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Title: Factors influencing the production of the antioxidant hydroxytyrosol during alcoholic fermentation: yeast strain, initial tyrosine concentration and initial must

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Abbreviations: AF alcoholic fermentation; HT hydroxytyrosol; NCE Normalized Collision Energy; RF Red Fruit; SM synthetic must; OD optical density; PRM Parallel Reaction Monitoring; YAN Yeast Assimilable Nitrogen; YPD yeast extract peptone dextrose

Abstract

Hydroxytyrosol is well known for its potent antioxidant activity and anticarcinogenic, antimicrobial, cardioprotective and neuroprotective properties. Main food sources are olive oil (formed from the hydrolysis of oleuropein) and wine. One possible explanation to its origin in wines is the synthesis from tyrosol, which in turn is produced from the Ehrlich pathway by yeasts. This work aims to explore the factors that could increase the content as the strain of yeast, the initial tyrosine concentrations as precursor and the effect of synthetic and sterilized natural grape musts.

Alcoholic fermentations in synthetic must showed that hydroxytyrosol is produced by all the yeast strains under study. Commercial *Saccharomyces cerevisiae* yeasts were those which produced higher concentrations, being the Red Fruit strain the biggest producer (6.12 ng/mL). Once the strain was selected, alcoholic fermentations were performed in synthetic must, with different tyrosine concentrations. The amount of hydroxytyrosol did not increase in a proportional way as tyrosine does. On the other hand, higher concentrations of hydroxytyrosol were obtained in natural grape musts (10.46 ng/mL) than in synthetic must (4.03 ng/mL). This work confirms the capacity of winemaking yeasts to produce the bioactive hydroxytyrosol.

Keywords: bioactive, tyrosine, *S. cerevisiae*, wine, alcoholic fermentation

1. Introduction

Hydroxytyrosol (HT) 2-(3,4-Dihydroxyphenyl) ethanol is a phenylethylalcohol known for its bioactivity (Fernández-Mar, Mateos, García-Parrilla, Puertas, & Cantos-Villar, 2012). Other biological properties that have been described are: anticarcinogenic, antimicrobial, cardioprotective (Yuri, Silvia, Giacomo, & Massimo, 2012) and neuroprotective (Hornedo-Ortega et al., 2018). Specifically, in relation to the cardioprotection, it has antithrombotic and anti-inflammatory properties (Robles-Almazan et al., 2018). In addition, HT avoids the oxidation of LDL particles, it can decrease total cholesterol and it can also increase HDL cholesterol (Robles-Almazan et al., 2018). Additionally, it presents a high bioavailability and degree of absorption (Echeverría, Ortiz, Valenzuela, & Videla, 2017). Its degree of absorption is around 99% in oil and 75% in aqueous solution since it depends on the delivery vehicle (Vilaplana-Pérez, Auñón, García-Flores, & Gil-Izquierdo, 2014).

Extra Virgin olive oil is the main source of HT in the diet (Fernández-Mar et al., 2012). The concentration of HT in extra virgin olive oil ranges between 50 mg/kg and 800 mg/kg (Carluccio et al., 2003). It is formed during the hydrolysis of oleuropein which is present in olives (Robles-Almazan et al., 2018). The content of HT in olive oil depends on certain factors such as the variety of olive, the cultivar and origin and most importantly, the olive oil elaboration process that results in the oil quality (Robles-Almazan et al., 2018). Furthermore, it has been described in fermented beverages such as wine, although the concentrations are lower (Piñeiro, Cantos-Villar, Palma, & Puertas, 2011; Fernadez-Mar et al., 2012). The concentration of HT in wine is approximately higher than 5 mg/L (Echeverría et al., 2017). Both red and white wines contain HT (Romboli, Mangani, Buscioni, Granchi, & Vincenzini, 2015). Some of the concentrations found in wines were: Tempranillo 1.84±1.56 mg/L; Petit Verdot 4.12±0.63 mg/L and Syrah 1.11±2.43 mg/L (Piñeiro et al., 2011); Blend 1.3±0.9 mg/kg^2; Chardonnay 2.3±1.3 mg/kg^2 and Fiano 1.1±0.3 mg/kg^2 (Ragusa et al., 2017).

It is well known that tyrosol is produced by yeasts during alcoholic fermentation (AF) (Romboli et al., 2015) through the Ehrlich pathway, which includes the transamination of tyrosine, the decarboxylation of p-hydroxyphenylpyruvate and the reduction of p-hydroxyphenylaldehyde (Mas et al., 2014). Some factors that have been reported to influence the AF are the pH, the temperature and the nature and composition of the medium. Specifically, they can affect the rate of fermentation and the production of metabolites (Vilela, 2019). Moreover, our group have recently proved that certain winemaking yeasts synthetize HT (M. Antonia Álvarez-Fernández, Fernández-Cruz, Cantos-Villar, Troncoso, & García-Parrilla, 2018) as it was unequivocally determined both in the extracellular and intracellular compartments.

The purpose of this work is to explore the production of HT by a wider set of different winemaking yeast strains, in the extracellular and intracellular compartments, in order to select the one with a higher production capacity. Since there are a wide range of yeast strains used for enology purpose, the main novelty of the present work is the demonstration, for the first time, that certain yeast strains *S. cerevisiae* and non *S. cerevisiae*, commercial and autochthonous yeasts (*S. cerevisiae* Enartis Ferm ES488, *S. cerevisiae* Lalvin ICV GRE, *S. cerevisiae* Uvaferm, *S. cerevisiae* Yero 2.23, *S. cerevisiae* Yero 2.24, and *Metschnikowia pulcherrima* Flavia MP 346) also produce HT. Additionally, there is a great interest on increasing the concentration of these bioactive by means of the conditions on the fermentation process. Since tyrosine is the precursor of HT, its initial concentration could be a key point. Therefore, once the most productive yeast strain is selected, we aim to evaluate if different initial tyrosine concentrations have an effect on the production of HT. As tyrosine is the aromatic amino acid precursor of HT, the hypothesis of the present work is the more content of tyrosine in must, the more production of HT. The last objective is to explore the

capacity of the selected yeast to produce HT in natural grape must to verify if the results are reproducible from synthetic to natural grape musts.

2. Materials and methods

2.1 Yeast strains

Eight different commercial wine yeast strains, including *S. cerevisiae* and non *S. cerevisiae*, were used in the fermentation of synthetic must (SM): *S. cerevisiae* Enartis Ferm Aroma White (Enartis), *S. cerevisiae* Enartis Ferm ES488 (Enartis), *S. cerevisiae* Lalvin ICV GRE (Lallemand), *S. cerevisiae* Lalvin YSEO QA23 (Lallemand), *S. cerevisiae* Enartis Ferm Red Fruit (RF) (Enartis), *S. cerevisiae* Uvaferm (Lallemand), *Torulaspora delbrueckii* Biodiva (Lallemand) and *Metschnikowia pulcherrima* Flavia MP 346 (Lallemand). Two *S. cerevisiae* autochthonous yeasts, isolated from the experimental vineyard of the Rancho de la Merced (IFAPA) in Jerez de la Frontera in 2016, were also tested: *S. cerevisiae* Yero 2.23 and *S. cerevisiae* Yero 2.24.

2.2 Synthetic Must

SM was used in two experiments: the first one (A) aimed to screen the strain with higher capacity to produce HT. The second experiment (B) was to test how different tyrosine concentrations could affect the production of HT.

SM used for experiment A was based on Riou, Nicaud, Barre & Gaillardin (1997), slightly modified. 3 L of SM were prepared, with the following composition: sugars (fructose 100 g/L and glucose 100 g/L), acids (malic acid 5 g/L, citric acid 0.5 g/L and tartaric acid 3 g/L), minerals (KH_2PO_4 0.75 g/L, K_2SO_4 0.5 g/L, $MgSO_4.7H_2O$ 0.25 g/L, NaCl 0.2 g/L and $CaCl_2$ 0.155 g/L), 0.46 g/L of NH_4Cl, 1 mL of trace elements, 13.09 mL of an amino acids solution (tyrosine 1.5 g/L, tryptophan 13.4 g/L, isoleucine 2.5 g/L, aspartic acid 3.4 g/L, glutamic acid 9.2 g/L, arginine 28.3 g/L, leucine 3.7 g/L, threonine 5.8 g/L, glycine 1.4 g/L, glutamine 38.4

115 g/L, alanine 11.2 g/L, valine 3.4 g/L, methionine 2.4 g/L, phenylalanine 2.9 g/L, serine 6 g/L,
116 histidine 2.6 g/L, lysine 1.3 g/L, cysteine 1.6 g/L and proline 46.1 g/L) and 10 mL of a
117 vitamins solution (myoinositol 2 g/L, calcium pantothenate 0.15 g/L, thyamine hydrochloride
118 0.025 g/L, nicotinic acid 0.2 g/L, pyridoxine 0.025 g/L and biotin 3 mL). The pH was
119 adjusted to 3.31 with NaOH.
120 5 L of two different SM were prepared for experiment B. In this case, tyrosine was the only
121 amino acid used as Yeast Assimilable Nitrogen (YAN) in order to force the yeast to use it. A
122 stock solution of this amino acid (1 g/L) was used to prepare the SM with a final tyrosine
123 concentration of 10 mg/L and 60 mg/L, respectively. The rest of the YAN was provided by
124 $(NH_4)_2SO_4$ to reach a final nitrogen concentration of 140 mg/L. The rest of the ingredients
125 such as sugars, acids, minerals and trace elements and vitamins solutions were identical as the
126 SM previously used for experiment A. The pH was adjusted to 3.25 with NaOH.

127 **2.3 Grape Must**

128 Palomino Fino and Chardonnay grape musts, belonged to the Rancho de la Merced (IFAPA)
129 in Jerez de la Frontera, Spain (longitude 06:00:58 W, latitude 36:45:29 N), were used in the
130 third part of the experimental process. Grapes were harvested, destemmed, crushed and
131 pressed. Subsequently, pectolytic enzymes (2.5 mL/hL Enartis ZYM; Enartis, Italy) and SO_2
132 (40 mg/L, Sulfosol, Sepsa-Enartis; Enartis) were added to the must for 24 h at 4°C. After that,
133 the dejuiced must was placed in a 100 L steel vessel.

134 **2.4 Inoculation**

135 Each yeast strain was rehydrated in a bath at 37 ºC for 30 min and plated on yeast extract
136 peptone dextrose (YPD) (1% yeast extract, 2% glucose and 2% peptone). For the third
137 experiment, the YPD was prepared with the difference of the addition of 2% of agar. Then,

they were incubated at 28ºC for 48 h. Subsequently, they were transferred into flasks with YPD to let the yeasts grow overnight before every experimental process.

For experiment A, flasks with 80 mL of SM were inoculated with 10^6 cells/mL and capped with plugs and syringes in order to release the carbon dioxide. AF were carried out in SM in triplicate. The fermentation was monitored by weighing the flasks daily, before and after sampling, from the first day of fermentation until a week, as well as the optical density (OD).

Regarding experiment B, flasks with 750 mL of SM at different tyrosine concentrations (10 mg/L, 60 mg/L) were inoculated in triplicate. The fermentation was monitored by weighing the flasks daily, before and after sampling, for 6 days and then at day 8 and 10 as well as the OD. Ethanol (Ethanol Assay Kit, Megazyme International, Ireland), residual sugars (D-Fructose and D-Glucose Assay Kit, Megazyme International, Ireland) and nitrogen (Primary Amino Nitrogen Assay Kit, Megazyme International, Ireland) were measured.

On the other hand, grape musts were sterilized at 121ºC for 20 min. Flasks with 750 mL of natural grape must were inoculated. AF were carried out in two natural musts (Palomino Fino and Chardonnay) in triplicate. The fermentation was monitored by weighing the flasks daily, before and after sampling, from the first day of fermentation until a week, as well as the OD.

2.5 Quenching and intracellular extraction

Regarding experiment A, samples from the last day of fermentation (day 7) were collected in tubes, once the AF was finished, in the required volume to have 10^9 cells. They were centrifuged at 3500 rpm, at room temperature for 3 min. Supernatants were filtered (syringes filters, cellulose acetate membrane, 0.2 µm, VWR) and they were stored at -20 ºC until the analysis. On the other hand, intracellular samples were subjected to a cold glycerol solution quenching according to the method conducted by Villas-Bôas and Bruheim (2007). Then, intracellular metabolites were extracted following the procedure of Álvarez-Fernández et al. (2019) with minor modifications. The resulting extracts were stored at -80 ºC until samples

cleaning.

During experiment B, samples from days 0, 1, 2, 3, 4, 5, 6, 8 and 10 were collected. Supernatants were filtered (syringes filters, nylon, 0.2 µm, VWR) and they were stored at -20 ºC until sample cleaning.

Regarding the experiment with grape musts, samples from days 0, 1, 2, 3, 4, 5, 6 and 7 were collected. Supernatants were filtered (syringes filters, nylon, 0.2 µm, VWR) and they were stored at -80 ºC until samples cleaning.

2.6 Samples treatment

Extracellular samples were cleaned up using C18 SPE cartridges (Variant, Aligent) conditioned with 2 mL of methanol and 2 mL of milliQ water. Then, 500 µl of sample were loaded and cartridges were washed with 2 mL of a 10% v/v methanol/water solution. Analytes were eluted with 1 mL of methanol. Samples were dried until total dryness, at 2000 rpm, at 30 ºC for 8 h, with a vacuum concentrator (HyperVAC-LITE, Gyrozen, Korea). Afterwards, samples were reconstituted with 167 µL of 0.1% v/v formic acid in 10% v/v methanol/water and they were stored at -80 ºC until the analysis.

On the other hand, the extracts of intracellular samples were cleaned up using phospholipid removal cartridges (Phree^{TM}, Phenomenex) with the purpose of removing the possible impurities that could affect the analysis. Then, 100 µL of the intracellular extracts described in section 2.5 were loaded. This process was repeated twice. The elution was performed according to manufacturer's instructions. Then, they were dried until total dryness, at 2000 rpm, at 30 ºC for 8 h, with a vacuum concentrator (HyperVAC-LITE, GyrozenKorea) (María Antonia Álvarez-Fernández et al., 2019). Afterwards, they were reconstituted with 167 µL of 0.1% v/v formic acid in 10% v/v methanol/water and they were stored at -80 ºC until the analysis.

2.7 UHPLC/HRMS parameters

The analysis was performed in a Thermo Scientific liquid chromatography system consisting of a binary UHPLC Dionex Ultimate 3000 RS, connected to a quadrupole orbitrap Qexactive hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), which was equipped with a heated electrospray ionization probe (HESI-II). The column used was a ZORBAX SB-C18 (2.1x 100 mm, 1.8-µm particle size) with a guard column (2.1 x 5 mm, 1.8-µm particle size). Both of them were purchased from Agilent Technologies (USA). The temperature used in this analysis was 40º C, the flow was 0.5 mL/min for HT and 0.4 mL/min for tyrosine and the injection volume was 5 µL. HT was analyzed following the described chromatographic conditions which consisted of two phases (A) aqueous formic acid solution 0.1% and (B) solution 0.1% of formic acid in methanol. The gradient was: 0-1 min (5% B); 1-7 min (100% B); 7-8.5 min (100% B); 8.6-10 min (5% B). A target MS^2 in negative mode was performed. Parallel Reaction Monitoring (PRM) of the $[M+H]^-$ at 153.05572 and normalized collision energy (NCE) was set at 25 eV. Xcalibur software and TraceFinder (version 3.3) software were used for instrument control and data acquisition.

The analysis of tyrosine presents slight differences regarding the gradient as follows: 0-1 min (5% B); 1-3 min (100% B); 3-4 min (100% B); 4.1-5 min (5% B). A target MS^2 in negative mode was performed for HT and in positive mode for tyrosine using the same software for data analysis. PRM of the [M+H]+ at 182.08117 and NCE was set at 40 eV.

2.8 Statistical Analysis

The data were subjected to ANOVA and Fisher's Least Significant Difference (LSD). The results were reported as the mean ± standard deviation (SD). Differences at $p<0.05$ were considered statistically significant. InfoStat version 2019 was used for data analysis.

3. Results

3.1 HT occurrence in SM by different yeast strains

Figure 1 shows HT is produced by all the yeast strains at the end of the fermentation of SM (day 7). Commercial *S. cerevisiae* yeast strains produce more HT than the non *S. cerevisiae* tested (*T. delbrueckii* and *M. pulcherrima*) and autochthonous one (*S. cerevisiae* Yero 2.23 and *S. cerevisiae* Yero 2.24), being the RF the biggest HT producer (6.12 ng/mL).

As RF synthetized the highest concentration, it was selected for further experiments.

3.2 HT occurrence in SM with different tyrosine concentrations

Figure 2 displays the production of HT in the extracellular compartment during AF of SM. Musts contained 10 mg/L or 60 mg/L of tyrosine and the yeast used was RF strain. At the beginning (t=0), HT is absent as expected and it was detected from the first day of AF in every sample. Results at 10 mg/L of tyrosine concentration show that HT is present from the very first day and no statistical differences are observed from day 2 to the following days. Conversely, when tyrosine concentration is 60 mg/L, the higher concentration is determined at day 3. Then it decreases slightly. Significant differences according to ANOVA are marked in figure 2.

HT derives from tyrosol, which is formed from tyrosine by the Ehrlich pathway (Mas et al., 2014). Therefore, the hypothesis of this work was that the more tyrosine concentration in the media, the higher HT content could be produced. However, despite tyrosine concentration was six times higher, HT was not determined in that proportion (Figure 2).

To confirm that yeast synthetizes HT, it was analyzed in the intracellular media. Concentrations are lower in the intracellular media than in the extracellular compartment. The highest concentrations detected were at day 6 and 5 when the must had 10 mg/L and 60 mg/L of tyrosine concentration, respectively (data not shown).

3.3 Tyrosine occurrence in SM with different tyrosine concentrations

Tyrosine was monitored along the fermentation to verify the yeast uses it. As it could be expected, tyrosine decreases in the extracellular media from day zero. In the must with an initial tyrosine concentration of 10 mg/L, the yeast consumes a 99.45% of tyrosine from day zero to the second day of fermentation. Afterwards, the fourth day tyrosine could be determined in noticeably quantities in the extracellular compartment (0.027 mg/L). On the other hand, when the must had 60 mg/L, the yeast consumes a 74.06% of tyrosine from the first day to the second day of fermentation. Afterwards, the eighth day the production increases (0.009 mg/L).

Figure 3 shows the occurrence of tyrosine in the intracellular compartment during AF. There are no significant differences regarding the initial tyrosine concentration, with the exception of day 8. For both conditions, the highest intracellular tyrosine concentration was measured at day 3.

3.4 HT occurrence in grape must

Figure 4 shows the production of HT in the extracellular compartment during AF of natural musts (Palomino Fino and Chardonnay varieties). Grape musts were sterilized in order to eliminate the microbial load and to observe the effects of the strain under study (RF).

Similarly to the experiments with SM, RF strain produces HT, excreting it in the extracellular media during AF. Indeed, HT is detected at the beginning of the AF in every sample. We observe higher HT concentrations with grape musts than SM. Specifically, grape musts concentrations are 2.6-4.4 times higher. If we compare both grape musts, the highest values are produced with the Chardonnay variety at the middle of the fermentation (days 3-4) as figure 4 shows.

3.5 Tyrosine occurrence in grape must

Regarding tyrosine occurrence in the extracellular media, concentrations with Palomino Fino must range between 0.004 mg/L (day 4) and 0.012 mg/L (day 2), while concentrations with

Chardonnay range between 0.005 mg/L (day 1) and 0.021 mg/mL (day 2) These values are similar to those obtained in the SM with an initial tyrosine concentration of 10 mg/L. There are no significant differences between the musts and neither between the days, with the exception of days 0 (Palomino Fino 0.117 mg/L and Chardonnay 0.109 mg/L) and 1 in both musts.

4. Discussion

Yeasts produce bioactive compounds such as HT deriving from tyrosine and melatonin deriving from tryptophan (Hornedo-Ortega, Cerezo, Troncoso, Garcia-Parrilla, & Mas, 2016). Melatonin follows a zig-zag pattern along the fermentation appearing and disappearing in SM that could reflect a role as signaling molecule (Fernández-Cruz, Álvarez-Fernández, Valero, Troncoso, & García-Parrilla, 2017); (Fernández-Cruz, Cerezo, Cantos-Villar, Troncoso, & García-Parrilla, 2018). Conversely, HT pattern is more reproducible and consistent through the different fermentations: it appears in all the cases and it remains almost constant from the first days and along the AF. Therefore, our data do not support a signaling role for HT but it is a metabolite produced from tyrosine, a nitrogen source. Some factors that have been reported to influence the AF are the pH, the temperature and the nature and composition of the medium. Specifically, they can affect the rate of fermentation and the production of metabolites (Vilela, 2019).

It is important that yeasts have available nitrogen in order to carry out the fermentation (Crepin, Nidelet, Sanchez, Dequin, & Camarasa, 2012). Specifically, 140 mg/L is the minimal concentration of YAN necessary for the fermentation (Vendramini et al, 2017). If the nitrogen available is insufficient, the fermentation becomes slower and there is a high risk of stucking (Bell & Henschke, 2005). Thus, this was the selected YAN for our experiments with SM in order to force the yeast to use tyrosine, a non-preferred source. However, no higher

284 intracellular concentrations were determined as tyrosine increased, showing that the capacity
285 of using tyrosine seems to be limited.
286 Some factors that can affect the YAN and amino acid profile, including tyrosine produced by
287 the must, are grape variety, grape berry ripening, grape processing, geographical origin and
288 climate (Tesnière, Brice, & Blondin, 2015). Henschke et al. (1993) observed that tyrosine
289 values in some white wines were: Chardonnay 6 mg/L; Riesling 3 mg/L; Sauvignon Blanc 24
290 mg/L and Traminer 5 mg/L (Henschke & Jiranek, 1993). Moreover, in another study the
291 average value of tyrosine in 9 white musts was 36.7 mg/L (Cabrita, Ratola, Laureano, &
292 Alves, 2007). Ünal et al. (2015) reported for white wines the following values: Emir
293 56.15±16 mg/L; Narince 28.85±2 mg/L and Sultaniye 35.16±11 mg/L (Ünal, Şener, Şen, &
294 Yilmaztekin, 2015). In our study, tyrosine values selected for SM fermentations are similar to
295 those described and results show that tyrosine initial concentration does not have an impact on
296 HT concentration. Therefore, it is advisable to study the yeast involved in winemaking.
297 Álvarez-Fernández et al. (2018) reported that yeast can synthetize HT as it was detected in the
298 intracellular medium. As it is well known that yeast synthesizes tyrosol through the Ehrlich
299 pathway, a possible way to explain HT synthesis is the hydroxylation of tyrosol which has
300 been recently evidence by Muñiz-Calvo et al. (2020). Our data shows that different strains
301 present varied capacities for the synthesis and their selection could have an impact on the
302 final concentration of this bioactive. In this paper, RF outstands among others strains. Table 1
303 shows a comparison of HT synthesis by different yeast strains. Furthermore, the must has also
304 a role as higher concentrations are achieved in natural grape musts if compared to SM. A
305 possible explanation could be that endogenous enzymes of grapes could catalyze the
306 hydroxylation of tyrosol to HT (Gerrini et al, 2018). Although olive oil and fermented
307 beverages are the main food sources of HT, its production has been investigated in other food
308 matrices. For instance, HT was the most abundant phenolic compound in sunflower-stalks

309 (3.79 mg/L) (Martínez-Cartas, Olivares, & Sánchez, 2019), in olive oil vinegar (1,019 mg/L)
310 (De Leonardis et al., 2018) and in green cracked table olives the concentrations were between
311 100-800 mg/L (Anagnostopoulos et al., 2020). Moreover, the values obtained in fermented
312 white musts (21.78 µg/L at maximum) are remarkably lower that those reported for red must,
313 specifically Tempranillo with higher concentrations of HT at day 2 (235 µg/L) (M. Antonia
314 Álvarez-Fernández et al., 2018). These results are consistent with already published data that
315 describe higher HT in red wines (3.66-4.20 mg/L) than in white ones (1.72-1.92 mg/L)
316 (Fernández-Mar et al., 2012). In the present work, we sterilized the must to ensure the
317 production of HT was due to the strain inoculated. Remarkably, lower values were obtained
318 after the elimination of the autochthonous flora; HT concentration after the fermentation of
319 sterilized must was 167 µg/L in Chardonnay and 89 µg/L in Palomino Fino. Grape microflora
320 could be responsible for the difference between reported values and those we obtained as we
321 sterilized grape musts. Indeed, tyrosine concentration does not seem to be a relevant factor
322 showing to be produced at a constant rate. Apparently, the presence of different strains could
323 be more important to achieve higher amounts.
324 HT concentrations obtained in this study are lower than those observed in a study conducted
325 by Piñeiro et al. (2011). In that paper, 15 red wine varieties were analyzed and concentrations
326 ranged between 0.28 mg/L and 5.02 mg/L. This fact leads out to think in another alternatives
327 pathways involving polyphenols as red wines are richer in HT than rosé and white ones
328 (Ragusa et al., 2017). In this paper, HT concentration in Chardonnay was 2.3±1.3 mg/kg^2.
329 As discussed before, tyrosine concentration does not seem to have a role in HT production
330 because, HT concentrations obtained were the same in both conditions (10 mg/L and 60
331 mg/L). Therefore, there might be other factors that affect HT production. On one hand,
332 different microorganism belonging to the autochthonous flora could also have the capacity to
333 produce HT increasing final concentration. Additionally, different metabolic pathways, apart

from the hydroxylation of tyrosol could be possible. For instance, the excision of macromolecules of polyphenolic nature in a similar way as HT derives from oleuropein in the olive. Further research is needed to explore these facts.

5. Conclusion

In conclusion, this study aimed to explore those factors related to the AF process influencing the production of HT. Yeast strain was the first one selected. HT was produced by all the yeast strains under study. Commercial *S. cerevisiae* yeast strains were those which produced higher concentrations, being the RF the most producer. The second one was the initial tyrosine concentration of the must. Our results reveal that distinct from what might be expected tyrosine initial concentration does not seem to have a role in HT production because, although we increased it six times in the SM, in general HT concentrations obtained were the same in both conditions. Thirdly, the must nature through the differences in synthetic and natural grape musts. HT was produced in higher amount in grape musts than in synthetic must, specifically, in Chardonnay.

Therefore, our results point out that efforts in other strategies should be to unravel those factors that might increase the production of HT in order to obtain wines richer in this bioactive compound.

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Conflicts of interest

Inmaculada Rebollo-Romero, Edwin Fernández-Cruz, Fernando Carrasco-Galán, Eva Valero, Emma Cantos-Villar, Ana Belén Cerezo Ana M. Troncoso and M. Carmen Garcia-Parrilla declare they have no conflicts of interest.

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466 strain of Saccharomyces cerevisiae Conclusions The contents of tyrosol and
467 hydroxytyrosol in wines fermented by a single strain of, (2006), 11689.

468 **Figure Captions**

469 Figure. 1. Hydroxytyrosol occurrence. Hydroxytyrosol ocurrence in synthetic must by ten
470 wine yeast strains, expressed in ng/mL

471 Figure. 2. Hydroxytyrosol occurrence in extracellular media in synthetic musts. Evolution of
472 hydroxytyrosol concentration during alcoholic fermentation in synthetic musts with
473 different tyrosine contents, by Red Fruit yeast strain, expressed in ng/mL. Significant
474 differences with $p<0.05$ are displayed with *, $p<0.01$with ** and $p<0.001$ with ***

475 Figure. 3. Tyrosine occurrence in intracellular media in synthetic musts. Evolution of tyrosine
476 concentration during alcoholic fermentation in synthetic musts with different tyrosine
477 contents, by Red Fruit yeast strain, expressed in ng/mL. Significant differences between
478 the two tyrosine concentrations are displayed with * for $p<0.05$.

479 Figure. 4. Hydroxytyrosol occurrence in extracellular media in grape musts. Evolution of
480 hydroxytyrosol concentration during alcoholic fermentation in natural grape musts
481 (Palomino Fino and Chardonnay), by Red Fruit yeast strain, expressed in ng/mL.
482 Significant differences with $p<0.05$ are displayed with *, $p<0.01$with ** and $p<0.001$
483 with ***

Table 1

Yeast strain	HT in SM (ng/mL)	HT in white must (ng/mL)	HT in red must (ng/mL)	Reference
T. delbrueckii	0.25			
Yero 2.23	0.32			
M. pulcherrima	0.55			
Yero 2.24	0.64			
QA23	1.19			
Aroma White	1.31			
Uvaferm	1.36			
ICV GRE	1.44			
ES 488	2.97			
Red Fruit	6.12 (screening), 3.31 (10 mg/L of tyrosine) and 4.74 (60 mg/L of tyrosine)	7.82 (Palomino Fino) and 13.09 (Chardonnay)		
Aroma White		89, 159, 173, 185, 238 and 288 (max)		Álvarez-Fernández et al., 2018
Red Fruit			235 (max)	Álvarez-Fernández et al., 2018
QA23			400 (max)	Álvarez-Fernández et al., 2018
U.C.L.M. S325			175	Bordiga et al., 2016

Table 1. Hydroxytyrosol concentration in musts fermented with different yeast strains

HT: hydroxytyrosol; SM: synthetic must

Figure

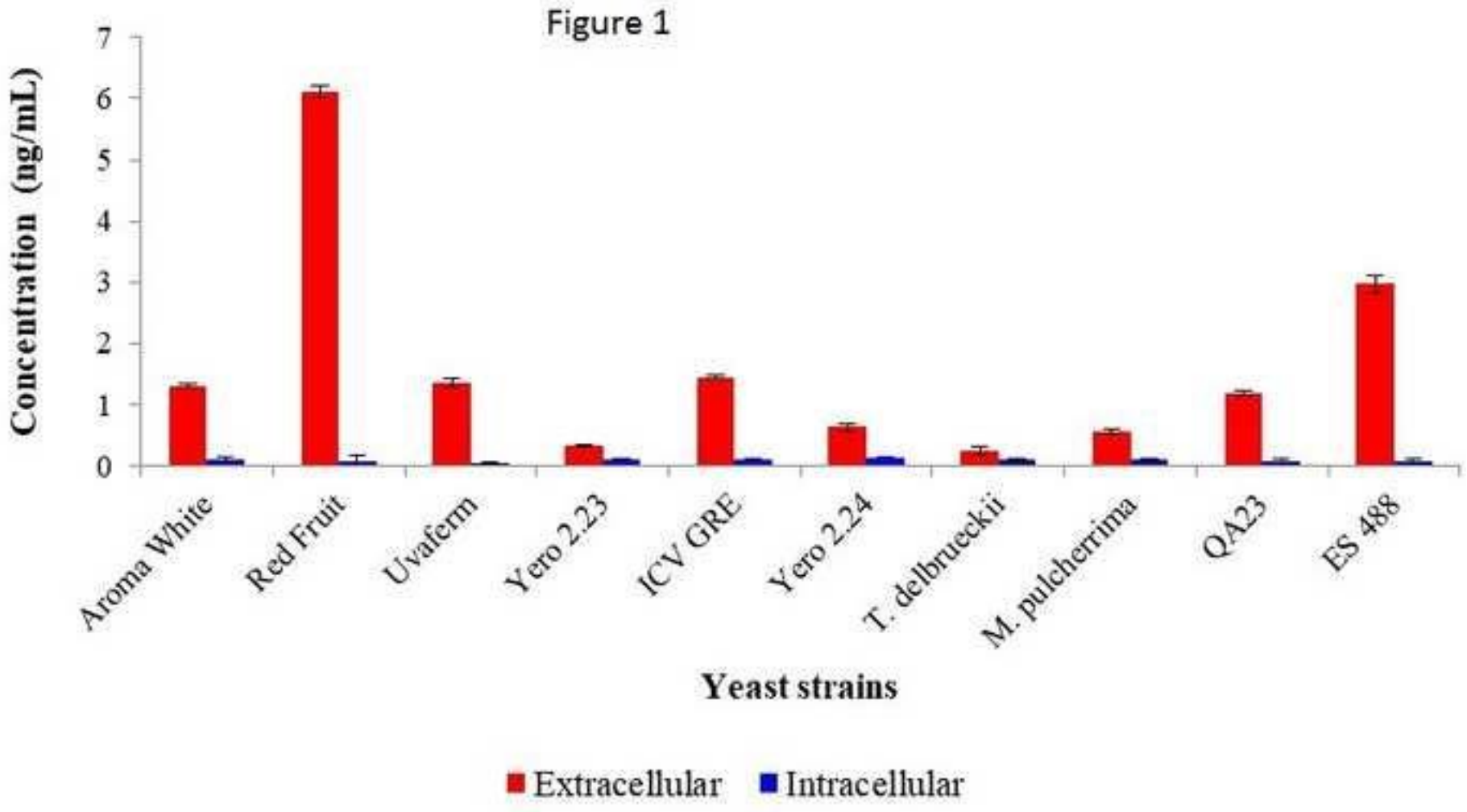
Figure 1
Concentration (ng/mL)
7
6
5
4
3
2
1
0
Aroma White
Red Fruit
Uvaferm
Yero 2.23
ICV GRE
Yero 2.24
T. delbrueckii
M. pulcherrima
QA23
ES 488
Yeast strains
Extracellular
Intracellular

Figure 2

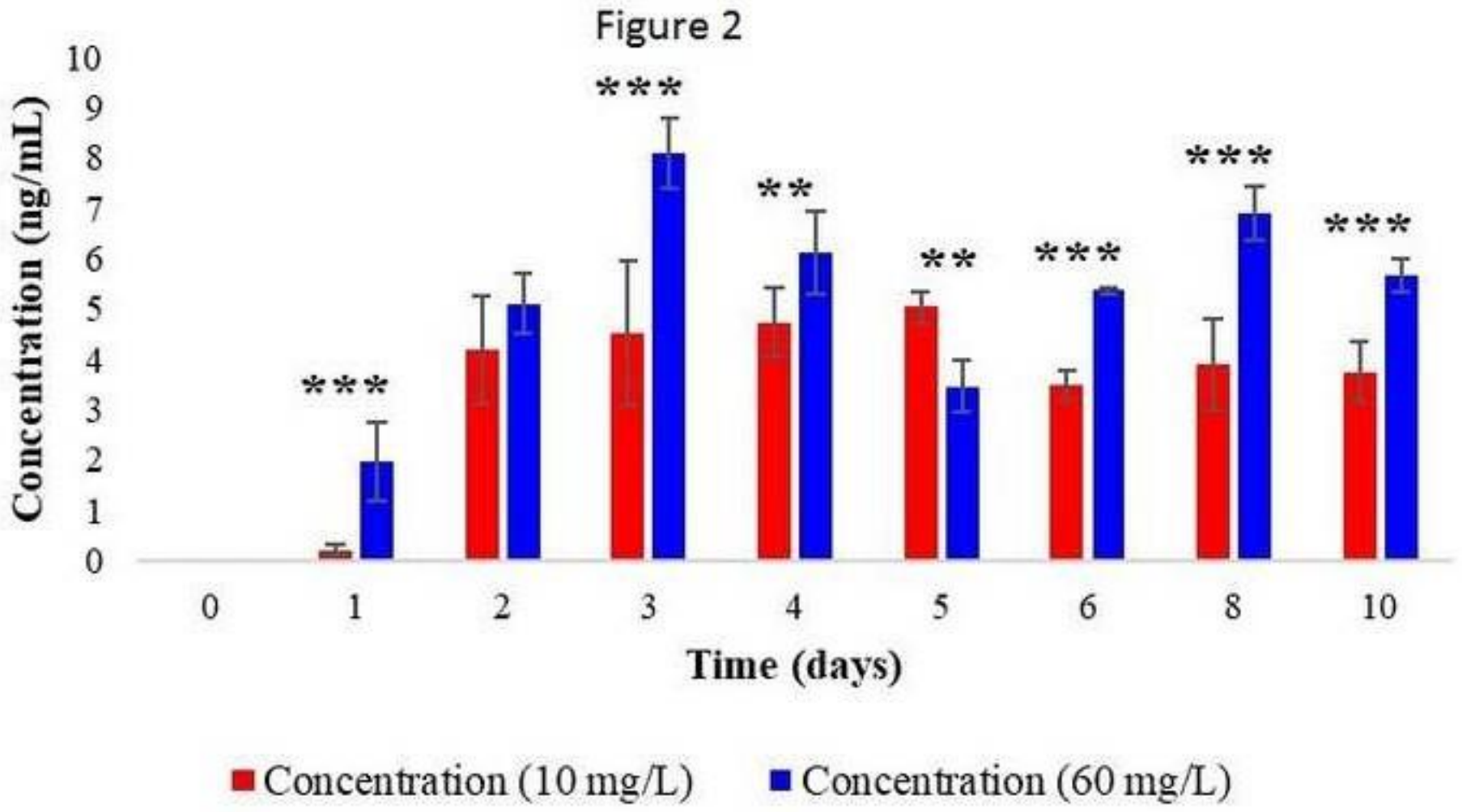
Figure 2
Concentration (ng/mL)
10
9
8
7
6
5
4
3
2
1
0
***
***
**
**
***
***
***
0
1
2
3
4
5
6
8
10
Time (days)
Concentration (10 mg/L)
Concentration (60 mg/L)

Figure 3

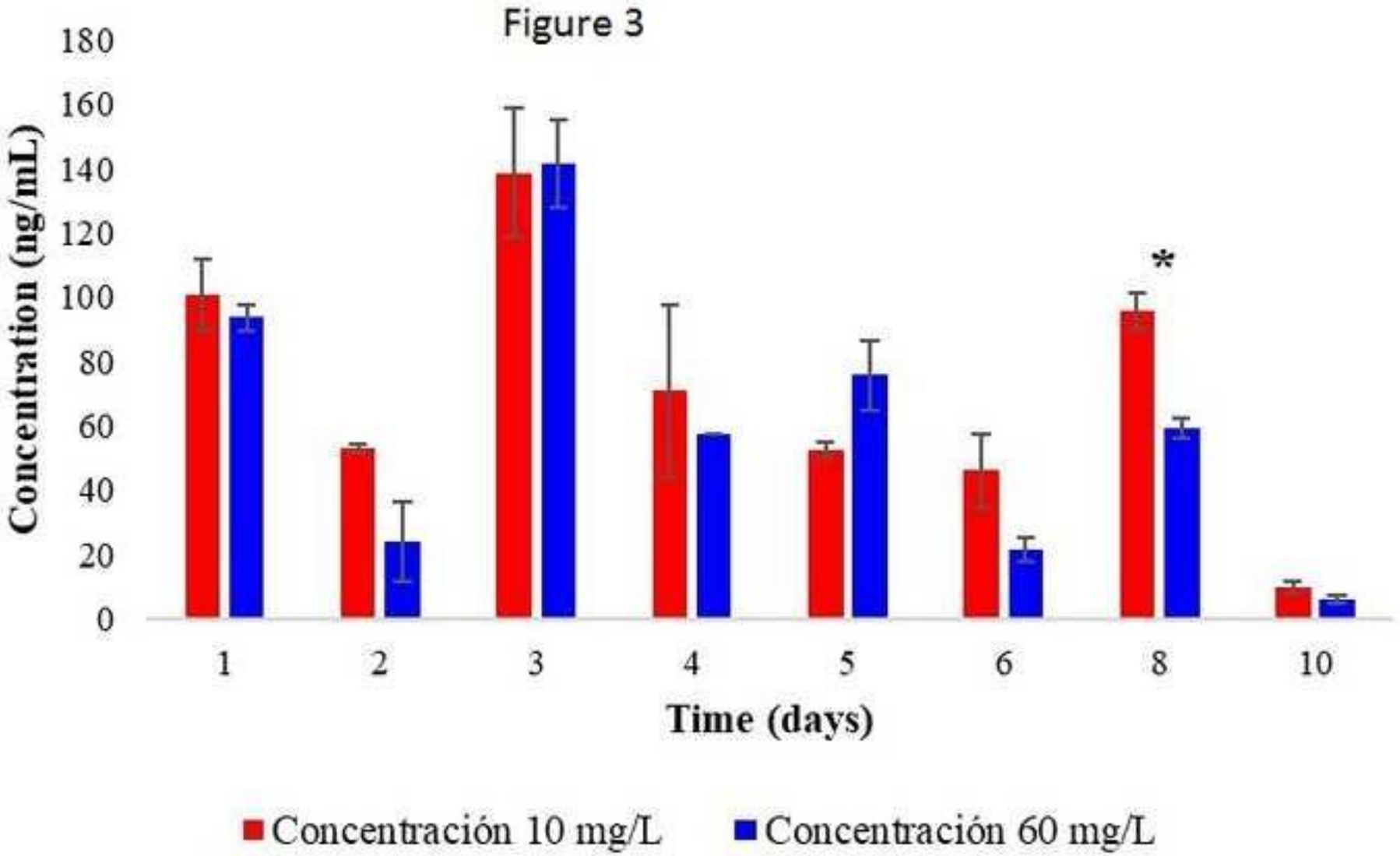
Figure 3
Concentration (ng/mL)
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
8
10
*
Time (days)
Concentración 10 mg/L
Concentración 60 mg/L

Figure 4

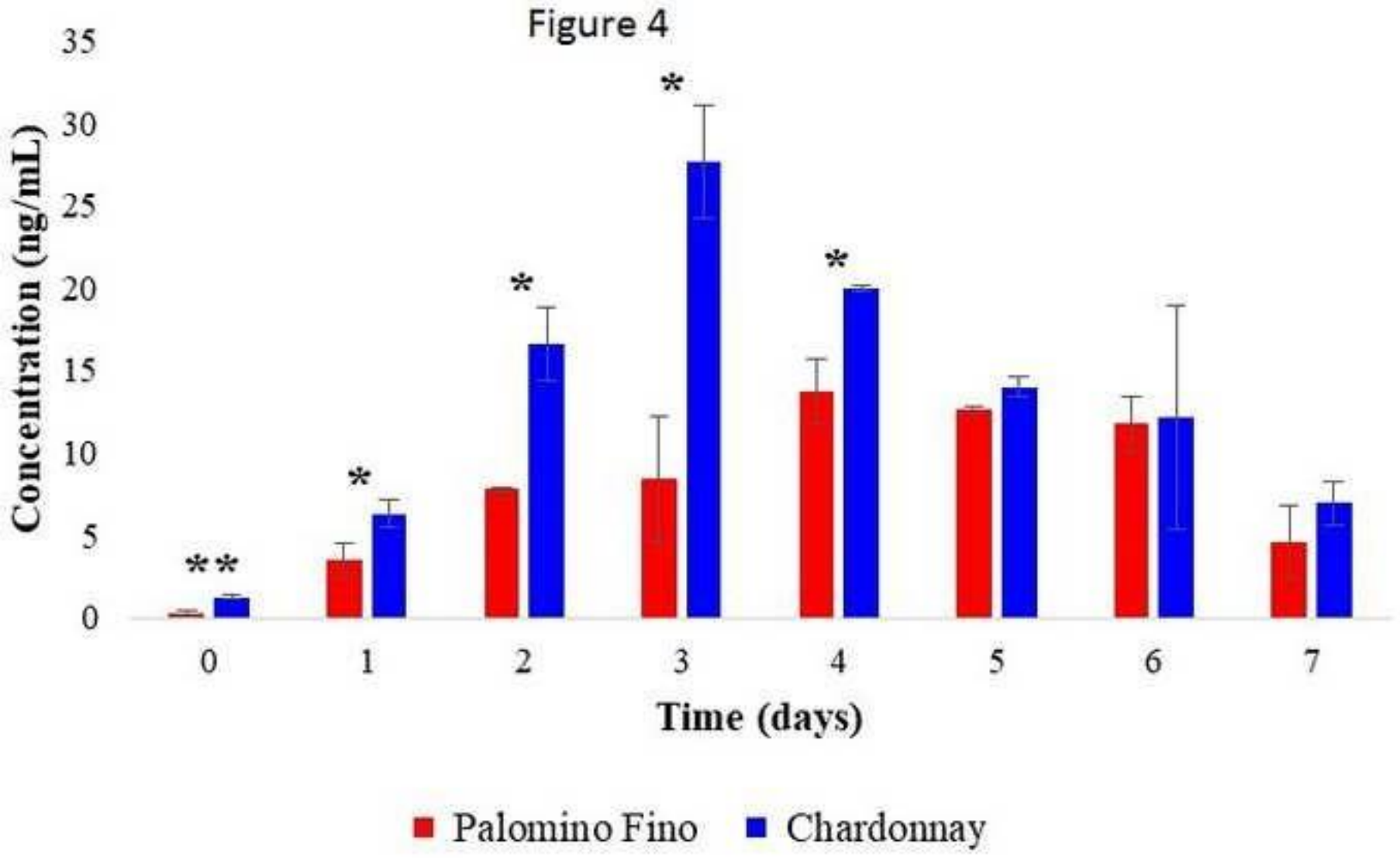
Figure 4
Concentration (ng/mL)
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
Time (days)
Palomino Fino
Chardonnay