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

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ABSTRACT

Semi-synthetic must containing standard nutrients, a phenolic and aromatic fraction extracted from Albariño grapes and synthetic precursors of 3-mercaptohexanol (MOH) and 4-mercapto-4-pentan-2-one (MMP) were fermented with three different selected commercial *S. cerevisiae* yeasts. Wines were subjected to anoxic aging at 50 °C for 1, 2, 5 and 8 weeks, and their volatile composition was comprehensively determined by the analysis of 86 different aroma compounds using five different GC methods. Yeasts exert a strong influence on wine aroma throughout the whole aging period. Their effects extend beyond the well-known actions on yeast secondary metabolites, including the formation of little amounts of Strecker aldehydes or the formation of acids precursors of fruity esters, and on the enzymatic actions on the different grape aroma precursors. Additionally, yeasts influence wine aroma; first, by producing SO_2 which reacts with β-damascenone and increases Strecker aldehyde production in fermentation; second, by inducing the differential accumulation of Strecker aldehydes during anoxic aging; and third, by determining the stability of MOH, likely because of its reaction with vinylphenols or other yeast-related electrophiles.

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 & 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, Bartowsky, Henschke, & Pretorius, 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, Ortiz-Julien, & Dequin, 2014). There is still an active research for strains minimizing the formation of hydrogen sulfide (Agarbati, Canonico, Comitini, & Ciani, 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, Hernández-Orte, Querol, & Ferreira, 2011; Lambrechts & Pretorius, 2000; Loscos, Hernandez-Orte, Cacho, & Ferreira, 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, Schneider, Razungles, & Cavelier, 2011; Swiegers & Pretorius, 2007) and particularly, for being able to transform 3-mercaptohexanol (MOH) 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, Julien, Rigou, Schneider, & Salmon, 2015; Ugliano, Rinaldi, Gambuti, & Moio, 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

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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 & 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, Straws, & Wilson, 1980). Additionally, recent research has also demonstrated that yeasts can modulate some aroma molecules formed only after long periods of aging such guaiacol (Denat, Pérez, Heras, Querol, & Ferreira, 2021; Oliveira & 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 constitute the backbone of the fruity perception in aged wines and for which the impact of yeast strain is already known (Gammacurta, Marchand, Albertin, Moine, & Revel, 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, Daran, Van Maris, Pronk, & Dickinson, 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., 2021). Additionally, Strecker aldehydes can be formed through the reaction between the amino acid precursor and different α-dicarbonyls (De Revel, Pripis-Nicolau, Barbe, & Bertrand, 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, Francis, Swiegers, & Curtin, 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 (Remy Romanet et al., 2019), mostly proteins and peptides (Rémy 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, Magiatis, & Waterhouse, 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.

2. Material and methods

2.1. Semi-synthetic must preparation

2.1.1. Mistelle and PAF extraction

Mistelle was prepared in the Instituto de las Ciencias de la Vid y el Vino (ICVV, Logroño, Spain) according to the method proposed by Alegre, Arias-Pérez, Hernández-Orte, and Ferreira (2020), adapted for white grapes. Fifteen kilograms of frozen Albariño grapes from Galicia (Spain) were destemmed and gently crushed. Potassium metabisulfite (0.05 g per kg of must) and ethanol (15% p/p) were added. Alcoholized Albariño must was divided into 5-L plastic jars. Headspace was removed by manual pressure and jars were hermetically closed with plastic screw caps. Maceration took place at 15 °C during 5 h, regularly homogenizing the jars manually. The mixture was then pressed using a hydraulic press and the liquid part was left in decantation for 1 week at 4 °C. The 12 L of mistelle obtained were bottled in 0.75-L green glass bottles, closed with corks (Diam, France). Then, 750 mL of mistelle were dealcoholized in a rotary evaporator (23 °C, 20 mbar) to less than 2% (v/v) of ethanol, and were further percolated through the pre-conditioned solid phase extraction cartridge (Sep Pak C18, 10 g). Phenolic and aromatic fractions (PAF) were obtained by elution with 100 mL of ethanol after previous cleaning and drying. The 1.6 L of PAF obtained from the 15 Kg of grapes were homogenized and stored together into hermetic 0.25-L glass containers closed with plastic screw caps and kept in the dark at −20 °C after oxygen removal with a flux of nitrogen.

2.1.2. Semi-synthetic must preparation

Semi-synthetic must, adapted from Hernandez-Orte, Bely, Cacho, and Ferreira (2006) was prepared with 105 g/L glucose, 105 g/L fructose, 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 $CaCl_2$), 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 (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). Synthetic glutathionylated (Glu) and cysteinilated (Cys) precursors of MOH and 4-mercapto-4-methylpentan-2-one (MMP) were added from a solution in MilliQ water, (0.1 mg/L Cys-MOH, 0.05 mg/L Cys-MMP, 1 mg/L Glu-MOH, 0.05 mg/L Glu-MMP). After pH adjustment to 3.5, semi-synthetic must was sterilized by filtration (0.45 μm) inside a vertical laminar flow chamber. Albariño PAF was dealcoholized, resuspended in sterile distilled water and added to semi-synthetic must at 10% (v/v).

2.2. Winemaking

The three *S. cerevisiae* yeast strains were Lalvin QA23™, Lalvin Sauvy™ and Affinity ECA5™ active dry yeast (ADY) from Lallemand Bio (Barcelona, Spain). ADY were rehydrated in 10 times their weigh of sterile distilled water and maintained in a 30 °C water bath during 20 min under agitation. They were dosed at 30 g/hL.

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 °C. Fermentations were monitored by daily weighing, and yeast cell viability was monitored by

plating diluted fermenting must on YPD solid media (2% glucose, 2% agar, 0.5% peptone, 0.5% yeast extract). At the end of fermentation, when the weight loss between two consecutive days was smaller than 0.1 g, wines were decanted during 24 h at 4 °C and then centrifuged at 10 °C, 4500 rpm during 10 min. Wines were bottled in 0.75-L green glass bottles, closed with a wine stopper (Vacu Vin, Spain) after displacement of air with a flux of nitrogen and stored at 8 °C up to their conditioning for aging.

Wines were conditioned for aging into a free-O_2 chamber Jacomex (Dagneux, France). Samples were placed into 18-mL glass tubes with metallic screw cap and bagged in high density plastic bags containing oxygen scavengers AnaeroGenTM (Thermo Scientific, USA). Samples were incubated at 50 °C during 1, 2, 5 and 8 weeks, and were then stored at 8 °C up to their analysis.

2.3. Wine analysis

2.3.1. Conventional oenological analysis

In the recently fermented wines, oenological parameters were measured using the analytical methods recommended by OIV (International Organisation of Vine and Wine, 2021) including reducing sugars, ethanol, pH, volatile and total acidity.

2.3.2. Free and total sulfur dioxide

Free and total SO_2 were analyzed following the procedure validated by Carrascon, Ontañón, Bueno, and Ferreira (2017) with some modifications. The internal standard (IS) was ethyl methyl sulfide (1 ppm). Analyses were carried out in an Agilent 7890B gas chromatograph (GC) with a sulfur chemiluminescent detector (SCD) 8355. The chromatographic system was identical to the one described in Ontañón, Vela, Hernández-Orte, and Ferreira (2019) without cryofocusing. The incubation temperature was set at 40 °C and 70 °C for free and total SO_2 respectively during 15 min. Injections of 400 and 200 μL of the sample headspace were carried out with a 1-mL syringe at 50 and 80 °C, with a split flow of 40 and 200 mL/min for free and total SO_2, respectively. Quantifications were based on a response factor calculated by the analysis of a synthetic wine with known amounts of SO_2.

2.3.3. Major compounds

Major secondary 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 a concentration above 0.2 mg/L, were analyzed following the method developed by Ortega, López, Cacho, and Ferreira (2001). Briefly, 3 mL of sample spiked with the IS solution (2-octanol, 4-methyl-2-pentanol, ethyl heptanoate and heptanoic acid, 30 mg/L each) were diluted with 7 mL of Milli-Q water, added with 4.1 g of ammonium sulfate, and extracted with 0.25 mL of dichloromethane (DCM) for 90 min of horizontal agitation. After centrifugation at 2500 rpm for 10 min, the organic phase was recovered with a syringe. Extracts were analyzed by GC-flame ionization detection (FID). Quantifications were carried out by using response factors calculated with the analyses of synthetic wines containing known amounts of analytes.

2.3.4. Trace compounds

Minor secondary metabolites of alcoholic fermentation (branched ethyl esters) and compounds related to specific precursors of grapes (terpenes, norisoprenoids, vanillin derivatives, volatile phenols), usually present in wine at concentrations around 0.1–200 μg/L, were analyzed following the protocol described by López, Aznar, Cacho, and Ferreira (2002) with the modifications of Oliveira and Ferreira (2019). Fifteen mL of sample, previously added with IS (2-octanol, 3-octanone and 3, 4-dimethoxyphenol, 250 μg/L each) were extracted by solid phase extraction (SPE) with 65 mg LiChrolut EN resin cartridges, previously conditioned with 2 mL of DCM, methanol and hydroalcoholic solution at 12% (v/v). The cartridge was further washed with 1.5 mL of a solution of water with 30% (v/v) methanol and 1% (m/v) of $NaHCO_3$, dried under vacuum and eluted with 0.6 mL of DCM containing 5% of methanol. The extracts were analyzed via GC-mass spectrometry (MS). Quantifications were based on the use of response factors calculated by the analysis of synthetic wines containing known amounts of the analytes.

2.3.5. Free and total aldehydes

Strecker aldehydes were quantified following the methods described and validated in Culleré, Cacho, and Ferreira (2004) and Ferreira, Culleré, Loscos, and Cacho (2006) with some modifications. Volatile compounds were purchased from Aldrich (Madrid, Spain) and PFBHA from Fluka (Madrid, Spain).

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 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 from Eptes Sarl, (Switzerland), 200 μg/L each) and sealed. The sealed vials were then taken out and incubated at 50 °C for 6 h to ensure equilibration. After this, 360 μL of a 10 g/L PFBHA solution were added and the reaction allowed to develop at 35 °C for 12 h. 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. Both free and total aldehydes were analyzed by GC-MS. Concentrations were obtained by using response factors calculated by the analysis of table wines spiked with known amounts of analytes.

2.3.6. PFMs

PFMs were determined by GC-GC-MS using negative chemical ionization (NCI) after derivatization with pentafluorobenzyl bromide (PFBBr), as described by Mateo-Vivaracho, Zapata, Cacho, and Ferreira (2010). The IS used for quantification were the deuterated analytes obtained from Eptes Sarl, (Switzerland). 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 (MOH-d5 at 700 ppt in wine, MHA-d5 at 200 ppt, MMP-d10 at 100 ppt, FFT-d2 at 70 ppt, BM-d5 at 40 ppt) were spiked and pure *O*-methylhydroxylamine was added to form the oximes of MMP. This oximation was performed at 55 °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 600 μL

of hexane 25% (v/v) in diethyl ether. After washing with brine and drying with anhydrous sodium sulfate, extracts were injected into the GC-GC-MS (NCI) system. Concentrations were obtained by using a response factor calculated by the analysis of table wines spiked with known amounts of the analytes.

2.4. Statistical analysis

The significance of the factors yeast and aging time were determined via 2-way ANOVA on the data collected after aging at 50 °C. One-way ANOVA and Tukey HSD test were also performed at each of the 5 sampling points independently (t0, t1-50 °C, t2-50 °C, t3-50 °C, t4-50 °C) in order to determine the specific differences between the yeasts. Those tests were realized using R software (v3.5.0), (R Core Team, 2020) via *anova* function from *car* package (v3.0.2) and *HSD.test* from *agricolae* package (v1.3.1) applied on *aov* function results. Principal Component Analysis (PCA) were performed and plotted using *factoextra* package (v1.0.5).

3. Results and discussion

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 °C for 5 different times. The complete set of results is given in the Supplementary material (Table S1). 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 S2).

The combined effects of yeast and time can be visualized in the PCA plot shown in Fig. 1. In order to simplify the variable biplot, only the compounds significantly affected by yeasts and/or aging time were conserved (Table S2). 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., 2021), 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., 2021; Oliveira & Ferreira, 2019) and/or with a more likely impact on wine sensory properties.

3.1. 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 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 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 Fig. 2a 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.

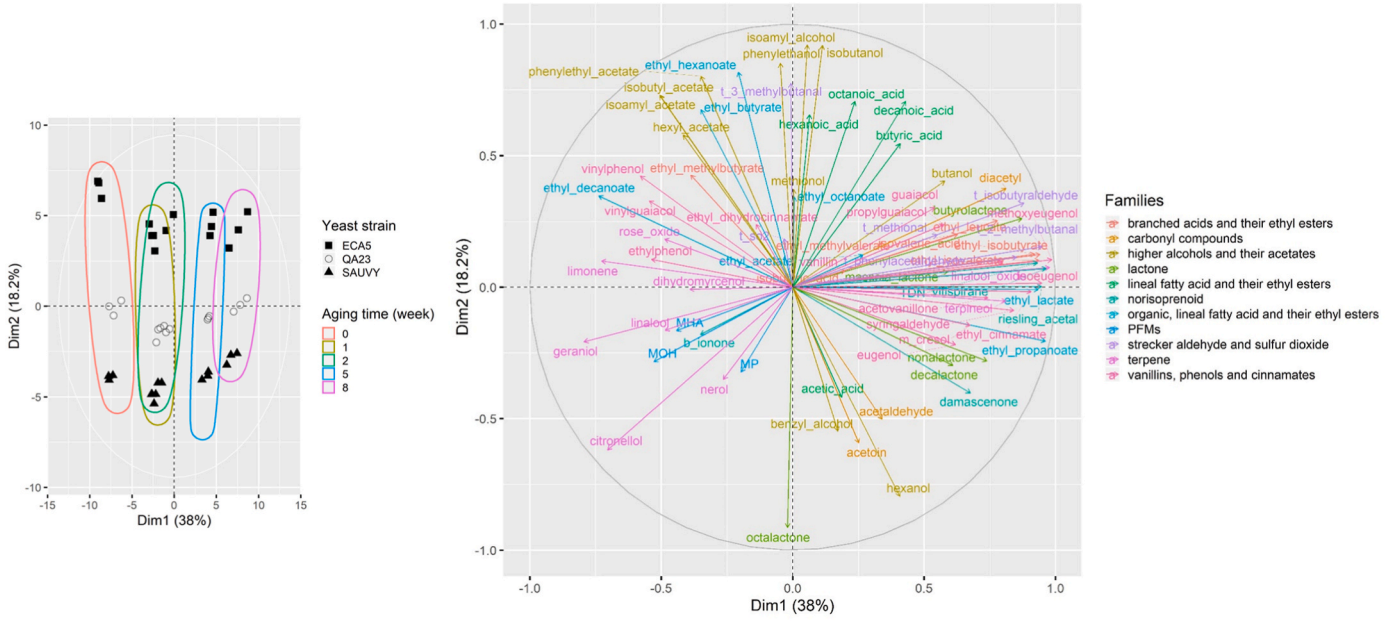

Fig. 1. PCA obtained with the 77 volatiles significantly influenced by yeast or time in the 45 samples (15 x 3 replicates) fermented by three *S. cerevisiae* strains and aged at 50 °C 5 different times.

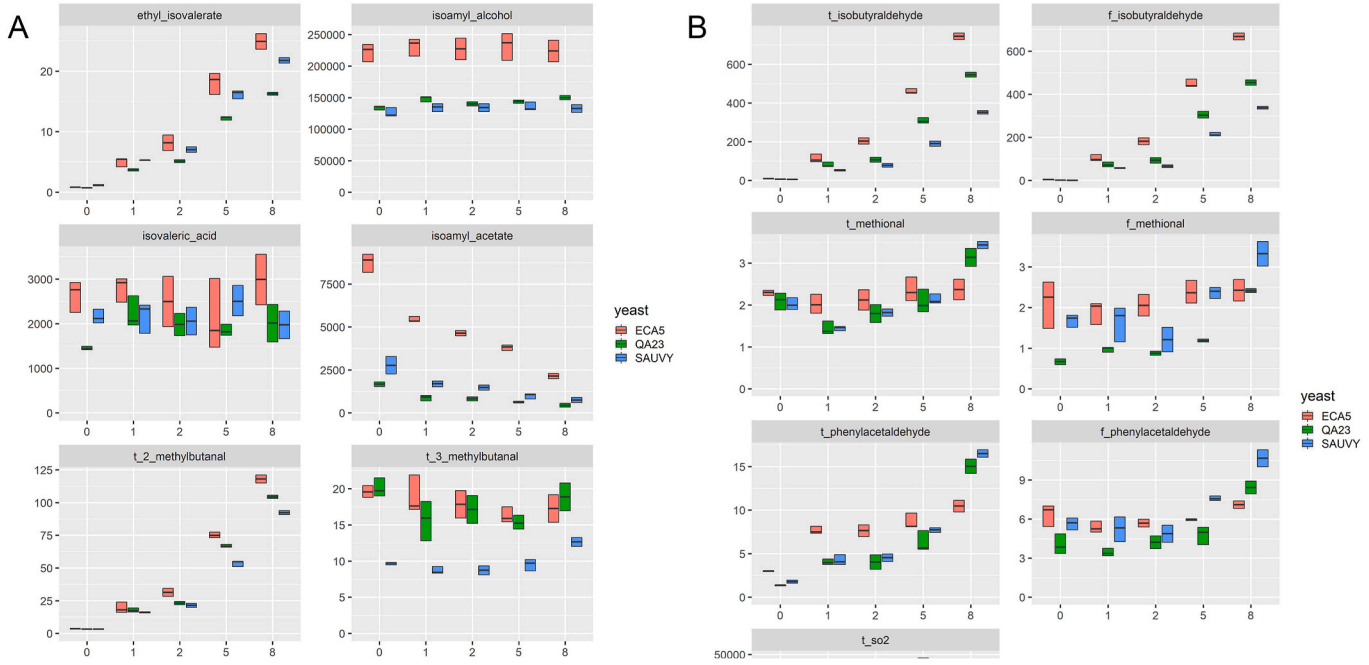

Fig. 2. a and b: Boxplots representing the evolution during aging of the levels of some compounds derived from Ehrlich pathway and SO_2. Y-axis in all cases is concentration (in μg/L) and X-axis is time of aging (in weeks).

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).

3.2. 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., 2021) 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 $p < 0.01$), for isobutyraldehyde/isobutanol, $R = 0.78$ (significant at $p < 0.05$), for methional/methionol, $R = 0.88$ (significant at $p < 0.05$) and for isobutyraldehyde/isobutyric acid, $R = 0.81$ (significant at $p < 0.05$). 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 & 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 contribution to the formation of Strecker aldehydes is the strong increases with aging of 2-methylbutanal (Fig. 2a) and isobutyraldehyde (Fig. 2b), the moderate increase of phenylacetaldehyde and the modest but significant increase of methional, also shown in Fig. 2b. 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 (Table S1) 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 °C has been recently demonstrated (Monforte, Martins, & Silva Ferreira, 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 (Fig. 2a) and isobutyraldehyde (2b) but not of methional and phenylacetaldehyde (Fig. 2b), and considering also that levels formed of isobutyraldehyde and 2-methylbutanal are correlated to levels of isobutanol and isoamyl alcohol ($p < 0.05$), 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 demonstrates that oxidation is not necessarily required to form Strecker aldehydes during wine aging, corroborating recent observations in model wines at 80 °C (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 affects 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, Zapata, & Ferreira, 2014). This can be seen in Fig. 2b 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.

3.3. Acid/alcohol/ester systems

Fig. 3 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, Franc, Cameleyre, & Barbe, 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., 2021) and of course for aged Bordeaux red wines, where it is involved into the perception of fresh blackberry notes (Falcao, Lytra, Darriet, & Barbe, 2012). This compound integrates within the other fruity ethyl esters in the fruity vector (Ferreira, De-la-Fuente-Blanco, & Sáenz-Navajas, 2021). 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 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

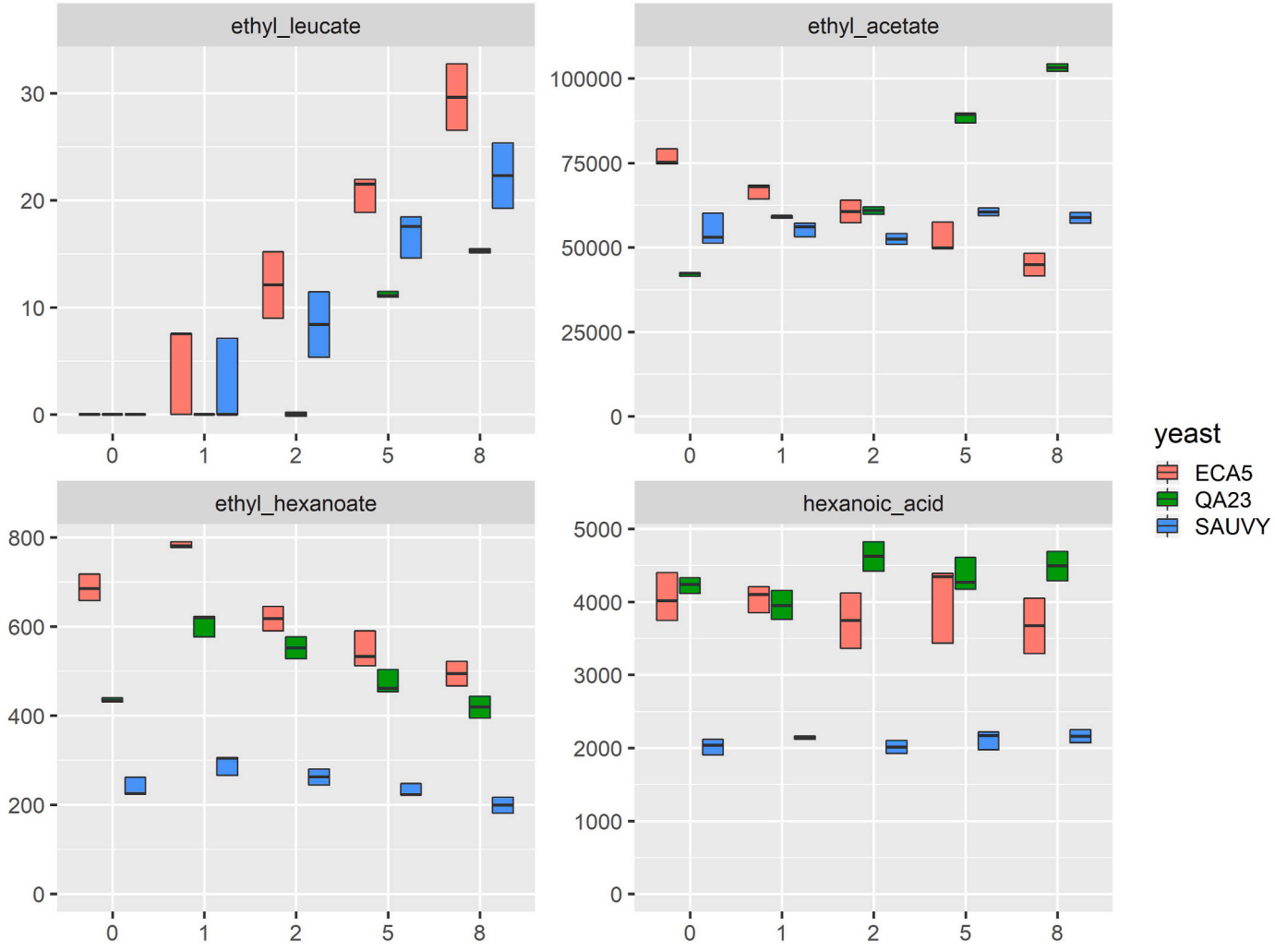

Fig. 3. Boxplots of a little selection of esters and acids formed in fermentation and representing different forms of yeast influence during aging. Y-axis in all cases is concentration (in μg/L) and X-axis is time of aging (in weeks).

minimal. However, as levels of acetic acid produced by QA23 in young wines were nearly 4 times higher than those produced by ECA5 (Table S1), 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.

3.4. 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 Fig. 4. 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., 2021; 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 SO_2, with which damascenone is known to react (Daniel, Elsey, Capone, Perkins, & Sefton, 2004; Sefton, Skouroumounis, Elsey, & Taylor, 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. The most stable α-terpineol and linalool oxide are weaker aromatically and accumulate during aging. In the present case, only linalool reached levels close to threshold after 1 or 2 weeks of aging, but 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 (Fig. 4). Transformation of these compounds catalyzed by yeasts have been well studied, demonstrating that yeasts not only liberate volatiles via glucosidase activity but could also modify the precursor or the volatile itself via reductase, oxidase, hydroxylase and acetyltransferase activity (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 (Table S1).

3.5. Vinylphenols

Fig. 4 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, Dubourdieu, Boidron, & Lavigne, 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, Zuker, Zehavi, & Rouseff, 1993; Turner, Mantick, & Carlson, 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 (Fig. 2b). 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., 2021). In Riesling and Garnacha semi-synthetic musts, similar tendencies were observed, with a reduced variability within yeasts along accelerated aging (I. Oliveira & 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.

3.6. Polyfunctional mercaptans

Polyfunctional mercaptans were produced from precursors spiked into the semi-synthetic must. As can be seen in Fig. 4, the levels of MOH 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 highest levels of this labile compound throughout the whole aging period. Levels in excess to 0.4 μg/L, a 70% increase, which should have a major sensory effect. Although SAUVY produced slightly more MHA which quickly hydrolyzed to MOH, 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 MOH are at least in part, related to vinylphenols. Other alternative reactive electrophiles could be α-dicarbonyls produced in fermentation (LoPachin & Gavin, 2014), such as glyoxal or methylglyoxal (Zeng & 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, Chichuc, Teissedre, & Darriet, 2010), and particularly of the most reactive MOH (Nikolantonaki et al., 2012), extends to its stability during aging. Further research to elucidate the causes of this result is required.

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 polyfunctional mercaptans, 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

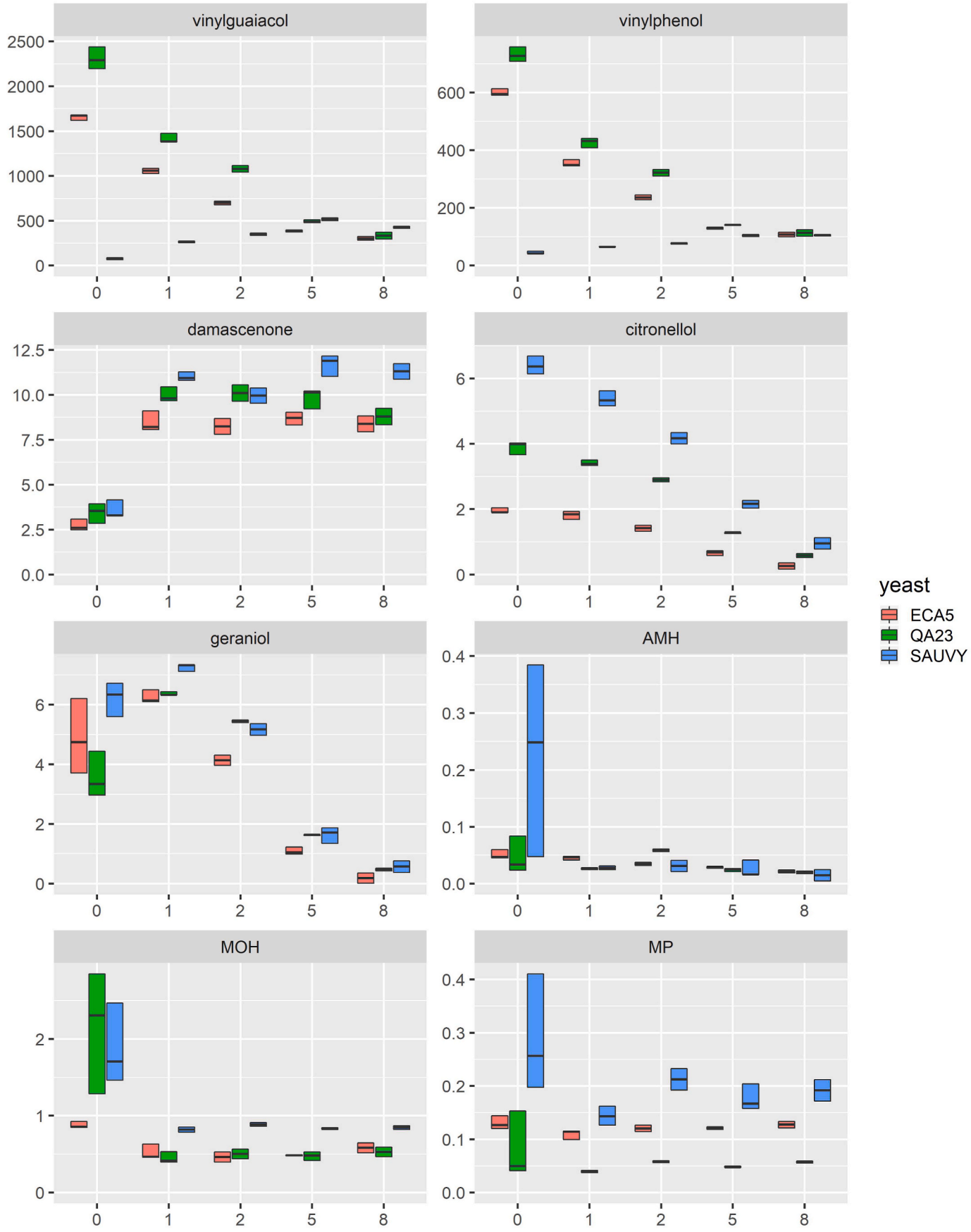

Fig. 4. Boxplots representing the evolution of some varietal compounds such as polyfunctional mercaptans, norisoprenoids, terpenes and volatile phenols. Y-axis in all cases is concentration (in μg/L) and X-axis is time of aging (in weeks).

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 3-mercaptohexanol.

Author declaration template

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

CRediT authorship contribution statement

M. Denat: has carried out physically the laboratory work, including all the sample preparation, fermentation and most of the analysis, She has also compiled all the quantitative data, carried out the data treatment and has also written out the first draft. **I. Ontañón:** has supervised all operations related to the quantitative analysis, ensuring that the instruments were in optimal conditions, adjusting and tuning spectrometers, and providing assistance with the different software and calibration solutions. He has also corrected the draft. **A. Querol:** has designed the microbiological part of the experiment and has directly supervised all fermentations, She has also participated in the discussion of results. **V. Ferreira:** has designed and supervised the whole experiment, has leaded data discussion and has finished and corrected the final draft of the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113111.

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