, it is not possible to provide a definitive reason to explain why all Strecker aldehydes but 3-methylbutanal increased and why the increases were strain-dependent. However, considering the fact that samples fermented with ECA5 accumulated maxima levels of 2-methylbutanal (Figure 5.3) and isobutyraldehyde (Figure 5.4) but not of methional and phenylacetaldehyde (Figure 5.4), and considering also that levels formed of isobutyraldehyde and 2-methylbutanal are correlated to levels of isobutanol and isoamyl alcohol (p-value < 0.05), it seems more likely that the different accumulation rates are the result of the differential residual amino acid profile present in each media, and that levels of the α-dicarbonyl are not limiting. Attending to this hypothesis, samples fermented with ECA5 should have highest levels of valine and isoleucine, while those fermented with SAUVY should have highest levels of phenylalanine and methionine. Residual levels of leucine should be very low in the three cases.

In any case, this finding may have extraordinary practical consequences, since it

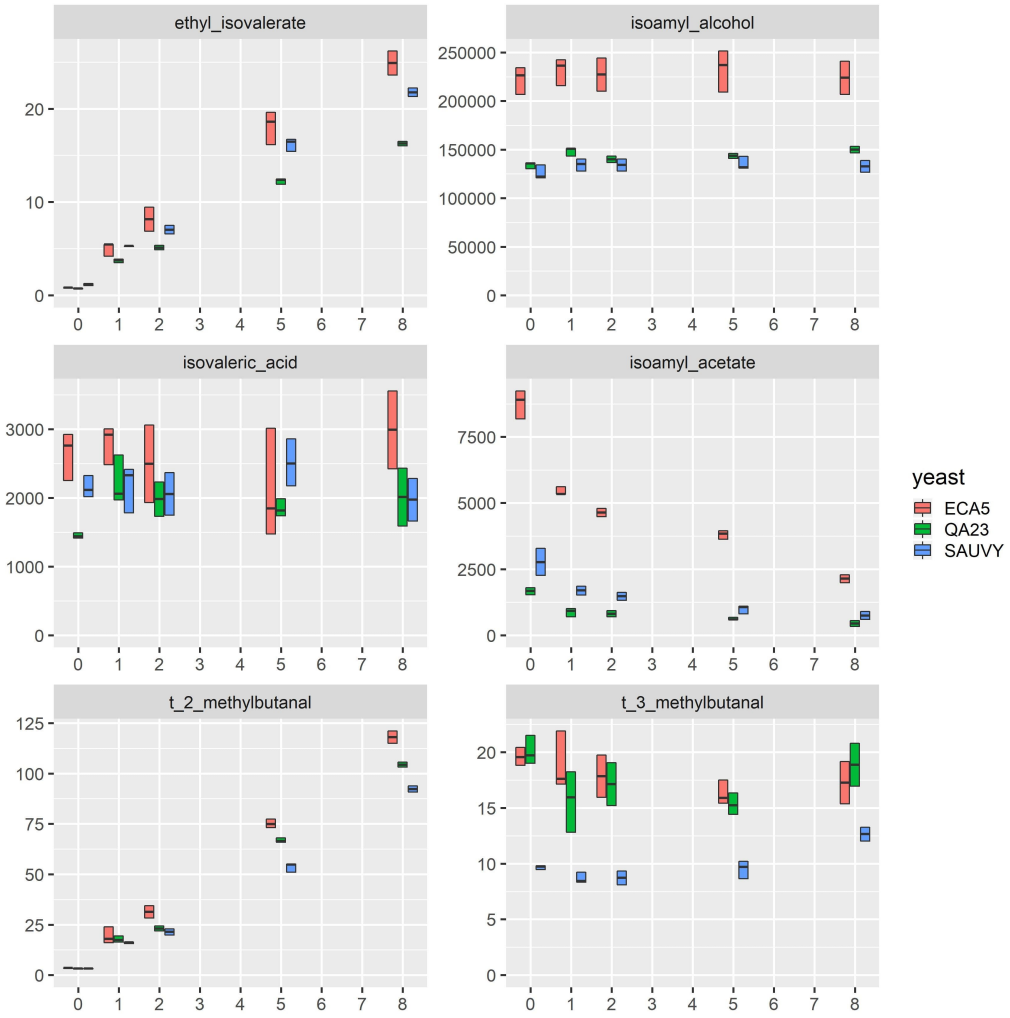


Figure 5.3: Boxplots representing the evolution during aging of some compounds concentration derived from Ehrlich pathway and SO_2, in y-axis concentration in μg/L, and x-axis time of aging in weeks.

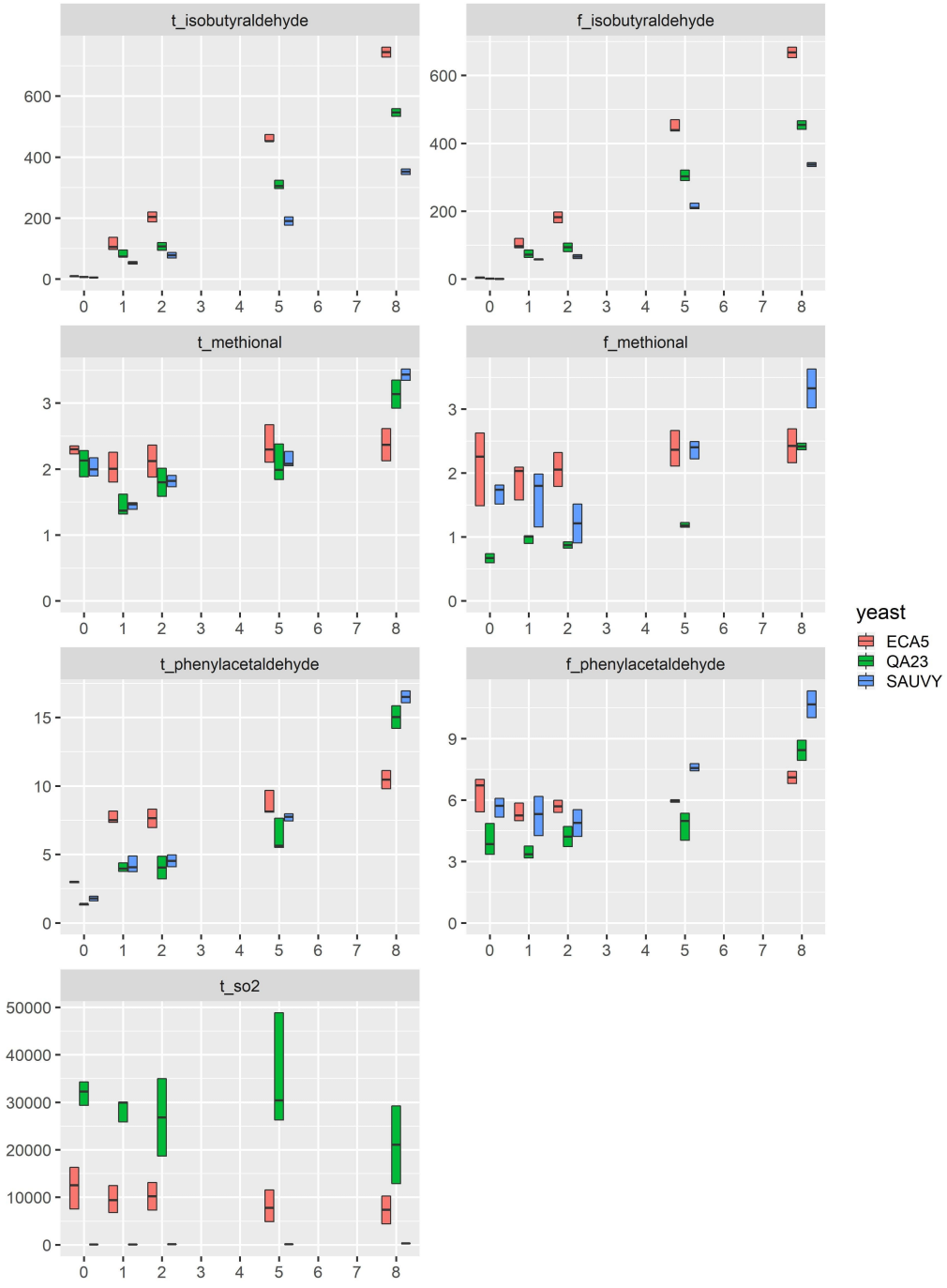


Figure 5.4: Boxplots representing the evolution during aging of some compounds concentration derived from Ehrlich pathway and SO_2, in y-axis concentration in μg/L, and x-axis time of aging in weeks.

demonstrates that oxidation is not necessarily required to form Strecker aldehydes during wine aging, corroborating recent observations in model wines at 80 ºC (Monforte et al., 2020). The formation of Strecker aldehydes will take place as long as reactive α-dicarbonyls become unprotected from SO_2. The exact nature of these reactive dicarbonyls and the different conditions leading to SO_2 depletion under anoxic conditions remain to be established.

Finally, levels of SO_2 also affect to the fraction of methional and phenylacetaldehyde in free form, as these aldehydes have strong formation constants for the formation of hydroxysulfonates with SO_2 (Bueno et al., 2014). This can be seen in Figure 5.4 by comparing plots of total and free aldehyde for the different yeasts. Both plots are equivalent for SAUVY, which did not produce SO_2; and were very similar for samples fermented with ECA5, which produced a little amount of SO_2, and free levels were clearly smaller for QA23, which produced maxima levels of SO_2. The effect is not observed for isobutyraldehyde, whose hydroxysulfonate complexing constants are lower.

5.3.1.3 Acid/alcohol/ester systems

Figure 5.5 shows a little selection of esters and acids formed in fermentation representing different forms of yeast influence during aging.

Ethyl leucate is not present after fermentation, but its levels increase during aging as it is formed by esterification of the corresponding acid, 2-hydroxy-4-methylvaleric acid (Lytra et al., 2017). It is evident that ECA5 produced the acid at levels more than twice those of SAUVY. Levels formed are much smaller than those recently reported for Tempranillo (Denat et al., 2021b) and of course for aged Bordeaux red wines, where it is involved into the perception of fresh blackberry notes (Falcao et al., 2012). This compound integrates within the other fruity ethyl esters in the fruity vector (Ferreira et al., 2021a). Similar patterns are found for ethyl propanoate, ethyl cinnamate, ethyl 4-methylvalerate, diethyl succinate or ethyl lactate.

In the case of ethyl hexanoate, initial levels were highest in ECA5 and minima

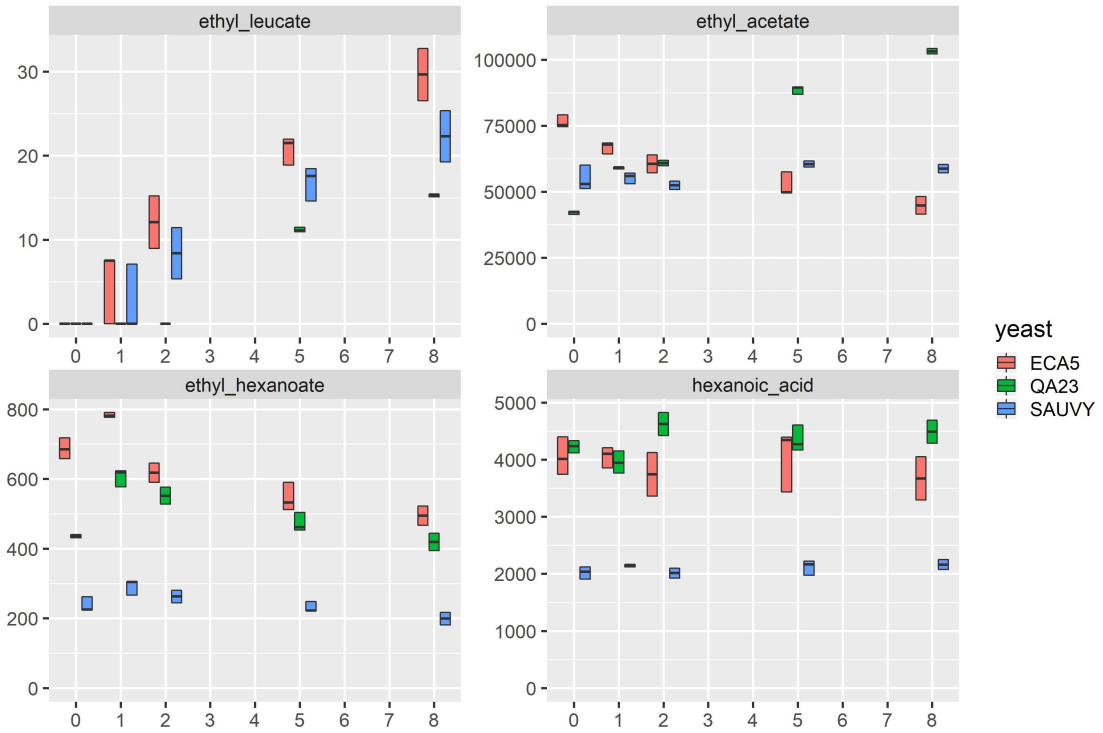


Figure 5.5: Boxplots of a little selection of esters and acids formed in fermentation and representing different forms of yeast influence during aging, in y-axis concentration in μg/L, and x-axis time of aging in weeks.

in SAUVY, and in all cases there was slight decrease during aging towards the concentration corresponding to the esterification equilibrium, so that after 8 weeks of accelerated aging, levels of this compound in ECA5 and QA23 were very close.

Finally, the case of ethyl acetate is worth mentioning because its evolution with time is completely different and strain-related. After fermentation, levels in ECA5 were maxima, consistently with the highest levels of acetates and also of ethyl esters produced by this strain, while those of QA23 were minimal. However, as levels of acetic acid produced by QA23 in young wines were nearly 4 times higher than those produced by ECA5 (Table D.7), levels of ethyl acetate in QA23 increased while those of ECA5 decreased and those of SAUVY remained constant. In the latter case, it can be hypothesized that at the end of the fermentation, levels were close to those of the corresponding esterification equilibrium. All these examples just confirm the need to control all the components of the acid/alcohol/ester system to predict the evolution with time.

5.3.1.4 Derivatives of glycosidic precursors

In the present case, both the accumulated levels of β-damascenone and their evolutions during aging are strain-related as can be seen in Figure 5.6.

This represents a difference with previous reports in which the differential action of yeast was limited to the time at which the maxima level of this compound was observed (Denat et al., 2021a; Loscos et al., 2007), which suggested that yeasts were just accelerating some of the reactions leading to the formation of this odorant from their multiple precursors. In the present case, however, samples fermented with SAUVY reached significantly highest levels above 11 μg/L and remained stable during aging, while those fermented with QA23 reached maxima levels around 10 μg/L and slightly but significantly decreased with aging. Wines made with ECA5 reached levels below 9 μg/L and remained stable throughout aging. It can be suggested that the decrease with aging observed with QA23 is due to the presence of SO2, with which damascenone is known to react (Daniel et al., 2004; Sefton

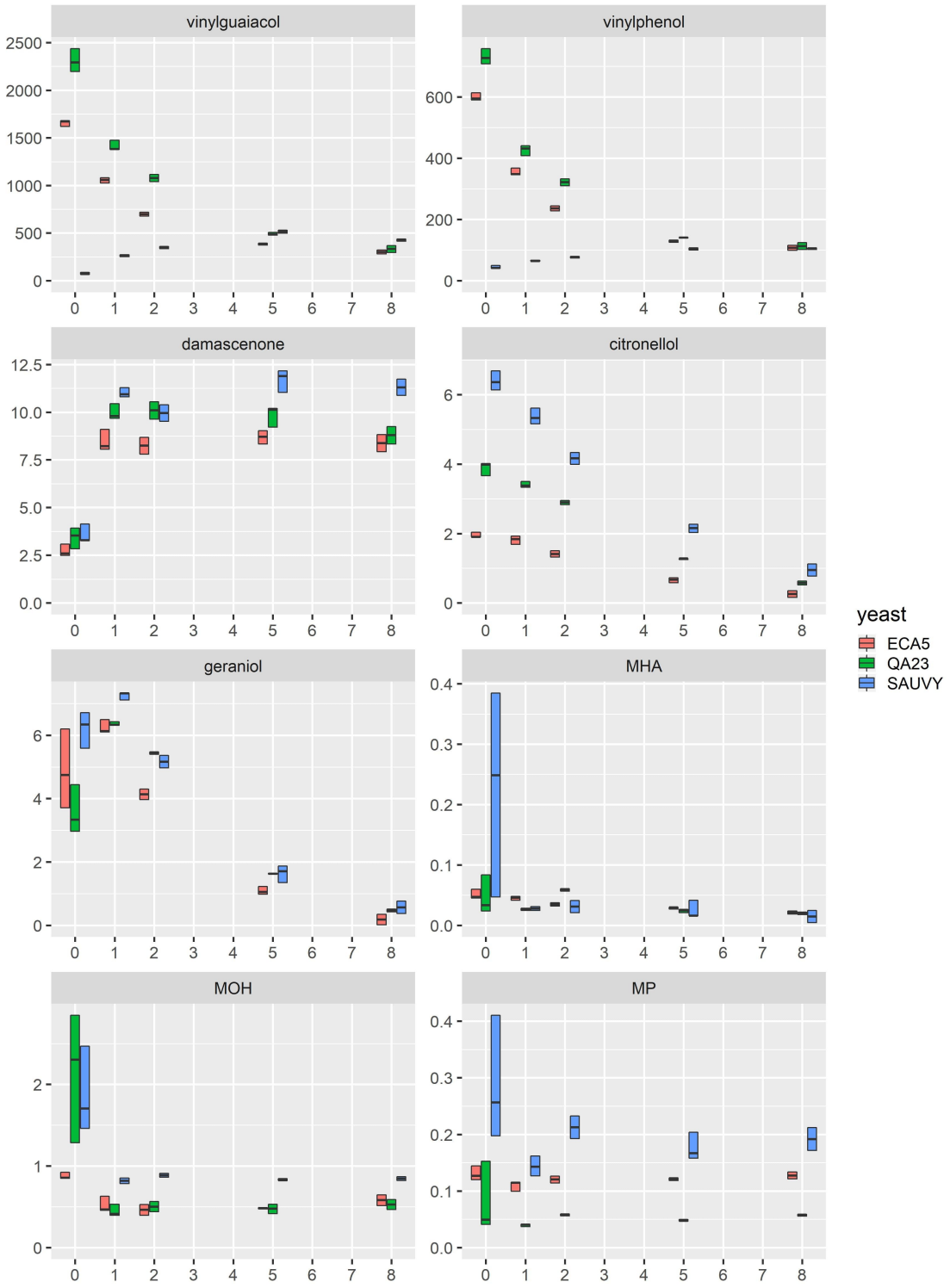


Figure 5.6: Boxplots representing the evolution of some varietal compounds such as PFMs, norisoprenoids, terpenes and volatile phenols, In y-axis concentration in μg/L, and x-axis time of aging in weeks.

et al., 2011), while the different maxima could be attributed to the differential yeast reductase activities able to reduce the diketone precursor of β-damascenone (Lloyd et al., 2011). Regarding terpenols, for which Albariño grapes are known to have relevant amounts of precursors, the effect of yeast was significant in some cases but, in general, were of little magnitude. The aromatically most relevant terpenols are the labile linalool, geraniol, (+)-rose oxide and to a lesser extent β-citronellol and nerol. α-terpineol and linalool oxide, which are more stable, are aromatically weaker and accumulate during aging. In the present case, only linalool reached levels close to threshold after 1 or 2 weeks of aging. However, in this family of compounds there is a clear cooperative action between the different members (Loscos et al., 2007) so that the maximum intensity of the flowery character derived from these compounds should be observed after 1 week of accelerated aging. The effect of yeast is particularly relevant in the levels of β-citronellol (Figure 5.6). The role of yeast in the transformation of these compounds has been well studied, and it has been demonstrated that yeasts not only liberate volatiles via glucosidase activities but can also modify the precursor or the volatile itself via reductase, oxidase, hydroxylase and acetyltransferase activities (Slaghenaufi et al., 2020).

Leaving aside vinylphenols, Albariño grapes contain very few amounts of precursors of volatile phenols as it is shown by the very low levels accumulated during aging of guaiacol, eugenol and the other volatile phenols (Tables D.2-D.6).

5.3.1.5 Vinylphenols

Figure 5.6 reveals that two of the strains, QA23 and ECA5, produced huge amounts of 4-vinylguaiacol and 4-vinylphenol most likely due to the decarboxylation of the corresponding phenolic acids (Chatonnet et al., 1993), while SAUVY produced just marginal levels during fermentation. The evolution with time of these two compounds is, however, paradigmatic. Levels in excess formed by yeast were completely eliminated during aging by reaction with unspecified wine nucleophiles. Attending to literature, these nucleophiles could be glutathione or cysteine (Naim

et al., 1993; Turner et al., 2005). However, giving the structural similarity between vinylphenols and other odorants with highly conjugated unsaturated systems, such as β-damascenone, a reaction with HSO_3^- could be also plausible, particularly considering that levels of SO_2 slightly decrease with aging in QA23 and ECA5 (Figure 5.4). By contrast, levels in SAUVY slowly increased likely by the hydrolysis of the glycosidic precursors with the result that levels of these compounds after aging were equivalent in all the samples. Similar results were obtained in semi-synthetic Tempranillo must, even if the starting levels and final levels were smaller (Denat et al., 2021a). In Riesling and Garnacha semi-synthetic musts, similar tendencies were observed, with a reduced variability within yeasts along accelerated aging (Oliveira and Ferreira, 2019). It seems that final levels are more dependent on the presence of anthocyanins in red wines or of other nucleophiles in white wines, than on the initial levels formed.

5.3.1.6 Polyfunctional mercaptans

PFMs were produced from precursors spiked into the semi-synthetic must. As can be seen in Figure 5.6, the levels of MH in the recently fermented samples were maxima in samples fermented by QA23 followed by those of SAUVY (differences non-significant) and were minima in those fermented by ECA5. However, during the first week of aging, levels in all cases decreased and then remained stable during aging, but the decrease was particularly strong in the case of QA23, so that SAUVY was able to keep significantly much higher levels of this labile compound throughout the whole aging period. Final levels in SAUVY exceed those of QA23 by 0.4 μg/L, a 70 % increase, a difference that will have a major sensory effect. Although SAUVY produced slightly more MHA, which quickly hydrolyzed to MH, the levels of the acetate formed are not large enough to explain such a difference. Given the aforementioned reported reactivity of vinylphenols towards mercaptans (Naim et al., 1993; Turner et al., 2005), and the minima levels of these compounds found in samples fermented with SAUVY, it can be hypothesized that the observed

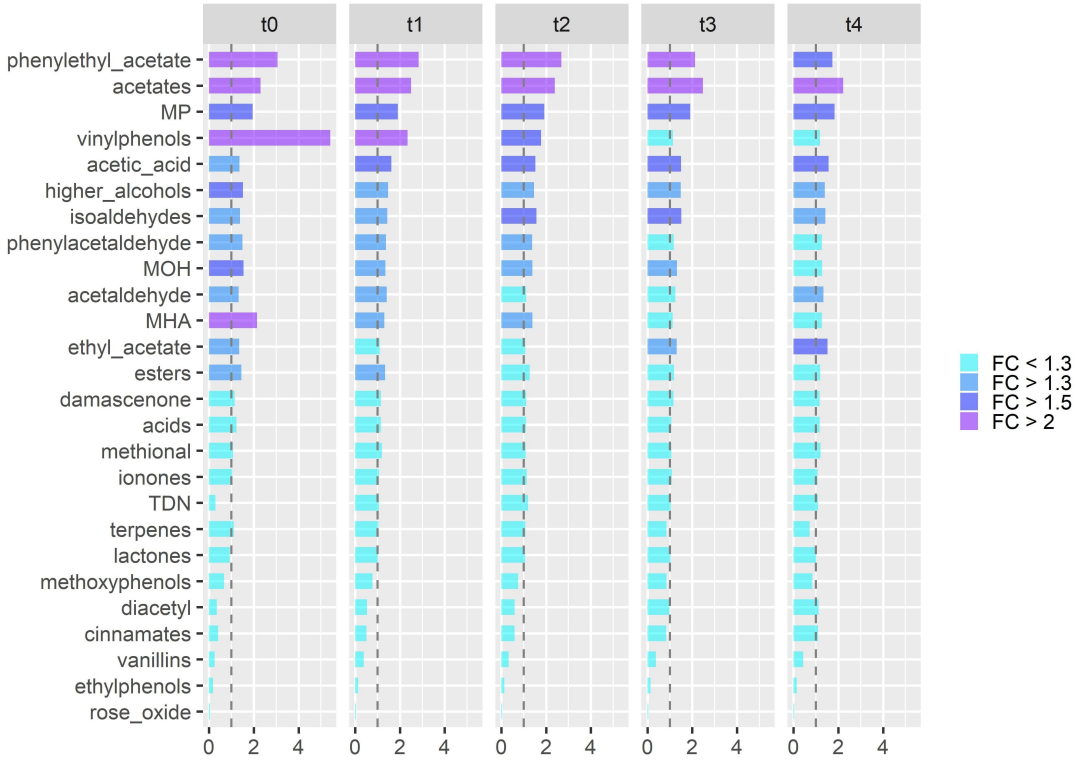
decreases in MH are at least in part, related to vinylphenols. Other alternative reactive electrophiles could be α-dicarbonyls produced in fermentation (LoPachin and Gavin, 2014), such as glyoxal or methylglyoxal (Zeng and Davies, 2005), but as discussed for Strecker aldehydes, these do not seem to be a major limiting factor. In any case, this result may be also relevant from the practical point of view, since demonstrates that the role of yeast on the wine levels of PFMs (Nikolantonaki et al., 2010), and particularly of the most reactive MH (Nikolantonaki et al., 2012), extends to its stability during aging. Further research to elucidate the causes of this result is required.

5.3.2 Potential sensory consequences

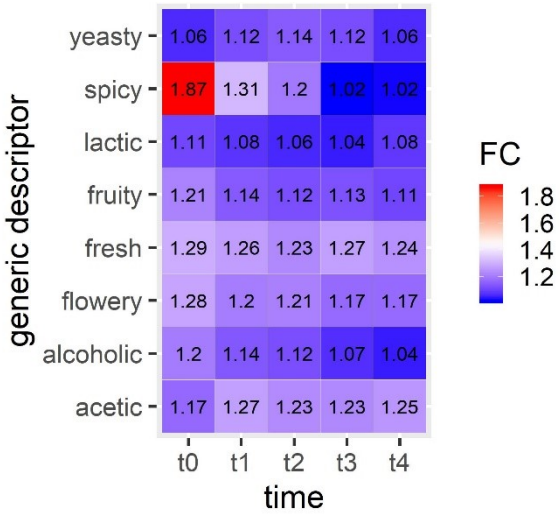
In the following part, the previous aroma composition data are processed using some assumptions derived from classical psychophysics in order to make an initial assessment about the potential sensory effects of the observed compositional differences introduced by yeast. As some of these assumptions have not been completely validated, and some others are based on numerical coefficients whose exact value is not known, the conclusions provide a mere orientation and would require verification by sensory analysis. There is, however, no major reason for not making this little study since can provide a reasonable assessment of the potential relevance of some of the observed chemical changes.

Concentrations were first normalized by odor thresholds into OAV. Second, compounds were gathered into aroma vectors attending to (bio)chemical and aromatic affinity, as detailed in Table D.1. The discriminant power of each compound or aroma vector was further estimated from the ratio $\mathrm{OAV}_{max}/\mathrm{OAV}_{min}$ in the group of samples considered, where OAV_{min} was set to 1 if < 1. These quotients for the wines of equivalent ages are represented Figure 5.7a.

The figure shows that quotients, except for vinylphenols in young wines, are always below 3, and that only for phenylacetate, acetates and MHA can be above 2. There are however, a relatively high number of relevant aroma vectors whose



(a)



(b)

Figure 5.7: Representations of the fold change evolution during wine aging between the 3 yeast strains in charge of fermentation, for each aroma vector (a) and generic descriptors (b). A vertical line was arbitrary placed at FC = 1.

ratios are within the 1.3-2.0 range, including MP, MH, acetic acid, ethyl acetate, higher alcohols, isoaldehydes, phenylacetaldehyde. Some of these ratios remain approximately constant during aging; there is a slightly increase in the cases of acetic acid and ethyl acetate, and a remarkable decrease in that of vinylphenols. Minor decreases with aging are also observed for esters, phenylacetaldehyde and MH. What these results suggest is that the impact of yeast on the sensory properties of wines, is relevant but not dramatic and that should be stable throughout aging. Only in recently fermented wines differences can be marked, mostly due to vinylphenols and MHA, but, in the rest of samples there are a consistent number of relevant differences of minor and moderate magnitude.

The discriminant power was also estimated for the generic descriptors, using quantitative data as explained in the Chapter 2. Results are presented in Figure5.7b, where it can be appreciated that only the attribute "spicy" in young wines shows a major difference, but that there are a number of relevant descriptors, such as fruity, fresh, acetic and flowery, whose ratios are in the 1.1-1.3 range, throughout the whole aging period.

5.3.3 Correlations with accelerated aging at 75 ºC

Samples were aged at 75 ºC in order to assess whether aging at this temperature maybe used to predict results at 50 ºC. This would be convenient, since it is apparent that in 24 h of hydrolysis at 75 ºC the amount of volatiles released is close to that seen after 5 weeks of aging at 50 ºC. In order to do that, results obtained at 75 ºC were compared by simple correlation with those obtained at 50 ºC. The complete table of correlations is given in the Table 5.1.

In order to interpret results in the correct context, it is necessary to take into consideration the original variability with time of the data set, as was assessed in Tables D.2-D.6 and in the figures 5.3 to 5.6. As can be seen in Table 5.1, results are highly promising, since results obtained at 75 ºC are closely correlated to those obtained at 50 ºC for most of the cases in which there is a significant

Table 5.1: Correlations between the concentrations obtained through accelerated aging at 75ºC (C75) and 50 ºC (C50), p-value indicating the significance of the correlation (Pearson), a coefficient associated with the linear regression C75 = a*C50. In bold, p-values inferior to 0.05.

group	variables	p-value	R	a
acetates	isobutyl acetate	**2,10E-17**	0,94	1,12
	β-phenylethyl acetate	**3,18E-21**	0,964	1,07
	isoamyl acetate	**8,95E-23**	0,971	1,6
	hexyl acetate	2,12E-01	0,213	0,66
acids	acetic acid	**2,14E-15**	0,92	0,73
	isobutyric acid	2,74E-01	0,187	0,356
	butyric acid	**6,63E-04**	0,541	0,621
	isovaleric acid	3,87E-01	0,149	0,107
	hexanoic acid	**2,59E-15**	0,919	0,921
	octanoic acid	**8,38E-10**	0,821	0,864
	decanoic acid	**6,05E-09**	0,797	1,27
alcohols	isobutanol	**9,26E-08**	0,757	0,9
	butanol	**4,69E-04**	0,553	0,694
	isoamyl alcohol	**6,26E-14**	0,902	1,03
	hexanol	**1,09E-10**	0,843	0,856
	benzyl alcohol	**2,85E-02**	-0,365	-0,641
	β-phenylethanol	**1,26E-22**	0,971	1,07
	methionol	**4,80E-11**	0,851	0,983
aldehydes	isobutyraldehyde	**1,43E-14**	0,91	4,5
	2-methylbutanal	**1,44E-19**	0,955	3,48
	3-methylbutanal	**5,02E-07**	0,727	0,912
	methional	**2,70E-02**	0,369	0,589
	phenylacetaldehyde	**1,00E-03**	0,525	0,356
branched esters	ethyl isobutyrate	**2,04E-14**	0,908	0,508
	ethyl 2-methylbutyrate	5,35E-01	-0,107	-0,0778
	ethyl isovalerate	**1,13E-21**	0,967	0,556
	ethyl 4-methylvalerate	**2,16E-04**	0,579	0,39
carbonyls	acetaldehyde	**4,08E-02**	0,343	0,265
	diacetyl	**7,20E-11**	0,847	1,04
	acetoin	**2,92E-02**	0,364	0,371
cinnamates	ethyl dihydrocinnamate	5,75E-01	-0,0968	-0,0981
	trans-ethyl cinnamate	**2,21E-20**	0,96	0,344
ethyl esters	ethyl leucate	**1,99E-12**	0,878	0,538
	ethyl acetate	**3,89E-04**	0,559	0,315
	ethyl propanoate	**1,69E-06**	0,704	0,598
	ethyl butyrate	**8,22E-04**	0,533	0,841
	ethyl hexanoate	**7,49E-10**	0,823	0,771
	ethyl lactate	**4,01E-13**	0,89	0,829
	ethyl octanoate	4,51E-01	0,13	0,175
	ethyl decanoate	2,14E-01	0,212	0,404
lactones	γ-octalactone	**4,67E-20**	0,958	0,877
	γ-nonalactone	1,37E-01	0,253	0,243
	δ-decalactone	4,99E-01	0,116	0,128
	massoia lactone	9,88E-01	0,00255	0,00273
	γ-butyrolactone	**2,58E-07**	0,739	1,36
norisoprenoids	vitispirane	**3,40E-25**	0,979	1,32
	Riesling acetal	**3,48E-23**	0,973	0,848
	TDN	**2,07E-23**	0,974	1,6
	β-damascenone	**9,69E-06**	0,665	0,83
	α-ionone	1,98E-01	0,219	0,176
	β-ionone	3,47E-01	-0,161	-0,0543
PFMs	FFT	**1,78E-02**	0,393	0,646
	MP	**4,83E-08**	0,767	1,14

	BM	4,55E-01	-0,129	-0,454
	MHA	8,42E-01	0,0345	0,103
	MH	**3,61E-05**	0,632	2,15
SO_2	SO_2	**5,48E-18**	0,944	0,885
terpenes	R-limonene	**1,16E-04**	0,598	1,57
	1,8-cineole	5,00E-02	0,329	0,879
	rose oxide	**1,01E-09**	0,819	0,781
	linalool oxide	**2,30E-25**	0,98	1,31
	dihydromyrcenol	**2,29E-02**	-0,378	-0,0721
	linalool	**6,35E-27**	0,984	0,933
	α-terpineol	**6,55E-13**	0,886	1,73
	β-citronellol	**5,13E-19**	0,952	0,998
	nerol	**2,47E-14**	0,907	1,14
	geraniol	**8,03E-24**	0,975	1,15
vanillins	vanillin	**2,57E-03**	0,487	0,26
	acetovanillone	5,47E-01	-0,104	-0,0689
	syringaldehyde	**2,29E-07**	0,742	3,18
volatile phenols	guaiacol	3,88E-01	-0,148	-0,142
	m-cresol	**1,15E-04**	0,599	0,836
	p-propylguaiacol	9,07E-02	-0,286	-0,107
	eugenol	**5,72E-09**	0,798	1,4
	4-ethylphenol	**1,34E-05**	-0,657	-14,2
	4-vinylguaiacol	**3,27E-17**	0,938	1,37
	trans-isoeugenol	**3,34E-06**	0,689	0,615
	4-vinylphenol	**3,90E-20**	0,959	1,22
	methoxyeugenol	**3,76E-25**	0,979	1,8

effect of time. This is certainly the case of the acetates of the higher alcohols, acetic acid, isobutyraldehyde, 2-methylbutanal, diacetyl, ethyl isobutyrate, and isovalerate, ethyl cinnamate and ethyl leucate, vitispirane, Riesling acetal and TDN, SO_2, linalool and the other terpenols, methoxyphenols and vinylphenols. In all these cases, the correlation coefficient is close or above to 0.9. Results were poorer in the case of phenylacetaldehyde and MH, (R = 0,52 and 0.63, respectively), but overall, the predictive ability at 75 ºC seems to be rather satisfactory.

It is however evident, that the kinetics of both processes in some cases need to be corrected. For instance, in the case of isobutyl and phenylethyl acetates, together with 3-methylbutanal, diacetyl, ethyl butyrate, hexanoate and lactate, Riesling acetal, linalool and the other monoterpenols, and 4-vinylphenol, the slope between both aging times is close to 1, meaning that there is a nearly perfect equivalence between the time periods taken at both times. i.e., that 1, 2, 5 and 8 weeks of aging at 50 ºC are equivalent to 12, 24, 48 and 96 hours at 75 ºC. In a group of cases, however, the hydrolysis at 75 ºC was proportionally more efficient, so that amounts at this temperature are above those measured at 50 ºC. This is the case of ethyl cinnamate, ethyl leucate, ethyl isobutyrate and isovalerate. On the contrary, in some other cases levels accumulated at 75 ºC are well below those observed at 50 ºC. This is the case of isoamyl acetate, isobutyraldehyde and 2-methylbutanal, TDN, MH or methoxyphenols.

5.4 Conclusion

Yeasts exert a most notable influence on wine aroma profile throughout the whole period of aging. This influence is exerted in different forms varying in complexity and in the way and time of action. The most direct, evident and well-known form of influence is through the ability of yeast to form aroma secondary metabolites, which affects to all major fermentation metabolites, including the differential formation of small amounts of Strecker aldehydes. A second more indirect but also well-known form of influence is the formation of acids able to form fruity esters by esterification

with ethanol, including branched acids, leucic (2-hydroxy-4-methylvaleric) and cinnamic acids. A third well-known form of influence is through the specific enzymatic transformation of precursors into aroma molecules. The decarboxylation of ferulic and coumaric acids to form vinylphenols, the cleavage of the glutathionyl and cysteinyl precursors of PFMs, the hydrolysis of glycoconjugates of terpenols or volatile phenols, or the reduction of ketonic nor-isoprenoids are within this category.

However, the present work has revealed that yeast exerts significant indirect effects on relevant aroma molecules. First, the yeast-related indigenous formation of SO_2 would affect the little levels of Strecker aldehydes formed in fermentation, the proportion of free forms of aldehydes and also the stability of β-damascenone. Second, either the yeast-related amino acid residues, or the different levels of dicarbonyls remaining after fermentation, induce the accumulation of Strecker aldehydes at different yeast-related rates during anoxic aging. Third, yeasts-related electrophiles, such as vinylphenols, may be related to the observed differential stability during anoxic aging of MH.

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Chapter 6

Influence of *Saccharomyces* wine strains on the aroma precursor fraction during fermentation. A preliminary metabolomic approximation.

6.1 Introduction

Wine varietal aroma is formed by compounds formed or liberated from specific grape-aroma precursors. Some of these compounds require relatively long periods of aging to form and accumulate, which makes the study of the influence of yeast difficult. The liberation/formation of these compounds from their specific precursors can occur spontaneously by acid catalyzed rearrangements or hydrolysis, which usually takes a long time, and can also occur quicker under the action of the yeast enzymatic activities. The modulation of some of these volatiles, particularly of those whose release is fast, by the fermenting yeast has been extensively studied and demonstrated, at genera, species and strain levels (Gammacurta et al., 2017; Oliveira and Ferreira, 2019; Swiegers et al., 2009). However, in the many cases in which the aroma compounds require certain aging time to form, the modulations exerted by yeast, and moreover, the mechanisms involved in such modulation, are no fully understood. This is the case of norisoprenoids such as TDN, β-damascenone, of PFMs such as MH, MP, MHA, or of volatile phenols such as 4-vinylphenol and 4-vinylguaiacol.

Difficulties arise from the existence of various different aroma-precursors to some of the previous aroma molecules, also from the existence of several formation steps involving both enzymatic and chemical mechanisms and hence multiple intermediaries, or from the existence of a specific reactivity towards other wine components, thus impacting the stability in wine of the aroma compounds. Yet, many of these aroma compounds are key for wine quality and longevity, which makes that understanding the mechanisms involved in their formation and persistence during wine aging is extremely important.

The difficulty in the direct analysis of the aroma precursors relies in their high diversity, multiplicity, in the lack of clear target compounds and, particularly in the lack of reference compounds. Because of that, most approaches use indirect strategies based on the extraction of the fraction of precursors, its subsequent enzymatic or acid catalyzed liberation, and the analyzed by GC-MS of the aroma

liberated (Loscos et al., 2009; Schneider et al., 2001). However, these indirect strategies cannot provide enough insight into the chemistry of the process. The direct target analysis of precursors require the use of LC-MS and the availability of reference compounds as it is the case for some PFMs precursors (Concejero et al., 2014; Vanzo et al., 2017). It is only recently that some LC-MS based untarget analysis have focused on the identification of grape aroma precursors (Caffrey et al., 2020; Cebrián-Tarancón et al., 2021; Flamini et al., 2014).

More recently, Bordet et al. (2021) combined untarget LC-MS, GC-MS and sensory analysis of a Chardonnay must fermented with different *S. cerevisiae* yeasts, identifying some strain-specific characteristic traits on both volatile and non-volatile metabolites, leading to sensory perceptible differences. Tufariello et al. (2021) obtained similar results in sparkling wine, differentiating several aroma liberation patterns according to the re-fermenting yeast. (Caffrey et al., 2021) specifically analyzed monoterpene glycosides during Riesling fermentation, performing an extraction of the precursor fraction and its subsequent untarget analysis. Combining HPLC fractionation and acid/enzymatic hydrolysis of the fraction, they identified several monoterpene precursors and could also observe their modulation by the fermenting yeast.

The objective of the present work is to study the interactions between the yeast in charge of fermentation and the grape aroma precursors and to assess how such interactions affect to the development of varietal aroma during aging and to wine longevity. For that, the aroma-precursor fraction was extracted before and after fermentation with different selected yeasts, and the extracts were comprehensively analyzed by untargeted LC-MS analysis. This study is complemented by the quantification of a wide range of wine aroma compounds in recently fermented wines and in wines aged different times. Additionally, the aroma precursor fraction of one of the wines made with a general-purpose yeast strain was extracted and analyzed by comprehensive untarget LC-MS analysis at the different aging times.

The yeast selected for the study were chosen attending to results obtained in

previous studies (Denat et al., 2022, 2021; Pérez et al., 2022a,b), in which the abilities of different yeasts to modulate wine varietal aroma from Tempranillo and Albariño was assessed. The four yeasts showing maxima divergence in their aroma profiles were selected: three *S. cerevisiae* strains, Lalvin QA23™, Lalvin Sauvy™ and Lalvin Rhône 2056®, and *S. kudriavzevii* CR89D1. In spite of the fact that they were not studied altogether, all of them show some remarkable particularities in their abilities to modulate aroma molecules derived from specific aroma precursors from grape.

In particular, CR89D1 revealed a high capacity to liberate MP; SAUVY formed high levels of MHA, while QA23 liberated low amounts of these two volatiles. In addition, QA23 and SAUVY liberated high quantities of linalool and geraniol, while the latter was also specifically characterized by a higher liberation of β-citronellol. RHONE and QA23 liberated high levels of vinylphenols, contrary to the other two strains. RHONE was also characterized by a superior liberation of TDN, even in young wines, while QA23 and SAUVY liberated lower quantities.

Therefore, this study will take advantage of the high diversity introduced by these yeasts on wine varietal aroma to obtain a preliminary assessment of the diversity of chemical effects introduced by yeasts on the fraction of grape aroma precursors during grape must fermentation.

6.2 Materials and Methods

6.2.1 Vinification

6.2.1.1 Must preparation

A mix of equal quantities of six mistelles of Chardonnay, Gewürztraminer, Macabeo, Riesling, Tempranillo and Garnacha was prepared and dealcoholized using a rotary evaporator. The mixture of the dealcoholized mistelles was diluted up to approximately 22 º Brix, (equivalent to 12 % of probable ethanol grade) by addition of sterile distilled water and nitrogen content was corrected by adding 50 mgN/L via ammonium phosphate addition.

6.2.1.2 Yeast strains and growth monitoring

Fermentations were carried out by 4 *Saccharomyces* wine yeasts: 3 *S. cerevisiae* commercial strains from Lallemand Bio (Barcelona) Lalvin QA23™, Lalvin Sauvy™, Lalvin Rhône 2056®; and the *S. kudriavzevii* CR89D1 strain from IATA-CSIC collection.

All the strains were pre-cultured in GPY broth overnight and inoculated at 1.10^6 cells/mL. During fermentation, yeast growth was monitored daily by optical density measurement of the appropriate dilution of fermenting must at 600 nm.

Implementation control was performed at the middle and at the end of fermentation. To that end, a mix of the three replicates of fermenting wine was plated on GPY agar plate at the appropriate dilution and incubated at 25 ºC for 48 hours. Ten colonies were taken from the grown plates and the implementation control was carried out following the protocol established byQuerol et al. (1992).

6.2.1.3 Fermentation monitoring and oenological analysis

Fermentations were carried out in triplicates in 200 mL Pyrex flasks containing 180 mL of must and tightly closed with Muller valves. Fermenters were constantly agitated at 200 rpm using a magnetic stirrer and incubated at 19 ºC. Fermentations were monitored by daily weighing; they were considered finished when the daily loss was inferior to 0.1 g for 2 consecutive days.

Initial and final glucose and fructose contents were determined by UV spectrometry using an enzymatic kit. Ethanol content in the recently fermented wines was determined by GC.

6.2.1.4 Wine accelerated aging

At the end of fermentation, the samples were centrifuged and conditioned for accelerated anoxic aging into 18 mL glass tubes at 75 ºC.

6.2.2 Experimental design

As represented in the Figure 6.1, five sampling times were considered in this study: t0 -must, t1 -wines recently fermented, t2 -wines after 12 hours of accelerated aging at 75 ^{o}C, t3 -wines after 24 hours of accelerated aging, t4 -wines after 96 hours of accelerated aging. The yeast strains were codified as follow: QA23, SAUVY, RHONE and CR89D1. The volatile compounds were quantified all along wine aging in order to follow their evolution (t0-t4). The precursor fraction was analysed before and after fermentation (t0, t1); and for one of the yeasts (QA23), it was also analysed during wine aging (t0-t4).

The experiment was divided into two batches. In the first vinification batch, fermentation was carried out only with QA23 in a common experiment with the PhD student Elayma Sánchez Acevedo, seeking to assess the effects of aging on the precursor fraction. The second batch of vinifications was carried out with the 4 yeast strains (QA23, SAUVY, RHONE, CR89D1) in order to assess their modulation on the precursor fraction.

Sample preparation, volatiles analysis and UPLC-QTOF-MS untargeted analysis were performed within the 2 months after the end of fermentations. Samples were stored at 4 ^{o}C after conditioning in anoxia, and extracts at -20 ^{o}C.

6.2.3 Volatiles quantification

6.2.3.1 Major compounds

Major compounds - including carbonyl compounds, higher alcohols and their acetates, fatty and organic acids and their ethyl esters - were analysed in the recently fermented wines (t1) by GC-FID on DCM micro-extracts as explained in the Chapter 2.

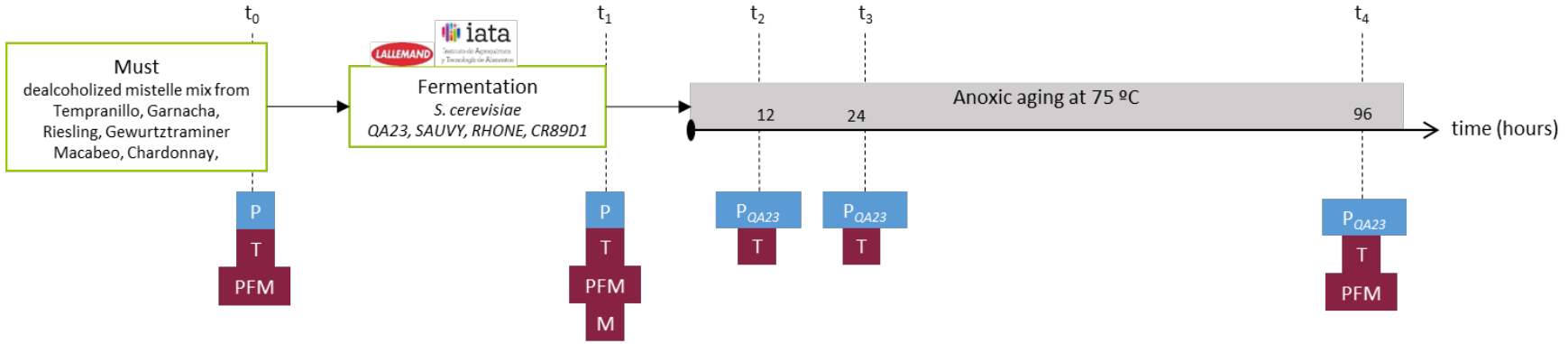

Figure 6.1: Experimental procedure - A must composed of a mix of 6 grapes varieties was fermented with 4 *Saccharomyces* yeasts. Wines were submitted to anoxic accelerated aging at 75 ºC. Wine aroma profile was analysed via volatiles quantifications in parallel with an untarget analysis of the phenolic and aroma precursors fraction. P: untarget analysis of precursors, M: major compound compounds analysis, T: trace compounds analysis, PFMs: polyfunctional mercaptans analysis.

6.2.3.2 Trace volatile compounds

Trace compounds - including ethyl esters of branched acids, terpenes, norisoprenoids, vanillin derivatives, lactones, volatile phenols - were analysed in wines the four sampling times (t1-t4), using SPE and further GC-MS following the strategy explained in the Chapter 2.

6.2.3.3 Polyfunctional mercaptans

PFMs - MH, MP, MHA, FFT, BM - were analysed by UHPLC-MS/MS after their derivatization with Ebselen as described in the Chapter 2. Analysis were carried out in the recently fermented wines (t1) and in the wines after 96 hours of accelerated aging (t4).

6.2.4 Metabolomics

Untarget analysis of aroma precursors was performed as described in Chapter 2, Materials and Methods. Analysis of the must (t0) and wines recently fermented (t1) by the 4 yeast strains were carried out. For the yeast strain QA23, the analysis was also performed during wine aging (t0-t4).

6.2.5 Statistical analysis

6.2.5.1 Data from aroma volatiles

One-way ANOVA was performed on the concentrations of major compounds in order to check for the effect of the yeast strain. Two-way ANOVA was performed on levels of PFMs and trace aroma compounds in order to check for the effect of the strain and aging time. PCAs were also carried out to obtain a hierarchical representation of effects.

Fold change is defined as the ratio between maxima and minima amounts of a volatile found in the set equivalent samples (at a unique sampling time). This ratio was calculated for each volatile and at each sampling time (t1-t4). If the compound

was not detected, the amount considered for the ratio was the detection limit.

6.2.5.2 Untarget analysis of aroma precursors

After checking for the proper clustering of the quality controls (QCs), these samples were excluded and one-way ANOVA analysis was performed on each dataset (the QA23 samples aged different times on the one hand, and the young wines fermented with the 4 different yeasts in the other hand) in order to check for the effects of aging time and of yeast, respectively, on the fraction of aromatic precursors and on other wine components detected in the experiment. Buckets significantly affected by the yeast strain (p-value < 0.05) were selected and as described in (Tufariello et al., 2021), the correlation matrix between their intensity and the volatile concentrations was calculated.

The SmartFormula algorithm was used in order to associate a chemical formula with a mass tolerance inferior of $\pm$ 5 ppm and buckets were compared with a list of already identified precursors from (Caffrey et al., 2020) for buckets in negative mode and (Bonnaffoux et al., 2017; Capone et al., 2011; Concejero et al., 2014; Vanzo et al., 2017) for PFMs precursors in positive mode.

6.3 Results and discussion

6.3.1 Fermentation, yeast growth and implantation

Fermentations lasted 7 days, the fermentative parameters and colorimetric measurements results are presented in Table 6.1. All the yeasts were well implanted apart from CR89D1. For this strain, the implantation control revealed a prevalence of 28 % at mid-AF and of 44 % at the end-AF. The implementation control revealed that these fermentations were contaminated by the strain RHONE. This result was expected, since *S. kudriavzevii* have less viability than their *S. cerevisiae* counterparts. In spite of this, the sample will be herein referred to as CR89D1, even if it was a mixed fermentation. The initial must contained 202 g/L of glucose and

fructose, and final wines contained around 0.05 g/L, except samples fermented with CR89D1 which contained approximately 0.2 g/L. The yeasts produced around 14 % (v/v) of ethanol and differences were not significant, contrary to the pH which was initially at 4.2 in the must and dropped to around 3.8 for CR89D1 and RHONE.

The PCA in Figure 2 was built with the concentration of major aroma compounds found in young wines. As major aroma compounds are essentially yeast metabolites, the plot gives a first approximation to the differences in metabolism between the four strains.

Quantitative results of major compounds (mean concentration ± standard deviation; n=3 replicates) are presented in Table E.1 in Annex E. Results of the one-way ANOVA (1st column) confirm that all major volatiles, except hexyl acetate and γ-butyrolactone are significantly modulated by the strains. Most surprisingly, samples inoculated with RHONE and CR89D1 are not clustered together, in spite of the fact that both strains co-existed in the CR89D1 samples because of contamination with RHONE. This should be attributed to the high particularities of *S. kudriavzevii* metabolism, neatly different to those of *S. cerevisiae* strains. CR89D1 samples, as can be observed in Table E.1 and in Figure 6.2, formed much higher levels of 2-phenylethanol than the rest of strains, and also of the three valine derivatives in the Ehrlich pathway (isobutanol, isobutyric acid and ethyl isobutyrate), plus of acetic acid, diethyl succinate and diacetyl, as previously reported (Minebois et al., 2020a,b; Pérez et al., 2021). Apart from this, CR89D1 samples also contain highest levels of butanol, methionol and acetoin, and minima levels of butyric and octanoic acids, and minima levels of ethyl acetate, ethyl propanoate, isoamyl acetate and of ethyl hexanoate. This suggests that this strain is particularly unable to synthesize ethyl esters and acetates.

The PCA plot also reveals that the second most different strain is SAUVY, which produced in general the smallest levels of all fermentative compounds, except of ethyl propanoate and ethyl acetate. In comparison, QA23 and RHONE have a more similar metabolism, including a strongly activated linear fatty acid metabolism,

Table 6.1: Fermentative parameters and colorimetric measurements of the must and wines fermented with 4 *Saccharomyces* yeasts. In case of significant effect of the yeast, p-values are in bold and letters indicate the results of Tukey's HSD test.

	must	p-value	CR89D1	QA23	RHONE	SAUVY
ethanol % (v/v)	-	5,40E-01	13.7 ± 0.5	13.7 ± 0.2	13.9 ± 0.5	14.1 ± 0.3
sugars (g/L)	202	**7,38E-09**	0.21 ± 0.01 a	0.051 ± 0.002 b	0.046 ± 0.003 b	0.048 ± 0.001 b
pH	4.23	**5,26E-07**	3.837 ± 0.003 c	3.92 ± 0.02 b	3.86 ± 0.02 c	4.05 ± 0.01 a

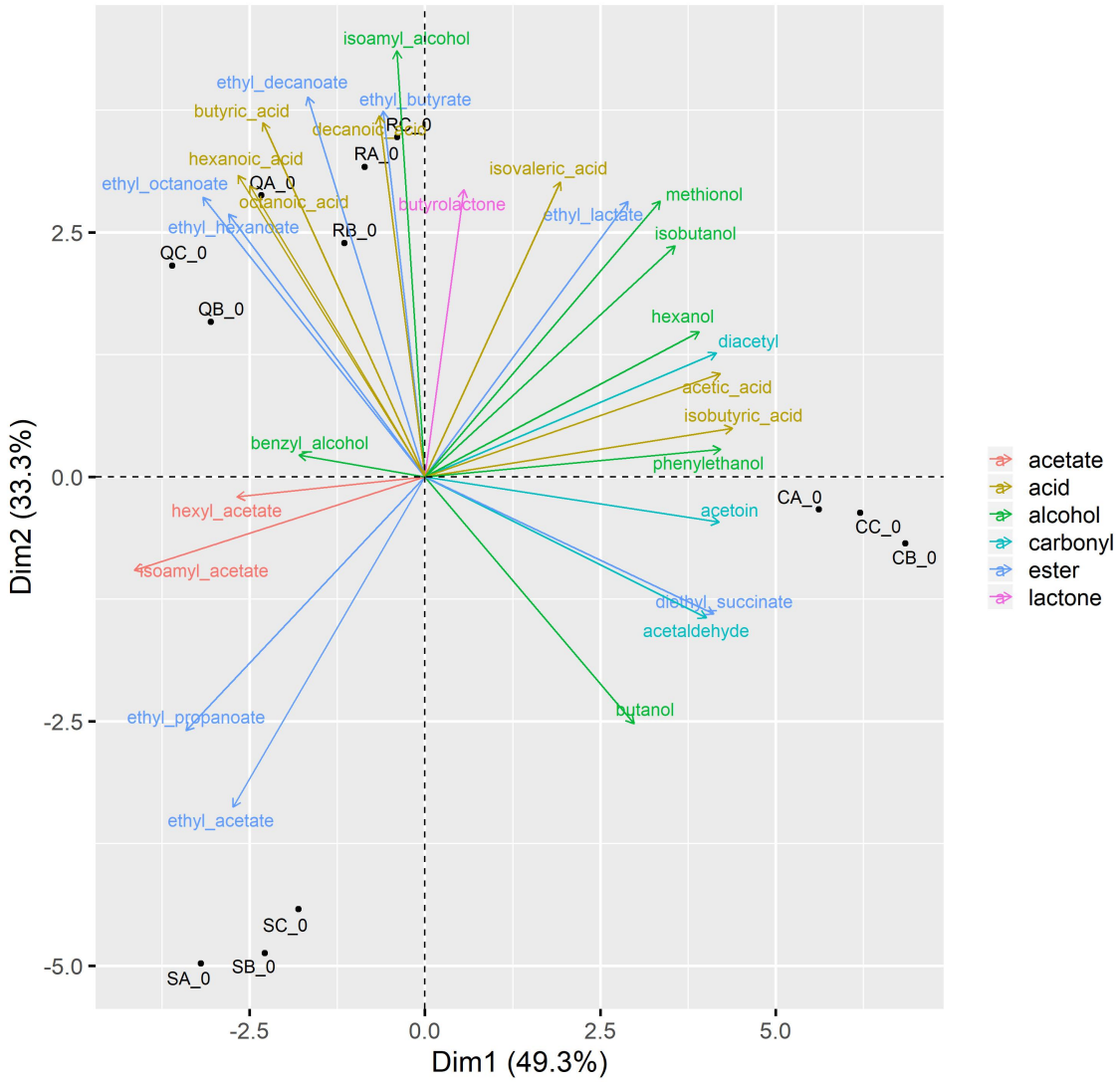

Figure 6.2: PCA representing the distribution of the samples fermented with 4 *Saccharomyces* yeasts according to the concentration of major compounds found in the young wines. QA, QB, QC: QA23 replicates; RA, RB, RC: RHONE replicates; SA, SB, SC: SAUVY replicates and CA, CB, CC: CR89D1 replicates.

and a high production of isoamyl alcohol. Such similarities make them appear in a closer area in the PCA plot, in spite of the fact that they have relevant differences in the levels of isobutyric acid, isobutanol, butanol or benzyl alcohol. Also, RHONE produces highest relative levels of ethyl butyrate plus butyric acid, while QA23 produces higher relative levels of hexanoic, octanoic and decanoic acids and of their ethyl esters. Those differences could be enough to produce significantly different aroma profiles.

6.3.2 Yeast modulation of volatiles during wine aging

Only trace volatiles were quantified throughout aging, since the evolution of most major compounds are of scarce interest for our purposes. Quantitative results of trace aroma compounds are presented in Annex E in Tables E.2 to E.5 (mean concentration ± standard deviation; n=3 replicates), while PFMs concentrations and statistical analysis are given in Tables E.7 and E.8. In the Tables E.6 and E.9, results of the significance of the factors yeast, time and their interaction obtained in the two-way ANOVA are displayed. It can be appreciated that yeasts exerted a significant modulation on most of the volatiles and in a number of cases also on their evolution with time. Only 8 compounds out of the 40 listed in Table E.6 and no one in Table E.9, were not significantly affected by yeast. These compounds were β-ionone, β-damascenone, guaiacol, o-cresol, R-limonene, 1,8-cineole, dihydromyrcenol, acetovanillone and syringaldehyde.

The magnitude of the effect can be assessed by means of the fold change, defined as the ratio between the maxima average concentration and the minima average concentration found in the wines fermented with each of the 4 strains. This parameter is represented in Figure 6.3. Significance of the factors yeast, time and their interaction are also indicated by the letters y, t and * respectively. Leaving aside ethyl dihydrocinnamate, all the volatiles were significantly affected by aging time.

In young wines, the volatiles more affected by yeast were, in this order,

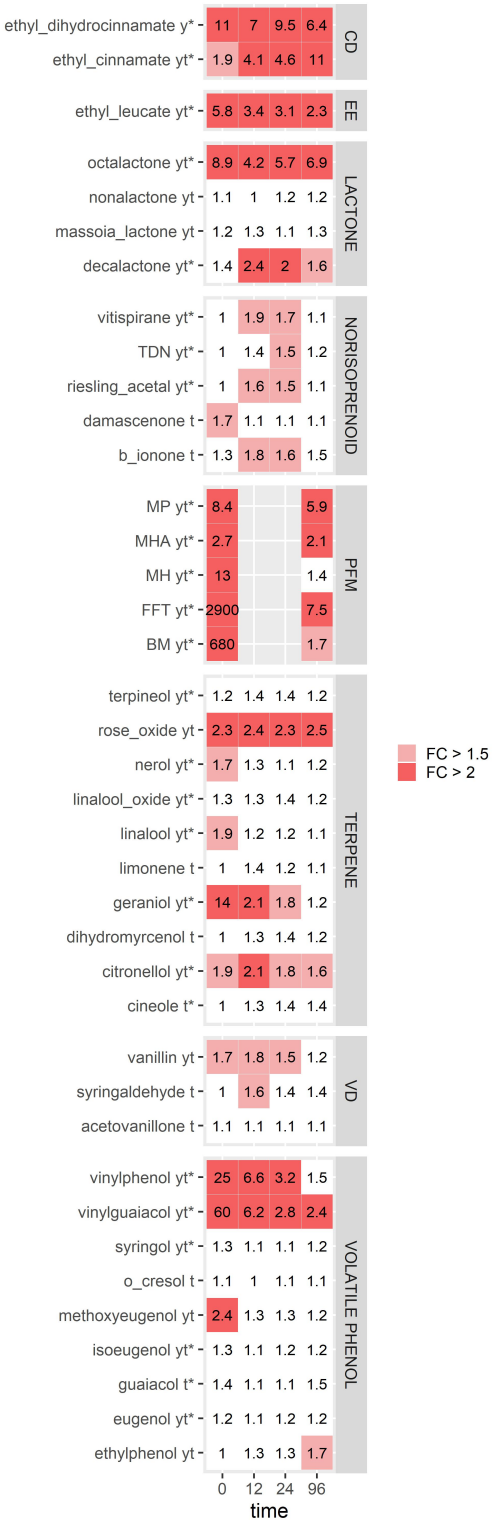

Figure 6.3: Fold change (FC), as the ratio between maxima and minima average amounts found in fermented samples, was calculated for each volatile, at each sampling time. If the compound was not detected, the amount considered was the detection limit. Significance of the factors yeast, time and their interaction is indicated by y, t and *respectively (p-value < 0.05). EE: ethyl ester, CD: cinnamate derivative, VP: vanillin derivative, PFMs: polyfunctional mercaptans.

vinylphenols, geraniol, MH, ethyl dihydrocinnamate, γ-octalactone, MP, ethyl leucate, MHA, methoxyeugenol and rose oxide, all them with a fold change superior to 2. A more moderate effect was observed on nerol, linalool, β-citronellol, ethyl cinnamate and vanillin with fold change values between 1.5 and 2.

During aging, the interaction between yeast and time was significant in many cases (31 volatiles out of 45, Tables E.6 and E.9). In some cases, the differences introduced by the yeast were of little magnitude and were not clearly kept during aging (methoxyeugenol, δ-decalactone, 4-ethylphenol, vanillin), mainly because of imprecision. In some other cases differences are kept constant, increase, or on the contrary are softened, or even disappear completely during aging. As differences introduced by yeast strain in many of these aroma molecules can help us to identify potential precursors or at least to learn about the possible actions of yeast on grape-related aroma, the different cases will be detailed in the following parts.

In some cases, it will be possible to formulate some hypothesis about the type of link or correlation between the aroma volatile and the different buckets detected in the metabolomic experiment. These hypotheses will be checked and discussed in part 6.3.4.2.

6.3.2.1 Differences maintained during aging

Rose oxide, γ-octalactone and ethyl dihydrocinnamate were globally little or not affected by aging time and the differences introduced by the yeast in recently fermented wines were maintained during aging. Highest amounts of these 3 volatiles were found in SAUVY samples at the 4 times of analysis. In the case of rose oxide, it can be suggested that if there is a specific precursor for this compound, it will have to be at smallest level in the samples fermented by SAUVY, since rose oxide is found at double level in wines made with SAUVY. Levels of rose oxide slightly increase in all cases during aging, reaching the maxima after 12 or 24 hours in the four cases.

The case of γ-octalactone is more complex, because it is not clear whether this

compound derives from a specific precursor already present in the grape or if it is formed by the yeast. Precursors for this compound can be the γ-hydroxyoctanoic acid (4-hydroxyoctanoic acid) or any of its glycosides. Only in the case that the precursor is a grape derived compound, we can reasonable expect that the precursor will be at minima levels in SAUVY.

Finally, ethyl dihydrocinnamate is an ester whose level is kept approximately constant during aging. This suggests that the acid precursor, dihydrocinnamic acid, is not an effective precursor, but that the compound is directly formed by yeast during fermentation by a likely metabolic transformation of cinnamic acid. This suggests that samples fermented by SAUVY should contain smaller levels of cinnamic acid, which is consistent with the smallest levels of ethyl cinnamate found after aging in these samples, as it will be later commented.

6.3.2.2 Differences attenuated during aging

Geraniol, linalool and nerol are labile terpenes, so that their levels and the differences introduced by the yeast were maxima after fermentation. As can be seen in Tables E.2 to E.5, SAUVY liberated highest amounts of linalool (tiny amounts, in fact) and of geraniol (together with RHONE) while minima amounts were found in CR89D1. These differences decreased and disappeared during aging as levels of aroma compounds decrease. β-citronellol is also in this category. Its concentration steadily decreases during aging. In this case CR89D1 liberates maxima levels and SAUVY minima (like nerol).

The clearest case is that of geraniol, as expected from its very high fold change (Figure 6.3). It can be seen that the maxima concentration is obtained in SAUVY and RHONE in the freshly fermented wine, and that this maxima level is kept for the two following aging times. By contrast, QA23 reaches the maximum in the second aging point (12 hours), while CR89D1 reaches it only in the last sampling point. This strongly suggests that CR89D1 has been more conservative regarding geraniol precursors, which have not been used, or at least not to produce geraniol. There is

then a chance that one or several precursors of geraniol are negatively correlated to the levels of this molecule found in young wines.

The case of ethyl leucate is a counter example, since concentrations of this compound increase with aging, but differences introduced by yeast decrease. Differences are maxima after fermentation (fold change of 5.8), and become of just 2.3 after the whole aging period (Fig. 6.3). In any case, maxima levels are found in samples fermented by RHONE while those made with CR89D1 contained minimal levels. In this case, levels of the precursor of this molecule (leucidic acid) in the young wines should be positively correlated with the average levels of ethyl leucate found in each grape variety.

Acetates from higher alcohols were also in this category, but they are of no interest for the present discussion (data not shown).

6.3.2.3 Differences accentuated during aging

TDN, vitispirane and Riesling acetal are in this category although in the present case results are, unfortunately, not as clear as they were in previous works (Denat et al., 2021; Oliveira and Ferreira, 2019). These compounds only accumulate after relatively long periods of aging, so that effects of yeast are not observed in young wines. However, and in clear contrast with results presented by Oliveira and Ferreira (2019), the effect of yeast becomes more noticeable at the second or third sampling points and in all cases is nearly completely cancelled in the last aging point. A comparison with pHs, suggests that the observed differences are simply caused by the different hydrolysis rates, which should be influenced by pH. SAUVY, which had a significant highest pH (4.05) produces smaller levels, while CR89D1 and RHONE, with minima pHs (3.84 and 3.86, Table 6.1), produce highest levels. This strongly suggests that in this case the yeasts were, in fact, unable to modulate the precursors of these compounds, so that no differences in their levels in young wines should be expected.

By contrast, ethyl cinnamate was liberated in much higher amounts by CR89D1

and this tendency increased during aging, from a fold change of 1.9 after fermentation up to 11 after 96 hours of accelerated aging. As aforementioned, this suggests that levels found at this last aging time should be positively correlated with the levels of cinnamic acid present in the corresponding young wines.

Ethyl esters of branched acids are also in this category, but they are not grape-related aroma compounds (data not shown).

6.3.2.4 Complex evolutions

Levels of vinylphenols (4-vinylphenol and 4-vinylguaiacol) were strongly modulated by the yeast. Immediately after fermentation levels in QA23 were maxima and were 25 and 60 times higher than those found in SAUVY. However, as wine is aged, differences decrease because maxima levels in QA23 and RHONE decrease, while minima levels in SAUVY increase.

This complex evolution has been previously observed and it is due to the high reactivity of these compounds towards different wine nucleophiles, such as mercaptans or SO_2, and to the existence of effective precursors releasing further aroma molecules by hydrolysis. Samples containing initial maxima levels cannot replace the molecules reacting with wine nucleophiles, so that levels decrease; while samples containing initially low levels, can accumulate them, so that final levels converge. Initial high levels may be attributed to the yeast ability to decarboxylate the precursors phenolic acids, so that these compounds (ferulic and coumaric acid, maybe with their tartrate esters caftaric and coutaric) should be strongly reduced by QA23 and RHONE, in comparison with SAUVY. Alternatively, the higher initial levels could be due to a much higher glycosidic activity towards the specific glycosidic precursors of those powerful aroma molecules. As in the present case, levels remaining in SAUVY and in CR89D1 are higher than those found in QA23 and RHONE, samples from these two strains should contain highest levels of the precursors, which would support the second alternative.

The five PFMs quantified in the study, MH, MHA, MP, FFT and BM were also

strongly and significantly modulated by the yeast, as can be seen in Table E.9. The effects of time were also highly significant in all cases, as it was the interaction yeast x time, confirming previous observations about the complexity of the evolution with time of these molecules. RHONE liberated the highest amounts of MH, nearly 5 times higher than CR89D1, which produced the second highest level, still more than twice levels found in SAUVY or QA23. RHONE also produced quite high levels of FFT and minor levels of BM, being the single strain in which these remarkable odor compounds were detected in young wines, and highest levels of MP, closely followed by CR89D1.

It is most remarkable, however, that the evolutions with aging of MH and MP, two of the most important varietal aroma compounds, are so different. In the case of MH, levels in all the samples except in RHONE strongly increase, so that after aging levels in CR89D1 are maxima, followed by SAUVY, while RHONE and QA23 have similar minima levels, but yet of relatively high magnitude. In the case of RHONE, this increasing evolution could be in part attributed to the higher levels of acetic acid and MH found in young wines, leading to the formation of the acetate. In the case of MP, the highest initial level of RHONE collapse, so that after aging, RHONE contain minima levels together with QA23, well below those in CR89D1 and even below those of SAUVY. This could be in part attributed to the initial high levels of vinylphenols found in RHONE, which could explain the fast decay. The reaction of PFMs with electrophilic polyphenols has been described (Nikolantonaki et al., 2010), including the recent identification of some reaction products (Suc et al., 2021). This would be consistent with the highest levels of MH and MP found in aged CR89D1 wine, which produced initially quite modest levels of vinylphenols. However, levels of vinylphenols in SAUVY were yet smaller, so that it will be of interest to measure the levels of precursors remaining in the initial wines.

6.3.3 A preliminary metabolomic study about the effects of yeast on varietal aroma modulation

The results of the untarget analysis are presented in 3 tables available online in Supplementary Material Tables A, B and C and the description and filtering of buckets is represented in the 3 corresponding Figures 6.4, 6.5 and 6.6.

The 3 data tables (negative buckets in the batch including aging, positive and negative buckets in the fermentations with 4 strains) were treated separately. Several classifications were proposed for the buckets detected. They were classified according to their intensity, their modulation by the fermentation, by the yeast or by the aging time. The statistical tools used for their classification will be detailed in each part. The tables also gather the chromatographic information and tentative identifications and finally the results of the study of correlation. A previous filtering of buckets was performed with the following criteria: RSD (must, 6 analytical replicates) < 50 % and RSD (wines, 3 biological replicates*2 analytical replicates) < 100 %.

6.3.3.1 The effect of fermentation on grape compounds

The tables of buckets in negative and positive mode from the fermentation with 4 *Saccharomyces* strains are presented in the Tables A and B respectively. The first column contains the bucket label composed of its M measured (Da) and retention time (s). The second column contains the p-value calculated performing an ANOVA between the must and the fermented samples altogether, in order to evaluate the significance of the modulations introduced during the alcoholic fermentation (AF). In the case of a significant modulation (p-value < 0.05), the ratio between the average intensities found in must and in wines was calculated (column mean(must)/mean(AF)).

According to their modulation during fermentation, they were classified in the column named “fate during AF” into the categories “not significant”, “decrease”, “decrease until 0”, “increase” and “increase from 0”. Since the latter were absent in the must, those buckets were most likely of fermentative origin and were not

considered in the present part. The following columns contain the classifications of buckets into groups according to their intensity in the must:

- "major" buckets with an intensity $\geq$ 10E6
- "moderate" between 10E5-10E6
- "minor" between 10E4-10E5
- "trace" between 10E3-10E4
- "ultra-trace" with an intensity $<$ 10E3
- "null"

The next column contains the p-value calculated with an ANOVA to evaluate the effect of the strain (excluding the must). In the case of a significant effect (p-value $<$ 0.05), the variability was evaluated by calculating the standard deviation and relative standard deviation of all the wines.

Then, the chromatographic information (retention time, m/z and M measured, the ions formed and the MS/MS acquisition) is gathered in the following column. The four next columns content a potential identification with the name of the compound, its molecular formula, the annotation source and the annotation confidence with the parameters "mzdev" and "mSigma". The former is the difference (in Da) between the m/z calculated for the molecular formula proposed and the m/z measured, while the latter indicates the divergence between the theoretical isotopic pattern for the chemical formula proposed with the experimental one (the smaller, the better).

The next columns contain the intensity of the buckets in each sample.

Finally, at the end of the tables, the correlation matrix between a selection of volatiles at the aging time at which major variability was observed, and the buckets significantly affected by the yeast strain is presented, with the corresponding p-value associated. Correlation coefficients will be briefly commented in the following parts.

As can be seen in the Figure A, a total number of 87796 buckets were detected in negative mode. A first filter was performed in order to eliminate the buckets with a too high variability mostly caused by undetection in some replicates. After this,

29247 buckets with RSDs < 50 % in musts and < 100 % in wines were selected. Among them, 25309 were detected in must, which implies that fermentation created 3938 new buckets. 12321 buckets out of the 25309 detected in the must were significantly modified during the fermentation (p-value < 0.05). In 7390 cases the fermentation made them decrease (average intensity in must $>$ average intensity in fermented samples) including 351 cases in which the buckets completely disappeared. Most remarkably, 4930 buckets previously found in must significantly increased during fermentation. Among the buckets decreasing with fermentation, levels of 2949 were significantly modulated by the fermenting strain (p-value < 0.05) and 25 of them formed a formic acid adduct.

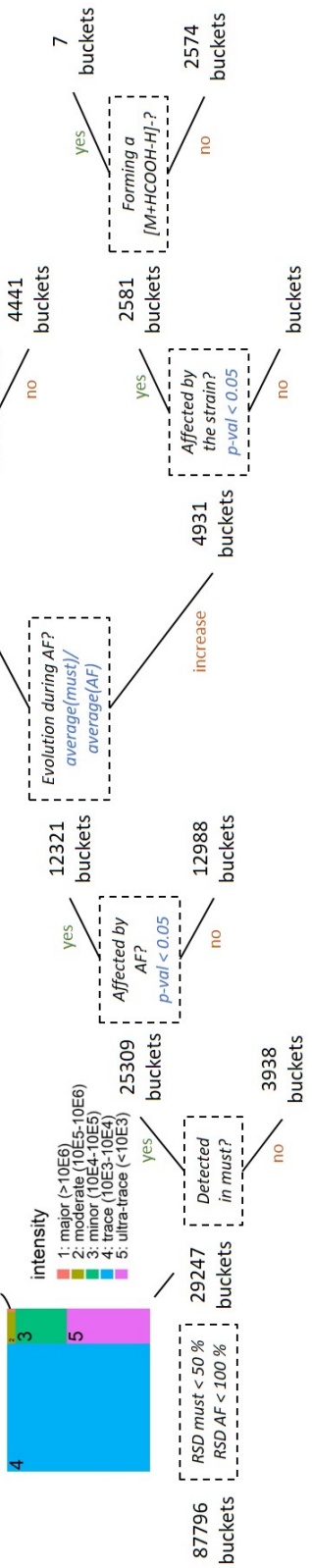

Figure 6.4: Overview of the data obtained via the LC-MS untarget analysis in negative mode of precursors in must and wines fermented with 4 *Saccharomyces* strains. RSD: relative standard deviation, AF: alcoholic fermentation.

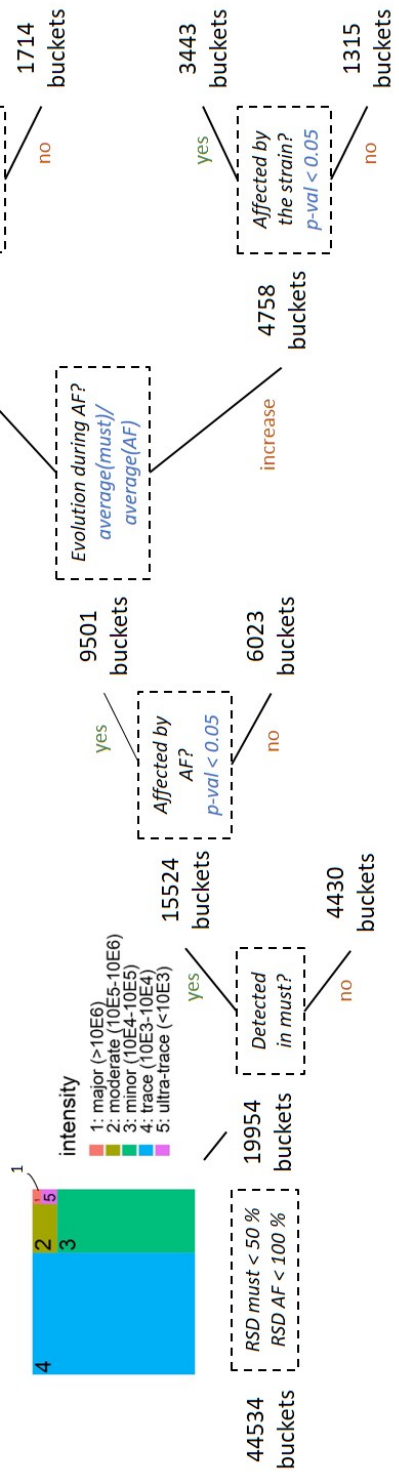

Figure 6.5: Overview of the data obtained via the LC-MS untarget analysis in positive mode of precursors in must and wines fermented with 4 *Saccharomyces* strains. RSD: relative standard deviation, AF: alcoholic fermentation.

Results obtained in positive mode are given in Figure B. In this case, 44534 buckets were initially found in total. This number was further reduced to 19954 applying the first filter criteria (RSD $<$ 50 % in must and $<$ 100 % in wines. 15524 were detected in the must and 4430 were exclusively detected in the wines. 9501 out of the 15524 buckets detected in the must were significantly affected by the fermentation, 4743 saw their levels reduced during this process and in 3029 cases, levels were also significantly modulated by the strain.

The loading plots provided by MetaboScape are available in Annex E (Figures E.1 and E.2 for the negative and positive modes, respectively). Plots were obtained with the total number of buckets detected. As can be seen, the must is very well separated from the fermented samples, as expected. Fermented samples are also clearly separated attending to the yeast. QA23 and RHONE are clustered closed together, as was previously observed with major compounds, while those of SAUVY and CR89D1 were clearly separated from the two previous ones, and could even show some similarity, which was not observed in the major aroma compounds.

6.3.3.2 The effect of aging on grape compounds

In the experiment including anoxic accelerated aging of the wines fermented with the yeast strain QA23, results are presented in Table C. Buckets were, as previously done, categorized into groups attending to the magnitude of their intensities. The next column contains the p-value calculated with ANOVA in order to assess the effect of aging. In the case of a significant effect of aging time, the variability introduced during aging was evaluated with the standard deviation and relative standard deviation between all the wines. The column "fate during aging" consists in a comparison between the first and last point of analysis, indicating if the compound globally increased or decreased during aging. Following, the chromatographic and identification information is gathered, including raw data and the potential candidate precursors as a result, of the study of correlation between volatiles and buckets.

Figure 6.6 summarizes the results obtained in negative mode in the aging experiment. In this case 21707 buckets were detected, and were further reduced to 14440 after applying the first filters (RSD $<$ 50 % in must and $<$ 100 % in wines). 10788 of the buckets detected in the experiment were detected in must. Of these, 7851 were significantly affected by aging time, and 60 of them formed a formate adduct. These samples were not analyzed in positive mode. The loading plot provided by MetaboScape is available in Annex E (Figure E.3). As expected, the must is the most different from the other samples. However, recently fermented wines are also well clustered together and are very dissimilar to the aged samples, which are well clustered by aging time, showing increasing differences to the recently fermented wine progressively with the aging time.

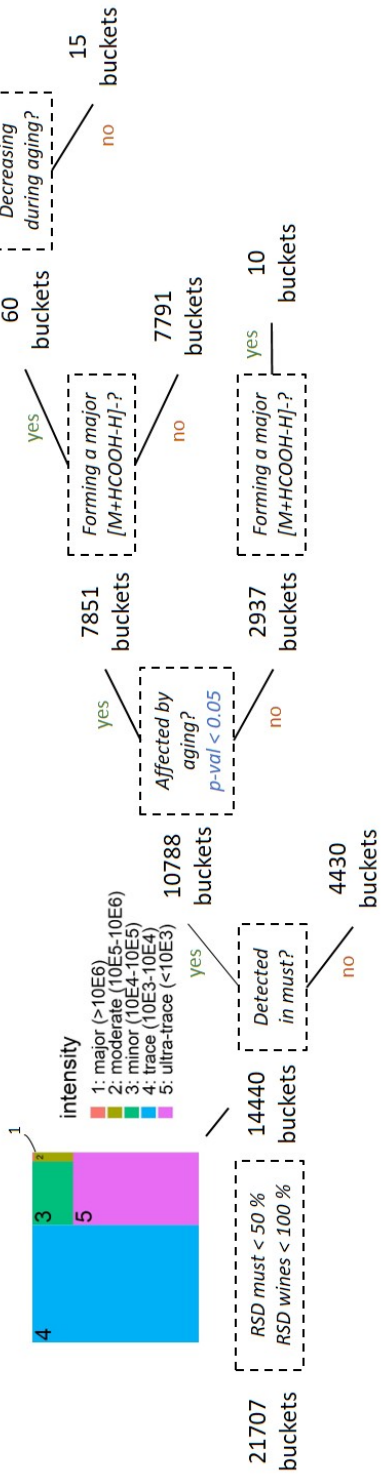

Figure 6.6: Overview of the data obtained via the LC-MS untarget analysis in negative mode of precursors in must and wines fermented with *S. cerevisiae* QA23 and submitted to accelerated anoxic aging at 75 ºC. RSD: relative standard deviation.

6.3.4 Potential precursors identification

6.3.4.1 Buckets forming formate adducts in negative mode (FANM)

Many precursors of aroma compounds are glycosides containing a polar but non-ionic part, the glucoside, and a rather non-polar and most often non-ionic aglycone part. These types of molecules have difficulties in forming stable ions in the different ion sources of the HPLC, so that very often the most abundant ion obtained in their MS analysis are adducts. In negative mode in the electrospray, they form majorly adducts with formate, as demonstrated in previous reports (Caffrey et al., 2020) and (Flamini et al., 2014). This is the case also of many oligosaccharides (Verardo et al., 2009). This property can be then exploited to make a first selection of buckets which could be glycosidic aroma precursors.

In the fermentation experiment with the 4 different yeast strains, 58 buckets formed a more intense adduct with formic acid. 55 of them were also detected in the must. Two of these 55 buckets were, by intensity, classified as major compounds, 19 of them reached moderate intensities, 19 minor and 15 were at trace level. 39 buckets out of the 55 were significantly reduced during fermentation and 25 were significantly modulated by the fermenting strain. Data from these buckets are compiled in Table A. All are common to all the yeasts.

In the aging experiment with the QA23 yeast strain, 73 buckets forming a more intense adduct with formic acid were detected, 70 of which were also detected in the must. Of these, 15 buckets were classified by intensity into the moderate category, 41 were minor compounds, 13 were at trace level and 1 was detected at ultra-trace level. An overwhelming majority (60) of all these buckets were significantly affected by aging time. Data from these buckets are included in Table C. All the buckets were detected in all the sampling times.

Aging experiment with samples fermented with QA23 - The correlation matrix between the 60 buckets forming a formate adduct and significantly affected by aging time and the levels of volatiles significantly affected by aging time were

calculated. The correlation matrix is represented in Figure 6.7. A hierarchical clustering was also displayed on the left and upper parts of the plot, to facilitate the identification of aging patterns between the different aroma compounds. These aging patterns were already discussed in part 6.3.2.

As can be seen in yellow in the Figure 6.7, volatile compounds in cluster 1 are those increasing during aging, such as α-terpineol, linalool-oxide, TDN, ethyl cinnamate, α-terpineol, vanillin or eugenol. The second cluster includes compounds reaching a plateau during aging, or those others reaching a maximum and then a decrease such as acetovanillone, δ-decalactone, γ-nonalactone, β-damascenone, γ-octalactone, rose oxide, linalool or geraniol. Cluster 3 only includes dihydromyrcenol and nerol which have unclear evolutions. Cluster 4 includes β-citronellol, R-limonene 4-vinylguaiacol and 4-vinylphenol, which in QA23 samples followed a continuously decreasing trend.

On the other hand, buckets can be separated into 6 clusters. As can be seen in green on the Figure 6.7, cluster 1 includes 6 buckets that increase continuously during aging, and those others presenting a maximum and then a decrease. Clusters 2, 3, 4 and 5 include 2, 3, 3 and 1 buckets, respectively, whose levels are globally kept constant, decrease but are affected by a high variability, or present a maximum and then decrease, also respectively. Finally, the majority of buckets (45) are in cluster 6 and are characterized by a decreasing tendency throughout aging.

As an aroma precursor is, by nature, a molecule which suffers a transformation to yield the aroma molecule, it can be suggested that the 45 buckets of the cluster 6 are possible candidates, as indicated in the Table C.

Moreover, as the levels of the precursor molecule should decrease while the levels of the aroma molecule increase (if it is not a very unstable molecule), a negative correlation (represented in blue on the heatmap) between the precursor and the aroma molecule should be expected. This statement is valid for volatiles in cluster 1, which are those ones whose concentration continuously increased throughout aging. The correlation matrix between volatiles in cluster 1 and buckets from cluster 6 is

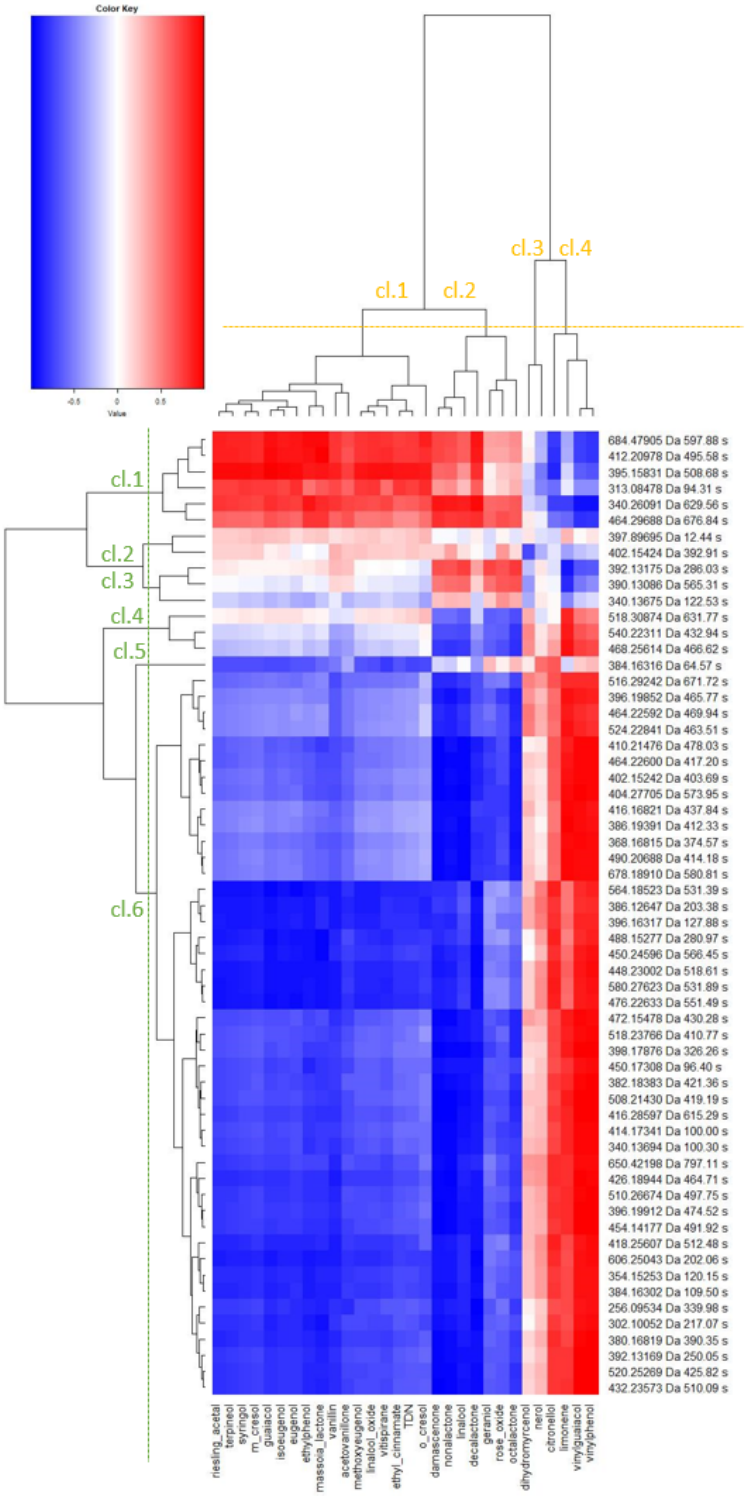

Figure 6.7: Representation of the correlation matrix between the buckets significantly affected by aging time and forming a formate adducts; and the volatiles found in the wines recently fermented by QA23 and significantly modulated by aging time. Correlation coefficients (R) from -1, in blue to 1, in red. Hierarchical clustering results are also displayed on the left and upper part of the heatmap.

given at the end of the Table C as well as the p-value associated. Since a relatively large number of volatiles and of buckets share similar accumulation/evolution patterns, this study just provides a relatively wide list of buckets some of which could be genuine aroma precursors to the aromas in cluster 1. It can be seen that some of them have been putatively identified as glycosylated precursors of sesquiterpenes, monoterpenes or of norisoprenoids.

Fermentation experiment with 4 different yeast strains - Correlations were calculated between the intensities of the 13192 buckets significantly affected by the fermenting yeast (Table A), and the concentrations of a selection of volatiles in the recently fermented wine. Volatiles selected for this study were those whose levels were significantly affected by the fermenting yeast and for which the modulation was maxima (t1 - in young wines: rose oxide, geraniol, ethyl dihydrocinnamate, γ-octalactone, linalool, nerol, β-citronellol, 4-vinylphenols and 4-vinylphenol; t2 - after 12 hours of accelerated aging: vitispirane, Riesling acetal; t3 - after 24 hours of accelerated aging: TDN t4 - after 96 hours of aging: ethyl cinnamate, ethyl leucate; discussed in the part 6.3.2).

These correlations are presented at the end of the Table A as well as their significance. A high negative correlation may indicate that the bucket could be a potential precursor of the aroma compound and suggest that the yeast is directly and positively affecting the liberation of the aroma compound. Remarkably and unfortunately, all the buckets showing statistical significance were among the many unknowns and no one of them was among those forming a formate adduct.

6.3.4.2 Specific hypothesis about links between aroma volatiles and buckets (enunciated in part 6.3.2)

Precursors for cis-rose oxide - As aforementioned, in some cases in which a high variability in an aroma volatile has been introduced by the yeast, it has been possible to formulate specific hypotheses about the potential links between the volatile and its precursors. Those hypotheses can help in the identification of potential candidates

between the many buckets detected in the metabolomic experiment.

Additionally, potential glycosides of those selected aroma volatiles, have been specifically searched. In these cases, as detailed in (Hjelmeland et al., 2015), the sugar moiety can be either a glucoside (sugar moiety: C6H12O6), a malonylated glucoside (sugar moiety: C9H14O9), a glucose-pentose glycoside (sugar moiety: C14H26O7), a glucose-deoxyhexose glycoside (sugar moiety: C12H22O11) and a glucose-pentose-glucose glycoside (sugar moiety: C18H32O16). Therefore, potential precursors should have a molecular mass equivalent to that of the aroma volatile, plus the glucoside minus a water molecule, lost in the glycosidic bond.

In the case of rose oxide, which is a cyclic ether, 4-hydroxy-2,6-dimethyl-2-octenol (C10H20O2) corresponds to the diol precursor. Such diol can then form glycosidic precursors with the sugar moieties previously described, so that, the following molecular formulae C16H30O7, C19H32O10, C24H44O8, C22H40O12, C28H50O17 will be expected.

Several buckets have been identified with the *m/z* and molecular formulas potentially corresponding to several putative precursors of cis-rose oxide: two corresponding to the diol precursor itself (C10H20O2), 2 to its glucoside (C16H30O7), 2 to its malonylated glucoside (C19H32O10), 2 to its glucose-pentose glycoside (C24H44O8) and 3 to its glucose-deoxyhexose precursor (C22H40O12). They were annotated (column "name", Table A). Several different buckets have been identified for the same compound, although the identification confidence (mzdev and mSigma) is better for one of them. In any case, a validation is necessary, by MS/MS acquisition and/or injection of pure reference compound.

On the other hand, the presence of isomers is not surprising since the hypothetical precursor has several sites available to bind the sugar moiety. All these putative precursors for cis-rose oxide, formed a [M-H]- ion and the formate form was not detected. This could be consistent with the presence of a hydroxyl in the aglycone part of the precursor. At this moment, MS/MS was acquired only for one of the annotated compounds, a putative glucose-deoxyhexose glycoside,

present at moderate level. The MS/MS spectra confirms the presence of a glucose moiety (fragments 161.04495 and 179.05693). However there is also a fragment characteristic of pentose (fragment 131.03519), while those ones characteristic of deoxyhexose are absent (Caffrey et al., 2020). The other candidates were present at trace and ultra-trace levels and at present it has not been possible to obtain their MS/MS spectra. Five out of the 10 putative glycosylated precursors decreased during fermentation while the other ones increased or were not significantly modified. Buckets increasing should not be discarded as putative precursors at this point of the study, since the yeast may have been involved in their formation from other glycosides. Only one bucket (putative malonylated glucoside, RT = 6.89 min) was differentially modulated by the fermenting strain. QA23 and CR89D1 contained approximately the same amount as the must, while RHONE contained minima and SAUVY maxima amounts.

The two putative direct precursors (not glycosylated) were significantly and positively affected by the fermentation, which make their levels increase. They were also significantly and differentially modulated by the yeasts. In both cases, samples fermented by SAUVY contained smaller amounts of both buckets. This would suggest that the yeast would not only form the diols but would also be involved in their internal dehydration to form cis-rose oxide. SAUVY, would be particularly efficient at this transformation, so that it contains minima amounts of the diols and maxima amounts of rose oxide.

Precursors for γ-octalactone - Regarding potential precursors of γ-octalactone. Several buckets with the m/z and molecular formula corresponding to one of its precursors have been identified and annotated (column "name", Table A). They were all [M-H]-. Only one of them, present in trace amounts, corresponds to the direct precursor (C8H16O3). Seven corresponds to the glucoside (C14H26O8), 6 of them were found at trace level, 1 at minor level, and one was absent in the must. Two more corresponded to its glucose-pentose glycoside (C22H40O9), one in moderate and the other one in minor amounts. The MS/MS spectra of the latter

was the only one acquired, but, there were not any fragment supporting the presence of a pentose-glucose moiety.

The direct precursor was the only one that increased during fermentation (from around 4000 to 70000 in average), and it was also differentially modulated by the fermenting strain. CR89D1 contained minima amounts, followed by RHONE, SAUVY and QA23; while the volatile was maxima in SAUVY. Six out of the 9 putative glycosylated precursors decreased, one increased from 0 (not detected in must) and two were not significantly modified during the fermentation. Among the decreasing buckets, only 2 were differentially modulated according to the fermenting strain: one of the glucosides (RT = 5.8 min) and one glucose-pentose glycosylated precursor (RT = 9.2 min). Both were found at minimal levels in SAUVY. This would support that the yeast has a specific role in the cleavage of the glucoside and maybe also in the cyclization.

Precursors for ethyl cinnamate - Five buckets with m/z and molecular formula corresponding to cinnamic acid (C9H8O2) have been identified (Table A). Four were present in trace levels and one of them in ultra-trace levels in the must. Two of them were not significantly modified during fermentation while 2 increased, and one decreased. Only one of the fermentation-increasing buckets (RT = 8.26 min) was differentially modulated by the fermenting strain and SAUVY had the lowest amounts, followed by QA23, RHONE and CR89D1. This is consistent with the minima levels of this molecule found in aged SAUVY, which would suggest that yeast forms the acid, and this molecule slowly esterifies with ethanol to form the ester.

Precursors for geraniol - Regarding geraniol precursors, 11 buckets with m/z and chemical formula corresponding to putative geraniol glycosides have been identified. 3 glucosides (C16H28O6), 3 malonylated glucoside (C19H30O9), 1 pentose-glucose glucoside (C24H42O7), 4 glucose-pentose-glucose glucoside (C28H48O16) and 3 geranic acid glycosides (C21H34O11, C22H36O12). They

were all detected in minor to ultra-trace amounts (column "name", Table A). The presence of several candidates with the same molecular formula was expected since geraniol, linalool and nerol are isomers.

Five of the buckets were not significantly modified during fermentation, seven decreased, including one which completely disappeared in all the samples, and 1 increased. Six out of the 11 were differentially affected by the fermenting strain, and 5 of those 6 (1 glucoside, 2 glucose-pentose-glucose glycoside and two geranic acid glycosides) were minima in CR89D1. As wines made with this strain contained smallest initial amounts of geraniol, this would suggest that yeast can transform the precursor into another molecule not further yielding geraniol. Most remarkably, wines made with CR89D1 contained maxima levels of nerol and β-citronellol. The former is a geometric isomer to geraniol (one double bond in cis instead of trans), and the latter is a reduced form of geraniol.

Precursors for ethyl leucate - Five buckets with m/z and chemical formula corresponding to leucidic acid (C6H12O3) have been identified (Table A). Five other buckets with m/z and chemical formula corresponding to putative different glycosides have been also found, including 2 glucosides C12H22O8, 1 malonylated glycoside C15H24O11, 1 glucose-pentose glycoside C20H36O9 and 1 glucose-pentose-glucose glycoside C24H42O18. In two cases, the MS/MS spectra was acquired but there was not any fragment characteristic of the presence of sugars. These buckets were present in moderate to trace amounts.

Three of the buckets with m/z and chemical formula consistent with those of leucidic acid increased during fermentation, and all of them were differentially affected by the fermenting yeast. Wines made with RHONE and CR89D1 contained in each case the maxima and minima amounts respectively, perfectly paralleling the levels of ethyl leucate in wines. This result strongly suggests that leucidic acid is mostly formed by yeast during fermentation.

Only two buckets with data consistent with glycosylated forms decreased during fermentation, and only one of them (the one with RT = 4.08 min) did it differentially

according to the fermenting yeast. Again, RHONE and CR89D1 contained, respectively, maxima and minima amounts, which suggests that leucidic acid is formed by cleavage of the glycosidic precursor, but that this precursor can be also metabolized to another molecule, particularly by CR89D1.

Precursors for 4-vinylguaiacol and 4-vinylphenol - Regarding direct precursors of 4-vinylguaiacol, buckets with data consistent with 2 putative glucosides (C15H20O7), one putative glucose-pentose glycoside (C23H34O8), one glucose-deoxyhexose glycoside (C21H30O12), one glucose-pentose-glucose glycoside (C27H40O17), have been found. Those buckets were present in trace and minor amounts and only two were differentially modulated by the fermenting strain. One of them (with RT = 10.95 min) contained maxima amounts in SAUVY and QA23 and the second one (with RT = 6.05 min), contained maxima levels in RHONE and QA23. After fermentation, the concentrations of 4-vinylguaiacol and 4-vinylphenol were maxima in QA23 and RHONE and during aging, the maxima amounts found initially in the recently fermented wines, decreased and vice-versa.

Five of the buckets found have data consistent with ferulic acid (C10H10O4); three more with its glucoside (C16H20O9), one with its malonylated glycoside (C22H30O14), one with its glucose-deoxyhexose glycoside (C22H30O14) and two more with its malonylated glucoside (C19H22O12). Two of them (RT = 6.58 and 7.54 min) was negatively correlated with levels of 4-vinylguaiacol in the initially fermented wines (ferulic acid glycoside and ferulic acid, $R < -0.9$, end of the Table A), suggesting that it could correspond to a precursor yielding 4-vinylguaiacol during fermentation. A second one formed a formate adduct, and it was found at minima levels in SAUVY and RHONE. MS/MS spectra was acquired for these two last buckets but no fragment characteristic of sugar was identified. Finally, three buckets with data consistent with caftaric acid glucoside (C19H22O14) and one with its glucose-deoxyhexose glycoside (C25H32O19) were found. However, none of them decreases during fermentation.

Concerning 4-vinylphenol, 24 buckets with *m/z* and chemical formula consistent

with a putative precursor have been identified, including glycosylated 4-vinylphenol, 2 consistent with its glucose-deoxyhexose (C20H28O11) glycoside, one with its glucose-pentose-glucose (C26H38O11) glycoside, one with its malonylated glucoside (C17H20O9). All of them were present in trace amounts, and only one was significantly affected by the fermenting yeast (RT = 8.17 min) and it was minima in RHONE and QA23, which produced initially maxima levels of this molecule. Six buckets had data consistent with those of coumaric acid (C9H8O3), three with coumaric acid glucoside (C15H18O8), one with its malonylated glycoside (C18H20O11) and 6 with coumaric acid glucose-deoxyhexose glycoside (C21H28O13). Two of the coumaric acid candidates (RT = 6.25 and 4.11 min) decreased and were minima in CR89D1 and SAUVY, suggesting that those candidates are not yielding 4-vinylphenol during fermentation, although they could form it during aging. One of them (coumaric acid, RT = 7.19 min) was negatively correlated with the amounts of 4-vinylphenol after fermentation ($R < 0.9$, end of Table A).

One bucket had data consistent with those of coutaric acid (C13H12O8), one with coutaric acid malonylated glycoside (C22H24O16) and 2 with coutaric acid glucoside (C19H22O13). Only one of them decreased and was significantly affected by the fermenting yeast. However, it was minima in SAUVY and RHONE, which suggests that it is not determinant in determining 4-vinylphenol levels of young or aged wines.

6.3.4.3 Correlations between levels of PFMs and positive buckets

Regarding PFMs, the correlation matrix was built between the intensities of the 19956 buckets in positive mode, affected by the strain and the concentrations of PFMs in the recently fermented wines and also after 96 hours of aging (indicated as compound_t1 and compound_t4 respectively). Such correlation matrix is at the end of the Table B as well as the significance of the correlation coefficients. In the following part, only the correlations inferior to -0.9 will be discussed, and with the

buckets decreasing during fermentation.

Regarding varietal thiols, MH (t1) was correlated only with one bucket forming a [M+H+H]2+ adduct and present in trace amounts; while after aging (t4), it was correlated with 6 different buckets present at minor and trace level, as can be seen in Table B. In the case of MHA (t1) 13 correlations were found, one of them corresponded to a bucket present at moderate intensity putatively identified as indole. This volatile is produced by the yeast from tryptophan during fermentation and if present at high levels, may be responsible for an off-flavor, conferring to wine some plastic notes (Capone et al., 2010). Its concentration increase during aging and it has also been hypothesized that indoles could be a key marker of aged wines (Arapitsas et al., 2018). After aging (t4), 14 negative correlations were found with buckets in moderate, minor and trace level, but none of them were identified. MP was correlated with 62 buckets at t1 and with 70 buckets at t4. 54 buckets were common and one of them was identified as indole-lactic acid.

6.3.4.4 Identification of precursors for varietal PFMs

The first precursors of MH and MP identified in grape must were the conjugated forms to cysteine (Tominaga et al., 1998) and glutathione (Des Gachons et al., 2002; Fedrizzi et al., 2009). The enzymatic conversion of Glu-MH into Cys-MH was initially suggested in grapes (Des Gachons et al., 2002), and during wine fermentation by yeasts (Grant-Preece et al., 2010). The cysteinylglycine (Capone et al., 2011) and glutamyl-cysteine (Bonnaffoux et al., 2017) conjugates were identified as intermediaries. The mechanisms involved in these transformations by the yeast during fermentation were recently studied (Bonnaffoux et al., 2018). The direct liberation by β-lyase yeast activity of MP and MH from all these precursors was observed; as well as the conversions involving carboxypeptidase: from Glu-MH to Glu-cys-MH and from Cys-Gly-MH to Cys-MH. In the present case, no target analysis was carried out, so that the precursors were generically quantified using the untarget method.

Two candidates were identified by comparison with the analysis of pure reference compounds and listed in Table B: the two diastereoisomers of 3-S-glutathionylhexan-1-ol (Glu-MH) and 3-S-cysteinylhexan-1-ol (Cys-MH).

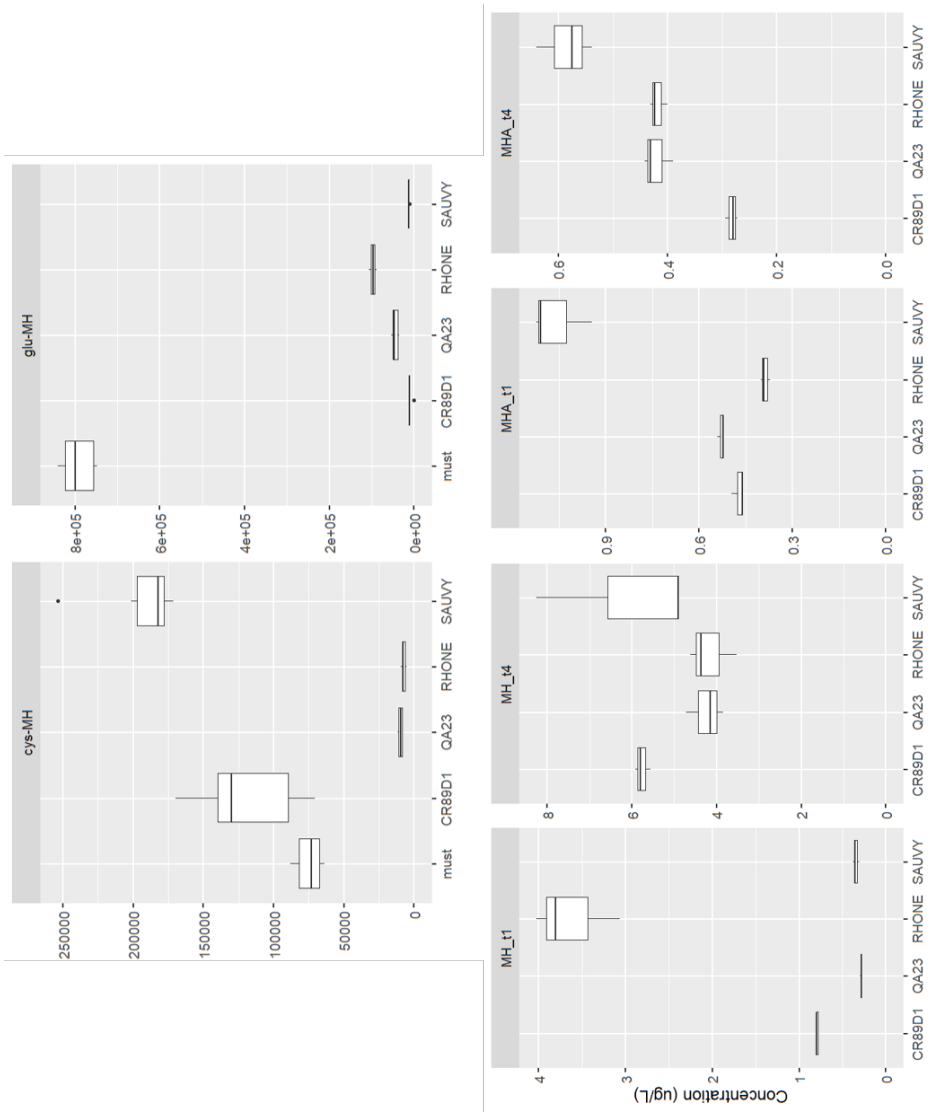

Figure 6.8: Intensity of PFMs precursors Cys-MH and Glu-MH and concentration of MH and MHA in μg/L.

As can be seen in the boxplots of Figure 6.8, only Glu-MH is strongly and generally metabolized by yeasts, which consume more than 95 % of this abundant precursor during fermentation (the intensity of [M+H]+, goes from 793.103 ± 40.103 in the must to 40.103 in average in the recently fermented wines). Cys-MH was, however, only metabolized by QA23 and RHONE. In any case, the aroma compound was not a major by-product of metabolization of the precursors. In fact, assuming that the must contained around 1 mg/L of Glu-MH (Concejero et al., 2014), the amount of precursor converted in the volatile (as sum of MH and MHA) represents less than 0.1 %, which is in accordance with previous studies (Alegre et al., 2017; Bonnaffoux et al., 2018; Capone et al., 2011). In fact, there seems to be a negative correlation between the metabolization of Glu-MH and the levels formed during fermentation (R = 0.86, Table B), since RHONE consumed smallest levels of the precursor and produced highest levels of the aroma volatile.

Cys-MH was contained in samples fermented by SAUVY at levels significantly higher than those originally present in the must, suggesting that in this particular case Cys-MH may have originated from the reaction between S-3-(hexanal) and/or its bisulfite adduct and cysteine (Thibon et al., 2016). This result may be relevant since, Cys-MH could remain as the nearly single source of MH during aging for wines fermented with CR89D1 and SAUVY.

Table 6.2: Correlations between precursors intensity and PFMs in the wines recently fermented (t1) and after 96 hours of accelerated aging at 75 ºC (t4). Significance (p-value < 0.05) is indicated by *.

R	MHA (t1)	MH (t1)	MP (t1)	MH+MHA (t1)	MHA (t4)	MH (t4)	MP (t4)	MH+MHA (t4)
Glu-MH	-0,5	0,8*	-0,7*	0,7*	0,002	-0,6*	-0,7*	-0,6*
Cys-MH	0,7*	-0,5	0,5	-0,3	0,3	0,6*	0,5	0,6*
all MH prec	0,8*	-0,2	0,3	-0,07	0,5	0,5	0,35	0,5

Correlations between PFMs precursors and volatiles were represented in the Table 6.2 . It can be seen that levels of MH found in recently fermented wines are positively correlated to the wine content in Glu-MH. This result has not an easy interpretation and we are not aware of the existence of similar previous observations. If it is not a statistical artifact, it may suggest that strains with more facility to transport and assimilate Glu-MH are also those more efficient at transforming it in products different to MH, such as a source of Glu or Cys. On the other hand, levels of MH found after aging are weakly but significantly and positively correlated to the levels of Cys-MH precursor found after fermentation. Considering the strong effect likely caused by initial levels of vinylphenols on the fate of free MH, this result strongly suggests that Cys-MH precursor maybe particularly relevant as an additional source of MH during aging, while Glu-MH will be of minor relevance in this extent. This is consistent with the higher levels of Cys-MH found after fermentation. In addition, Glu-MH may be more stable to hydrolysis than Cys-MH, which is more hydrophobic and is sterically less hindered. This statement, however, would require experimental confirmation.

Precursors of MH are liberated through β-lyase activity, which is highly strain dependent (Ruiz et al., 2021), and this cleavage occurs into the cell, requiring amino acids and glutathione transporters. Other enzymes are also involved into the cleavage of precursors, such as γ-glutamate transpeptidase involved into the transformations of Glu-MH in Cys-Gly-MH and minorly of γ-Glu-Cys-MH in Cys-MH; or carboxypeptidase from Cys-Gly-MH to Cys-MH (Cordente et al., 2019). Finally, MHA is formed via the action of alcohol acetyl transferase. In the case of MP, the pathway leading to this volatile is still unknown, mainly because of the very low concentrations of precursors (Bonnaffoux et al., 2018). The great variability in MP liberation by yeasts was mainly attributed to the presence or absence of a deletion in the IRC7 gene, encoding a cysteine-S-conjugate β-lyase enzyme (Dufour et al., 2013).

6.4 Conclusions

In this study, the variability introduced by the strain of yeast in charge of fermentation has been used as a means to induce differences in the aroma composition of wine throughout its aging. Such variability should provide some additional clues helping to identify, by untarget HPLC-MS strategies, varietal aroma precursors and also some of the processes linked to varietal aroma formation during fermentation and aging.

This approach has made it possible to introduce notable differences in levels of vinylphenols, geraniol and rose oxide, MH and MP, ethyl dihydrocinnamate, ethyl cinnamate, ethyl leucate, γ-octalactone and methoxyeugenol. However, the approach failed in introducing enough variability for nor-isoprenoid aroma compounds, such as β-damascenone, β-ionone and TDN. As this study is complementary to a second one carried out by a research colleague (Elayma Sánchez-Acevedo) is expected that these compounds could be covered in that second work.

The metabolomic study has revealed that alcoholic fermentation introduces a most notable change on must chemical composition, since levels of more than half of the compounds detected in the grape must (as HPLC-MS buckets) change during the process. The yeast in charge of fermentation also has a most notable influence, so that around a 50 % of the compounds changing are significantly influenced by the strain. By contrast, the direct effect of the strain of yeast on those compounds forming intense adducts with formic acid (glycosides with non-polar aglycones, putative aroma precursors) is marginal (less than 0.5 %).

The metabolomic study has also revealed that aging itself exerts a notable effect on must chemical composition, so that nearly 75 % of all the compounds detected in must suffered compositional changes during aging, including compound forming a major adducts with forming acid. Most notably, another 85 % of the few compounds forming strong adducts with formic acid, decreased during aging, which supports the relevance of aging for producing varietal aroma derived from glycosidic precursors.

The study has also made it possible to detect putative precursors for the aroma compounds showing more variability. At the expense of confirmation by MS-MS and other additional identification studies, a relevant group of chemicals putative precursors for cis-rose oxide, γ-octalactone, ethyl cinnamate, geraniol, ethyl leucate and 4-vinylphenols, have been detected and annotated. The study has finally made it possible to obtain some potentially interesting clues about the fate of varietal PFMs which deserve further research. These are the possibility that yeast strain could enhance the levels of Cys-MH during fermentation. This can have relevance since correlation studies support that this precursor is the most important source of MH during aging.

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Chapter 7

General conclusions

The fundamental conclusion of the present thesis is that fermentation, and in particular the fermenting yeast, not only determine the composition of fermentative aroma, but also strongly influence wine aroma evolution and hence its longevity and sensory quality. Yeasts can modulate wine aroma profile in at least three different ways:

1. Acting on primary varietal aroma and its evolution during wine aging
   (a) Accelerating precursors hydrolysis, improving aroma formation but without altering the final amount of aroma formed:
      – directly (via enzymatic activity, for example for β-damascenone, Ch. 3 and 4 or geraniol, Ch. 6)
      – indirectly (acidifying pH, for example TDN, Ch. 6)
   (b) Metabolizing or transforming the aroma precursor in other compound, and hence reducing the final quantity of aroma formed (guaiacol, massoia lactone, TDN, Ch. 3)
   (c) Metabolizing some related grape molecules into aroma precursors, and hence increasing the aroma formed (for example for vanillin, methoxyeugenol, Ch. 3; rose oxide, β-citronellol Ch. 5 and 6)
   (d) Forming by itself varietal aroma (*de novo* formation)
   (e) Forming reactive species that destroy varietal aroma:
      – Vinylphenols reactive toward polyfunctional mercaptans (Ch. 5 and 6)
      – SO_2 reactive toward β-damascenone (Ch. 5)
2. Modulating Strecker aldehydes in at least 2 different ways:
   (a) Modulating the amounts produced during fermentation (Ch. 5)
   (b) Producing the necessary reactive medium with residual aminoacids and dicarbonyls for their formation in anoxia at different yeast-related rates (Ch. 5)

3. Producing or modulating precursors of fermentative aroma relevant in aged wines (fruity ethyl esters of branched acids, leucidic acid and cinnamic acids, Ch. 3-6)

Moreover, this thesis leaves relevant experimental observations that could lead to new technological developments:

1. Some yeasts may partially metabolize some glycosidic precursors related with aging related off-odors such as TDN, massoia lactone and guaiacol (Ch. 3)

2. The de novo formation of relevant amounts of linalool and geraniol by the yeast strain IONYS, which was only observed in certain fermentative context (Ch. 3), which suggests a complex regulation.

3. Varietal PFM precursors remaining after fermentation may play a relevant role in the amounts of volatiles in wine during aging (Ch. 5 and 6)

Finally, the preliminary metabolomic study completing this thesis confirmed that fermentation affects intensely grapes components, apart from glycosides of aroma precursors only minorly affected; and that during aging a strong transformation of grape components was observed, including varietal aroma precursors (Ch. 6).

List of abbreviations

ATP adenosine triphosphate.

BM benzylmercaptan.

CDD carotenoid cleavage dioxygenase.

CFU colony-forming unit.

CIS cryo-cooled injection system.

Cys-MH cysteine-3-mercaptohexan-1-ol.

Cys-MP cysteine-4-mercapto-4- methyl-2-pentanone.

D.O. Ca Denominación de Origen Calificada.

DBU 1,8-diazabicyclo[5.4.0]undec-7-ene.

DCM dichloromethane.

DL detection limit.

DMS dimethylsulfide.

EDTA ethylenediaminetetraacetic acid.

ESI electrospray ionization.

FAN free ammonia nitrogen.

FFT 2-furfurylthiol.

FID flame ionization detector.

FP flash profile.

GC gas chromatography.

GC-O gas chromatography-olfactometry.

Glu-MH glutathione-3-mercaptohexan-1-ol.

Glu-MP glutathione-4-mercapto-4-methyl- 2-pentanone.

GMO genetically modified organism.

GPA generalized procrustes analysis.

GPY glucose peptone yeast.

HCA hierarchical clustering analysis.

IATA Instituto de Agroquímica y Tecnología de Alimentos, Valencia.

ICMA Instituto de Ciencia de Materiales de Aragón, Zaragoza.

ICVV Instituto de Ciencias de la Vid y el Vino, Logroño.

IS internal standard.

LAAE Laboratorio de Análisis del Aroma y Enología, Zaragoza.
LC liquid chromatography.

MDS multidimensional scaling.
MF modified frequency.
MH 3-mercaptohexanol.
MHA 3-mercaptohexyl acetate.
MP 4-methyl-4-mercaptopentan-2-one.
MRM multiple reaction monitoring.
MS mass spectrometry.
MVA mevalonate pathway.

NAD^+ nicotinamide adenine dinucleotide.
NADH nicotinamide adenine dinucleotide hydrogenase.

OAV odor activity value.
OT odour threshold.

PAF phenolic and aroma precursors fraction.
PCA principal component analysis.
PDMS polydimethylsiloxane.
PFBBr pentafluorobenzyl bromide.
PFBHA O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride.
PFMs polyfunctional mercaptans.
pFPD pulsed flame photometric detector.
PTFE polytetrafluoroethylene.
PTV programmable temperature vaporization.

QC quality control.
QTOF quadrupole time-of-flight.

RI retention index.

SBSE stir bar sorptive extraction.
SCD sulfur chemiluminescence detector.
SIM single ion monitoring.
SPE solid phase extraction.

TCA tricarboxylic acid cycle or Krebs cycle.
TDN 1,1,6-trimethyl-1,2-dihydronaphthalene.
TDU thermal desoprtion unit.
TIMS trapped ion mobility spectrometry.

UHPLC ultra-high performance liquid chromatography.

VSC volatile sulfur compound.

YAN yeast assimilable nitrogen.
YPD yeast extract peptone dextrose.

List of figures

List of tables

Annexes

Annexes A

Supplementary material related to the Chapter 2

Table A.1: Standards used with their provider, purity (in %), CAS retention times (in minutes) and detection limits (in mg/L) of the analytes determined in major compounds analysis (GC-FID).

group	compound	provider	purity	CAS	tr	DL
carbonyls	acetaldehyde	Sigma-Aldrich	99	75-07-0	1.27	0.033
	diacetyl	Sigma-Aldrich	99	431-03-8	5.57	0.0050
	acetoin	Sigma-Aldrich	98	513-86-0	23.92	0.10
acids	acetic acid	PanReac	99.5	64-19-7	35.42	1.5
	butyric acid	Polyscience	99.5	107-92-6	45.76	0.026
	hexanoic acid	Polyscience	99.5	142-62-1	57.40	0.0075
	octanoic acid	Polyscience	98.5	124-07-2	68.83	0.0051
	decanoic acid	Polyscience	99.5	334-48-5	74.30	0.0041
	isobutyric acid	Sigma-Aldrich	99	79-31-2	42.23	0.023
	isovaleric acid	Sigma-Aldrich	99	503-74-2	47.81	0.25
alcohols	1-butanol	PanReac	99.5	71-36-3	14.74	0.0052
	1-hexanol	Sigma-Aldrich	99	111-27-3	28.38	0.010
	isobutanol	Merck	99	78-83-1	11.46	0.0097
	isoamyl alcohol	Sigma-Aldrich	99	123-51-3	18.95	0.012
	2-phenylethanol	Fluka	99	60-12-8	61.15	0.0037
	methionol	Sigma-Aldrich	98	505-10-2	50.00	0.0094
	cis-3-hexenol	Sigma-Aldrich	98	928-96-1	30.40	0.0053
	benzyl alcohol	Sigma-Aldrich	99	100-51-6	58.96	0.0035
acetates	hexyl acetate	Chemservice	99	142-92-7	22.98	0.00088
	isoamyl acetate	Chemservice	99	123-92-2	13.22	0.0051
esters	ethyl acetate	Polyscience	99.5	141-78-6	2.71	0.017
	ethyl propanoate	Fluka	99	105-37-3	4.47	0.020
	ethyl butyrate	Sigma-Aldrich	99	105-54-4	8.18	0.0040
	ethyl hexanoate	Polyscience	99.5	123-66-0	20.37	0.013
	ethyl octanoate	Polyscience	99.5	106-32-1	33.26	0.0096
	ethyl decanoate	Polyscience	99.5	110-38-3	45.27	0.014
	ethyl lactate	Sigma-Aldrich	99	97-64-3	27.97	0.0077
	diethyl succinate	Fluka	99	123-25-1	47.80	0.00063
lactone	γ-butyrolactone	Sigma-Aldrich	99	96-48-0	44.82	0.017
IS	2-octanol	Merck	98	123-96-6	32.57	
	4-methyl-2-pentanol	Merck	99	108-11-2	16.14	
	ethyl heptanoate	Merck	99	106-30-9	26.88	
	heptanoic acid	Merck	99	111-14-8	64.04	

Table A.2: Standards used with their provider, purity (in %). CAS, retention times (in min), *m/z* ratios and detection limits (in *mu*g/L) of the analytes determined in trace compounds analysis. The first *m/z* ratio was the one used for quantification (GC-MS).

group	compound	provider	purity	CAS	tr	*m/z*	DL
acetates	isobutyl acetate	Chemservice	99	110-19-0	7.40	56, 101, 73	0.056
	2-phenylethyl acetate	Chemservice	98.5	103-45-7	66.53	89, 91	1.7
esters	ethyl isobutyrate	Sigma-Aldrich	99	97-62-1	5.50	71, 116	0.10
	ethyl 2-methylbutyrate	Sigma-Aldrich	99	7452-79-1	9.30	57, 115, 102	0.056
	ethyl isovalerate	Sigma-Aldrich	98	108-64-5	10.25	88, 115, 70	0.14
	ethyl 4-methylvalerate	Sigma-Aldrich	97	25415-67-2	19.53	88, 115, 101	0.10
	ethyl cyclohexanoate	Alfa Aesar	98	3289-28-9	41.00	83, 101, 156	0.016
	ethyl D/L-leucate	Fluka	99	10348-47-7	50.66	69, 117, 87	0.11
cinnamates	trans-ethyl cinnamate	Fluka	98	103-36-6	82.36	131, 103, 176	0.025
	ethyl dihydrocinnamate	Fluka	98	2021-28-5	70.21	178, 149, 133	0.18
vinylphenols	4-vinylguaicol	Lancaster	97	7786-61-0	85.41	150, 135	0.10
	4-vinylphenol	Lancaster	10	2628-17-3	94.06	120, 91	0.75
ethylphenols	o-cresol	Sigma-Aldrich	99	95-48-7	76.44	108, 107, 79	0.077
	m-cresol	Sigma-Aldrich	99	108-39-4	80.57	108, 90, 79	0.031
	4-ethylguaiacol	Lancaster	98	2785-89-9	77.57	137, 122, 152	0.080
	4-ethylphenol	Sigma-Aldrich	99	123-07-9	84.53	107, 122	0.025
methoxyphenols	guaiacol	Sigma-Aldrich	97	90-05-1	68.91	109, 125, 124	0.064
	eugenol	Sigma-Aldrich	99	97-53-0	84.07	164, 149	0.038
	p-propylguaiacol	Lancaster	98	2785-87-7	81.37	137, 122, 166	0.024
	syringol	Sigma-Aldrich	99	91-10-1	88.55	154, 139	0.10
	trans-isoeugenol	Lancaster	98	97-54-1	92.12	164, 131, 149	0.65
	methoxyeugenol	Sigma-Aldrich	90	6627-88-9	100.13	194, 179, 119	0.21
vanillin derivatives	vanillin	Polyscience	99	121-33-5	100.87	152, 151, 123	0.063
	acetovanillone	Sigma-Aldrich	98	498-02-2	103.88	166, 151, 123	0.32
	syringaldehyde	Sigma-Aldrich	98	134-96-3	117.70	182, 181, 167	0.044
lactones	R/S-γ-octalactone	Lancaster	98	104-50-7	71.79	85, 114, 100	0.015
	γ-nonalactone	Sigma-Aldrich	97	104-61-0	77.48	85, 128, 100	0.016

	δ-decalactone	Lancaster	98	706-14-9	83.03	85, 128, 100	0.026
	massoia lactone	Sigma-Aldrich	95	54814-64-1	87.05	97, 139, 68	0.10
	cis/trans-whiskylactone	Sigma-Aldrich	98	39212-23-2	70.38	99, 128, 114	0.068
terpenes	linalool	Sigma-Aldrich	97	78-70-6	51.22	71, 93, 121	0.069
	nerol	Fluka	90	106-25-2	66.04	93, 121, 68	0.26
	geraniol	Fluka	98	106-24-1	68.62	69, 139, 123	0.12
	β-citronellol	Sigma-Aldrich	98	106-22-9	64.25	69, 81, 123	0.19
	(+)-cis/trans-rose oxide	Sigma-Aldrich	99	16409-43-1	35.32	139, 140, 154	0.031
	α-terpineol	Fluka	99	98-55-5	60.11	93, 121, 136	0.072
	cis/trans-linalool oxide (furan)	Sigma-Aldrich	98	60047-17-8	43.18	94, 59, 111	0.30
	dihydromyrcenol	Sigma-Aldrich	99	18479-58-8	45.94	59, 123, 55	0.043
	1,8-cineole	Merck	99	470-82-6	20.22	108, 139, 154	0.083
	R-(+)-limonene	Sigma-Aldrich	97	5989-27-5	19.39	107, 136, 121	0.71
norisoprenoids	α-ionone	Sigma-Aldrich	90	127-41-3	68.40	121, 93, 192	0.034
	β-ionone	Sigma-Aldrich	98	201-224-3	72.97	177, 135, 192	0.014
	β-damascenone	Firmenich	98	23726-93-4	66.73	190, 175, 69	0.031
	TDN	Synchem UG&Co	80	30364-38-6	62.00	157, 142, 172	0.12
	Riesling acetal			129601-94-1	57.00	138, 125, 133	
	vitispirane A and B			65416-59-3	48.60	192, 93, 136, 121	
IS	3-octanone	Merck	99	106-68-3	25.00	57, 99	
	2-octanol	Merck	99.5	5978-70-1	42.16	45, 55, 97	
	3,4-dimethylphenol	Merck	99	95-65-8	86.53	107, 122	

Table A.3: Standards used with their provider, purity (in %), CAS, retention times (in minutes), *m/z* ratios and detection limits (in ng/L) of the analytes determined in PFMs analysis by GC-GC-MS and UHPLC-MS/MS. Within the TDU-GC-GC-MS part, the first *m/z* ratio was the one used for quantification. In the UHPLC-MS/MS part, the first *m/z* was used into the first quadrupole and second *m/z* in the third quadrupole. The latter corresponds to the selenyl moiety ($[C_{13}H_{10}ONSe]^+$, *m/z* 276) coming from the breakup of the selenyl-sulfide bond. PG: propylene glycol.

					TDU-GC-GC-MS			**UHPLC-MS/MS**			
group	**compound**	**provider**	**purity**	**CAS**	**tr**	***m/z***	**DL**	**tr**	***m/z* (Q1)**	***m/z* (Q3)**	**DL**
PFMs	MP	Lancaster	1% in PG	19872-52-7	16.72	160, 194, 213	0.25	4.96	452, 450	276, 274	0.028
	MH	Lancaster	98	51755-83-0	18.78	133, 194, 213	3.1	5.53	457, 455	276, 274	0.061
	MHA	Oxford Chemicals	98	136954-20-6	18.50	175, 194, 213	1.0	8.36	400, 398	276, 274	0.020
	BM	Fluka	98	100-53-8	17.97	284, 213, 162	-	7.80	405, 403	276, 274	0.002
	FFT	Lancaster	98	98-02-2	15.47	274, 213, 194	0.050	5.83	390, 388	276, 274	0.046
IS	MP-d_{10}	Eptes	99.9		16.84	170		4.88	392, 390	276, 274	
	MH-d_5	Eptes	97		18.70	138		5.48	410, 408	276, 274	
	MHA-d_5	Eptes	98		18.56	180		8.31	415, 413	276, 274	
	BM-d_5	Eptes	99		18.02	289		7.72	408, 406	276, 274	
	FFT-d_2	Eptes	98		15.48	275		5.70	418, 416	276, 274	

Table A.4: Standards used with their provider, purity (in %), CAS, retention times (in minutes), *m/z* ratios and detection limits (in *mu*g/L) of the analytes determined in Strecker aldehydes analysis. The first *m/z* ratio was the one used for quantification (GC-MS).

group	compound	provider	purity	CAS	tr	*m/z*	DL
Strecker aldehydes	isobutyraldehyde	Sigma-Aldrich	99	78-84-2	14.95	250, 195, 239	0.014
	2-methylbutanal	Sigma-Aldrich	95	96-17-3	16.01	239, 253, 195	0.027
	3-methylbutanal	Sigma-Aldrich	97	590-86-3	16.33	239, 196, 266	0.025
	methional	Sigma-Aldrich	99	3268-49-3	22.61	299, 252, 181	0.29
	phenylacetaldehyde	Sigma-Aldrich	90	122-78-1	24.31	297, 91, 181	0.15
IS	2-methylpentanal	Sigma-Aldrich	98	123-15-9	16.90	253, 266, 195	
	methional-d_3	Eptes	90	1849-29-2	22.58	302, 252, 181	
	phenylacetaldehyde-d_5	Eptes	95		24.21	301, 96, 181	
	2,3,6-trichloroanisole	Sigma-Aldrich	99	50375-10-5	20.63	167	

Instrucciones

Juez _____ Fecha _____

En la mesa se presentan 6 copas de vino. Cada una está codificada con un número de tres cifras.

Le pedimos que huela cada muestra por vía orto-nasal y que constituya grupos de vino basándose en la similitud en el aroma:

- En primer lugar, le pedimos que huela las muestras de izquierda a derecha. A continuación, puede proceder libremente.
- Puede oler cada muestra tantas veces como desee y puede utilizar todo el tiempo que necesite para realizar este test.
- Los grupos los debe hacer sobre la mesa, moviendo las copas de vino y agrupándolas físicamente, en función de su similitud aromática.
- Un grupo puede, eventualmente, estar constituido por una sola muestra.

Tabla de respuesta

Se entregará después de que hayan generado los grupos de copas sobre la mesa, nunca antes. Transcribir los números de las muestras.

Nombre:

Grupo	Muestras
1	
2	
3	
4	
5	

Figure A.1: Instructions for the sorting task.

PARTE 1/2

Juez _____ Fecha _____

En la mesa se presentan 6 copas codificadas con un número de tres cifras. Le pedimos que HUELA cada muestra y que anote los atributos (aroma, y sensaciones queméstesicas) que según su criterio diferencian las muestras.

1. Le pedimos que huela las muestras de izquierda a derecha. A continuación, puede proceder libremente.

2. Anote tantos atributos como desee.

3. Los atributos que cite han de ser descriptivos (por ej. floral, fruta pasa, ácido, alcohólico…).

4. Los atributos que cite no podrán tener un carácter hedónico (por ej. alta calidad, me gusta, es mi preferido, etc…)

Cite a continuación los atributos que diferencian las muestras:

-

-

-

PARTE 2/2

Para CADA ATRIBUTO citado en la actividad anterior, sitúe las muestras de menor y mayor intensidad en la escala, marcando su posición (con una X o |) y escribiendo su código de 3 cifras.

A continuación, le pedimos que evalué la intensidad para cada muestra, comparándolas con las situadas en los extremos. Marque su posición (con una X o |) y su código de 3 cifras en la escala tal y como se muestra en el ejemplo siguiente.

Ejemplo:

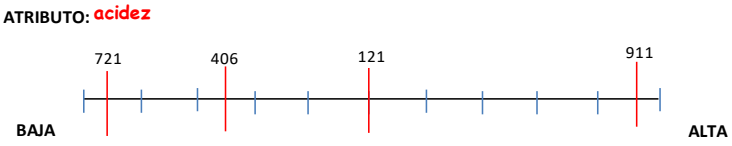

Figure A.2: Instructions for the flash profile.

Juez _____ Fecha _____

A continuación, se presentan **10 test**, formado cada uno por 3 copas codificadas con un número de tres cifras.

- Le pedimos que HUELA las tres copas que forman cada test **DE IZQUIERDA A DERECHA Y DE DELANTE HACIA ATRÁS.**

- Anote los códigos de las copas en la serie correspondiente en el orden que se les presenta, de izquierda a derecha

- RODEE en cada test cuál de las copas presenta un aroma diferente.

- Pase al siguiente test.

Serie 1. _____ _____ _____

Serie 2. _____ _____ _____

Serie 3. _____ _____ _____

Serie 4. _____ _____ _____

Serie 5. _____ _____ _____

Serie 6. _____ _____ _____

Serie 7. _____ _____ _____

Serie 8. _____ _____ _____

Serie 9. _____ _____ _____

Serie 10. _____ _____ _____

Figure A.3: Instructions for the OT determination.

Annexes B

Supplementary material related to the Chapter 3

Table B.1: Major and trace compounds concentration in μg/L found in recently fermented wines with 10 *S. cerevisiae* yeasts with PAF (average ± standard deviation). The letter indicates the results of Tukey's HSD test.*Y indicates that the compound production was significantly affected by the factor yeast (p-value < 0.05). n.d. indicates that the compound was not detected (below detection limits).

compound*	CLOS	IONYS	71B	BDX	D254	D80	HPS	OKAY	PERSY	RHONE
carbonyls										
acetaldehyde	8008 ± 7000	17910 ± 10000	9170 ± 900	8469 ± 6000	6646 ± 3000	8729 ± 4000	10267 ± 8000	9657 ± 4000	10636 ± 2000	13072 ± 4000
diacetyl Y	39 ± 30 bc	69 ± 10 bc	816 ± 70 a	101 ± 10 bc	34 ± 30 bc	111 ± 30 b	62 ± 20 bc	82 ± 30 bc	103 ± 10 b	85 ± 20 bc
acetoin	308 ± 200	790 ± 500	564 ± 60	300 ± 200	252 ± 100	283 ± 100	372 ± 300	385 ± 200	633 ± 100	610 ± 200
esters										
ethyl acetate Y	20985 ± 800 de	50338 ± 3000 a	18345 ± 2000 e	23796 ± 600 cde	23051 ± 3000 cde	24684 ± 2000 cd	24654 ± 3000 cd	28687 ± 2000 bc	32911 ± 3000 b	22829 ± 1000 cde
isobutyl acetate Y	8 ± 0.4 b	76 ± 20 a	11 ± 1 b	22 ± 1 b	14 ± 3 b	15 ± 10 b	19 ± 4 b	10.7 ± 0.3 b	18 ± 1 b	14 ± 1 b
isoamyl acetate Y	43 ± 10 bc	540 ± 100 a	115 ± 20 bc	99 ± 20 bc	70 ± 30 bc	121 ± 100 bc	98 ± 50 bc	102 ± 30 bc	210 ± 40 b	115 ± 20 bc
β–phenylethyl acetate Y	81 ± 20 b	1509 ± 30 a	256 ± 80 b	204 ± 20 b	83 ± 30 b	200 ± 200 b	117 ± 50 b	182 ± 30 b	283 ± 30 b	140 ± 30 b
ethyl propanoate Y	32 ± 4 b	263 ± 100 a	n.d. b	17 ± 10 b	26 ± 20 b	27 ± 5 b	32 ± 10 b	34 ± 10 b	59 ± 4 b	28 ± 2 b
ethyl butyrate Y	n.d. b	6 ± 1 a	n.d. b	n.d. b	n.d. b	n.d. b	n.d. b	n.d. b	n.d. b	n.d. b
ethyl hexanoate Y	51 ± 6 abc	63 ± 10 a	37 ± 3 c	47 ± 10 abc	49 ± 4 abc	59 ± 9 ab	44 ± 6 abc	40 ± 2 bc	43 ± 1 abc	43 ± 6 abc
ethyl octanoate	n.d.	12 ± 20	17 ± 4	9 ± 10	n.d.	11 ± 20	11 ± 20	9 ± 8	18 ± 2	17 ± 10
ethyl decanoate	192 ± 70	247 ± 100	132 ± 50	107 ± 40	151 ± 90	178 ± 20	138 ± 50	86 ± 80	148 ± 70	172 ± 100
ethyl isobutyrate Y	6.9 ± 0.2 ab	5 ± 2 abc	2.4 ± 0.7 c	7.1 ± 0.9 ab	8 ± 0.6 ab	7 ± 2 ab	9 ± 1 a	5 ± 0.5 bc	5 ± 1 bc	6 ± 2 ab
ethyl 2-methylbutyrate Y	0.56 ± 0.04 abc	0.8 ± 0.2 a	0.21 ± 0.07 c	0.34 ± 0.06 bc	0.7 ± 0.2 ab	0.5 ± 0.1 abc	0.6 ± 0.2 ab	0.5 ± 0.1 abc	0.5 ± 0.2 abc	0.5 ± 0.1 abc
ethyl isovalerate Y	0.83 ± 0.08 ab	0.7 ± 0.2 ab	0.28 ± 0.04 c	0.7 ± 0.1 ab	0.83 ± 0.09 ab	0.8 ± 0.3 ab	1 ± 0.2 a	0.89 ± 0.09 ab	0.5 ± 0.08 bc	0.75 ± 0.06 ab
ethyl leucate	10 ± 4	28 ± 8	6.7 ± 0.8	31 ± 10	9 ± 6	23 ± 30	17 ± 9	21 ± 3	23 ± 4	10 ± 2
ethyl dihydrocinnamate Y	0.021 ± 0.002 c	0.058 ± 0.008 a	0.018 ± 0.004 c	0.023 ± 0.005 bc	0.021 ± 0.001 c	0.021 ± 0.007 c	0.026 ± 0.001 bc	0.036 ± 0.011 b	0.023 ± 0.005 bc	0.022 ± 0.003 bc
ethyl lactate	378 ± 50	278 ± 80	360 ± 90	478 ± 50	441 ± 200	516 ± 300	585 ± 300	355 ± 40	538 ± 50	494 ± 70
diethyl succinate Y	3774 ± 3000 ab	6938 ± 1000 a	3660 ± 400 ab	4566 ± 800 ab	5086 ± 1000 ab	665 ± 60 b	5799 ± 3000 a	5781 ± 3000 a	3848 ± 100 ab	5011 ± 500 ab
alcohols										
1-butanol Y	201 ± 30 b	967 ± 400 a	1135 ± 300 a	185 ± 20 b	165 ± 40 b	160 ± 40 b	179 ± 90 b	234 ± 20 b	294 ± 40 b	186 ± 20 b
isobutanol Y	28613 ± 3000 c	33352 ± 3000 bc	22289 ± 1000 c	61885 ± 9000 a	36045 ± 6000 bc	50817 ± 10000 ab	37738 ± 8000 bc	20366 ± 2000 c	20029 ± 4000 c	32779 ± 3000 c
isoamyl alcohol Y	194852 ± 20000 ab	217380 ± 40000 ab	205485 ± 20000 ab	306583 ± 30000 a	173993 ± 40000 ab	266544 ± 1e+05 ab	191365 ± 70000 ab	187005 ± 20000 ab	161094 ± 6000 b	189807 ± 20000 ab
1-hexanol Y	25 ± 6 a	17 ± 1 b	25.5 ± 0.5 a	24 ± 1 ab	23 ± 3 ab	22 ± 3 ab	20 ± 3 ab	23 ± 1 ab	21 ± 1 ab	21.9 ± 0.9 ab

benzyl alcohol	109 ± 40	78 ± 60	123 ± 70	101 ± 20	61 ± 10	101 ± 20	93 ± 30	105 ± 40	104 ± 4	126 ± 40
β-phenylethanol	21393 ± 10000	43029 ± 20000	45053 ± 20000	34184 ± 10000	14929 ± 8000	40498 ± 20000	19262 ± 7000	29549 ± 6000	34806 ± 20000	26471 ± 3000
methionol	4880 ± 2000	5370 ± 3000	11721 ± 4000	8741 ± 3000	4154 ± 1000	8873 ± 3000	6413 ± 4000	6125 ± 2000	5066 ± 2000	6230 ± 2000
acids										
acetic acid Y	426171 ± 40000 a	35446 ± 5000 b	407025 ± 70000 a	372495 ± 70000 a	494160 ± 70000 a	548806 ± 2e+05 a	427292 ± 60000 a	462674 ± 2e+05 a	453883 ± 2e+05 a	611486 ± 1e+05 a
isobutyric acid Y	3864 ± 800 bcd	2931 ± 200 bcde	1878 ± 200 de	3895 ± 800 bcd	5455 ± 400 ab	7190 ± 3000 a	4921 ± 200 abc	2150 ± 600 cde	2249 ± 500 cde	4310 ± 1000 bcd
isovaleric acid	141 ± 100	242 ± 60	89 ± 20	129 ± 70	223 ± 80	214 ± 2	116 ± 40	85 ± 70	88 ± 20	149 ± 50
hexanoic acid	290 ± 100	182 ± 90	355 ± 200	244 ± 60	317 ± 300	329 ± 30	284 ± 100	231 ± 200	322 ± 80	367 ± 80
octanoic acid	111 ± 4	141 ± 20	129 ± 20	102 ± 10	112 ± 20	154 ± 20	128 ± 20	67 ± 50	159 ± 40	146 ± 20
decanoic acid Y	194 ± 30 ab	n.d. b	420 ± 80 a	358 ± 100 a	216 ± 60 ab	421 ± 300 a	312 ± 100 ab	168 ± 100 ab	n.d. b	261 ± 60 ab
lactones										
γ-butyrolactone Y	533 ± 100 b	928 ± 200 a	200 ± 50 cde	387 ± 50 bcd	455 ± 100 bc	326 ± 70 bcde	495 ± 200 bc	102 ± 9 de	481 ± 30 bc	454 ± 40 bc
γ-octalactone Y	0.29 ± 0.09 b	2 ± 1 ab	0.4 ± 0.1 b	0.79 ± 0.08 ab	0.7 ± 0.2 b	1 ± 1 ab	0.9 ± 0.5 ab	2.3 ± 0.2 a	1.3 ± 0.1 ab	0.33 ± 0.09 b
γ-nonalactone	2 ± 0.07	2.3 ± 0.4	1.9 ± 0.2	2.1 ± 0.2	2 ± 0.1	2 ± 0.6	2.1 ± 0.3	2 ± 0.2	2.2 ± 0.3	2.1 ± 0.1
δ-decalactone Y	0.9 ± 0.1 bcd	1.9 ± 0.1 a	0.88 ± 0.09 bcd	0.65 ± 0.08 d	0.82 ± 0.03 cd	0.77 ± 0.01 d	0.9 ± 0.1 bcd	0.85 ± 0.08 bcd	1.1 ± 0.2 b	1.1 ± 0.1 bc
massoia lactone	0.57 ± 0.05	0.6 ± 0.2	0.5 ± 0.1	0.6 ± 0.2	0.58 ± 0.06	0.52 ± 0.04	0.68 ± 0.06	0.6 ± 0.1	0.6 ± 0.2	0.8 ± 0.1
norisoprenoids										
β-damascenone Y	2.8 ± 0.7 b	4.3 ± 0.7 a	1.5 ± 0.2 cd	1.82 ± 0.09 bcd	2 ± 0.2 bcd	2 ± 0.6 bcd	2.4 ± 0.5 bc	1.1 ± 0.3 d	2.1 ± 0.3 bcd	2.1 ± 0.3 bcd
β-ionone	0.208 ± 0.046	0.27 ± 0.22	0.21 ± 0.16	0.151 ± 0.096	0.14 ± 0.004	0.28 ± 0.11	0.30 ± 0.19	0.120 ± 0.047	0.054 ± 0.023	0.163 ± 0.009
TDN Y	2.6 ± 0.2 abc	2 ± 1 abc	2 ± 1 abc	1.6 ± 0.9 abc	2.9 ± 0.4 ab	2.6 ± 0.2 abc	2.6 ± 0.4 abc	1.2 ± 0.6 bc	0.9 ± 0.5 c	3.2 ± 0.1 a
Terpenes										
linalool Y	4 ± 0.3 b	22 ± 6 a	1.5 ± 0.2 b	4 ± 0.6 b	3.61 ± 0.03 b	5 ± 1 b	4 ± 1 b	1.75 ± 0.01 b	2.29 ± 0.05 b	3.9 ± 0.3 b
linalool oxide	0.5 ± 0.4	0.39 ± 0.05	0.32 ± 0.06	0.29 ± 0.05	0.3 ± 0.03	0.32 ± 0.04	0.46 ± 0.09	0.26 ± 0.05	0.27 ± 0.08	0.22 ± 0.06
β-citronellol Y	2.3 ± 0.3 bc	4 ± 1 ab	3.4 ± 0.2 b	3.12 ± 0.08 bc	2.2 ± 0.1 bc	3 ± 0.9 bc	2.4 ± 0.3 bc	5 ± 0.6 a	1.6 ± 0.2 c	2.3 ± 0.2 bc
geraniol Y	6.2 ± 0.5 b	21 ± 2 a	3.9 ± 0.4 c	3.3 ± 0.4 c	2.9 ± 0.5 c	4.1 ± 0.9 c	2.5 ± 0.1 c	3.7 ± 0.4 c	3.99 ± 0.07 c	4 ± 0.5 c
nerol Y	0.9 ± 0.1 ab	0.97 ± 0.08 a	0.77 ± 0.02 ab	0.85 ± 0.06 ab	0.72 ± 0.01 ab	0.8 ± 0.2 ab	0.8 ± 0.1 ab	0.75 ± 0.03 ab	0.67 ± 0.02 b	0.74 ± 0.06 ab
vanillin derivatives										
vanillin	5 ± 2	6 ± 3	10 ± 6	10 ± 3	6 ± 2	5 ± 1	7 ± 1	11 ± 5	9 ± 3	4 ± 1
acetovanillone	36 ± 4	37 ± 3	38 ± 1	40 ± 3	35 ± 2	35 ± 7	35 ± 2	39 ± 2	39 ± 2	33.1 ± 0.1
syringaldehyde	17 ± 20	11 ± 9	64 ± 30	39 ± 20	42 ± 7	10 ± 2	36 ± 20	66 ± 40	54 ± 20	11 ± 10

volatile phenols										
syringol	4 ± 1	4 ± 2	3.6 ± 0.5	3.3 ± 0.2	3.4 ± 0.5	3.1 ± 0.7	3.11 ± 0.09	3.46 ± 0.08	3.3 ± 0.1	3.4 ± 0.9
guaiacol	0.8 ± 0.4	1.1 ± 0.9	0.6 ± 0.1	0.58 ± 0.01	0.7 ± 0.2	0.6 ± 0.2	0.62 ± 0.05	0.65 ± 0.01	0.53 ± 0.03	0.7 ± 0.3
4-ethylguaiacol Y	0.108 ± 0.009 b	0.37 ± 0.08 a	0.076 ± 0.002 b	0.027 ± 0.002 b	0.08 ± 0.02 b	0.12 ± 0.04 b	0.06 ± 0.04 b	0.06 ± 0.01 b	0.1 ± 0.02 b	0.07 ± 0.06 b
4-ethylphenol	0.17 ± 0.04	0.16 ± 0.01	0.154 ± 0.003	0.163 ± 0.006	0.152 ± 0.008	0.14 ± 0.04	0.178 ± 0.008	0.134 ± 0.005	0.17 ± 0.01	0.17 ± 0.01
4-vinylguaicol Y	28 ± 7 ab	40 ± 30 a	27 ± 8 ab	14 ± 6 ab	28 ± 9 ab	29 ± 9 ab	22 ± 3 ab	3.7 ± 0.8 b	18 ± 4 ab	39 ± 4 a
4-vinylphenol Y	163 ± 50 ab	280 ± 90 a	199 ± 9 ab	118 ± 20 bc	111 ± 20 bc	177 ± 90 ab	96 ± 10 bc	25 ± 0.8 c	106 ± 20 bc	220 ± 7 ab
eugenol	0.42 ± 0.05	0.53 ± 0.07	0.41 ± 0.06	0.49 ± 0.02	0.48 ± 0.02	0.4 ± 0.1	0.51 ± 0.04	0.51 ± 0.05	0.43 ± 0.04	0.43 ± 0.04
methoxyeugenol	1.2 ± 0.3	1.1 ± 0.3	1.1 ± 0.5	1.3 ± 0.5	1 ± 0.3	0.9 ± 0.4	1.1 ± 0.5	1.2 ± 0.4	1 ± 0.4	1.1 ± 0.4
trans-isoeugenol	0.7 ± 0.1	0.8 ± 0.2	0.6 ± 0.1	0.65 ± 0.06	0.7 ± 0.2	0.6 ± 0.2	0.7 ± 0.1	0.64 ± 0.03	0.59 ± 0.07	0.8 ± 0.1
4-propylguaiacol Y	0.1 ± 0.02 ab	0.101 ± 0.008 ab	0.08 ± 0.02 b	0.081 ± 0.005 b	0.09 ± 0.01 b	0.095 ± 0.009 ab	0.12 ± 0.01 ab	n.d. ab	0.13 ± 0.03 a	0.1 ± 0.01 ab

Table B.2: Trace compounds concentration in μg/L found in the unfermented control, young and aged wines fermented with 2 *S. cerevisiae* yeasts (average ± standard deviation) comparing the effect of PAF addition. Amounts of vitispirane and Riesling acetal are expressed in relative area since pure reference compounds were not available.*Y, T and P indicate that the compound was significantly affected by yeasts, time and PAF addition, respectively (p-value < 0.05). n.d., indicates that the compound was not detected (below detection limits).

	MUST		CLOS				IONYS			
compound*	young+PAF	aged+PAF	young-PAF	young+PAF	aged-PAF	aged+PAF	young-PAF	young+PAF	aged-PAF	aged+PAF
esters										
isobutyl acetate Y	n.d.	n.d.	6.3 ± 0.7	8 ± 0.4	33 ± 5	33 ± 4	58 ± 20	76 ± 20	18 ± 6	26 ± 9
β-phenylethyl acetate YTP	n.d.	n.d.	32 ± 4	81 ± 20	23 ± 4	51 ± 9	886 ± 100	1509 ± 30	315 ± 40	585 ± 100
ethyl isobutyrate YT	n.d.	n.d.	6 ± 2	6.9 ± 0.2	383 ± 50	432 ± 70	8 ± 3	5 ± 2	372 ± 90	237 ± 30
ethyl isovalerate YTP	n.d.	n.d.	0.42 ± 0.08	0.83 ± 0.08	29 ± 4	41 ± 20	0.51 ± 0.01	0.7 ± 0.2	54 ± 9	58 ± 20
ethyl 2-methylbutyrate YT	n.d.	n.d.	0.72 ± 0.05	0.56 ± 0.04	36 ± 7	60 ± 10	0.7 ± 0.2	0.8 ± 0.2	68 ± 10	49 ± 3
ethyl leucate YTP	n.d.	n.d.	n.d.	10 ± 4	46 ± 10	73 ± 20	n.d.	28 ± 8	36 ± 10	139 ± 9
ethyl dihydrocinnamate YP	n.d.	n.d.	n.d.	0.021 ± 0.003	n.d.	0.020 ± 0.003	n.d.	0.058 ± 0.009	n.d.	0.0633 ± 0.004
lactones										
γ-octalactone YP	n.d.	n.d.	0.4 ± 0.06	0.29 ± 0.09	0.37 ± 0.04	0.3 ± 0.1	1.22 ± 0.02	2 ± 1	1.1 ± 0.2	2.5 ± 0.6
γ-nonalactone YTP	n.d.	n.d.	0.68 ± 0.02	2 ± 0.07	1 ± 0.1	2.6 ± 0.2	0.42 ± 0.07	2.3 ± 0.4	0.69 ± 0.04	2.86 ± 0.09
δ-decalactone	n.d.	n.d.	1.4 ± 0.2	0.9 ± 0.1	1.43 ± 0.05	0.8 ± 0.3	1.6 ± 0.5	1.9 ± 0.1	11 ± 20	2 ± 0.3
massoia lactone YTP	0.74 ± 0.09	2.2 ± 0.7	n.d.	0.57 ± 0.05	n.d.	0.14 ± 0.02	n.d.	0.6 ± 0.2	n.d.	0.129 ± 0.003
norisoprenoids										
β-damascenone YTP	0.4 ± 0.2	8 ± 2	n.d.	2.8 ± 0.7	n.d.	8.3 ± 0.7	n.d.	4.3 ± 0.7	n.d.	9.1 ± 0.9
β-ionone P	n.d.	n.d.	n.d.	0.21 ± 0.05	n.d.	n.d.	n.d.	0.27 ± 0.23	n.d.	n.d.
TDN YTP	n.d.	156 ± 40	n.d.	2.6 ± 0.2	n.d.	132 ± 10	n.d.	2 ± 1	n.d.	105 ± 10
vitispirane YTP	n.d.	0.55 ± 0.08	n.d.	n.d.	n.d.	0.46 ± 0.05	n.d.	n.d.	n.d.	0.39 ± 0.02
Riesling acetal YTP	n.d.	0.22 ± 0.02	n.d.	n.d.	n.d.	0.17 ± 0.03	n.d.	n.d.	n.d.	0.21 ± 0.02
terpenes										
linalool YTP	0.977 ± 0.005	n.d.	2.5 ± 0.1	4 ± 0.3	n.d.	0.52 ± 0.08	77 ± 20	22 ± 6	2 ± 1	1.8 ± 0.7
linalool oxide YTP	n.d.	13 ± 4	n.d.	0.5 ± 0.4	n.d.	8 ± 1	n.d.	0.39 ± 0.05	2.3 ± 0.4	6.2 ± 0.2
β-citronellol YTP	n.d.	n.d.	1.36 ± 0.06	2.3 ± 0.3	n.d.	0.5 ± 0.1	2 ± 0.2	4 ± 1	0.1 ± 0.1	0.6 ± 0.3
geraniol YTP	n.d.	n.d.	4.7 ± 0.3	6.2 ± 0.5	n.d.	n.d.	47 ± 7	21 ± 2	n.d.	n.d.
nerol YTP	n.d.	n.d.	0.92 ± 0.04	0.9 ± 0.1	n.d.	n.d.	3.7 ± 0.9	0.97 ± 0.08	n.d.	n.d.
vanillin derivatives										
vanillin YP	6.90 ± 0.26	22 ± 10	n.d.	5 ± 2	n.d.	21 ± 1	n.d.	6 ± 3	n.d.	20 ± 0.7
acetovanillone YP	3.9 ± 0.9	5.8 ± 0.7	n.d.	36 ± 4	n.d.	36.5 ± 0.2	n.d.	37 ± 3	n.d.	38 ± 2
syringaldehyde YTP	236 ± 5	267 ± 60	0.1 ± 0.3	17 ± 20	n.d.	57 ± 10	n.d.	11 ± 9	n.d.	57 ± 30
volatile phenols										
syringol YTP	3.5 ± 0.2	143 ± 20	n.d.	4 ± 1	n.d.	73 ± 10	n.d.	4 ± 2	n.d.	57 ± 9
guaiacol YTP	n.d.	7 ± 1	n.d.	0.8 ± 0.4	n.d.	4 ± 0.6	n.d.	1.1 ± 0.9	n.d.	4.4 ± 0.2
4-ethylguaiacol	n.d.	n.d.	n.d.	0.108 ± 0.009	n.d.	n.d.	n.d.	0.37 ± 0.08	n.d.	n.d.
4-ethylphenol YTP	n.d.	0.28 ± 0.08	n.d.	0.17 ± 0.04	0.1 ± 0.1	0.45 ± 0.06	n.d.	0.16 ± 0.01	n.d.	0.327 ± 0.004
4-vinylguaiacol P	9 ± 3	21 ± 2	n.d.	28 ± 7	n.d.	19 ± 5	n.d.	40 ± 30	n.d.	50 ± 20

4-vinylphenol YP	6.5 ± 0.6	172 ± 10	n.d.	163 ± 50	n.d.	137 ± 30	n.d.	280 ± 90	n.d.	194 ± 40
eugenol YP	n.d.	n.d.	n.d.	0.42 ± 0.05	n.d.	0.57 ± 0.01	n.d.	0.53 ± 0.07	n.d.	0.54 ± 0.06
methoxyeugenol YTP	0.6 ± 0.1	5 ± 1	n.d.	1.2 ± 0.3	n.d.	6.4 ± 0.3	n.d.	1.1 ± 0.3	n.d.	5.2 ± 0.6
trans-isoeugenol TP	0.5 ± 0.2	0.23 ± 0.08	n.d.	0.7 ± 0.1	n.d.	0.58 ± 0.07	n.d.	0.8 ± 0.2	n.d.	0.7 ± 0.2
4-propylguaiacol	n.d.	n.d.	n.d.	0.1 ± 0.02	n.d.	0.093 ± 0.007	n.d.	0.101 ± 0.008	n.d.	0.11 ± 0.02

Table B.3: ANOVA results for the factors yeast, aging time, PAF addition and their interactions on trace compounds concentrations in the fermentations carried our with and without PAF with 2 *S. cerevisiae* strains. In bold, p-values < 0.05.

compound	p-value (yeast)	p-value (time)	p-value (PAF)	p-value (yeast x time)	p-value (yeast x PAF)	p-value (time x PAF)	p-value (yeast x time x PAF)
isobutyl acetate	**2.19E-06**	6.48E-02	1.44E-01	**1.24E-06**	1.97E-01	5.17E-01	6.40E-01
β-phenylethyl acetate	**7.41E-18**	**4.99E-12**	**2.54E-09**	**4.94E-12**	**3.56E-08**	**5.75E-04**	**1.61E-03**
ethyl isobutyrate	**8.38E-07**	**5.30E-13**	2.37E-01	**1.33E-06**	**1.72E-02**	2.55E-01	**2.00E-02**
ethyl isovalerate	**9.73E-08**	**1.50E-14**	**3.10E-03**	**1.42E-07**	**2.18E-04**	**4.43E-03**	**2.41E-04**
ethyl 2-methylbutyrate	**3.44E-04**	**6.21E-09**	7.63E-01	**4.34E-04**	4.05E-01	7.69E-01	3.91E-01
ethyl leucate	**1.01E-07**	**7.93E-12**	**2.36E-09**	**1.15E-05**	**9.82E-06**	**1.15E-05**	**1.69E-03**
ethyl dihydrocinnamate	**4.91E-10**	2.08E-01	**4.31E-11**	**1.87E-02**	**1.13E-08**	7.69E-01	5.44E-01
γ-octalactone	**7.44E-08**	4.30E-01	**9.29E-03**	6.48E-01	**2.90E-03**	1.81E-01	2.51E-01
γ-nonalactone	**7.19E-13**	**3.43E-06**	**1.47E-16**	**4.76E-02**	**3.00E-04**	**1.60E-02**	9.18E-01
δ-decalactone	3.07E-01	3.37E-01	2.89E-01	5.33E-01	4.06E-01	3.04E-01	3.17E-01
massoia lactone	**3.99E-10**	8.09E-01	**9.57E-05**	**2.57E-07**	9.03E-01	**5.73E-03**	8.30E-01
β-damascenone	**1.26E-03**	**3.74E-11**	**4.58E-15**	**1.25E-06**	**3.41E-02**	**8.54E-09**	4.67E-01
β-ionone	6.62E-02	9.35E-01	**1.86E-02**	2.69E-01	4.88E-01	9.33E-02	4.08E-01
TDN	**1.06E-06**	**1.32E-12**	**8.87E-11**	**7.70E-07**	1.48E-01	**1.71E-10**	1.62E-01
vitispirane	**1.07E-09**	**3.18E-16**	**4.35E-14**	**1.07E-09**	1.11E-01	**4.35E-14**	1.11E-01
Riesling acetal	**1.60E-07**	**4.94E-15**	**3.53E-13**	**1.60E-07**	1.43E-01	**3.53E-13**	1.43E-01
linalool	**1.57E-08**	**1.48E-08**	**3.40E-05**	**5.12E-08**	**1.44E-05**	**3.32E-05**	**2.19E-05**
linalool oxide	**9.75E-06**	**4.28E-10**	**1.74E-06**	**4.66E-06**	**3.60E-02**	**1.33E-05**	**4.91E-02**
β-citronellol	**1.51E-05**	**2.48E-09**	**7.45E-05**	**3.26E-04**	3.32E-01	6.54E-02	4.23E-01
geraniol	**1.04E-11**	**2.31E-13**	**5.76E-06**	**1.04E-11**	**1.24E-06**	**5.76E-06**	**1.24E-06**
nerol	**1.84E-06**	**3.15E-10**	**2.25E-05**	**1.84E-06**	**2.42E-05**	**2.25E-05**	**2.42E-05**
vanillin	**1.10E-03**	**3.37E-06**	**1.35E-08**	2.26E-01	9.93E-01	**3.49E-05**	6.32E-01
acetovanillone	**4.08E-10**	6.19E-01	**1.85E-20**	6.71E-01	4.34E-01	8.97E-01	9.00E-01
syringaldehyde	**2.67E-14**	**3.70E-03**	**1.68E-04**	8.70E-01	8.58E-01	**9.28E-03**	8.39E-01
syringol	**6.92E-11**	**9.74E-13**	**4.09E-10**	**1.05E-10**	2.01E-01	**3.17E-09**	1.72E-01
guaiacol	**2.27E-08**	**1.25E-11**	**2.41E-11**	**1.55E-09**	3.94E-01	**3.02E-08**	8.89E-01
4-ethylguaiacol	**7.94E-06**	**1.46E-08**	**4.45E-09**	**7.94E-06**	**2.00E-05**	**4.45E-09**	**2.00E-05**
4-ethylphenol	**1.39E-08**	**7.46E-09**	**4.06E-16**	**3.89E-04**	**6.73E-03**	**2.24E-09**	**1.11E-02**
4-vinylguaiacol	1.43E-01	6.68E-01	**3.10E-06**	4.68E-01	5.39E-02	9.39E-01	3.72E-01
4-vinylphenol	**3.58E-02**	9.82E-01	**2.60E-10**	**5.55E-04**	**1.14E-02**	8.66E-02	3.42E-01
eugenol	**1.62E-10**	**1.38E-02**	**1.58E-18**	**3.39E-02**	1.54E-01	**8.60E-03**	**1.66E-02**
methoxyeugenol	**5.83E-05**	**3.66E-13**	**1.41E-14**	**7.04E-05**	6.53E-02	**1.26E-11**	1.12E-01
trans-isoeugenol	5.40E-01	1.14E-01	**1.88E-11**	1.41E-01	2.97E-01	4.35E-01	4.74E-01
4-propylguaiacol	**1.59E-07**	8.66E-01	**9.79E-15**	5.28E-01	**5.12E-03**	8.55E-01	2.66E-01

Table B.4: Trace compounds concentration in μg/L found in wines fermented with 10 *S. cerevisiae* strains with PAF (mean ± standard deviation) after accelerated aging. Amounts of vitispirane and Riesling acetal are expressed in relative area since pure reference compounds were not available. Letters indicates Tukey's HSD test results. *Y indicates that the compound was significantly affected by yeasts (p-value < 0.05). n.d., indicates that the compound was not detected or below detection limits.

compound*	CLOS	IONYS	71B	BDX	D254	D80	HPS	OKAY	PERSY	RHONE
esters										
isobutyl acetate Y	33 ± 4 bcd	26 ± 9 cd	21 ± 2 d	43 ± 3 abcd	51 ± 10 ab	65 ± 20 a	48 ± 9 abc	25 ± 2 d	25 ± 6 d	55 ± 4 ab
β-phenylethyl acetate Y	51 ± 9 b	585 ± 100 a	137 ± 30 b	91 ± 6 b	53 ± 20 b	109 ± 60 b	64 ± 20 b	110 ± 20 b	137 ± 20 b	85 ± 10 b
ethyl isobutyrate Y	432 ± 70 abc	237 ± 30 bc	122 ± 10 c	282 ± 20 bc	531 ± 90 ab	775 ± 300 a	518 ± 100 ab	169 ± 50 bc	190 ± 50 bc	468 ± 70 abc
ethyl 2-methylbutyrate Y	41 ± 20 abc	58 ± 20 a	16 ± 0.2 c	25 ± 5 bc	52 ± 7 ab	53 ± 9 ab	48 ± 7 ab	29 ± 5 abc	23 ± 3 bc	45 ± 8 abc
ethyl isovalerate Y	60 ± 10 abcd	49 ± 3 bcde	23 ± 2 e	38 ± 1 cde	64 ± 20 abc	85 ± 20 a	64 ± 10 abc	45 ± 5 bcde	33 ± 3 de	69 ± 9 ab
ethyl leucate	73 ± 20	139 ± 9	69 ± 2	121 ± 3	73 ± 20	118 ± 40	120 ± 60	70 ± 3	115 ± 10	124 ± 20
ethyl dihydrocinnamate Y	0.020 ± 0.002 c	0.063 ± 0.004 a	0.0220 ± 0.001 bc	0.020 ± 0.002 c	0.020 ± 0.001 bc	0.036 ± 0.009 b	0.028 ± 0.006 bc	0.053 ± 0.003 a	0.027 ± 0.004 bc	0.032 ± 0.007 bc
lactones										
γ-octalactone Y	0.3 ± 0.1 c	2.5 ± 0.6 a	0.54 ± 0.04 c	0.63 ± 0.05 c	0.7 ± 0.3 c	0.8 ± 0.8 c	0.9 ± 0.4 c	2.1 ± 0.3 ab	1.3 ± 0.3 bc	0.43 ± 0.07 c
γ-nonalactone	2.6 ± 0.2	2.86 ± 0.09	2.6 ± 0.1	2.5 ± 0.3	2.7 ± 0.4	3 ± 0.1	2.7 ± 0.3	2.68 ± 0.02	2.9 ± 0.3	2.8 ± 0.1
δ-decalactone Y	0.8 ± 0.3 bc	2 ± 0.3 a	0.94 ± 0.01 bc	0.67 ± 0.03 c	0.91 ± 0.06 bc	0.88 ± 0.09 bc	0.92 ± 0.06 bc	0.93 ± 0.04 bc	1 ± 0.1 bc	1.12 ± 0.02 b
massoia lactone	0.14 ± 0.02	0.129 ± 0.003	0.1 ± 0.01	0.13 ± 0.02	0.14 ± 0.04	0.11 ± 0.06	0.15 ± 0.02	0.15 ± 0.008	0.138 ± 0.006	0.15 ± 0.01
norisoprenoids										
β-damascenone	8.3 ± 0.7	9.1 ± 0.9	7.72 ± 0.06	7.5 ± 0.4	8 ± 1	9.1 ± 0.7	8 ± 1	8.1 ± 0.4	8.8 ± 0.1	9.1 ± 0.3
TDN Y	132 ± 10 a	105 ± 10 abc	55 ± 2 e	96 ± 8 bcd	108 ± 20 abc	130 ± 10 a	113 ± 9 abc	71 ± 5 de	87 ± 6 cd	125 ± 9 ab
vitispirane Y	0.46 ± 0.05 ab	0.39 ± 0.02 abcd	0.277 ± 0.008 e	0.36 ± 0.02 cde	0.43 ± 0.07 abcd	0.49 ± 0.03 a	0.45 ± 0.04 abc	0.34 ± 0.03 de	0.38 ± 0.02 bcd	0.49 ± 0.02 a
Riesling acetal Y	0.17 ± 0.03 ab	0.21 ± 0.02 ab	0.166 ± 0.002 b	0.17 ± 0.02 ab	0.19 ± 0.03 ab	0.22 ± 0.02 a	0.2 ± 0.02 ab	0.19 ± 0.02 ab	0.2 ± 0.01 ab	0.212 ± 0.009 ab
terpenes										
linalool Y	0.52 ± 0.08 c	1.8 ± 0.7 a	1.35 ± 0.04 ab	1 ± 0.2 abc	0.54 ± 0.08 c	0.7 ± 0.2 bc	0.48 ± 0.03 c	1 ± 0.1 abc	1 ± 0.2 abc	0.46 ± 0.02 c
linalool oxide Y	8 ± 1 ab	6.2 ± 0.2 abcd	4.18 ± 0.08 e	5.6 ± 0.3 cde	7 ± 1 abcd	7.4 ± 0.4 abc	7 ± 1 abcd	5.4 ± 0.4 de	5.9 ± 0.4 bcde	8 ± 0.3 a
β-citronellol	0.5 ± 0.1	0.6 ± 0.3	0.96 ± 0.05	0.6 ± 0.1	0.5 ± 0.2	0.9 ± 0.3	0.6 ± 0.2	1 ± 0.1	0.5 ± 0.1	0.7 ± 0.2
vanillin derivatives										
vanillin	21 ± 1 bcd	20 ± 0.7 cd	26 ± 2 ab	28 ± 1 a	23.6 ± 0.7 abc	23 ± 1 abcd	22 ± 1 bcd	18 ± 3 d	25 ± 4 abc	19.5 ± 0.8 cd
acetovanillone	36.5 ± 0.2	38 ± 2	38 ± 1	40 ± 2	37 ± 2	39 ± 4	35 ± 2	38 ± 2	38 ± 1	36 ± 2
syringaldehyde	57 ± 10	57 ± 30	113 ± 20	97 ± 30	75 ± 10	52 ± 20	63 ± 10	105.4 ± 0.8	99 ± 50	72 ± 10
volatile phenols										
syringol	73 ± 10	57 ± 9	81.8 ± 0.2	79 ± 4	81 ± 10	71 ± 20	74 ± 20	80 ± 8	78 ± 10	55 ± 3
guaiacol Y	4 ± 0.6 bc	4.4 ± 0.2 bc	6.59 ± 0.07 a	5 ± 1 ab	4.9 ± 0.2 ab	4.6 ± 0.8 bc	4.8 ± 0.6 b	6 ± 1 ab	5 ± 0.6 ab	3 ± 0.2 c
4-ethylphenol Y	0.45 ± 0.06 a	0.327 ± 0.004 b	0.258 ± 0.002 b	0.3 ± 0.02 b	0.43 ± 0.05 a	0.51 ± 0.05 a	0.47 ± 0.02 a	0.27 ± 0.04 b	0.31 ± 0.01 b	0.48 ± 0.03 a
4-vinylguaicol Y	19 ± 5 c	50 ± 20 ab	29 ± 1 bc	20 ± 6 c	17 ± 4 c	24 ± 5 c	17 ± 4 c	53 ± 20 a	22 ± 2 c	19 ± 2 c
4-vinylphenol	137 ± 30	194 ± 40	170 ± 5	152 ± 30	135 ± 30	189 ± 40	133 ± 20	179 ± 40	148 ± 20	145 ± 10
eugenol Y	0.57 ± 0.01 abc	0.54 ± 0.06 bc	0.467 ± 0.006 c	0.6 ± 0.06 ab	0.65 ± 0.03 a	0.62 ± 0.03 ab	0.63 ± 0.01 ab	0.63 ± 0.02 ab	0.57 ± 0.04 abc	0.59 ± 0.06 ab
methoxyeugenol Y	6.4 ± 0.3 a	5.2 ± 0.6 abc	4.4 ± 0.4 c	5.8 ± 0.3 ab	5.9 ± 0.4 ab	6.1 ± 0.4 ab	5.6 ± 0.7 ab	5 ± 0.2 bc	5.2 ± 0.5 abc	6 ± 0.2 ab
trans-isoeugenol	0.58 ± 0.07	0.7 ± 0.2	0.6 ± 0.1	0.62 ± 0.02	0.6 ± 0.1	0.65 ± 0.04	0.55 ± 0.06	0.56 ± 0.02	0.61 ± 0.09	0.5 ± 0.03
4-propylguaiacol	0.093 ± 0.007	0.11 ± 0.02	0.12 ± 0.01	0.11 ± 0.02	0.09 ± 0.03	0.11 ± 0.02	0.093 ± 0.009	0.111 ± 0.005	0.132 ± 0.005	0.111 ± 0.005

Table B.5: ANOVA results for the factors yeast, aging time and their interactions on trace compounds concentrations in the fermentations performed with PAF with 10 *S. cerevisiae* strains. p-values in bold are inferior to 0.05.

compound	p-value (yeast)	p-value (time)	p-value (time x yeast)
isobutyl acetate	**2.16E-08**	**4.99E-11**	**1.90E-11**
β-phenylethyl acetate	**2.88E-27**	**6.83E-13**	**6.92E-15**
ethyl isobutyrate	**1.36E-06**	**1.19E-18**	**1.82E-06**
ethyl isovalerate	**7.32E-09**	**5.17E-29**	**1.12E-08**
ethyl 2-methylbutyrate	**7.29E-05**	**7.53E-22**	**1.05E-04**
ethyl leucate	**5.17E-04**	**1.62E-19**	5.94E-02
ethyl dihydrocinnamate	**2.20E-16**	6.02E-02	**1.45E-02**
β-damascenone	**6.18E-07**	**8.71E-36**	**9.59E-03**
β-ionone	3.55E-01	**6.15E-03**	1.28E-01
TDN	**1.71E-11**	**3.07E-38**	**6.73E-11**
vitispirane	**4.18E-09**	**2.50E-42**	**4.18E-09**
Riesling acetal	**1.02E-02**	**8.74E-39**	**1.02E-02**
geraniol	**8.09E-30**	**2.03E-35**	**8.09E-30**
linalool	**6.38E-16**	**2.36E-15**	**1.20E-14**
linalool oxide	**1.25E-07**	**4.26E-37**	**4.28E-07**
β-citronellol	**7.56E-09**	**1.64E-23**	**1.93E-05**
nerol	**5.75E-03**	**5.78E-37**	**5.75E-03**
γ-octalactone	**1.52E-09**	7.13E-01	9.13E-01
γ-nonalactone	2.52E-01	**5.14E-13**	8.57E-01
δ-decalactone	**4.64E-18**	2.97E-01	8.99E-01
massoia lactone	3.01E-01	**9.57E-23**	6.90E-01
vanillin	**7.37E-04**	**2.76E-24**	**3.36E-02**
acetovanillone	**2.75E-02**	5.77E-01	8.61E-01
syringaldehyde	**3.19E-05**	**1.70E-09**	9.58E-01
syringol	**4.35E-02**	**4.26E-31**	**3.05E-02**
guaiacol	**2.81E-04**	**3.80E-30**	**1.70E-05**
4-ethylguaiacol	**1.23E-11**	**2.97E-18**	**1.23E-11**
4-ethylphenol	**8.04E-12**	**2.21E-29**	**7.07E-11**
4-vinylguaiacol	**2.56E-03**	3.80E-01	**1.44E-04**
4-vinylphenol	**4.19E-06**	3.87E-01	**2.14E-04**
eugenol	**4.00E-04**	**2.02E-12**	9.05E-02
methoxyeugenol	**4.44E-03**	**1.38E-34**	**2.62E-03**
trans-isoeugenol	5.30E-01	**2.46E-02**	6.18E-01
4-propylguaiacol	**4.83E-04**	5.32E-02	7.92E-01

Annexes C

Supplementary material related to the Chapter 4

Table C.1: Volatiles concentration (average ± standard deviation) found in wines fermented with 2 *S. cerevisiae* strains in wines recently fermented and after accelerated aging. Units are specified for each family of compounds. Significance of the factors yeast (IONYS or D254), aging (young or aged wines) and their interaction are indicated with a Y, T and * respectively (p-value < 0.05).

compound	young wines		aged wines	
	D254	**IONYS**	**D254**	**IONYS**
acids (mg/L)				
acetic acid YT	391 ± 20	106 ± 10	604 ± 100	323 ± 30
butyric acid	0.47 ± 0.04	0.50 ± 0.02	0.476 ± 0.007	0.48 ± 0.04
isobutyric acid Y	5.1 ± 0.4	3.1 ± 0.2	5.3 ± 0.8	2.9 ± 0.4
isovaleric acid YT	3.9 ± 0.1	3.5 ± 0.4	3.7 ± 0.3	2.7 ± 0.5
hexanoic acid Y	2.10 ± 0.04	2.3 ± 0.2	2.14 ± 0.06	2.2 ± 0.1
octanoic acid YT	1.67 ± 0.05	1.7 ± 0.1	1.50 ± 0.08	1.57 ± 0.09
decanoic acid Y	1.13 ± 0.07	3.0 ± 0.5	1.2 ± 0.1	3.2 ± 0.5
alcohols (mg/L)				
butanol Y	0.403 ± 0.003	0.64 ± 0.08	0.47 ± 0.07	0.65 ± 0.08
isobutanol Y	57 ± 1	39 ± 4	61 ± 10	37 ± 4
isoamyl alcohol Y	267 ± 4	295 ± 8	274 ± 20	285 ± 20
hexanol	1.42 ± 0.08	1.44 ± 0.02	1.4 ± 0.1	1.41 ± 0.08
c-3-hexenol Y	0.31 ± 0.02	0.282 ± 0.005	0.32 ± 0.01	0.282 ± 0.008
benzyl alcohol YT	0.108 ± 0.008	0.11 ± 0.01	0.17 ± 0.04	0.17 ± 0.03
β-phenylethanol Y	38.7 ± 0.5	77 ± 3	40 ± 2	75 ± 6
methionol YT	1.06 ± 0.09	2.3 ± 0.1	1.6 ± 0.2	2.8 ± 0.2
carbonyls (mg/L)				
acetaldehyde Y	4.5 ± 0.7	7 ± 1	4.9 ± 0.5	6.5 ± 0.6
acetoin Y*	1.2 ± 0.2	0.4 ± 0.1	0.7 ± 0.2	0.4 ± 0.1
diacetyl T*	0.4 ± 0.2	0.21 ± 0.06	0.12 ± 0.04	0.14 ± 0.02
γ-butyrolactone YT	3.8 ± 0.3	5.65 ± 0.04	9 ± 1	11.5 ± 0.9
esters (mg/L)				
ethyl acetate YT*	42.6 ± 0.5	40.6 ± 0.6	71 ± 4	31 ± 3
ethyl propanoate YT	0.096 ± 0.002	0.19 ± 0.02	0.15 ± 0.01	0.21 ± 0.01
ethyl butyrate YT	0.09 ± 0.01	0.088 ± 0.003	0.100 ± 0.005	0.113 ± 0.002
isoamyl acetate YT	0.29 ± 0.01	0.29 ± 0.05	0.17 ± 0.03	0.14 ± 0.01
ethyl hexanoate YT	0.22 ± 0.02	0.25 ± 0.03	0.19 ± 0.01	0.22 ± 0.02
hexyl acetate	n.d.	n.d.	n.d.	n.d.
ethyl octanoate YT	0.23 ± 0.03	0.21 ± 0.01	0.045 ± 0.003	0.05 ± 0.01
ethyl decanoate	n.d.	n.d.	n.d.	n.d.
ethyl lactate YT*	8 ± 3	1.72 ± 0.04	26 ± 6	6.0 ± 0.7
diethyl succinate YT*	0.34 ± 0.02	0.45 ± 0.05	4.9 ± 0.5	6.7 ± 0.4
esters (μg/L)				
ethyl isobutyrate YT*	90 ± 9	67 ± 9	384 ± 27	277 ± 41
isobutyl acetate YT*	53 ± 2	42 ± 1	55 ± 4	23 ± 2
ethyl 2-methylbutyrate YT	8.7 ± 0.6	8.5 ± 0.7	44 ± 5	42 ± 5
ethyl isovalerate YT	12.5 ± 0.7	11.1 ± 0.6	64 ± 6	58 ± 5
ethyl 4-methylvalerate	n.d.	n.d.	n.d.	n.d.
β-phenylethyl acetate T	665 ± 200	932 ± 80	136 ± 3	139 ± 10
ethyl leucate YT*	57 ± 6	77 ± 7	177 ± 9	235 ± 30
ethyl cyclohexanoate	n.d.	n.d.	n.d.	n.d.
cinnamates (μg/L)				
t-ethyl cinnamate YT	2.0 ± 0.4	1.6 ± 0.2	8 ± 2	7 ± 1
ethyl dihydrocinnamate Y	0.24 ± 0.02	0.34 ± 0.04	0.24 ± 0.03	0.350 ± 0.008
lactones (μg/L)				
γ-octalactone	n.d.	n.d.	n.d.	n.d.
γ-nonalactone Y	7 ± 1	7.2 ± 0.3	7 ± 1	7.3 ± 0.6
δ-decalactone	n.d.	n.d.	n.d.	n.d.
massoia lactone Y	1.1 ± 0.4	3 ± 1	2.0 ± 0.4	2.5 ± 0.4
t/c-whiskylactone	n.d.	n.d.	n.d.	n.d.
furaneol	n.d.	n.d.	n.d.	n.d.
norisoprenoids (μg/L)				
(+)-rose oxide YT	0.061 ± 0.003	0.071 ± 0.006	0.049 ± 0.003	0.06 ± 0.01
α-ionone	0.08 ± 0.01	0.065 ± 0.009	0.067 ± 0.006	0.071 ± 0.009
β-ionone T	0.31 ± 0.03	0.28 ± 0.03	0.24 ± 0.01	0.25 ± 0.02
β-damascenone Y	2.8 ± 0.2	3.4 ± 0.4	3.1 ± 0.3	3.5 ± 0.3
TDN YT*	0.07 ± 0.02	0.07 ± 0.01	1.6 ± 0.1	2.5 ± 0.2
vitispirane YT* (relative area)	n.d.	n.d.	0.31 ± 0.03	0.39 ± 0.02
Riesling acetal YT (relative area)	n.d.	0.021 ± 0.004	0.35 ± 0.02	0.39 ± 0.03
terpenes (μg/L)				
R-limonene *	21 ± 2	18 ± 2	19.3 ± 0.6	20 ± 1
nerol YT*	0.90 ± 0.08	1.18 ± 0.05	0.49 ± 0.05	0.53 ± 0.04
1,8-cineole *	0.74 ± 0.07	0.58 ± 0.07	0.62 ± 0.04	0.71 ± 0.08

β-citronellol YT*	5.0 ± 0.2	9.5 ± 0.4	1.3 ± 0.1	1.8 ± 0.4
α-terpineol YT*	0.99 ± 0.07	1.8 ± 0.2	4.08 ± 0.08	8.8 ± 0.6
geraniol YT*	3.5 ± 0.1	12 ± 1	n.d.	n.d.
linalool YT*	2.9 ± 0.1	7.6 ± 0.6	1.4 ± 0.1	1.7 ± 0.3
linalool oxide YT	0.38 ± 0.05	0.44 ± 0.06	5.6 ± 0.2	6.6 ± 0.7
vanillins (μg/L)				
vanillin YT	8.1 ± 0.5	9 ± 2	13.3 ± 0.8	13 ± 1
acetovanillone Y	48 ± 4	55 ± 6	48 ± 2	53 ± 5
syringaldehyde Y*	36 ± 10	35 ± 5	53 ± 5	33 ± 4
volatile phenols (μg/L)				
4-ethylguaiacol	n.d.	n.d.	n.d.	n.d.
4-vinylguaicol YT	4.6 ± 0.8	4.6 ± 0.7	9.9 ± 0.7	10.7 ± 0.5
4-vinylphenol YT*	12 ± 1	12 ± 2	25 ± 2	35 ± 3
4-ethylphenol	n.d.	n.d.	n.d.	n.d.
eugenol	1.2 ± 0.1	1.0 ± 0.3	1.15 ± 0.07	1.0 ± 0.3
guaiacol T	1.4 ± 0.1	1.5 ± 0.2	5.7 ± 0.4	5.3 ± 0.4
m-cresol YT	0.101 ± 0.008	0.18 ± 0.04	0.26 ± 0.04	0.41 ± 0.08
methoxyeugenol YT*	0.8 ± 0.1	0.99 ± 0.08	3.51 ± 0.04	4.6 ± 0.7
o-cresol Y	0.74 ± 0.05	0.81 ± 0.06	0.81 ± 0.02	0.83 ± 0.06
p-propylguaiacol	n.d.	n.d.	n.d.	n.d.
syringol	n.d.	n.d.	n.d.	n.d.
t-isoeugenol YT	0.28 ± 0.06	0.35 ± 0.03	0.24 ± 0.02	0.294 ± 0.005

Table C.2: Average sum of OAVs for each aroma vector (average ± standard deviation) in wines recently fermented with 2 *S. cerevisiae* yeasts and after accelerated aging. Significance of the factors aging (young or aged), yeast (IONYS or D254) and their interaction is indicated by T, Y and * respectively (p-value < 0.05).

	Young wines		Aged wines	
	D254	**IONYS**	**D254**	**IONYS**
acetic acid TY	1.30 ± 0.08	0.35 ± 0.03	2.0 ± 0.3	1.1 ± 0.1
ethyl acetate TY*	3.46 ± 0.04	3.30 ± 0.05	5.7 ± 0.3	2.5 ± 0.2
higher alcohols Y	15.1 ± 0.1	19.5 ± 0.6	16 ± 1	20 ± 1
cinnamates T	1.9 ± 0.4	1.7 ± 0.1	8 ± 2	7 ± 1
ionones T	3.4 ± 0.3	3.1 ± 0.3	2.7 ± 0.1	2.8 ± 0.2
β-phenylethyl acetate T	2.7 ± 0.9	3.7 ± 0.3	0.54 ± 0.01	0.55 ± 0.04
terpenes 1 TY	1.7 ± 0.1	2.0 ± 0.1	1.23 ± 0.05	1.4 ± 0.1
acetates T	9.7 ± 0.4	10 ± 2	6 ± 1	4.8 ± 0.4
β-damascenone Y	55 ± 3	69 ± 8	62 ± 6	71 ± 6
ethyl esters TY	15.5 ± 0.4	14.0 ± 0.3	54 ± 4	45 ± 5
lactones TY	0.34 ± 0.04	0.42 ± 0.02	0.48 ± 0.08	0.57 ± 0.03
branched acids Y	119 ± 4	109 ± 12	115 ± 9	82 ± 15
diacetyl T	4 ± 2	2.1 ± 0.6	1.2 ± 0.4	1.4 ± 0.2
linear fatty acids Y	12.2 ± 0.2	14.7 ± 0.3	12.0 ± 0.2	14.2 ± 0.7
methoxyphenols T	0.49 ± 0.04	0.48 ± 0.07	1.00 ± 0.04	1.0 ± 0.1
TDN TY*	0.04 ± 0.01	0.037 ± 0.004	0.81 ± 0.06	1.25 ± 0.08
acetaldehyde Y	9 ± 1	14 ± 2	10 ± 1	13 ± 1

Table C.3: Results of ANOVA to evaluate the factors aging (young or aged), yeast (IONYS or D254) and their interaction, indicated with a Y, T and * (p-values < 0.05, indicated in bold).

	aging	**yeast**	**aging*yeast**
acetic acid TY	**1.27E-04**	**1.79E-05**	9.39E-01
ethyl acetate TY*	**1.51E-04**	**3.70E-07**	**8.15E-07**
higher alcohols Y	4.01E-01	**8.14E-05**	4.26E-01
cinnamates T	**9.19E-06**	2.14E-01	4.18E-01
ionones T	**4.55E-03**	2.94E-01	1.64E-01
β-phenylethyl acetate T	**1.46E-05**	9.40E-02	1.01E-01
terpenes 1 TY	**3.58E-05**	**6.37E-03**	1.54E-01
acetates T	**6.45E-05**	4.04E-01	4.30E-01
β-damascenone Y	2.44E-01	**1.56E-02**	5.03E-01
ethyl esters TY	**5.59E-08**	**2.30E-02**	8.87E-02
lactones TY	**1.07E-03**	**1.76E-02**	7.93E-01
branched acids Y	1.55E-01	**2.46E-04**	1.68E-01
diacetyl T	**6.01E-03**	8.24E-02	5.37E-02
linear fatty acids Y	2.32E-01	**7.58E-06**	5.49E-01
methoxyphenols T	**1.17E-06**	8.60E-01	9.32E-01
TDN TY*	**5.18E-10**	**5.44E-05**	**5.72E-05**
acetaldehyde Y	8.95E-01	**1.95E-03**	2.86E-01

Annexes D

Supplementary material related to the Chapter 5

Table D.1: Aroma vector composition, their generic and specific aroma descriptors in isolation. In grey, the aroma vectors with scores inferior to 0.2.

generic descriptor	**aroma vector**	**compounds**	**specific descriptor**
acetic	acetic acid	acetic acid	acetic, vinegar
alcoholic, solvent	ethyl acetate	ethyl acetate	glue
	higher alcohols	benzyl alcohol 1-butanol 1-hexanol isoamyl alcohol isobutanol methionol β-phenylethanol	harsh, spirit, solvent
lactic, acid	diacetyl	diacetyl	buttery, milky, yogurt
	acids	butyric acid hexanoic acid octanoic acid decanoic acid isobutyric acid isovaleric acid	soapy, sweaty, cheese
yeasty, oxidized	acetaldehyde	acetaldehyde	green apple, oxidized
	isoaldehydes	2-methylbutanal 3-methylbutanal isobutyraldehyde	yeasty, malty
	methional	methional	potato, overripe oxidized
	phenylacetaldehyde	phenylacetaldehyde	honey, oxidized
flowery	β-phenylethyl acetate	β-phenylethyl acetate	floral, sweet, rose
	cinnamates	trans-ethyl cinnamate ethyl dihydrocinnamate	sweet, balsamic
	ionones	α-ionone	violets, berry

		β-ionone	
	terpenes	β-citronellol dihydromyrcenol geraniol linalool nerol 1,8-cineole R-limonene α-terpineol cis/trans-linalool oxide	jasmine, orange blossom, muscat
	rose oxide	(+)-cis/trans-rose oxide	rose, litchi
fruity	esters	ethyl propanoate ethyl butyrate ethyl hexanoate ethyl octanoate ethyl decanoate ethyl isobutyrate ethyl isovalerate ethyl 2-methylbutyrate ethyl 4-methylvalerate ethyl D/L-leucate ethyl lactate diethyl succinate	fruits, apple, strawberry, blackberry, anise
	β-damascenone	β-damascenone	baked apple, dry plum
	acetates	hexyl acetate isoamyl acetate isobutyl acetate	banana
	lactones	γ-butyrolactone δ-decalactone γ-nonalactone γ-octalactone massoia lactone	peach

fresh, citric, green	MH	MH	grapefruit
	MP	MP	box tree, fresh
	MHA	MHA	passion fruit
spicy, woody	vanillins	acetovanillone syringaldehyde vanillin	vanilla, nutmeg
	ethylphenols	4-ethylphenol m-cresol	animal
	vinylphenols	4-vinylguaiacol 4-vinylphenol	medicinal
	methoxyphenols	eugenol guaiacol trans-isoeugenol methoxyeugenol p-propylguaiacol	clove, smoky
	TDN	TDN	kerosene

Table D.2: Volatiles concentration (average ± standard, μg/L) in samples recently fermented with 3 *S. cerevisiae* strains. Significance (p-value < 0.05) of the factors yeast, aging time and their interaction is indicated by the letters y, t and * respectively.

	wine recently fermented		
compound	**ECA5**	**QA23**	**SAUVY**
hexyl acetate yt*	14 ± 1 a	n.d. b	n.d. b
isoamyl acetate yt*	8774 ± 500 a	1664 ± 100 c	2769 ± 500 b
isobutyl acetate yt*	388 ± 30 a	78 ± 3 b	109 ± 20 b
β-phenylethyl acetate yt*	2347 ± 300 a	241 ± 20 bc	443 ± 100 b
acetic acid yt	152495 ± 30000 c	558283 ± 10000 a	264422 ± 10000 b
butyric acid t	368 ± 30 a	340 ± 40 a	188 ± 10 b
decanoic acid yt*	1205 ± 40 b	1571 ± 100 a	611 ± 40 c
hexanoic acid y	4051 ± 300 a	4228 ± 100 a	2019 ± 100 b
isobutyric acid y	1405 ± 100 a	1002 ± 20 b	1172 ± 50 b
isovaleric acid y	2647 ± 300 a	1449 ± 40 b	2153 ± 200 a
octanoic acid yt	5983 ± 30 a	5353 ± 3000 a	2940 ± 100 ab
benzyl alcohol y*	60 ± 3 b	65 ± 3 b	58 ± 3 b
butanol yt	557 ± 20 a	581 ± 30 a	415 ± 30 b
hexanol yt*	45 ± 0.8 c	70 ± 1 b	69.4 ± 0.8 b
hexenol	n.d.	n.d.	n.d.
isoamyl alcohol y	222557 ± 10000 a	134150 ± 3000 b	125806 ± 7000 b
isobutanol y	24874 ± 2000 a	16833 ± 400 b	13872 ± 900 c
methionol y	5072 ± 700 a	1358 ± 70 c	3838 ± 200 b
phenylethanol y	49910 ± 10000 a	13531 ± 400 b	17576 ± 1000 b
ethyl isobutyrate yt*	8.4 ± 0.2 b	6.4 ± 0.6 c	12 ± 1 a
ethyl isovalerate yt*	0.8 ± 0.02 b	0.72 ± 0.02 b	1.1 ± 0.1 a
ethyl2-methylbutyrate	0.50 ± 0.05 b	0.36 ± 0.05 b	0.7 ± 0.1 a
ethyl4-methylvalerate t	n.d.	n.d.	n.d.
acetaldehyde yt*	636 ± 100 b	993 ± 100 a	1114 ± 90 a
acetoin y	189 ± 20 b	257 ± 20 a	230 ± 10 a
diacetyl yt*	12 ± 2 a	9 ± 3 ab	4 ± 4 bc
ethyl cinnamate yt*	n.d.	n.d.	n.d.
ethyl dihydrocinnamate y	n.d. b	n.d. b	0.21 ± 0.01 a
ethyl acetate yt*	76364 ± 2000 a	42133 ± 600 c	54778 ± 5000 b
ethyl butyrate yt	112 ± 10 a	125 ± 6 a	74 ± 5 b
ethyl decanoate yt	135 ± 70 a	89 ± 10 ab	88 ± 10 ab
ethyl hexanoate yt*	687 ± 30 a	435 ± 5 b	237 ± 20 c
ethyl octanoate y	215 ± 10 a	137 ± 10 b	151 ± 30 b
ethyl propanoate yt	39 ± 5 c	60 ± 3 b	75 ± 5 a
ethyl leucate yt*	n.d.	n.d.	n.d.
γ-butyrolactone yt	518 ± 10 a	429 ± 8 b	352 ± 30 c
δ-decalactone t	9.3 ± 0.3 a	9.9 ± 0.8 a	10.5 ± 0.9 a
massoia lactone t	n.d.	n.d.	n.d.
γ-nonalactone t	17.3 ± 0.9 a	19 ± 2 a	20 ± 2 a
γ-octalactone y	0.017 ± 0.002 c	0.42 ± 0.02 b	1.9 ± 0.2 a
whiskylactone	n.d.	n.d.	n.d.
diethyl succinate yt*	n.d.	n.d.	n.d.
ethyl lactate yt*	987 ± 40 b	670 ± 20 c	1075 ± 30 a
ethyl cyclohexanoate	n.d.	n.d.	n.d.
α-ionone t	n.d.	n.d.	n.d.
beta-ionone t	0.13 ± 0.01 a	0.13 ± 0.01 a	0.13 ± 0.01 a
β-damascenone t	2.7 ± 0.3 ab	3.4 ± 0.6 ab	3.6 ± 0.5 a
riesling acetal t	n.d.	n.d.	n.d.
TDN yt*	n.d.	n.d.	n.d.
vitispirane t	0.6 ± 0.5	0.3 ± 0.5	0.5 ± 0.4
AMH t	0.051 ± 0.008	0.05 ± 0.03	0.2 ± 0.2

BM	0.05 ± 0.01	0.3 ± 0.3	0.4 ± 0.3
FFT	0.032 ± 0.004	0.4 ± 0.5	0.3 ± 0.3
MOH yt	0.88 ± 0.04 bc	2.1 ± 0.8 a	1.9 ± 0.5 ab
MP y	0.13 ± 0.01 ab	0.08 ± 0.06 b	0.3 ± 0.1 a
free 2-methylbutanal yt*	1,5 ± 0,2	0,2 ± 0,3	2 ± 1
free 3-methylbutanal yt	18 ± 2 a	15 ± 1 ab	13 ± 1 b
free isobutyraldehyde yt*	4 ± 2 b	0,9 ± 0,5 b	0,6 ± 0,5 b
free methional yt*	2,1 ± 0,6 a	0,67 ± 0,07 b	1,7 ± 0,2 a
free phenylacetaldehyde yt*	6,4 ± 0,8 b	4 ± 0,8 b	5,7 ± 0,5 b
total 2-methylbutanal yt*	3.49 ± 0.08 a	3.2 ± 0.2 a	3.2 ± 0.2 a
total 3-methylbutanal y*	19.6 ± 0.8 a	20 ± 1 a	9.7 ± 0.2 b
total isobutyraldehyde yt*	8.8 ± 0.3 b	6.6 ± 0.3 bc	4.9 ± 0.2 c
total methional t*	2.29 ± 0.06	2.1 ± 0.2	2 ± 0.1
total phenylacetaldehyde t*	2.98 ± 0.05 a	1.35 ± 0.04 c	1.8 ± 0.2 b
free SO_2 y	225 ± 200 ab	351 ± 200 a	7 ± 3 ab
total SO_2 y	12117 ± 4000 b	31943 ± 2000 a	40 ± 4 c
(+)-rose oxide yt	0.14 ± 0.02 ab	0.17 ± 0.05 a	0.16 ± 0.05 a
1,8-cineole	1.94 ± 0.08 c	3.9 ± 0.2 b	6.4 ± 0.3 a
β-citronellol yt*	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.1
dihydromyrcenol t	5 ± 1 ab	3.6 ± 0.8 b	6.2 ± 0.6 a
geraniol t	1.8 ± 0.7	1.4 ± 0.8	1.8 ± 0.5
R-limonene t	6.84 ± 0.08 b	6.3 ± 0.1 c	7.97 ± 0.04 a
linalool t	0.38 ± 0.02 ab	0.36 ± 0.02 bc	0.3 ± 0.02 c
linalool oxide yt	0.54 ± 0.03 b	0.65 ± 0.06 b	0.71 ± 0.02 b
nerol t	0.109 ± 0.007 a	0.071 ± 0.006 b	0.11 ± 0.009 a
α-terpineol t	2.81 ± 0.03 a	2.65 ± 0.09 a	2.84 ± 0.03 a
acetovanillone t	35.4 ± 0.8 a	37 ± 2 a	35.2 ± 0.6 a
syringaldehyde t*	24 ± 2	33 ± 7	33 ± 4
vanillin t	21 ± 2 a	21.6 ± 0.5 a	12 ± 2 b
4-ethylguaiacol	n.d.	n.d.	n.d.
4-ethylphenol t	0.5 ± 0.2	0.6 ± 0.2	0.5 ± 0.2
eugenol yt*	1.8 ± 0.1 bc	2.22 ± 0.07 a	2.02 ± 0.06 ab
guaiacol t	0.79 ± 0.07 a	0.73 ± 0.08 a	0.73 ± 0.06 a
trans-isoeugenol t	n.d.	n.d.	n.d.
m-cresol t	0.328 ± 0.005 a	0.312 ± 0.002 a	0.35 ± 0.02 a
methoxyeugenol yt	n.d.	n.d.	n.d.
o-cresol yt*	n.d.	n.d.	n.d.
p-propylguaiacol t*	n.d.	n.d.	n.d.
syringol	n.d.	n.d.	n.d.
4-vinylguaiacol yt*	1657 ± 30 b	2308 ± 100 a	75 ± 10 c
4-vinylphenol yt*	599 ± 10 b	731 ± 30 a	44 ± 5 c

Table D.3: Volatiles concentration (average ± standard, μg/L) in samples fermented with 3 *S. cerevisiae* strains and submitted to 1 week of accelerated anoxic aging at 50 ^{o}C. Significance (p-value < 0.05) of the factors yeast, aging time and their interaction is indicated by the letters y, t and * respectively.

	1 week		
compound	**ECA5**	**QA23**	**SAUVY**
hexyl acetate yt*	n.d.	n.d.	n.d.
isoamyl acetate yt*	5433 ± 200 a	875 ± 200 c	1690 ± 200 b
isobutyl acetate yt*	303 ± 20 a	61 ± 3 b	84 ± 20 b
β-phenylethyl acetate yt*	2014 ± 300 a	206 ± 10 b	363 ± 70 b
acetic acid yt	188959 ± 30000 c	780290 ± 10000 a	350590 ± 20000 b
butyric acid t	512 ± 7 a	565 ± 10 a	295 ± 50 b
decanoic acid yt*	1305 ± 70 a	1331 ± 200 a	661 ± 10 b
hexanoic acid y	4053 ± 200 a	3953 ± 200 a	2138 ± 20 b
isobutyric acid y	1625 ± 100 a	1391 ± 6 b	1542 ± 40 a
isovaleric acid y	2803 ± 300	2219 ± 400	2175 ± 400
octanoic acid yt	5827 ± 300 a	6194 ± 400 a	2944 ± 60 b
benzyl alcohol y*	60 ± 2 b	61 ± 2 b	68 ± 2 a
butanol yt	656 ± 50 a	759 ± 40 a	489 ± 50 b
hexanol yt*	49 ± 4 b	69.7 ± 0.9 a	73 ± 5 a
hexenol	n.d.	n.d.	n.d.
isoamyl alcohol y	231562 ± 10000 a	148390 ± 4000 b	134446 ± 6000 b
isobutanol y	26678 ± 2000 a	21302 ± 200 b	15523 ± 1000 c
methionol y	5294 ± 400 a	1538 ± 100 c	4276 ± 200 b
β-phenylethanol y	47442 ± 7000 a	12009 ± 700 b	17418 ± 1000 b
ethyl isobutyrate yt*	40 ± 5 a	31 ± 2 b	42 ± 1 a
ethyl isovalerate yt*	5 ± 0.7 a	3.7 ± 0.2 b	5.26 ± 0.03 a
ethyl 2-methylbutyrate	2.8 ± 0.3 b	2.1 ± 0.1 c	3.3 ± 0.1 a
ethyl 4-methylvalerate t	0.01 ± 0.02	n.d.	0.03 ± 0.03
acetaldehyde yt*	794 ± 100 b	1571 ± 200 a	1186 ± 80 ab
acetoin y	205 ± 20 c	333 ± 30 a	270 ± 20 b
diacetyl yt*	28 ± 10 a	16 ± 6 ab	9 ± 2 b
ethyl cinnamate yt*	0.1 ± 0.03	0.12 ± 0.05	0.15 ± 0.03
ethyl dihydrocinnamate y	n.d.	n.d.	0.1 ± 0.1
ethyl acetate yt*	66893 ± 2000 a	59095 ± 400 b	55411 ± 2000 b
ethyl butyrate yt	84 ± 8 a	78 ± 10 a	50 ± 5 b
ethyl decanoate yt	82 ± 20 a	84 ± 8 a	30 ± 10 b
ethyl hexanoate yt*	783 ± 7 a	606 ± 30 b	292 ± 20 c
ethyl octanoate y	572 ± 50 a	525 ± 8 a	306 ± 10 b
ethyl propanoate yt	77 ± 5 c	98 ± 10 b	118 ± 2 a
ethyl leucate yt*	5 ± 4	n.d.	2 ± 4
γ-butyrolactone yt	2618 ± 100 a	2484 ± 200 a	1671 ± 100 b
δ-decalactone t	11.2 ± 0.2	11.3 ± 0.3	11.6 ± 0.2
massoia lactone t	0.93 ± 0.03 ab	0.85 ± 0.05 b	0.98 ± 0.07 a
γ-nonalactone t	25.5 ± 0.2 b	26.2 ± 0.5 ab	26.8 ± 0.3 a
γ-octalactone y	0.045 ± 0.003 c	0.8 ± 0.1 b	2.1 ± 0.1 a
whiskylactone	n.d.	n.d.	n.d.
diethyl succinate yt*	813 ± 30 a	280 ± 30 c	603 ± 20 b
ethyl lactate yt*	5575 ± 300 a	4188 ± 200 b	6102 ± 200 a
ethyl cyclohexanoate	n.d.	n.d.	n.d.
α-ionone t	0.03 ± 0.06	0.07 ± 0.01	0.02 ± 0.03
β-ionone t	0.177 ± 0.009	0.19 ± 0.01	0.2 ± 0.02
β-damascenone t	8.5 ± 0.6 b	10 ± 0.4 a	11 ± 0.2 a
riesling acetal t	0.048 ± 0.002	0.047 ± 0.002	0.048 ± 0.001
TDN yt*	2.6 ± 0.09	2.8 ± 0.2	2.95 ± 0.03
vitispirane t	0.098 ± 0.003	0.099 ± 0.006	0.104 ± 0.002

AMH t	0.045 ± 0.003 a	0.026 ± 0.002 b	0.028 ± 0.003 b
BM	n.d.	0.02 ± 0.02	n.d.
FFT	0.03 ± 0.01	0.06 ± 0.03	0.059 ± 0.004
MOH yt	0.5 ± 0.1 b	0.45 ± 0.07 b	0.82 ± 0.03 a
MP y	0.11 ± 0.009 b	0.04 ± 0.002 c	0.14 ± 0.02 a
free 2-methylbutanal yt*	16 ± 2 a	12,39 ± 0,07 b	15,3 ± 0,3 a
free 3-methylbutanal yt	15,7 ± 0,9 a	12,1 ± 0,5 b	8,95 ± 0,07 c
free isobutyraldehyde yt*	103 ± 10 a	73 ± 10 b	58 ± 2 b
free methional yt*	1,9 ± 0,3 a	0,97 ± 0,07 b	1,6 ± 0,4 ab
free phenylacetaldehyde yt*	5,4 ± 0,4 a	3,4 ± 0,3 b	5 ± 1 a
total 2-methylbutanal yt*	19 ± 4	18 ± 2	16.1 ± 0.4
total 3-methylbutanal y*	19 ± 3 a	16 ± 3 a	8.7 ± 0.5 b
total isobutyraldehyde yt*	113 ± 20 a	80 ± 10 ab	53 ± 4 b
total methional t*	2 ± 0.2 a	1.4 ± 0.2 b	1.45 ± 0.05 b
total phenylacetaldehyde t*	7.7 ± 0.4 a	4 ± 0.3 b	4.2 ± 0.6 b
free SO_2 y	153 ± 100 b	1366 ± 400 a	n.d. b
total SO_2 y	9528 ± 3000 b	28601 ± 2000 a	73 ± 10 c
(+)-rose oxide yt	0.088 ± 0.004 a	0.06 ± 0.003 b	0.09 ± 0.01 a
1,8-cineole	0.33 ± 0.02	0.34 ± 0.01	0.35 ± 0.07
β-citronellol yt*	1.8 ± 0.1 c	3.4 ± 0.09 b	5.4 ± 0.2 a
dihydromyrcenol t	0.9 ± 0.1	0.96 ± 0.08	1 ± 0.1
geraniol t	6.2 ± 0.2 b	6.36 ± 0.06 b	7.3 ± 0.1 a
R-limonene t	0.84 ± 0.09	1.04 ± 0.05	1 ± 0.1
linalool t	22.6 ± 0.2 a	20.6 ± 0.4 b	21.6 ± 0.5 a
linalool oxide yt	4.2 ± 0.2	4.2 ± 0.2	4.21 ± 0.06
nerol t	1.17 ± 0.009 c	1.291 ± 0.008 b	1.34 ± 0.03 a
α-terpineol t	17 ± 0.3 a	16.2 ± 0.4 b	17 ± 0.1 a
acetovanillone t	75 ± 1 a	76 ± 2 a	70 ± 1 b
syringaldehyde t*	100 ± 20	126 ± 20	103 ± 20
vanillin t	70 ± 8	72 ± 7	54 ± 9
4-ethylguaiacol	n.d.	n.d.	n.d.
4-ethylphenol t	0.108 ± 0.006	0.102 ± 0.007	0.102 ± 0.001
eugenol yt*	1.96 ± 0.04 b	2.31 ± 0.03 a	2.03 ± 0.02 b
guaiacol t	0.9 ± 0.1	0.9 ± 0.1	0.73 ± 0.04
trans-isoeugenol t	0.6 ± 0.01 b	0.69 ± 0.03 a	0.524 ± 0.007 c
m-cresol t	0.31 ± 0.02	0.31 ± 0.03	0.35 ± 0.02
methoxyeugenol yt	0.28 ± 0.02 a	0.26 ± 0.01 ab	0.236 ± 0.005 b
o-cresol yt*	n.d.	n.d.	n.d.
p-propylguaiacol t*	0.01 ± 0.03 b	0.07 ± 0.006 a	0.041 ± 0.005 ab
syringol	n.d.	n.d.	n.d.
4-vinylguaiacol yt*	1055 ± 30 b	1412 ± 50 a	262 ± 9 c
4-vinylphenol yt*	354 ± 10 b	426 ± 20 a	65 ± 1 c

Table D.4: Volatiles concentration (average ± standard, μg/L) in the samples fermented with 3 *S. cerevisiae* strains and submitted to 2 weeks of accelerated anoxic aging at 50 ^{o}C. Significance (p-value < 0.05) of the factors yeast, aging time and their interaction is indicated by the letters y, t and * respectively.

	2 weeks		
compound	**ECA5**	**QA23**	**SAUVY**
hexyl acetate yt*	9.155946 ± 0 a	n.d. b	n.d. b
isoamyl acetate yt*	4636 ± 200 a	811 ± 100 c	1471 ± 100 b
isobutyl acetate yt*	239 ± 20 a	52 ± 2 b	68 ± 10 b
β-phenylethyl acetate yt*	1806 ± 200 a	199 ± 9 b	305 ± 60 b
acetic acid yt	196163 ± 20000 c	691425 ± 8000 a	372657 ± 40000 b
butyric acid t	37 ± 10 a	13 ± 6 a	17 ± 5 b
decanoic acid yt*	1403 ± 70 a	1251 ± 100 a	706 ± 60 b
hexanoic acid y	3741 ± 400 b	4623 ± 200 a	2012 ± 90 c
isobutyric acid y	1620 ± 200	1343 ± 30	1664 ± 40
isovaleric acid y	2496 ± 600	1981 ± 300	2058 ± 300
octanoic acid yt	5624 ± 300 a	6214 ± 300 a	2933 ± 200 b
benzyl alcohol y*	56 ± 3 c	68 ± 2 a	61 ± 2 b
butanol yt	644 ± 40 a	663 ± 30 a	513 ± 30 b
hexanol yt*	50 ± 3 b	77 ± 1 a	72 ± 3 a
hexenol	n.d.	n.d.	n.d.
isoamyl alcohol y	227282 ± 20000 a	140037 ± 3000 b	134082 ± 6000 b
isobutanol y	27616 ± 2000 a	17770 ± 500 b	15797 ± 900 b
methionol y	5242 ± 800 a	1579 ± 80 b	4263 ± 100 a
β-phenylethanol y	42958 ± 9000 a	14012 ± 600 b	16270 ± 1000 b
ethyl isobutyrate yt*	61 ± 10 a	40 ± 6 b	52 ± 3 ab
ethyl isovalerate yt*	8 ± 1 a	5.1 ± 0.2 b	7 ± 0.5 ab
ethyl 2-methylbutyrate	4.3 ± 0.9 a	2.8 ± 0.3 b	4.8 ± 0.1 a
ethyl 4-methylvalerate t	n.d.	n.d.	n.d.
acetaldehyde yt*	930 ± 100	999 ± 200	1136 ± 60
acetoin y	216 ± 10 b	307 ± 30 a	276 ± 10 a
diacetyl yt*	556 ± 60 a	558 ± 30 b	333 ± 40 ab
ethyl cinnamate yt*	0.25 ± 0.04 a	0.14 ± 0.05 b	0.23 ± 0.05 ab
ethyl dihydrocinnamate y	n.d.	n.d.	n.d.
ethyl acetate yt*	60584 ± 3000 a	60885 ± 1000 a	52459 ± 2000 b
ethyl butyrate yt	82 ± 7 a	82 ± 10 a	52 ± 4 b
ethyl decanoate yt	59 ± 20 ab	77 ± 10 a	39 ± 8 b
ethyl hexanoate yt*	618 ± 30 a	552 ± 20 b	263 ± 20 c
ethyl octanoate y	516 ± 50 a	549 ± 20 a	267 ± 10 b
ethyl propanoate yt	102 ± 6	117 ± 7	119 ± 8
ethyl leucate yt*	12 ± 3 a	n.d. b	8 ± 3 a
γ-butyrolactone yt	3633 ± 300 a	3158 ± 100 a	2038 ± 90 b
δ-decalactone t	11.4 ± 0.2 ab	11.7 ± 0.3 a	10.9 ± 0.3 b
massoia lactone t	1 ± 0.4	0.84 ± 0.08	0.67 ± 0.07
γ-nonalactone t	25.7 ± 0.4 b	27.7 ± 0.5 a	25.3 ± 0.5 b
γ-octalactone y	0.065 ± 0.004 c	0.75 ± 0.07 b	2 ± 0.1 a
whiskylactone	n.d.	n.d.	n.d.
diethyl succinate yt*	1000 ± 200 a	386 ± 30 b	1049 ± 40 a
ethyl lactate yt*	8833 ± 500 a	5579 ± 200 b	8758 ± 200 a
ethyl cyclohexanoate	n.d.	n.d.	n.d.
α-ionone t	n.d.	0.067 ± 0.008	n.d.
β-ionone t	0.097 ± 0.007 a	0.119 ± 0.009 a	0.09 ± 0.01 a
β-damascenone t	8.2 ± 0.4 b	10.1 ± 0.5 a	9.9 ± 0.4 a
riesling acetal t	0.092 ± 0.003 a	0.076 ± 0.003 b	0.07 ± 0.005 b
TDN yt*	6.9 ± 0.2	4.9 ± 0.8	5 ± 1
vitispirane t	0.241 ± 0.002 a	0.18 ± 0.02 b	0.17 ± 0.03 b

AMH t	0.034 ± 0.003 b	0.058 ± 0.002 a	0.03 ± 0.01 b
BM	n.d. b	0.03 ± 0.01 a	0.013 ± 0.004 b
FFT	0.03 ± 0.02 b	0.08 ± 0.02 a	0.066 ± 0.008 ab
MOH yt	0.46 ± 0.07 b	0.5 ± 0.06 b	0.88 ± 0.02 a
MP y	0.12 ± 0.006 b	0.058 ± 0.002 c	0.21 ± 0.02 a
free 2-methylbutanal yt*	26 ± 1 a	19,9 ± 0,9 b	24 ± 1 a
free 3-methylbutanal yt	15 ± 1 a	12,9 ± 0,9 b	8,5 ± 0,2 c
free isobutyraldehyde yt*	182 ± 20 a	93 ± 10 b	66 ± 6 b
free methional yt*	2,1 ± 0,3 a	0,87 ± 0,05 b	1,2 ± 0,3 b
free phenylacetaldehyde yt*	5,7 ± 0,3 a	4,2 ± 0,5 b	4,9 ± 0,7 ab
total 2-methylbutanal yt*	31 ± 3 a	23 ± 1 b	21 ± 2 b
total 3-methylbutanal y*	18 ± 2 a	17 ± 2 a	8.7 ± 0.6 b
total isobutyraldehyde yt*	203 ± 20 a	107 ± 10 b	78 ± 9 b
total methional t*	2.1 ± 0.2	1.8 ± 0.2	1.82 ± 0.09
total phenylacetaldehyde t*	7.6 ± 0.7 a	4 ± 0.8 b	4.5 ± 0.4 b
free SO_2 y	208 ± 200	221 ± 300	n.d.
total SO_2 y	10193 ± 3000 b	26795 ± 8000 a	84 ± 8 b
(+)-rose oxide yt	0.085 ± 0.007 a	0.059 ± 0.003 b	0.079 ± 0.009 a
1,8-cineole	0.2 ± 0.02	0.19 ± 0.02	0.2 ± 0.04
β-citronellol yt*	1.41 ± 0.09 c	2.89 ± 0.06 b	4.2 ± 0.2 a
dihydromyrcenol t	0.27 ± 0.07	0.28 ± 0.06	0.25 ± 0.08
geraniol t	4.1 ± 0.2 b	5.44 ± 0.04 a	5.2 ± 0.2 a
R-limonene t	0.9 ± 0.1	0.76 ± 0.05	0.9 ± 0.1
linalool t	14.1 ± 0.2 b	16.4 ± 0.3 a	16 ± 0.4 a
linalool oxide yt	7.7 ± 0.3 a	6.1 ± 0.3 b	5.7 ± 0.5 b
nerol t	0.84 ± 0.08 b	1.13 ± 0.01 a	1.05 ± 0.03 a
α-terpineol t	22.3 ± 0.4 a	19.9 ± 0.4 b	19.5 ± 0.5 b
acetovanillone t	65.8 ± 0.8 a	66 ± 2 a	59 ± 2 b
syringaldehyde t*	86 ± 10	64 ± 20	63 ± 20
vanillin t	43 ± 6 a	27 ± 6 a	30 ± 6 a
4-ethylguaiacol	n.d.	n.d.	n.d.
4-ethylphenol t	0.112 ± 0.006	0.109 ± 0.005	0.099 ± 0.005
eugenol yt*	1.87 ± 0.03 c	2.28 ± 0.02 a	1.93 ± 0.02 b
guaiacol t	0.68 ± 0.07	0.6 ± 0.2	0.56 ± 0.03
trans-isoeugenol t	0.66 ± 0.02 a	0.68 ± 0.03 a	0.57 ± 0.02 b
m-cresol t	0.26 ± 0.02 b	0.33 ± 0.02 a	0.33 ± 0.01 a
methoxyeugenol yt	0.35 ± 0.01 a	0.32 ± 0.01 a	0.27 ± 0.03 b
o-cresol yt*	n.d.	n.d.	n.d.
p-propylguaiacol t*	n.d.	n.d.	n.d.
syringol	n.d.	n.d.	n.d.
4-vinylguaiacol yt*	697 ± 20 b	1078 ± 40 a	347 ± 10 c
4-vinylphenol yt*	236 ± 8 b	321 ± 10 a	76 ± 3 c

Table D.5: Volatiles concentration (average ± standard, μg/L) in samples fermented with 3 *S. cerevisiae* strains and submitted to 5 weeks of accelerated anoxic aging at 50 ºC. Significance (p-value < 0.05) of the factors yeast, aging time and their interaction is indicated by the letters y, t and * respectively.

	5 weeks		
compound	**ECA5**	**QA23**	**SAUVY**
hexyl acetate yt*	n.d.	n.d.	n.d.
isoamyl acetate yt*	3807 ± 200 a	619 ± 50 c	989 ± 200 b
isobutyl acetate yt*	150 ± 10 a	44 ± 2 b	49 ± 6 b
β-phenylethyl acetate yt*	1142 ± 200 a	136 ± 6 b	237 ± 50 b
acetic acid yt	204530 ± 9000 c	676254 ± 6000 a	365631 ± 50000 b
butyric acid t	544 ± 90 a	483 ± 40 a	317 ± 10 b
decanoic acid yt*	1920 ± 80 a	1520 ± 60 b	714 ± 90 c
hexanoic acid y	4056 ± 500 a	4348 ± 200 a	2121 ± 100 b
isobutyric acid y	1513 ± 400	1195 ± 40	1518 ± 50
isovaleric acid y	2112 ± 800	1849 ± 100	2513 ± 300
octanoic acid yt	6908 ± 400 a	6680 ± 300 a	2898 ± 300 b
benzyl alcohol y*	61 ± 4 b	64 ± 2 b	71 ± 2 a
butanol yt	674 ± 20 b	728 ± 10 a	549 ± 8 c
hexanol yt*	56 ± 3 b	76 ± 1 a	76 ± 1 a
hexenol	n.d.	n.d.	n.d.
isoamyl alcohol y	232512 ± 20000 a	143564 ± 3000 b	135247 ± 7000 b
isobutanol y	26734 ± 3000 a	18672 ± 700 b	15734 ± 800 b
methionol y	5349 ± 1000 a	1501 ± 50 b	4290 ± 30 a
β-phenylethanol y	48121 ± 10000 a	13272 ± 600 b	17345 ± 1000 b
ethyl isobutyrate yt*	127 ± 20 a	87 ± 9 b	108 ± 5 ab
ethyl isovalerate yt*	18 ± 2 a	12.3 ± 0.3 b	16.2 ± 0.7 a
ethyl 2-methylbutyrate	10 ± 1 a	7 ± 0.4 b	10.3 ± 0.2 a
ethyl 4-methylvalerate t	0.07 ± 0.02	0.04 ± 0.03	0.04 ± 0.03
acetaldehyde yt*	851 ± 100 b	1312 ± 100 a	1157 ± 40 a
acetoin y	205 ± 9 b	300 ± 30 a	267 ± 6 a
diacetyl yt*	97 ± 20 a	43 ± 7 b	45 ± 8 b
ethyl cinnamate yt*	0.5 ± 0.05	0.56 ± 0.06	0.65 ± 0.07
ethyl dihydrocinnamate y	n.d.	n.d.	0.1 ± 0.1
ethyl acetate yt*	52321 ± 5000 c	88671 ± 2000 a	60513 ± 1000 b
ethyl butyrate yt	92 ± 6 a	88 ± 7 a	44 ± 5 b
ethyl decanoate yt	26 ± 9 a	16 ± 10 ab	n.d. b
ethyl hexanoate yt*	545 ± 40 a	473 ± 30 a	231 ± 10 b
ethyl octanoate y	356 ± 60 a	306 ± 20 a	162 ± 20 b
ethyl propanoate yt	161 ± 7	175 ± 3	170 ± 10
ethyl leucate yt*	21 ± 2 a	11.2 ± 0.3 c	17 ± 2 b
γ-butyrolactone yt	4404 ± 500 a	3921 ± 60 a	2616 ± 60 b
δ-decalactone t	10.9 ± 0.2 b	11.3 ± 0.3 ab	11.9 ± 0.3 a
massoia lactone t	0.6 ± 0.5	0.9 ± 0.1	1.1 ± 0.08
γ-nonalactone t	25.2 ± 0.6 b	27 ± 0.5 a	28 ± 0.6 a
γ-octalactone y	0.079 ± 0.006 c	0.75 ± 0.02 b	2.1 ± 0.2 a
whiskylactone	n.d.	n.d.	n.d.
diethyl succinate yt*	2272 ± 300 a	1267 ± 40 b	1602 ± 60 b
ethyl lactate yt*	14816 ± 600 a	10118 ± 200 b	15545 ± 200 a
ethyl cyclohexanoate	n.d.	n.d.	n.d.
α-ionone t	0.062 ± 0.004	0.068 ± 0.005	0.05 ± 0.05
β-ionone t	0.116 ± 0.006 b	0.131 ± 0.004 ab	0.138 ± 0.007 a
β-damascenone t	8.7 ± 0.3 b	9.8 ± 0.5 b	11.7 ± 0.6 a
riesling acetal t	0.128 ± 0.004	0.129 ± 0.003	0.132 ± 0.007
TDN yt*	21.4 ± 0.3	20 ± 1	20 ± 2
vitispirane t	1.000 ± 0.001	0.50 ± 0.03	0.51 ± 0.05

AMH t	0.029 ± 0.002	0.024 ± 0.003	0.02 ± 0.01
BM	n.d.	0.008 ± 0.001	0.016 ± 0.006
FFT	0.14 ± 0.03 a	0.073 ± 0.004 b	0.11 ± 0.01 ab
MOH yt	0.482 ± 0.007 b	0.47 ± 0.06 b	0.83 ± 0.01 a
MP y	0.121 ± 0.002 b	0.048 ± 0.001 c	0.18 ± 0.02 a
free 2-methylbutanal yt*	65,4 ± 0,6 a	59 ± 1 b	49 ± 2 c
free 3-methylbutanal yt	14 ± 1 a	13 ± 1 a	9,5 ± 0,3 b
free isobutyraldehyde yt*	448 ± 20 a	304 ± 20 b	213 ± 9 c
free methional yt*	2,4 ± 0,3 a	1,19 ± 0,04 b	2,4 ± 0,1 a
free phenylacetaldehyde yt*	5,94 ± 0,07 b	4,8 ± 0,7 c	7,6 ± 0,2 a
total 2-methylbutanal yt*	75 ± 2 a	67 ± 1 b	54 ± 2 c
total 3-methylbutanal y*	16 ± 1 a	15 ± 1 a	9.5 ± 0.8 b
total isobutyraldehyde yt*	459 ± 10 a	308 ± 10 b	190 ± 10 c
total methional t*	2.4 ± 0.3	2.1 ± 0.3	2.1 ± 0.1
total phenylacetaldehyde t*	8.6 ± 0.9 a	6 ± 1 b	7.7 ± 0.3 ab
free SO_2 y	406 ± 300 ab	719 ± 50 a	n.d. b
total SO_2 y	8044 ± 3000 b	35149 ± 10000 a	82 ± 3 b
(+)-rose oxide yt	0.09 ± 0.01 a	0.06 ± 0.002 b	0.089 ± 0.007 a
1,8-cineole	0.38 ± 0.02 a	0.31 ± 0.03 b	0.303 ± 0.008 b
β-citronellol yt*	0.66 ± 0.07 c	1.27 ± 0.02 b	2.2 ± 0.1 a
dihydromyrcenol t	0.447 ± 0.006	0.43 ± 0.02	0.42 ± 0.01
geraniol t	1.1 ± 0.1 b	1.63 ± 0.01 a	1.6 ± 0.3 a
R-limonene t	0.9 ± 0.1	0.77 ± 0.05	0.8 ± 0.2
linalool t	4 ± 0.2 b	4.2 ± 0.3 ab	4.8 ± 0.3 a
linalool oxide yt	14.4 ± 0.3	13.8 ± 0.4	13.4 ± 0.8
nerol t	0.5 ± 0.1	0.63 ± 0.02	0.67 ± 0.03
α-terpineol t	25 ± 0.5	23.9 ± 0.4	25.2 ± 0.8
acetovanillone t	70.4 ± 0.8	75 ± 2	73 ± 2
syringaldehyde t*	121 ± 5	109 ± 30	152 ± 20
vanillin t	67 ± 4	65 ± 7	69 ± 2
4-ethylguaiacol	n.d.	n.d.	n.d.
4-ethylphenol t	0.146 ± 0.006	0.14 ± 0.004	0.133 ± 0.007
eugenol yt*	2.14 ± 0.01 b	2.47 ± 0.02 a	2.18 ± 0.02 b
guaiacol t	1.17 ± 0.02	1.1 ± 0.2	1.03 ± 0.03
trans-isoeugenol t	1.07 ± 0.02 ab	1.12 ± 0.02 a	1.01 ± 0.03 b
m-cresol t	0.38 ± 0.03	0.381 ± 0.007	0.37 ± 0.01
methoxyeugenol yt	0.649 ± 0.006 a	0.585 ± 0.009 b	0.55 ± 0.04 b
o-cresol yt*	n.d.	n.d.	n.d.
p-propylguaiacol t*	0.09 ± 0.07	0.05 ± 0.02	0.06 ± 0.01
syringol	n.d.	n.d.	n.d.
4-vinylguaiacol yt*	383 ± 10 b	491 ± 10 a	515 ± 20 a
4-vinylphenol yt*	129 ± 3 b	140.4 ± 0.9 a	103 ± 4 c

Table D.6: Volatiles concentration (average ± standard, μg/L) in samples fermented by 3 *S. cerevisiae* strains and submitted to 8 weeks of accelerated anoxic aging at 50 ºC. Significance (p-value < 0.05) of the factors yeast, aging time and their interaction is indicated by the letters y, t and * respectively.

	8 weeks		
compound	**ECA5**	**QA23**	**SAUVY**
hexyl acetate yt*	n.d.	n.d.	n.d.
isoamyl acetate yt*	2136 ± 200 a	438 ± 100 b	742 ± 100 b
isobutyl acetate yt*	83 ± 20 a	32 ± 2 b	29 ± 10 b
β-phenylethyl acetate yt*	765 ± 200 a	98 ± 9 b	155 ± 60 b
acetic acid yt	193042 ± 20000 c	738681 ± 8000 a	375360 ± 40000 b
butyric acid t	484 ± 60 a	555 ± 30 a	339 ± 40 b
decanoic acid yt*	2019 ± 70 a	1730 ± 100 b	998 ± 60 c
hexanoic acid y	3670 ± 400 b	4489 ± 200 a	2160 ± 90 c
isobutyric acid y	1315 ± 200	1313 ± 30	1616 ± 40
isovaleric acid y	2991 ± 600	2011 ± 400	1973 ± 300
octanoic acid yt	6988 ± 300 a	6566 ± 300 a	3381 ± 200 b
benzyl alcohol y*	51 ± 3 b	68 ± 2 a	69 ± 2 a
butanol yt	691 ± 40 b	843 ± 30 a	588 ± 30 c
hexanol yt*	63 ± 3 b	79 ± 1 a	80 ± 3 a
hexenol	n.d.	n.d.	n.d.
isoamyl alcohol y	223905 ± 20000 a	149937 ± 3000 b	132692 ± 6000 b
isobutanol y	25777 ± 2000 a	20039 ± 500 b	15299 ± 900 c
methionol y	4820 ± 800 a	1665 ± 80 b	4463 ± 100 a
β-phenylethanol y	42538 ± 9000 a	13989 ± 600 b	17639 ± 1000 b
ethyl isobutyrate yt*	150 ± 10 a	103 ± 6 b	120 ± 3 b
ethyl isovalerate yt*	25 ± 1 a	16.3 ± 0.2 c	21.8 ± 0.5 b
ethyl 2-methylbutyrate	13.2 ± 0.9 a	9.1 ± 0.3 b	13.5 ± 0.1 a
ethyl 4-methylvalerate t	0.11 ± 0.02	0.07 ± 0.02	0.09 ± 0.03
acetaldehyde yt*	923 ± 100 b	1644 ± 200 a	1040 ± 60 b
acetoin y	197 ± 10 c	329 ± 30 a	271 ± 10 b
diacetyl yt*	125 ± 10 a	58.92746 ± 0 b	74.58299 ± 0 b
ethyl cinnamate yt*	0.99 ± 0.04 b	0.82 ± 0.05 c	1.2 ± 0.05 a
ethyl dihydrocinnamate y	n.d.	n.d.	n.d.
ethyl acetate yt*	44874 ± 3000 c	103239 ± 1000 a	58788 ± 2000 b
ethyl butyrate yt	90 ± 7 a	75 ± 10 a	46 ± 4 b
ethyl decanoate yt	36 ± 20 a	26 ± 10 ab	n.d. b
ethyl hexanoate yt*	494 ± 30 a	419 ± 20 b	199 ± 20 c
ethyl octanoate y	306 ± 50 a	280 ± 20 a	134 ± 10 b
ethyl propanoate yt	174 ± 6 b	206 ± 7 a	204 ± 8 a
ethyl leucate yt*	30 ± 3 a	15.3 ± 0.2 c	22 ± 3 b
γ-butyrolactone yt	4241 ± 300 a	4775 ± 100 a	2850 ± 90 b
δ-decalactone t	11.6 ± 0.2	11.1 ± 0.3	11.3 ± 0.3
massoia lactone t	1.1 ± 0.4	0.87 ± 0.08	1.01 ± 0.07
γ-nonalactone t	26.2 ± 0.4	26.3 ± 0.5	27 ± 0.5
γ-octalactone y	0.114 ± 0.004 c	0.49 ± 0.07 b	1.7 ± 0.1 a
whiskylactone	n.d.	n.d.	n.d.
diethyl succinate yt*	3528 ± 200 a	2202 ± 30 b	3245 ± 40 a
ethyl lactate yt*	17753 ± 500 b	14004 ± 200 c	20462 ± 200 a
ethyl cyclohexanoate	n.d.	n.d.	n.d.
α-ionone t	0.05 ± 0.04	0.054 ± 0.008	0.06 ± 0.04
β-ionone t	0.1 ± 0.007	0.091 ± 0.009	0.1 ± 0.01
β-damascenone t	8.4 ± 0.4 b	8.8 ± 0.5 b	11.3 ± 0.4 a
riesling acetal t	0.123 ± 0.003	0.121 ± 0.003	0.126 ± 0.005
TDN yt*	38.2 ± 0.2 a	32 ± 0.8 b	34 ± 1 b
vitispirane t	0.733 ± 0.002 a	0.68 ± 0.02 b	0.72 ± 0.03 ab

AMH t	0.021 ± 0.003	0.02 ± 0.002	0.01 ± 0.01
BM	n.d. a	0.01 ± 0.01 b	0.016 ± 0.004 b
FFT	0.2 ± 0.02 a	0.13 ± 0.02 b	0.114 ± 0.008 b
MOH yt	0.58 ± 0.07 b	0.53 ± 0.06 b	0.85 ± 0.02 a
MP y	0.127 ± 0.006 b	0.057 ± 0.002 c	0.19 ± 0.02 a
free 2-methylbutanal yt*	100 ± 1 a	97,2 ± 0,9 b	86 ± 1 c
free 3-methylbutanal yt	14 ± 1 b	16,7 ± 0,9 a	11,8 ± 0,2 c
free isobutyraldehyde yt*	668 ± 20 a	454 ± 10 b	337 ± 6 c
free methional yt*	2,4 ± 0,3 b	2,42 ± 0,05 b	3,3 ± 0,3 a
free phenylacetaldehyde yt*	7,1 ± 0,3 c	8,4 ± 0,5 b	10,7 ± 0,7 a
total 2-methylbutanal yt*	118 ± 3 a	104 ± 1 b	92 ± 2 c
total 3-methylbutanal y*	17 ± 2 a	19 ± 2 a	12.6 ± 0.6 b
total isobutyraldehyde yt*	744 ± 20 a	546 ± 10 b	352 ± 9 c
total methional t*	2.4 ± 0.2 b	3.1 ± 0.2 a	3.43 ± 0.09 a
total phenylacetaldehyde t*	10.5 ± 0.7 b	15 ± 0.8 a	16.5 ± 0.4 a
free SO_2 y	89 ± 200	48 ± 300	n.d.
total SO_2 y	7335 ± 3000 b	21042 ± 8000 a	274 ± 8 b
(+)-rose oxide yt	0.074 ± 0.007 a	0.052 ± 0.003 b	0.073 ± 0.009 a
1,8-cineole	0.35 ± 0.02	0.34 ± 0.02	0.29 ± 0.04
β-citronellol yt*	0.26 ± 0.09 c	0.57 ± 0.06 b	0.9 ± 0.2 a
dihydromyrcenol t	0.26 ± 0.07	0.26 ± 0.06	0.24 ± 0.08
geraniol t	0.2 ± 0.2 b	0.47 ± 0.04 ab	0.6 ± 0.2 a
R-limonene t	0.5 ± 0.1	0.61 ± 0.05	0.6 ± 0.1
linalool t	1.3 ± 0.2	1.6 ± 0.3	1.6 ± 0.4
linalool oxide yt	20.5 ± 0.3 a	18.9 ± 0.3 b	19 ± 0.5 b
nerol t	0.6 ± 0.08	0.7 ± 0.01	0.61 ± 0.03
α-terpineol t	21.1 ± 0.4 a	19.4 ± 0.4 b	21.1 ± 0.5 a
acetovanillone t	71.5 ± 0.8 a	72 ± 2 a	67 ± 2 b
syringaldehyde t*	137 ± 10 c	284 ± 20 a	214 ± 20 b
vanillin t	67 ± 6 b	104 ± 6 a	66 ± 6 b
4-ethylguaiacol	n.d.	n.d.	n.d.
4-ethylphenol t	0.172 ± 0.006	0.159 ± 0.005	0.163 ± 0.005
eugenol yt*	2.25 ± 0.03 b	2.51 ± 0.02 a	2.19 ± 0.02 c
guaiacol t	0.94 ± 0.07	1 ± 0.2	0.82 ± 0.03
trans-isoeugenol t	1.07 ± 0.02 a	1.06 ± 0.03 a	0.95 ± 0.02 b
m-cresol t	0.4 ± 0.02	0.4 ± 0.02	0.39 ± 0.01
methoxyeugenol yt	0.9 ± 0.01 a	0.82 ± 0.01 b	0.76 ± 0.03 c
o-cresol yt*	n.d.	n.d.	n.d.
p-propylguaiacol t*	0.12 ± 0.05 a	0.11 ± 0.01 ab	0.05 ± 0.01 b
syringol	n.d.	n.d.	n.d.
4-vinylguaiacol yt*	302 ± 20 b	332 ± 40 b	425 ± 10 a
4-vinylphenol yt*	107 ± 8	113 ± 10	105 ± 3

Table D.7: ANOVA results for the factors yeast, aging time and their interaction. In bold significant effect (p-value < 0.05).

compound	p (yeast)	p (time)	p (y*t)
hexyl acetate yt*	**2,90E-05**	**4,08E-03**	**4,94E-04**
isoamyl acetate yt*	**1,49E-18**	**3,64E-11**	**4,78E-07**
isobutyl acetate yt*	**1,79E-24**	**2,05E-17**	**7,70E-14**
phenylethyl acetate yt*	**1,93E-28**	**3,25E-14**	**3,81E-13**
acetic acid yt	**8,31E-26**	**6,90E-03**	5,72E-01
butyric acid t	**1,93E-09**	**7,44E-03**	8,36E-01
decanoic acid yt*	**5,16E-21**	**4,46E-12**	**3,62E-05**
hexanoic acid y	**3,30E-24**	6,70E-01	1,10E-01
isobutyric acid y	**5,25E-04**	4,42E-01	6,74E-02
isovaleric acid y	**2,85E-04**	6,10E-01	8,68E-01
octanoic acid yt	**1,54E-15**	**4,75E-03**	4,25E-01
benzyl alcohol y*	**6,15E-07**	1,84E-01	**1,42E-03**
butanol yt	**2,37E-13**	**7,29E-09**	1,71E-01
hexanol yt*	**8,92E-25**	**1,67E-13**	**8,97E-03**
hexenol	n.d.	n.d.	n.d.
isoamyl alcohol y	**1,74E-26**	2,73E-01	5,98E-01
isobutanol y	**5,24E-20**	2,64E-01	7,17E-01
methionol y	**9,09E-24**	4,90E-01	2,80E-01
phenylethanol y	**3,32E-21**	5,68E-01	4,74E-01
ethyl isobutyrate yt*	**2,82E-05**	**4,66E-24**	**1,90E-03**
ethyl isovalerate yt*	**2,40E-11**	**1,79E-35**	**8,66E-09**
ethyl 2-methylbutyrate	**2,73E-10**	**2,40E-32**	**1,47E-06**
ethyl 4-methylvalerate t	7,43E-02	**2,59E-12**	9,09E-02
acetaldehyde yt*	**7,22E-08**	**1,62E-02**	**2,01E-02**
acetoin y	**2,08E-13**	5,68E-02	3,34E-01
diacetyl yt*	**1,57E-12**	**1,12E-23**	**1,97E-08**
ethyl cinnamate yt*	**5,80E-06**	**2,98E-34**	**3,47E-06**
ethyl dihydrocinnamate y	**1,61E-03**	1,81E-01	1,70E-01
ethyl acetate yt*	**6,13E-12**	**4,43E-09**	**4,11E-23**
ethyl butyrate yt	**1,48E-09**	**1,93E-03**	3,79E-01
ethyl decanoate yt	**2,42E-03**	**1,76E-08**	8,78E-01
ethyl hexanoate yt*	**9,54E-21**	**3,92E-07**	**4,82E-03**
ethyl octanoate y	**7,46E-04**	1,27E-01	9,66E-01
ethyl propanoate yt	**2,23E-05**	**4,82E-24**	3,66E-01
ethyl leucate yt*	**1,72E-09**	**6,67E-23**	**6,22E-04**
γ-butyrolactone yt	**1,55E-04**	**8,94E-12**	1,29E-01
δ-decalactone t	3,87E-01	**3,33E-03**	4,55E-01
Massoia lactone t	9,04E-01	**2,30E-04**	8,86E-01
γ-nonalactone t	3,77E-01	**1,29E-04**	9,39E-01
γ-octalactone y	**2,91E-29**	2,59E-01	1,29E-01
whiskylactone	n.d.	n.d.	n.d.
diethyl succinate yt*	**1,11E-11**	**9,80E-32**	**1,47E-06**
ethyl lactate yt*	**3,54E-08**	**8,38E-28**	**3,88E-04**
ethyl cyclohexanoate	n.d.	n.d.	n.d.
α-ionone t	6,99E-02	**1,19E-03**	5,61E-01
β-ionone t	6,39E-01	**7,30E-04**	8,97E-01
β-damascenone t	5,32E-02	**3,75E-04**	5,94E-01
Riesling acetal t	9,20E-01	**3,58E-14**	9,22E-01
TDN yt*	**3,65E-03**	**5,19E-39**	**2,03E-03**
vitispirane t	5,55E-02	**8,99E-36**	3,63E-01
MHA t	2,77E-01	**1,07E-02**	1,13E-01
BM	5,50E-01	5,15E-02	1,09E-01
FFT	4,83E-01	6,56E-01	8,85E-02
MH yt	**3,55E-02**	**5,11E-03**	2,59E-01

MP y	**8,23E-11**	3,09E-01	4,46E-01
free 2-methylbutanal yt*	**1,08E-09**	**4,70E-49**	**3,00E-10**
free 3-methylbutanal yt	**3,52E-09**	8,56E-01	**6,13E-03**
free isobutyraldehyde yt*	**1,38E-24**	**2,88E-43**	**1,78E-21**
free methional yt*	**2,87E-09**	**2,79E-11**	**6,62E-04**
free phenylacetaldehyde yt*	**5,19E-06**	**1,05E-12**	**2,58E-05**
total 2-methylbutanal yt*	**1,90E-13**	**2,81E-46**	**1,08E-10**
total 3-methylbutanal y*	**4,39E-15**	8,25E-01	**1,44E-02**
total isobutyraldehyde yt*	**1,77E-25**	**2,41E-43**	**1,53E-22**
total methional t*	6,13E-01	**7,64E-09**	**9,68E-04**
total phenylacetaldehyde t*	7,96E-02	**5,62E-19**	**1,95E-05**
free SO_2 y	**2,39E-04**	1,62E-01	1,95E-01
total SO_2 y	**6,24E-18**	1,03E-01	4,30E-01
(+)-rose oxide yt	**1,95E-10**	**7,63E-06**	4,17E-01
1,8-cineole	6,78E-01	6,29E-01	7,23E-01
β-citronellol yt*	**9,28E-24**	**7,48E-28**	**7,63E-14**
dihydromyrcenol t	9,69E-01	**4,35E-04**	9,77E-01
geraniol t	9,66E-02	**9,97E-16**	2,86E-01
R-limonene t	7,56E-01	**9,79E-06**	8,13E-01
linalool t	9,48E-01	**1,49E-06**	9,79E-01
linalool oxide yt	**1,32E-02**	**1,25E-37**	2,75E-01
nerol t	2,17E-01	**1,59E-03**	8,20E-01
α-terpineol t	8,62E-01	**3,65E-06**	9,67E-01
acetovanillone t	6,34E-01	**2,28E-04**	1,00E+00
syringaldehyde t*	8,49E-02	**5,12E-13**	**7,41E-03**
vanillin t	1,87E-01	**7,46E-08**	1,56E-01
4-ethylguaiacol	n.d.	n.d.	n.d.
4-ethylphenol t	9,23E-01	**1,72E-02**	8,77E-01
eugenol yt*	**5,67E-18**	**5,23E-15**	**7,51E-03**
guaiacol t	1,40E-01	**8,59E-04**	8,87E-01
trans-isoeugenol t	4,51E-01	**5,02E-12**	9,35E-01
m-cresol t	6,42E-02	**4,05E-09**	1,15E-01
methoxyeugenol yt	**1,17E-02**	**3,44E-28**	1,69E-01
o-cresol yt*	n.d.	n.d.	n.d.
p-propylguaiacol t*	3,42E-01	**5,45E-08**	**3,23E-02**
syringol	n.d.	n.d.	n.d.
4-vinylguaiacol yt*	**8,17E-10**	**3,49E-10**	**1,88E-09**
4-vinylphenol yt*	**1,35E-09**	**7,35E-10**	**1,42E-07**

Annexes E

Supplementary material related to the Chapter 6

Table E.1: Major compounds concentration in μg/L in wines recently fermented with 4 *Saccharomyces* strains. In the case of a significant effect of the yeast (p-values <0.05, in bold) letters indicate Tukey's HSD test results. n.d.: not detected or below the detection limits.

compound	pvalue	CR89D1	QA23	RHONE	SAUVY
acetic acid	**5,84E-05**	497 ± 100 a	157 ± 20 bc	261 ± 20 b	95 ± 20 c
isobutyric acid	**5,64E-08**	2.7 ± 0.2 a	0.69 ± 0.07 c	1.2 ± 0.1 b	0.6 ± 0.1 c
butyric acid	**1,34E-05**	0.74 ± 0.02 b	1.7 ± 0.2 a	1.9 ± 0.1 a	0.9 ± 0.1 b
isovaleric acid	**2,28E-02**	1.2 ± 0.2 ab	1 ± 0.2 ab	1.3 ± 0.2 a	0.7 ± 0.1 b
hexanoic acid	**6,37E-08**	1.6 ± 0.1 c	3.7 ± 0.1 a	2.7 ± 0.1 b	1.7 ± 0.1 c
octanoic acid	**9,95E-06**	2.3 ± 0.3 c	5.7 ± 0.3 a	3.7 ± 0.4 b	2.4 ± 0.5 c
decanoic acid	**6,68E-03**	1.4 ± 0.5 ab	2.1 ± 0.4 a	1.9 ± 0.3 a	0.8 ± 0.1 b
isobutanol	**1,04E-05**	44 ± 2 a	26 ± 3 b	39 ± 2 a	19 ± 4 c
butanol	**4,18E-07**	1.31 ± 0.05 a	0.75 ± 0.05 c	0.43 ± 0.02 d	0.98 ± 0.08 b
isoamyl alcohol	**2,18E-06**	151 ± 4 b	189 ± 10 a	199 ± 6 a	112 ± 9 c
hexanol	**1,29E-05**	0.061 ± 0.004 a	0.061 ± 0.001 b	0.044 ± 0.002 b	0.034 ± 0.002 c
methionol	**8,18E-06**	0.73 ± 0.02 a	0.46 ± 0.09 b	0.55 ± 0.05 b	0.15 ± 0.02 c
benzyl alcohol	**2,34E-02**	0.65 ± 0.03 b	0.78 ± 0.07 a	0.63 ± 0.03 b	0.7 ± 0.05 ab
β-phenylethanol	**6,07E-09**	34 ± 2 a	13.4 ± 0.8 b	12.2 ± 0.6 b	9.3 ± 0.5 c
acetaldehyde	**2,55E-05**	4.6 ± 0.7 a	1.09 ± 0.08 b	0.89 ± 0.04 b	1.9 ± 0.5 b
diacetyl	**1,25E-08**	0.64 ± 0.04 a	0.27 ± 0.02 b	0.32 ± 0.01 b	0.133 ± 0.007 c
acetoin	**5,97E-05**	22 ± 6 a	0.2 ± 0.02 b	0.12 ± 0.01 b	0.6 ± 0.1 b
ethyl acetate	**2,71E-10**	48 ± 1 d	94 ± 4 b	78 ± 4 c	203 ± 6 a
ethyl propanoate	**1,84E-05**	n.d. c	0.07 ± 0.003 b	0.044 ± 0.007 b	0.14 ± 0.03 a
ethyl butyrate	**8,79E-05**	0.22 ± 0.04 bc	0.3 ± 0.05 b	0.5 ± 0.07 a	0.12 ± 0.02 c
isoamyl acetate	**1,51E-04**	0.8 ± 0.1 b	5.1 ± 0.9 a	4.3 ± 0.9 a	6.1 ± 0.8 a
ethyl hexanoate	**5,83E-08**	0.288 ± 0.005 c	0.74 ± 0.04 a	0.47 ± 0.01 b	0.34 ± 0.02 c
hexyl acetate	2,82E-01	n.d.	0.011 ± 0.002	0.011 ± 0.004	0.011 ± 0.001
ethyl lactate	**3,36E-03**	0.7 ± 0.02 a	0.57 ± 0.07 ab	0.7 ± 0.06 a	0.49 ± 0.05 b
ethyl octanoate	**3,42E-06**	0.37 ± 0.05 c	1.04 ± 0.06 a	0.82 ± 0.06 b	0.52 ± 0.07 c
ethyl decanoate	**1,57E-04**	0.32 ± 0.08 b	0.56 ± 0.05 a	0.55 ± 0.05 a	0.26 ± 0.02 b
diethyl succinate	**5,80E-06**	0.112 ± 0.006 a	0.021 ± 0.004 c	0.03 ± 0.008 bc	0.05 ± 0.01 b
γ-butyrolactone	1,94E-01	0.59 ± 0.04	0.59 ± 0.09	0.66 ± 0.07	0.53 ± 0.05

Table E.2: Trace compounds concentration in μg/L in wines recently fermented with 4 *Saccharomyces* strains. In the case of a significant effect of yeast, letters of Tukey's HSD test results were added. n.d.: not detected or below the detection limits. n.d.: not detected or below the detection limits.

	wine recently fermented			
compound	**CR89D1**	**QA23**	**RHONE**	**SAUVY**
ethyl dihydrocinnamate	0.05 ± 0.01 b	0.04 ± 0.01 b	0.037 ± 0.003 b	0.393 ± 0.007 a
ethyl cinnamate	0.47 ± 0.02 a	0.27 ± 0.01 c	0.25 ± 0.02 c	0.33 ± 0.01 b
ethyl isobutyrate	6 ± 0.1 a	2.1 ± 0.3 c	3.9 ± 0.1 b	3.5 ± 0.4 b
isobutyl acetate	25 ± 3 c	29 ± 1 bc	35 ± 1 b	57 ± 3 a
ethyl 2-methylbutyrate	0.19 ± 0.03 b	0.173 ± 0.009 b	0.275 ± 0.006 a	0.171 ± 0.002 b
ethyl isovalerate	0.29 ± 0.04 c	0.554 ± 0.007 b	0.82 ± 0.06 a	0.32 ± 0.02 c
ethyl 4-methylvalerate	n.d.	n.d.	n.d.	n.d.
ethyl leucate	1.1 ± 0.1 c	3.2 ± 0.2 b	6.5 ± 0.5 a	3.6 ± 0.5 b
β-phenylethyl acetate	365 ± 30 b	269 ± 6 c	239 ± 10 c	533 ± 20 a
γ-octalactone	0.09 ± 0.01 c	0.166 ± 0.006 b	0.179 ± 0.007 b	0.83 ± 0.02 a
γ-nonalactone	1.25 ± 0.05 ab	1.2 ± 0.01 b	1.3 ± 0.02 a	1.26 ± 0.01 ab
δ-decalactone	0.23 ± 0.03 a	0.16 ± 0.01 b	0.18 ± 0.01 ab	0.18 ± 0.01 b
massoia lactone	3.4 ± 0.6	3.8 ± 0.8	3.79 ± 0.09	3 ± 1
vitispirane	n.d.	n.d.	n.d.	n.d.
riesling acetal	n.d.	n.d.	n.d.	n.d.
β-damascenone	0.14 ± 0.01	0.23 ± 0.08	0.16 ± 0.02	0.15 ± 0.04
β-ionone	0.027 ± 0.003 ab	0.028 ± 0.003 a	0.025 ± 0.002 ab	n.d. b
TDN	n.d.	n.d.	n.d.	n.d.
guaiacol	2.2 ± 0.3	2.6 ± 0.4	2.8 ± 0.5	2 ± 0.4
o-cresol	0.3 ± 0.006 b	0.313 ± 0.005 ab	0.32 ± 0.01 a	0.32 ± 0.006 a
eugenol	0.53 ± 0.02 b	0.54 ± 0.02 ab	0.59 ± 0.03 a	0.5 ± 0.007 b
4-ethylphenol	n.d.	n.d.	n.d.	n.d.
4-vinylguaiacol	85 ± 20 c	525 ± 20 a	455 ± 20 b	9 ± 2 d
syringol	3.2 ± 0.2 ab	3.3 ± 0.2 ab	4 ± 0.5 a	3.1 ± 0.3 b
isoeugenol	5.6 ± 0.6 ab	6 ± 0.3 a	6.3 ± 0.3 a	4.9 ± 0.4 b
4-vinylphenol	28 ± 4 c	102 ± 3 a	90 ± 1 b	4 ± 1 d
methoxyeugenol	0.7 ± 0.2	1.7 ± 0.7	1.2 ± 0.3	0.7 ± 0.3
R-limonene	n.d.	n.d.	n.d.	n.d.
1,8-cineole	n.d.	n.d.	n.d.	n.d.
rose oxide	0.35 ± 0.02 bc	0.42 ± 0.01 b	0.31 ± 0.008 c	0.72 ± 0.06 a
linalool oxide	0.48 ± 0.02	0.59 ± 0.07	0.5 ± 0.07	0.46 ± 0.04
dihydromyrcenol	n.d.	n.d.	n.d.	n.d.
linalool	0.77 ± 0.02 d	0.917 ± 0.008 c	1.11 ± 0.03 b	1.5 ± 0.03 a
α-terpineol	0.159 ± 0.001 b	0.164 ± 0.002 b	0.19 ± 0.005 a	0.19 ± 0.01 a
β-citronellol	6.9 ± 0.3 a	4.5 ± 0.4 bc	5.2 ± 0.2 b	3.7 ± 0.6 c
nerol	2.81 ± 0.05 a	2.3 ± 0.07 b	2.5 ± 0.1 b	1.7 ± 0.1 c
geraniol	1.1 ± 0.4 c	10.6 ± 0.3 b	15 ± 1 a	14.5 ± 0.7 a
vanillin	8 ± 2 ab	12 ± 2 a	10 ± 1 ab	7 ± 2 b
acetovanillone	26 ± 3	28 ± 0.6	25.8 ± 0.2	26 ± 3
syringaldehyde	n.d.	n.d.	n.d.	n.d.

Table E.3: Trace compounds concentration in μg/L in wines fermented with 4 *Saccharomyces* strains and submitted to acceerated aging for 12 hours at 75 ºC. In the case of a significant effect of yeast, letters of Tukey's HSD test results were added. n.d.: not detected or below the detection limits. n.d.: not detected or below the detection limits.

	12 h at 75 ºC			
compound	**CR89D1**	**QA23**	**RHONE**	**SAUVY**
ethyl dihydrocinnamate	0.005 ± 0.001 b	0.06 ± 0.01 b	0.05 ± 0.008 b	0.35 ± 0.01 a
ethyl cinnamate	0.93 ± 0.05 a	0.23 ± 0.03 c	0.25 ± 0.01 c	0.37 ± 0.03 b
ethyl isobutyrate	11.5 ± 0.7 a	3.8 ± 0.6 c	6.9 ± 0.2 b	4.6 ± 0.2 c
isobutyl acetate	21.5 ± 0.5 c	25 ± 3 bc	30 ± 1 b	50 ± 2 a
ethyl 2-methylbutyrate	0.38 ± 0.06 b	0.29 ± 0.01 c	0.55 ± 0.01 a	0.22 ± 0.01 c
ethyl isovalerate	0.85 ± 0.05 c	1.18 ± 0.07 b	1.75 ± 0.08 a	0.661 ± 0.007 d
ethyl 4-methylvalerate	n.d.	n.d.	n.d.	n.d.
ethyl leucate	2.2 ± 0.2 c	4.48 ± 0.09 b	7.6 ± 0.6 a	4.7 ± 0.7 b
β-phenylethyl acetate	325 ± 10 b	255 ± 20 c	224 ± 2 c	488 ± 20 a
γ-octalactone	0.18 ± 0.04 b	0.162 ± 0.005 b	0.162 ± 0.002 b	0.68 ± 0.02 a
γ-nonalactone	1.47 ± 0.03	1.5 ± 0.2	1.53 ± 0.07	1.48 ± 0.05
δ-decalactone	0.5 ± 0.2	0.23 ± 0.03	0.26 ± 0.01	0.2 ± 0.01
massoia lactone	2.8 ± 0.4	3.4 ± 0.4	3.5 ± 0.4	2.8 ± 0.1
vitispirane	0.036 ± 0.007 ab	0.038 ± 0.006 a	0.045 ± 0.004 a	0.024 ± 0.003 b
riesling acetal	0.033 ± 0.005 a	0.035 ± 0.003 a	0.04 ± 0.002 a	0.025 ± 0.002 b
β-damascenone	1 ± 0.1	1.07 ± 0.08	1.01 ± 0.05	0.95 ± 0.04
β-ionone	0.03 ± 0.01	0.026 ± 0.005	0.019 ± 0.003	0.018 ± 0.005
TDN	1.2 ± 0.1	1.1 ± 0.2	1.3 ± 0.1	0.92 ± 0.04
guaiacol	2.09 ± 0.04	2.3 ± 0.1	2.1 ± 0.1	2.11 ± 0.02
o cresol	0.32 ± 0.02	0.318 ± 0.002	0.331 ± 0.009	0.332 ± 0.004
eugenol	0.55 ± 0.01	0.51 ± 0.04	0.535 ± 0.008	0.493 ± 0.008
4-ethylphenol	0.03 ± 0.004	0.034 ± 0.005	0.04 ± 0.01	0.032 ± 0.002
4-vinylguaiacol	75 ± 10 c	224 ± 5 a	196 ± 10 b	35.9 ± 0.5 d
syringol	7.5 ± 0.5	7 ± 0.4	7 ± 0.2	7.2 ± 0.3
isoeugenol	5.5 ± 0.2 a	6.1 ± 0.2 a	6 ± 0.4 a	5.4 ± 0.2 a
4-vinylphenol	19 ± 2 c	55 ± 5 a	46.1 ± 0.5 b	8.4 ± 0.4 d
methoxyeugenol	0.94 ± 0.04 ab	1.12 ± 0.04 a	1.1 ± 0.1 a	0.88 ± 0.05 b
R-limonene	1 ± 0.3	1.4 ± 0.2	1.2 ± 0.2	1.38 ± 0.06
1,8-cineole	0.16 ± 0.05	0.19 ± 0.02	0.16 ± 0.02	0.21 ± 0.04
rose oxide	0.43 ± 0.03 c	0.52 ± 0.02 b	0.38 ± 0.01 c	0.91 ± 0.05 a
linalool oxide	3.6 ± 0.5 a	3.6 ± 0.2 a	3.7 ± 0.1 a	2.7 ± 0.1 b
dihydromyrcenol	0.16 ± 0.04	0.14 ± 0.02	0.116 ± 0.006	0.16 ± 0.02
linalool	14 ± 1 b	15 ± 1 ab	17.6 ± 0.5 a	15.2 ± 0.2 ab
α-terpineol	2.2 ± 0.2 b	2.5 ± 0.3 ab	2.8 ± 0.2 a	1.97 ± 0.06 b
β-citronellol	7.3 ± 0.4 a	4.3 ± 0.7 b	4.7 ± 0.4 b	3.5 ± 0.6 b
nerol	2.82 ± 0.09 a	2.5 ± 0.1 b	2.61 ± 0.08 ab	2.21 ± 0.05 c
geraniol	7.8 ± 0.9 b	14 ± 1 a	15 ± 1 a	16.2 ± 0.7 a
vanillin	18 ± 0.8 b	24 ± 2 ab	30 ± 7 a	16 ± 1 b
acetovanillone	24.3 ± 0.8	24.8 ± 0.5	26 ± 1	25.1 ± 0.6
syringaldehyde	441 ± 20 b	637 ± 40 ab	691 ± 200 a	426 ± 50 b

Table E.4: Trace compounds concentration in μg/L in wines fermented with 4 *Saccharomyces* strains and submitted to accelerated aging for 24 hours at 75 ºC. In the case of a significant effect of yeast, letters of Tukey's HSD test results were added. n.d.: not detected or below the detection limits. n.d.: not detected or below the detection limits.

	24 h at 75 ºC			
compound	**CR89D1**	**QA23**	**RHONE**	**SAUVY**
ethyl dihydrocinnamate	0.05 ± 0.01 b	0.04 ± 0.005 b	0.05 ± 0.02 b	0.38 ± 0.01 a
ethyl cinnamate	1.42 ± 0.03 a	0.31 ± 0.02 d	0.4 ± 0.03 c	0.56 ± 0.03 b
ethyl isobutyrate	19 ± 3 a	5.59 ± 0.06 c	10.8 ± 0.6 b	5.7 ± 0.3 c
isobutyl acetate	22 ± 2 c	26 ± 1 c	32 ± 2 b	51 ± 4 a
ethyl 2-methylbutyrate	0.67 ± 0.08 b	0.47 ± 0.02 c	0.85 ± 0.05 a	0.312 ± 0.007 d
ethyl isovalerate	1.45 ± 0.06 c	1.79 ± 0.07 b	2.5 ± 0.1 a	0.88 ± 0.08 d
ethyl 4-methylvalerate	n.d.	n.d.	n.d.	n.d.
ethyl leucate	3.6 ± 0.6 c	6.5 ± 0.8 b	11 ± 1 a	6.5 ± 0.5 b
β-phenylethyl acetate	333 ± 40 b	252 ± 7 c	232 ± 10 c	493 ± 30 a
γ-octalactone	0.2 ± 0.1 b	0.14 ± 0.02 b	0.18 ± 0.02 b	0.81 ± 0.07 a
γ-nonalactone	1.8 ± 0.2	1.9 ± 0.2	2.2 ± 0.1	2 ± 0.3
δ-decalactone	0.21 ± 0.01 b	0.31 ± 0.05 b	0.43 ± 0.05 a	0.28 ± 0.03 b
massoia lactone	3.4 ± 0.6	3.3 ± 0.2	3.5 ± 0.3	3.4 ± 0.3
vitispirane	0.117 ± 0.006 b	0.121 ± 0.008 b	0.15 ± 0.01 a	0.085 ± 0.003 c
riesling acetal	0.077 ± 0.005 bc	0.084 ± 0.007 ab	0.1 ± 0.008 a	0.065 ± 0.003 c
β-damascenone	1.3 ± 0.2	1.4 ± 0.1	1.5 ± 0.2	1.4 ± 0.2
β-ionone	0.024 ± 0.002 ab	0.029 ± 0.004 a	0.03 ± 0.003 a	0.019 ± 0.002 b
TDN	3.9 ± 0.6 a	3.7 ± 0.2 ab	4.4 ± 0.4 a	2.9 ± 0.2 b
guaiacol	3 ± 0.3	3.3 ± 0.7	2.9 ± 0.3	3.1 ± 0.4
o cresol	0.33 ± 0.02	0.34 ± 0.02	0.35 ± 0.02	0.352 ± 0.007
eugenol	0.54 ± 0.03 ab	0.61 ± 0.06 a	0.608 ± 0.004 a	0.5 ± 0.008 b
4-ethylphenol	0.034 ± 0.004	0.04 ± 0.002	0.043 ± 0.009	0.04 ± 0.004
4-vinylguaiacol	109 ± 10 bc	226 ± 50 a	184 ± 20 ab	82 ± 8 c
syringol	12 ± 0.3	13 ± 2	12.3 ± 0.3	12.3 ± 0.4
isoeugenol	7 ± 1	8 ± 1	7.2 ± 0.4	6.5 ± 0.3
4-vinylphenol	23 ± 5 b	51 ± 8 a	45 ± 5 a	16 ± 4 b
methoxyeugenol	1.5 ± 0.4	1.7 ± 0.3	1.6 ± 0.1	1.3 ± 0.1
R-limonene	1.5 ± 0.4	1.2 ± 0.3	1.4 ± 0.3	1.4 ± 0.2
1,8-cineole	0.15 ± 0.06	0.13 ± 0.07	0.13 ± 0.08	0.17 ± 0.08
rose oxide	0.43 ± 0.01 bc	0.52 ± 0.04 b	0.4 ± 0.03 c	0.93 ± 0.07 a
linalool oxide	6.7 ± 0.2 bc	7.2 ± 0.7 ab	8.2 ± 0.7 a	5.6 ± 0.3 c
dihydromyrcenol	0.12 ± 0.04	0.09 ± 0.03	0.09 ± 0.04	0.12 ± 0.02
linalool	17.8 ± 0.8 b	19.6 ± 0.7 ab	21.3 ± 0.4 a	20 ± 1 ab
α-terpineol	4.2 ± 0.5 b	4.6 ± 0.2 ab	5.3 ± 0.3 a	3.8 ± 0.4 b
β-citronellol	6 ± 0.1 a	3.9 ± 0.4 bc	4.3 ± 0.2 b	3.3 ± 0.4 c
nerol	2.2 ± 0.1	2.3 ± 0.06	2.3 ± 0.1	2.3 ± 0.1
geraniol	10 ± 2 b	14.5 ± 0.6 a	16.3 ± 0.5 a	16.8 ± 0.8 a
vanillin	26 ± 10	29 ± 2	29 ± 5	19.5 ± 0.5
acetovanillone	27 ± 5	27 ± 1	29 ± 3	28 ± 3
syringaldehyde	669 ± 400	492 ± 200	464 ± 200	462 ± 100

Table E.5: Trace compounds concentration in μg/L in wines fermented with 4 *Saccharomyces* strains and submitted to accelerated aging for 96 hours at 75 ºC. In the case of a significant effect of yeast, letters of Tukey's HSD test results were added. n.d.: not detected or below the detection limits. n.d.: not detected or below the detection limits.*indicates that only two replicates were considered for statistical treatment.

	96 h at 75 ºC			
compound	**CR89D1**	**QA23**	**RHONE**	**SAUVY***
ethyl dihydrocinnamate	0.062 ± 0.004 b	0.06 ± 0.002 b	0.058 ± 0.002 b	0.36 ± 0.02 a
ethyl cinnamate	3.7 ± 0.2 a	0.49 ± 0.05 b	0.55 ± 0.09 b	0.3 ± 0.1 b
ethyl isobutyrate	56 ± 3 a	17.3 ± 0.8 c	32 ± 2 b	13.5 ± 0.2 c
isobutyl acetate	16.4 ± 0.5 c	17.2 ± 0.8 c	20.8 ± 0.9 b	36 ± 1 a
ethyl 2-methylbutyrate	2.8 ± 0.2 b	2.1 ± 0.1 c	3.4 ± 0.3 a	1.2 ± 0.1 d
ethyl isovalerate	5.3 ± 0.4 c	6.5 ± 0.4 b	8.4 ± 0.3 a	3 ± 0.1 d
ethyl 4-methylvalerate	n.d. b	n.d. b	0.116 ± 0.004 a	n.d. b
ethyl leucate	11 ± 1 c	17 ± 1 b	25.8 ± 0.5 a	14 ± 1 bc
β-phenylethyl acetate	250 ± 20 b	171 ± 2 c	153 ± 2 c	360 ± 20 a
γ-octalactone	0.09 ± 0.05 b	0.116 ± 0.003 b	0.125 ± 0.004 b	0.6 ± 0.1 a
γ-nonalactone	1.5 ± 0.2	1.65 ± 0.03	1.8 ± 0.09	1.7 ± 0.1
δ-decalactone	0.38 ± 0.02 a	0.27 ± 0.03 bc	0.32 ± 0.02 b	0.22 ± 0.02 c
massoia lactone	4 ± 0.2 c	4.5 ± 0.2 b	5.28 ± 0.09 a	3.91 ± 0.07 c
vitispirane	0.63 ± 0.03 a	0.66 ± 0.03 a	0.7 ± 0.02 a	0.54 ± 0.01 b
riesling acetal	0.229 ± 0.005 a	0.227 ± 0.007 a	0.235 ± 0.006 a	0.208 ± 0.005 b
β-damascenone	1.4 ± 0.1	1.47 ± 0.06	1.44 ± 0.07	1.5 ± 0.2
β-ionone	0.04 ± 0.02	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01
TDN	48 ± 8 ab	48 ± 3 ab	57 ± 4 a	35 ± 3 b
guaiacol	7.6 ± 0.2 ab	7.2 ± 0.7 ab	6.5 ± 0.4 b	8.7 ± 0.5 a
o cresol	0.4 ± 0.06	0.43 ± 0.03	0.43 ± 0.02	0.39 ± 0.05
eugenol	0.56 ± 0.03 a	0.61 ± 0.02 a	0.63 ± 0.03 a	0.54 ± 0.04 a
4-ethylphenol	0.06 ± 0.03	0.078 ± 0.002	0.09 ± 0.01	0.07 ± 0.004
4-vinylguaiacol	306 ± 20 a	180 ± 9 b	150 ± 10 b	336 ± 8 a
syringol	35 ± 2	39 ± 3	36 ± 2	40.46 ± 0.07
isoeugenol	14.5 ± 0.3 a	12.2 ± 0.6 b	11.9 ± 0.3 b	11.9 ± 0.5 b
4-vinylphenol	38 ± 2 a	32 ± 1 b	29.8 ± 0.3 b	40 ± 4 a
methoxyeugenol	3.9 ± 0.2 bc	4.5 ± 0.4 ab	4.7 ± 0.3 a	3.5 ± 0.2 c
R-limonene	2.6 ± 0.4	2.45 ± 0.06	2.7 ± 0.2	2.5 ± 0.1
1,8-cineole	0.38 ± 0.02 c	0.46 ± 0.03 b	0.53 ± 0.03 a	0.33 ± 0.02 c
rose oxide	0.41 ± 0.02 b	0.49 ± 0.04 b	0.36 ± 0.02 b	0.9 ± 0.1 a
linalool oxide	30 ± 2 bc	33 ± 2 ab	36 ± 2 a	25 ± 1 c
dihydromyrcenol	0.19 ± 0.04	0.17 ± 0.04	0.19 ± 0.04	0.202 ± 0.001
linalool	17.8 ± 0.6 b	16.4 ± 0.04 c	16.4 ± 0.5 c	19.4 ± 0.6 a
α-terpineol	17.9 ± 0.6 b	18.9 ± 0.7 b	21.3 ± 0.5 a	16.1 ± 0.1 c
β-citronellol	3.5 ± 0.3 a	2.3 ± 0.3 b	2.42 ± 0.05 b	2.3 ± 0.9 b
nerol	1.74 ± 0.06	1.6 ± 0.04	1.59 ± 0.07	1.9 ± 0.3
geraniol	11 ± 2	11.3 ± 0.6	11.3 ± 0.7	13 ± 3
vanillin	50 ± 20	48 ± 6	52 ± 10	40 ± 10
acetovanillone	27 ± 3	24.5 ± 0.4	26 ± 2	26 ± 2
syringaldehyde	1235 ± 500	912 ± 100	914 ± 200	1029 ± 400

Table E.6: Significance of the factor yeast, time and their interaction on the modulation of trace compounds concentration (p-value < 0.05 in bold).

	p-value		
compound	**yeast**	**time**	**yeast*time**
ethyl dihydrocinnamate	**5,91E-38**	2,27E-01	**2,01E-04**
ethyl cinnamate	**4,18E-31**	**4,52E-26**	**2,70E-27**
ethyl isobutyrate	**7,57E-26**	**1,42E-31**	**1,64E-21**
isobutyl acetate	**2,88E-26**	**5,23E-16**	**2,12E-03**
ethyl 2-methylbutyrate	**2,84E-19**	**9,63E-33**	**1,07E-15**
ethyl isovalerate	**9,12E-24**	**3,24E-36**	**7,07E-18**
ethyl 4-methylvalerate	**6,25E-38**	**1,88E-38**	**1,51E-42**
ethyl leucate	**1,01E-22**	**7,71E-30**	**2,93E-11**
β-phenylethyl acetate	**2,18E-25**	**2,62E-14**	9,67E-02
γ-octalactone	**4,63E-27**	**3,02E-05**	**2,42E-04**
γ-nonalactone	**9,18E-03**	**5,32E-13**	4,88E-01
δ-decalactone	**7,53E-04**	**1,40E-04**	**1,95E-04**
massoia lactone	**1,55E-03**	**2,95E-07**	4,13E-01
vitispirane	**4,59E-17**	**7,68E-43**	**1,13E-08**
riesling acetal	**1,98E-16**	**1,46E-42**	**3,51E-05**
β-damascenone	1,31E-01	**5,92E-24**	9,56E-01
β-ionone	7,64E-02	**5,43E-09**	1,51E-01
TDN	**1,20E-07**	**2,06E-30**	**1,52E-05**
guaiacol	2,98E-01	**6,71E-25**	**7,99E-04**
o-cresol	1,86E-01	**3,98E-11**	8,84E-01
eugenol	**1,68E-07**	**3,93E-05**	**4,43E-02**
4-ethylphenol	**4,41E-03**	**1,21E-17**	1,77E-01
4-vinylguaiacol	**2,88E-22**	**3,80E-18**	**1,08E-23**
syringol	**1,18E-03**	**8,77E-36**	**3,34E-03**
isoeugenol	**6,94E-07**	**1,01E-25**	**8,71E-05**
4-vinylphenol	**7,69E-25**	**2,35E-17**	**3,49E-20**
methoxyeugenol	**2,05E-07**	**1,14E-22**	1,12E-01
R-limonene	6,30E-01	**1,02E-21**	2,98E-01
1,8-cineole	9,59E-02	**1,59E-20**	**2,25E-03**
rose oxide	**1,12E-23**	**2,46E-07**	2,26E-01
linalool oxide	**1,94E-12**	**4,13E-36**	**1,26E-07**
dihydromyrcenol	2,20E-01	**2,70E-16**	8,15E-01
linalool	**1,40E-04**	**8,12E-35**	**2,93E-06**
α-terpineol	**5,69E-18**	**1,08E-43**	**6,31E-11**
β-citronellol	**1,78E-15**	**4,18E-15**	**4,93E-03**
nerol	**3,17E-08**	**6,11E-18**	**4,96E-10**
geraniol	**2,68E-17**	**5,07E-09**	**6,11E-09**
vanillin	**4,95E-03**	**1,66E-12**	8,99E-01
acetovanillone	9,57E-01	**3,57E-02**	8,10E-01
syringaldehyde	3,90E-01	**3,00E-11**	5,07E-01

Table E.7: PFMs concentration in μg/L in wines recently fermented with 4 *Saccharomyces* strains. In the case of a significant effect of the factor yeast, letters of Tukey's HSD test results were added. n.d.: not detected or below the detection limits. *indicates that only two replicates were considered for statistical treatment. n.d.: not detected or below the detection limits.

	wine recently fermented			
	CR89D1	**QA23***	**RHONE**	**SAUVY**
MHA	0.47 ± 0.02 b	0.52 ± 0.01 b	0.39 ± 0.02 b	1.1 ± 0.1 a
BM	n.d. b	n.d. b	0.0014 ± 0.0001 a	n.d. b
FFT	n.d. b	n.d. b	0.14 ± 0.02 a	n.d. b
MH	0.79 ± 0.02 b	0.289 ± 0.007 b	3.6 ± 0.5 a	0.35 ± 0.04 b
MP	0.0135 ± 0.0005 a	0.0020 ± 0.0008 c	0.0192 ± 0.0002 c	0.00506 ± 0.0003 b

Table E.8: PFMs concentration in μg/L in wines fermented with 4 *Saccharomyces* strains and submitted to accelerated aging for 96 hours at 75 ^{o}C. In the case of a significant effect of the factor yeast, letters of Tukey's HSD test results were added. n.d.: not detected or below the detection limits. *indicates that only two replicates were considered for statistical treatment. n.d.: not detected or below the detection limits.

	96 h at 75 ^{o}C			
	CR89D1	**QA23**	**RHONE**	**SAUVY***
MHA	0.28 ± 0.01 c	0.42 ± 0.03 b	0.42 ± 0.02 b	0.59 ± 0.07 a
BM	0.0012 ± 0.0003 ab	0.0014 ± 0.0001 a	0.0014 ± 0.0001 a	0.0008 ± 0.0001 b
FFT	0.069 ± 0.003 b	0.24 ± 0.04 a	0.18 ± 0.03 a	0.0325 ± 0.0009 b
MH	5.8 ± 0.2 a	4.2 ± 0.4 b	4.2 ± 0.6 b	4.898 ± 0.001 ab
MP	0.0094 ± 0.0005 a	0.0016 ± 0.0002 c	0.0018 ± 0.0002 c	0.0040 ± 0.0003 b

Table E.9: Significance of the factors yeast, time and their interaction on the modulation of PFMs concentrations (p-value < 0.05 in bold).

	p-value		
	yeast	**time**	**yeast*time**
MHA	**1,38E-10**	**4,62E-07**	**3,40E-06**
BM	**2,17E-08**	**6,13E-10**	**2,81E-06**
FFT	**2,68E-08**	**1,35E-07**	**8,40E-06**
MH	**4,94E-06**	**1,40E-12**	**8,54E-08**
MP	**3,60E-15**	**3,32E-06**	**3,67E-06**

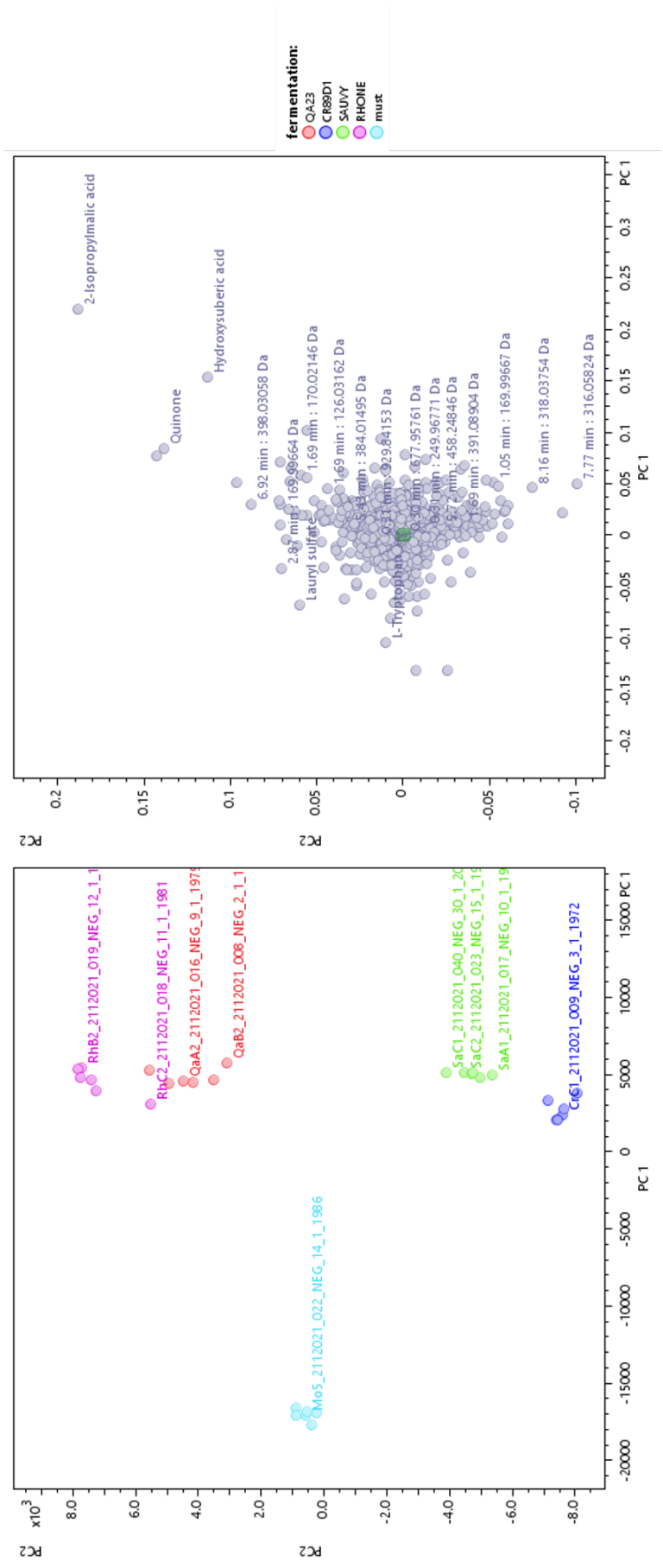

Figure E.1: Loading plot provided by Metaboscape representing samples (on the left) and buckets (on the right) distribution detected in negative mode, in the must and wines recently fermented by 4 *Saccharomyces* strains.

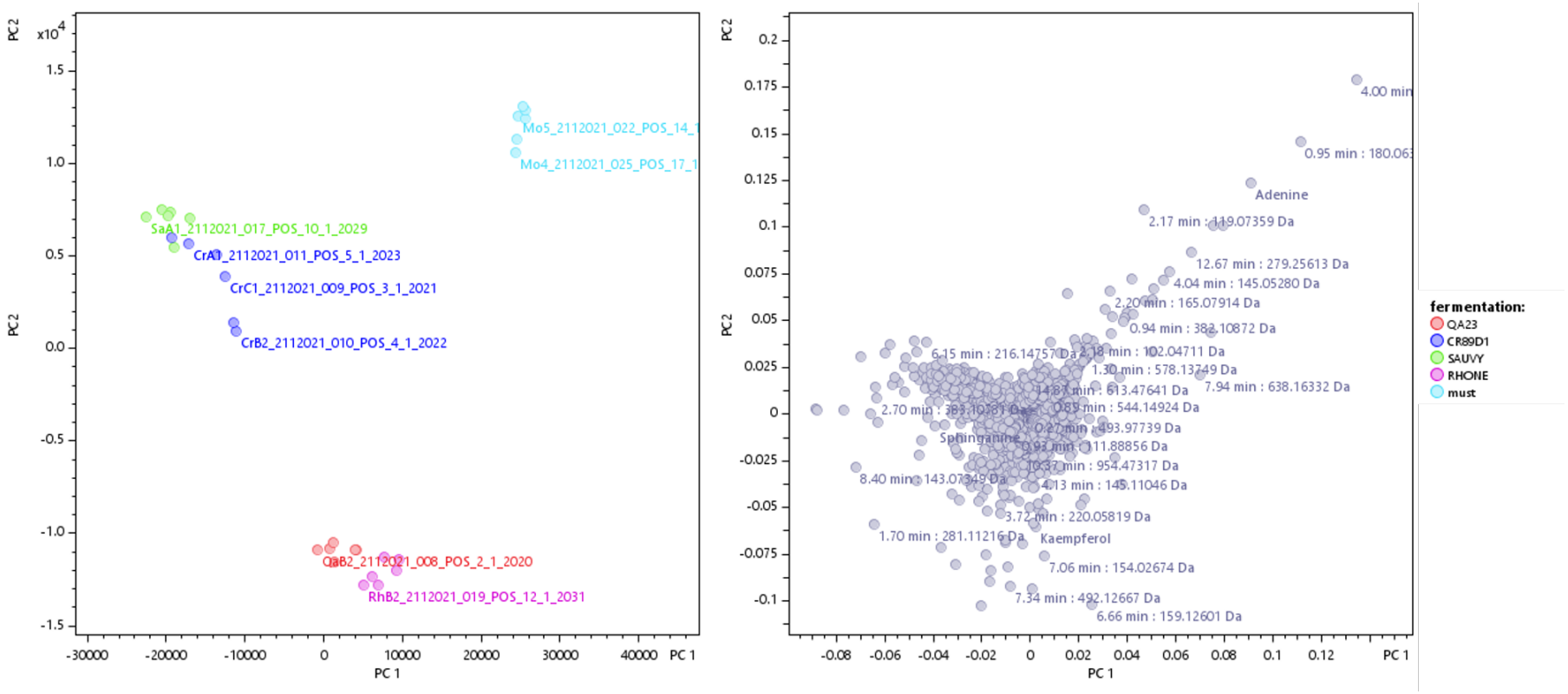

Figure E.2: Loading plot provided by Metaboscape representing samples (on the left) and buckets (on the right) distribution detected in positive mode, in the must and wines recently fermented by 4 *Saccharomyces* strains.

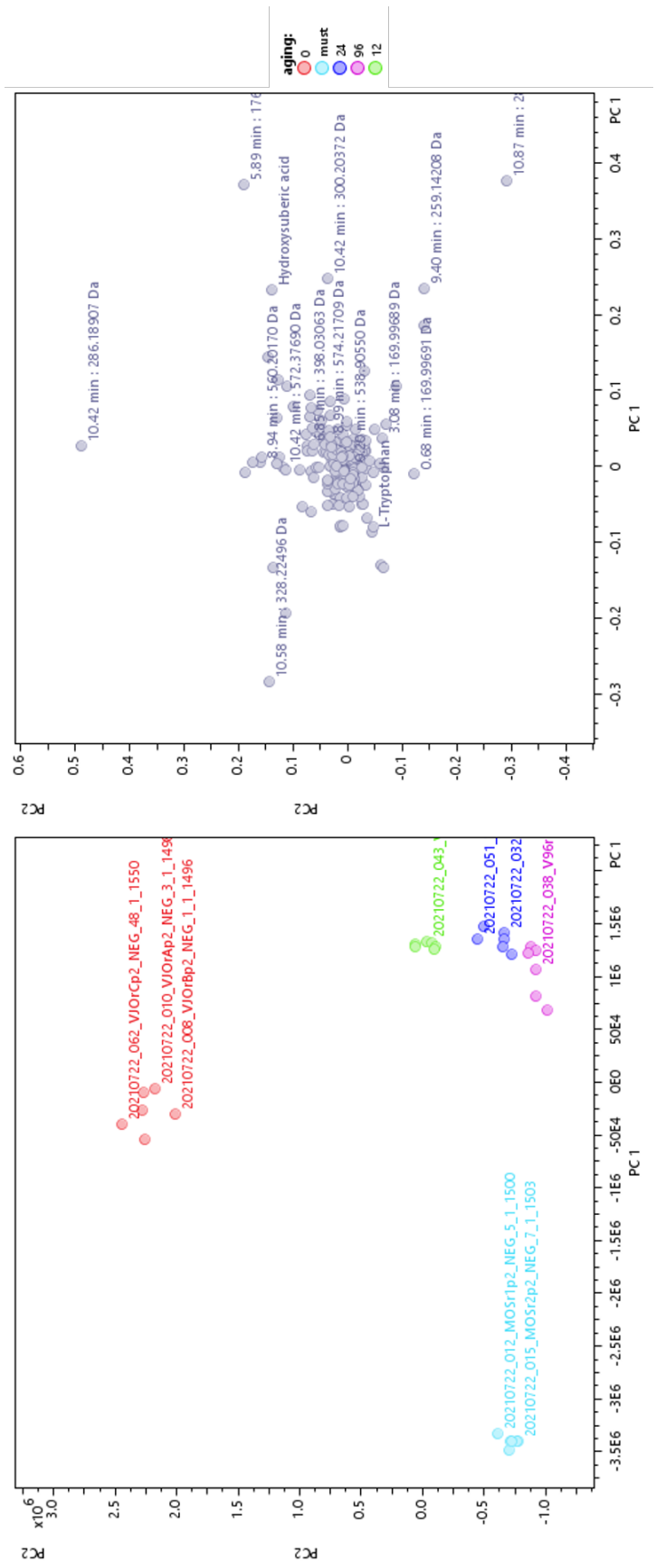

Figure E.3: Loading plot provided by Metaboscape representing samples (on the left) and buckets (on the right) distribution detected in negative mode, in the must and wines fermented with QA23 and submitted to accelerated anoxic aging at 75 ºC.