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Contribution of critical doses of fungicides on the generation of volatile compounds from Monastrell-based wines

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Abstract:	Grapes from Vitis vinifera var. Monastrell of Jumilla (South-East Spain) were vinified after the addition of three fungicides (iprovalicarb, mepanipyrim, and tetraconazole) at concentrations corresponding to twice and five times their maximum residue levels (MRLs) in grapes. These fungicides are commonly applied on vineyards to control downy mildew, powdery mildew, and botrytis diseases. The fungicide effect throughout winemaking on the volatile composition of the final wines was evaluated and the obtained results were critically discussed. The most significant variations at both doses of the active substances with respect to the control wine (without fungicides) were observed for the concentrations of two acetates (isoamyl acetate and 2-phenylethyl acetate) and esters derived from linear fatty acids (especially ethyl caproate and caprylate). As a consequence of the modifications on the content of some aromatic compounds, wines obtained under the presence of fungicides showed a higher global odorant intensity, with increased fresh fruit notes.
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HIGHLIGHTS

- Winemaking process contributed to decrease fungicide residues.
- Two variables were selected: type of active substance and its concentration on the must.
- Significant variations were observed for acetate and ethyl esters families in fungicide-treated wines.
- Wines treated with tetraconazole and those treated with mepanipyrim and iprovalicarb were clearly separated.

TITLE

Contribution of critical doses of fungicides on the generation of volatile compounds from Monastrell-based wines

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ABSTRACT

Grapes from *Vitis vinifera* var. Monastrell of Jumilla (South-East Spain) were vinified after the addition of three fungicides (iprovalicarb, mepanipyrim, and tetraconazole) at concentrations corresponding to twice and five times their maximum residue levels (MRLs) in grapes. These fungicides are commonly applied on vineyards to control downy mildew, powdery mildew, and botrytis diseases. The fungicide effect throughout winemaking on the volatile composition of the final wines was evaluated and the obtained results were critically discussed. This study focuses on two variables, the type of active substance and its concentration level on the must.

The most significant variations at both doses of the active substances with respect to the control wine (without fungicides) were observed for the concentrations of two acetates (isoamyl acetate and 2-phenylethyl acetate) and esters derived from linear fatty acids (especially ethyl caproate and caprylate). As a consequence of the modifications on the content of some aromatic compounds, wines obtained under the presence of fungicides showed a higher global odorant intensity, with increased fresh fruit notes.

Keywords**:** iprovalicarb; mepanipyrim; tetraconazole; critical conditions; aromatic profile.

1. INTRODUCTION

The proper protection of wine grapes is the most critical factor to get an excellent wine. Nowadays, fungal diseases remain one of the main problems for the wine sector, and the application of antifungal treatments is the commonly adopted measure to fight against them. However, fungicides are also modulators of the biochemical activity of yeasts. Therefore, knowing their effects on fermentation is of the utmost importance to control the quality of wines.

Negative effects can occur even though the doses of fungicides and the safety periods have been respected and even when the levels of fungicides are reduced to traces during the winemaking process (González-Rodríguez, Cancho-Grande, & Simal-Gándara, 2009; González-Rodríguez, Cancho-Grande, Torrado-Agrasar, Simal-Gándara, & Mazaira-Pérez, 2009; González-Rodríguez, González-Barreiro, et al., 2011; González-Rodríguez, Noguerol-Pato, González-Barreiro, Cancho-Grande,& Simal-Gándara, 2011). Several studies have demonstrated that fungicides can limit the viability of wine yeasts (González-Rodríguez, González-Barreiro, et al., 2011), induce changes in the fermentation process (González-Rodríguez, González-Barreiro, et al., 2011; Noguerol-Pato, Torrado-Agrasar, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2014), and also alter the secondary metabolism of yeast (Dzedze, Breda, Hart, & Wyk, 2019). In this sense, our research group has contributed to a great extent on the knowledge related to the effects caused by the residues of several fungicides in the secondary metabolism of yeast, such as the alteration of the biosynthesis of fermentative volatile compounds or the release of varietal and pre-fermentative compounds during the fermentation process (González-Álvarez, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2012; González-Rodríguez, Noguerol-Pato, et al., 2011; González Álvarez, Noguerol-Pato, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2012; Noguerol-Pato, González-Rodríguez, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2011; Noguerol-Pato, Sieiro-Sampedro, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2015; Noguerol-Pato et al., 2014; Noguerol-Pato et al., 2016; Noguerol-Pato, Sieiro-Sampedro, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2014; Oliva et al., 2015; Oliva, Navarro, Barba, Navarro, & Salinas, 1999; Oliva, Zalacain, Payá, Salinas, & Barba, 2008; Sieiro-Sampedro et al., 2020; Sieiro-Sampedro, Figueiredo-González, et al., 2019; Sieiro-Sampedro, Pose-Juan, et al., 2019).

The effect of adding mepanipyrim as an active substance (Noguerol-Pato et al., 2014) or a commercial formulation (Sieiro-Sampedro, Pose-Juan, et al., 2019) has been tested previously on the volatile composition of an ecological must fermented at laboratory scale. This fungicide caused a significant decrease in the concentration level of isoamyl alcohols and alterations in the content of esters at doses corresponding to its MRL in grapes (set by the Regulation (EC) No 396/2005 and later amendments) and at critical concentrations (twice higher than its MRL). Several experiments have also been carried out to study the repercussion of applying a commercial formulation of mepanipyrim to vines, under Good Agricultural Practices (GAP), or directly to the must in the cellar on the aroma profile of wines from Graciano and Tempranillo varieties (Noguerol-Pato et al., 2015; Noguerol-Pato et al., 2016). Noticeable modifications in the floral nuances of the treated wines, provided mainly by alterations on the content of esters and C_{13}-norisoprenoids, were found. The effect of this active substance and its commercial formulation were also tested on Mencía musts at MRL levels and twice this value (Sieiro-Sampedro, Figueiredo-González, et al., 2019). In this case, the fungicide modified mainly some varietal compounds such as benzene derivatives. Only the commercial product caused a remarkable increase in the content of the fermentative compound 2-phenylethanol. Furthermore, the addition of the fungicide tetraconazole as an active substance or a commercial formulation at two concentration levels (MRL and 2MRL) was tested at laboratory scale using pasteurized ecological musts (Sieiro-Sampedro et al., 2020) and at medium scale in an experimental cellar, with grapes from Mencía cultivar (Sieiro-Sampedro, Figueiredo-González, et al., 2019). These studies showed a clear modification on ethyl esters content regarding control wines, mainly when the commercial formulation was applied. To the best of our knowledge, the effect of iprovalicarb was only evaluated jointly with other active substances by González-Rodríguez, Noguerol-Pato, et al., (2011). This fungicide was applied as a commercial formulation (fosetyl-al 37.1 %, mancozeb 28.6 %, and iprovalicarb 3.4 %) in a Godello vineyard under GAP. The obtained wines showed a lower content of terpenes and higher alcohols while the concentration of some acetates and esters increased. In this vein, other researchers found alterations in the content of many volatiles, mainly acetate and ethyl esters or some varietal compounds such as C_{13}-norisoprenoids, on Monastrell wines after the application of some commercial formulations based on diverse antifungal active substances (fenarimol, mancozeb, vinclozolin, metalaxyl, fenhexamide, fluquiconazole, quinoxyfen, kresoxim-methyl, and trifloxystrobin) at different concentration levels (Oliva et al., 2015, 1999; Oliva et al., 2008). At this point, as general conclusion that can be drawn from all the studies mentioned above is that the modifications in the content of both varietal and fermentative volatile compounds are multifactor-dependent (for instance, type of fungicide, fungicide concentration, grape variety, yeast strains, and winemaking conditions are some of the most studied variables).

In the present study, we will focus only on two variables: type of active substance and its concentration level on the must. The main reason for this selection was to ascribe a concrete effect to a particular variable (cause), reducing possible interactions or synergistic effects among multiple variables. Thus, different batches of destemmed and crushed grapes of Monastrell cultivar were supplemented before alcoholic fermentation with three active fungicide substances commonly applied on vineyards (iprovalicarb, mepanipyrim, and tetraconazole) at two critical concentration levels, corresponding to twice and five-times their MRLs in grapes, to obtain wines under rigorous winemaking supervision. Besides, we will try to explain the changes produced by these substances on the aromatic profile taking as a basis our experience in previous assays. To date, the effect of these fungicides on the aromatic profile of Monastrell-based wines was not studied, and the knowledge of their effects on other cultivars is scarce. Monastrell is one of the leading Spanish grape varieties and the most representative of the Designation of Origin (DO) Jumilla (Murcia, Southeast of Spain). The main attributes that highlight Monastrell wines are fruity notes of black fruits, ripe, and plum, among others (Pliego de Condiciones de la DOP “Jumilla”, 2021).

The fungicide mepanipyrim (abbreviated with Mep from now on) is used against *Botrytis cinerea*. It belongs to the anilino-pyrimidine chemical family, and its biochemical mode of action in target phytopathogenic fungi affects the methionine biosynthesis and hydrolases involved in the infection process (FRAC, 2021). Tetraconazole (abbreviated with Tetra from now on) is another widely used triazole fungicide, which acts against *Uncinula necator*, altering the sterol biosynthesis in cell membranes (FRAC, 2021). Finally, iprovalicarb (abbreviated with Ipro from now on) is a widely used valinamide carbamate fungicide very effective against *Plasmopara viticola*. Its biochemical mode of action in target phytopathogenic fungi focuses on the cellulose synthase in cell wall biosynthesis (FRAC, 2021).

2. MATERIALS AND METHODS

2.1. Grape characterization

Red grapes of *Vitis vinifera* var. Monastrell grown in Jumilla (Murcia, Spain) were harvested in 2016. Amino acid profile of the grape must was determined through the method described by Oliva, Garde-Cerdán, Martínez-Gil, Salinas, & Barba, (2011) and the obtained results were as follows: 6.5 g/kg of total amino acids concentration and a content of free amino acids lower than 4 g/kg. Glutamic acid (2.2 g/kg), proline (1.1 g/kg), and arginine (1.0 g/kg) were the primary amino acids present in the grape juice. Grape characterization was conducted by the determination of basic and essential components concentration of grapes using an Enological Multiparametric Analyzer Bacchus FTIR-Vis-UV MultiSpec (Tecnología Difusión Ibérica, Barcelona, Spain): sugar content (13.5 %); 3.27 of pH; total acidity (4.7 g/L); malic acid (2.41 g/L); and < 0.01 g/L of gluconic acid (Briz-Cid, Rial-Otero, Cámara, Oliva, & Simal-Gandara, 2019).

2.2. Fungicide experiments

Different micro-vinification assays (Control, A, B, C, D, E, and F) were performed in the experimental cellar in triplicate. The control experiment, made with uncontaminated destemmed and crushed grapes, was used for comparative purposes. Experiments A, C, and E were carried out with grape must fortified with Ipro, Mepa, and Tetra, respectively, at concentration levels corresponding to twice their MRLs (2MRL) on grapes (4, 4, and 1 mg/kg, respectively). Finally, in experiments B, D, and F, grape musts were spiked with the same fungicides at five times their MRLs (5MRL) on grapes (10, 10, and 2.5 mg/kg, respectively). Active fungicide substances were purchased as Pestanal Grade standards of certified purity > 99 % from Supelco (Bellefonte, PA, USA).

2.3. Winemaking process and wine sampling

The winemaking process was developed under the same conditions for all experiments. Briefly, red destemmed and crushed grapes (8 kg) were placed in separate metallic vessels (15 L) and supplied with SO_2 at 80 mg/L. After 24 h of fungicide addition, the commercial *Saccharomyces cerevisiae* Lalvin T73™ yeast strain (Lallemand Inc, Montreal, Canada) was inoculated at 25 g/hL.

During alcoholic fermentation-maceration, which took place at temperatures below 18 ± 2 ^{o}C (controlled by recirculating water) for ten days, the mixtures were homogenized once a day. Temperature and density (sugar percentage) were measured to control the fermentation evolution and possible stoppages or delays in the fermentation process. After this period, the wines were strained off, and grape residues pressed. Then, the wines were moved to other metallic vessels and left to ferment for another four days. After seven days of sedimentation, the wines were transferred to other clean vessels, discarding lees. A clarification step was developed with bentonite (40 g/hL) and gelatin (8 g/hL) for six days, and then, the wines were filtered (0.45 μm). In order to stabilize the obtained wines, SO_2 (30 mg/L) was added before bottling.

Oenological parameters of the final wines (alcoholic degree, total and volatile acidity, pH, malic and lactic acid content, glucose/fructose ratio, dry extract, and total polyphenols index (TPI)) were measured using an Enological Multiparametric Analyzer Bacchus FTIR-Vis-UV MultiSpec as is described in Briz-Cid et al., (2019).

2.4. Volatile determination

Chemical standards of volatile compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA). Individual standard solutions of appropriate concentrations were prepared in ethanol absolute from Scharlau (Barcelona, Spain) according to Noguerol-Pato, González-Barreiro, Cancho-Grande, & Simal-Gándara, (2009), and secondary standard solutions were also prepared by dilution in ethanol of the individual standard solutions. All of them were stored in the darkness at -20 ^{o}C. The internal standards considered, 2-octanol (used for minor compounds), 4-methyl-2-pentanol, and 4-hydroxy-4-methyl-2-pentanone (used for major compounds), were also purchased from Sigma-Aldrich.

Major compounds were determined by direct injection of red wines in a gas chromatograph equipped with a flame ionization detector (GC-FID) from Thermo Fisher Scientific (Waltham, MA, USA) and an HP-INNOWAX (60 m x 0.25 mm i.d., 0.25 μm) analytical column from Agilent Technologies (Santa Clara, CA, United States) following the method described by Peinado, Moreno, Muñoz, Medina, & Moreno, (2004). Chromatographic conditions and the oven temperature programme was previously described on González-Álvarez et al., (2012).

Minor compounds were extracted from wines by a solid-phase extraction procedure described on González-Álvarez et al., (2012), using 4-nonanol as a surrogate (Sigma-Aldrich). Volatile compounds were separated and identified on a gas chromatograph Trace GC 2000 Series from Thermo Scientific (Waltham, Massachusetts, USA) equipped with a PolarisQ ion trap mass selective detector (ITMS) and a ZB-WAX Zebron Phenomenex polyethylene glycol capillary column (60 m x 0.25 mm i.d., 0.25 µm). Chromatographic conditions and the oven temperature programme were previously described on González-Álvarez et al., (2012). Quantification was performed in SIM mode by choosing specific m/z values of each volatile compound from the full-scan mode (Noguerol-Pato et al., 2009) (**Table 1S** of Supplementary material).

2.5. Statistical analyses

One-way ANOVA and a Tukey's HSD test were performed in order to determine the statistically significant differences ($p < 0.05$) among A, B, C, D, E, and F spiked wines and the uncontaminated wine (control). Analyses were carried out using the software package Statgraphics Centurion XVI from StatPoint Technologies Inc.

Principal component analysis (PCA) was performed on the autoscaled data (38 samples and 21 variables) using the Statgraphics software package to provide partial visualization of the data set in a reduced dimension. PCA was employed to examine the natural grouping of the samples according to the type, and critical concentration of fungicide in two-dimensional principal components (PCs) plans, where each PC was a linear correlation of the original variables (latent variables).

3. RESULTS AND DISCUSSION

3.1. An oenological overview of Monastrell based-wines elaborated under critical doses of fungicides

Once in the cellar, the three selected fungicides (Ipro, Mepa, and Tetra) were directly and individually added in the form of active substances to destemmed and crushed grapes at concentrations corresponding to 2MRL and 5MRL in wine grapes, respectively. In a recent work, our research group verified that during the winemaking process, the dissipation of fungicide residues happened, reaching at the end of the process reductions of 97 % (in mass units) for Mepa, 91–92 % for Tetra, and 72–74 % for Ipro (Briz-Cid et al., 2019). As expected, fungicides removal was dependent on their physicochemical properties and their stability in the ethanolic medium.

Although the application of the different fungicide treatments did not promote fermentative stoppages, statistically significant differences were observed in some oenological parameters, probably due to modifications in the viability and metabolism of yeasts (**Table 2S** of Supplementary Material). Specifically, it was possible to verify how a spontaneous malolactic fermentation occurred in those wines elaborated with grapes supplemented with fungicides in a dose-dependent manner, especially with Ipro and Mepa. Also, in the case of Tetra, a reduction of malic acid concentration and an increment of lactic acid content occurred, although at a lower level. Besides, the volatile acidity increased in all wines (between 4.0 and 8.6 times for Ipro and Mepa, and around 1.6-2.6 times for Tetra). This fact could be related to the presence of fungicides in the medium. Under these conditions, the extension of *S. cerevisiae* T73™ lag phase could increase, and another opportunistic microbiota (such as lactic and acetic acid bacteria) could be developed, altering the composition of wine.

3.2. Impact of iprovalicarb, mepanipyrim, and tetraconazole on the volatile composition of Monastrell based-wines

Average concentration values of forty-six volatile compounds resulting either from the transformation of volatile grape precursors or the metabolism of yeasts are listed in **Table 1**. One-way ANOVA and a Tukey's HSD test ($p < 0.05$) were chosen as the statistical techniques to find similarities and differences among the aroma profile of treated wines and the control wine.

Although many statistically significant differences were observed between treated and control wines, only those variations on the concentration of volatiles higher than 30 % (remarked values in **Table 1**) could be exclusively attributed to the presence of fungicides (Sieiro-Sampedro, Figueiredo-González, et al., 2019).

3.2.1. Varietal compounds resulting from the biotransformation of grape precursors

Three monoterpenes, two C_{13}-norisoprenoids, five alcohols with six carbon atoms (C_6-alcohols), and nine benzene derivatives were identified and included in the group of varietal compounds (**Table 1**). Monoterpenoids are biosynthesized from acetyl-CoA, taking part as intermediates the five-carbon precursors isopentenyl diphosphate and dimethylallyl diphosphate (Maicas & Mateo, 2005). The synthesis of carotenoid-derived volatiles, such as the C_{13} ketones β-ionone and β-damascenone, is carried out by dioxygenases that cleavage double bonds in carotenoids (Rambla et al., 2016). C_6-alcohols are mainly generated through the enzymatic breakdown of C_{18} polyunsaturated fatty acids contained in plant membranes. Four enzymes are sequentially involved in this pathway. First, an acyl-hydrolase frees the fatty acids from membrane lipids. Next, a lipoxygenase catalyzes the fixation of oxygen. The peroxides obtained are then split into C_6-aldehydes by a hydroperoxide lyase. Finally, some of them may be reduced to their corresponding alcohols by alcohol dehydrogenase (Mozzon, Savini, Boselli, & Thorngate, 2016). Although the complete metabolic pathways of volatile benzenoids are still not totally understood, it is known that benzyl alcohol is formed in plants during the phenylpropanoid synthesis by the phenylalanine ammonia-lyase (Martin et al., 2016). This enzyme catalyzes the conversion of phenylalanine to *trans*-cinnamic acid, which is subsequently converted into benzyl alcohol and other derived compounds. As a result of all the cited biotransformations, these compounds can be present in grape berries as free volatiles, or most of them as glycosidically conjugated forms, comprising the free aroma compound (an aglycone) linked to one or more sugar moieties (the glycone) (Baumes, 2009). These grape aroma glycosides can be released during the winemaking process by glycosidase enzymes produced by grapes, yeasts, and bacteria (Belda et al., 2017).

In general, the concentration of monoterpenes and C_{13}-norisoprenoids detected in Monastrell-based wines did not change after fungicide supplementation (**Table 1**), except for β-citronellol that decreased its content (about 50 %) in all experiments, regardless of the type and concentration of fungicide added. In the case of C_6-alcohols, the *trans*-3-hexen-1-ol content increased significantly (26 - 32 %) after the addition of Tetra, while the *cis*-3-hexen-1-ol concentration increased (32 %) with the highest concentration of Mepa (5MRL). In the group of benzene derivatives, four compounds underwent important changes. The concentration of benzyl alcohol exhibited increments between 22 and 31 % after the addition of Tetra. However, the benzaldehyde content increased with all antifungal treatments, being these increments statistically significant (between 26 and 45 %) at the lowest concentration assayed (2MRL). In addition, the concentration of ethyl vanillate increased (between 37 % and 70 %) with the three tested fungicides at both critical concentration levels. Contrary to this uptrend, the concentration of syringol diminished (37-57 %) in those wines treated with both levels of Tetra and the highest concentration of Ipro and Mepa (5MRL). Also, a slight decrement in the concentration of methyl vanillate and acetovainillone (17-22 %) was observed in the presence of Ipro.

The effect of Mepa and Tetra on the aromatic composition of wines has been previously studied by our research group in the past, albeit emphasizing other conditions (*viz.* fungicide added as active substance or commercial vineyard protection product considering, in this case, other ingredients of the formulation; fermentation laboratory-scale assays or medium-scale assays in the winery; different grape varieties (Tempranillo, Graciano, Garnacha, and Mencía); spontaneous fermentation with endogenous yeasts or inoculation of *S. cerevisiae*) (Noguerol-Pato et al., 2015; Noguerol-Pato et al., 2016; Sieiro-Sampedro et al., 2020; Sieiro-Sampedro, Figueiredo-González, et al., 2019; Sieiro-Sampedro, Pose-Juan, et al., 2019).

For comparative purposes, **Table 2** summarizes the results obtained in those more similar studies to this one, where the active substances Mepa and Tetra were added over the grapes must and then inoculated with the yeast strain *S. cerevisiae* T73™. As observed in this table, no fungicide effects were previously observed over the C_{13}-norisoprenoids and C_6-alcohols at fungicide concentrations corresponding with the MRL and 2MRL. The decrease observed in the levels of β-citronellol with both fungicides was not coincident with previous studies, although the content of other monoterpenoids was altered. On the contrary, the effect of both fungicides over the concentration of some benzene derivatives was previously registered in medium-scale assays using Mencía (Sieiro-Sampedro, Figueiredo-González, et al., 2019). Fungicide residues could promote or decline the activity of endogenous grape-derived glycosidases, exogenous yeast-derived glycosidases, and bacterial glycosidases during the fermentation process. It is known that the impact of glycosidases on the release of aroma molecules from precursors is dependent on their stability and activity in the juice or wine medium (Robinson et al., 2014). This theory is also sustained by other studies, in which the application of fungicides was in the vineyard. Noguerol-Pato and coworkers observed significant variations in varietal compounds' content in Graciano and Tempranillo wines elaborated from grapes treated under GAP with boscalid+kresoxim-methyl and metrafenone, separately (Noguerol-Pato et al., 2016, 2014). Higher concentrations of terpenoids (nerolidol and damascenone) and benzaldehyde were registered in Monastrell wines from grapes treated with kresoxim-methyl, famoxadone, fluquinconazole, and fenhexamid under GAP and CAP (Oliva et al., 2008). In a later study, the same authors observed increased concentrations of nerolidol and farnesol after applying fenhexamid and famoxadone treatments under CAP, respectively, in Monastrell wines obtained from inoculated yeast UCLM S377 (Oliva et al., 2015).

3.2.2. Fermentation derived volatile aroma compounds

In the following subsections, the changes observed in the principal families of the fermentation-derived volatile aroma compounds are commented. These flavour metabolites produced by yeast during fermentation process are generated *de novo* or by transforming and volatilizing the precursor compounds present in the starting material (Hirst & Richter, 2016).

3.2.2.1. Higher alcohols and their associated aldehydes and acids

The importance of higher alcohols (also known as fusel alcohols) and their derived aldehydes and acids lie in being the most abundant volatile components produced during fermentation, so they significantly impact on the final flavour profile of wines even at low concentrations (Belda et al., 2017). Most of them are formed from the sugar metabolism of yeasts, producing α-keto acid precursors from pyruvate and acetyl-CoA via the tricarboxylic acid (TCA) cycle (Robinson et al., 2014). Alternatively, higher alcohols are also produced by yeasts from the amino acid catabolism via the *Ehrlich pathway*. First, aldehydes are generated by transamination and decarboxylation steps. Then, depending on the cell´s redox state, aldehydes can be reduced to fusel alcohols or oxidized to their corresponding acids (Dzialo, Park, Steensels, Lievens, & Verstrepen, 2017; Hirst & Richter, 2016).

As is shown in **Table 1**, the addition of critical levels of the tested active substances (Ipro, Mepa, and Tetra) to Monastrell grapes did not promote changes bigger than 30 % in the most important volatiles of this family (*i.e.*, isoamyl alcohols, 2-phenylethanol, and isovaleric acid). Besides, three minor compounds, 1-octanol, 3-methyl-3-buten-1-ol, and phenylacetaldehyde, were not modified either.

In our previous studies, when different grape varieties (and consequently different microbial ecosystem and medium composition) were contaminated after harvesting with Mepa and Tetra active substances, isoamyl alcohols remain unchanged (**Table 2**). However, in laboratory-scale assays with pasteurized must, the addition of Mepa decreased the content of isoamyl alcohols (Noguerol-Pato et al., 2014; Sieiro-Sampedro, Pose-Juan, et al., 2019). On the contrary, García and coworkers observed an increment of their content in laboratory-scale assays done in the presence of cyprodinil and fludioxonil (two fungicides with the same mode of action as Mep) (García et al., 2004). Moreover, increments in the content of isoamyl alcohols were registered in Monastrell wines obtained from grapes treated in the vineyard with commercial formulations of fenhexamid and fluquinconazole (fungicides with the same mode of action as Tetra) (Oliva et al., 2008). However, no variation in the content of isoamyl alcohols was previously found with other new-generation fungicides in wines from Godello, Tempranillo, Graciano and Chenin blanc grapes treated under GAP (Dzedze et al., 2019; González-Rodríguez, González-Barreiro, et al., 2011; Noguerol-Pato et al., 2015; Noguerol-Pato et al., 2016), and also Monastrell grapes treated under CAP (Oliva et al., 2015).

The biosynthesis of 2-phenylethanol depends on the grape variety and the type of fungicide applied (**Table 2**). For instance, its content was stimulated in the presence of critical doses of Tetra (2MRL) in Mencía wines (Sieiro-Sampedro, Figueiredo-González, et al., 2019). In addition, González-Álvarez et al., (2012) observed an increment in the content of 2-phenylethanol in Godello-based wines after applying a mandipropamid commercial formulation on vineyards, a fungicide with the same mode of action as Ipro (FRAC, 2021). A significant increment in the content of 2-phenylethanol was also found in this work after applying the highest dose of Ipro assayed (5MRL), although this rise was lower than 30 % compared to the control wine. In part, this effect could be related to differences in grape composition, especially in the content of the amino acids.

On the contrary, the biosynthesis of three alcohols (*i.e.*, 1-butanol, 3-methyl-1-pentanol, and 4-methyl-1-pentanol) was clearly affected by the presence of all tested fungicides (**Table 1**), registering increments between 122 % and 255 % for 1-butanol, between 43 % and 84 % for 3-methyl-1-pentanol, and between 30 % and 53 % for 4-methyl-1-pentanol. For methionol, only the highest dose assayed (5MRL) was substantially effective, increasing its concentration between 179 % and 220 % (**Table 1**). Methionol production is related to methionine concentration. Since methionine is found in relatively low concentration in Monastrell grape must (<0.09 % w/w), yeasts are required to assimilate inorganic sulfur via the sulfate reduction pathway, where methionine is re-metabolized to produce methionol via the Ehrlich pathway (through transamination to form the α-keto-γ-(methylthio)-butyrate, subsequent production of methional and finally methionol) (Dzialo et al., 2017). Lactic acid bacteria can also metabolize methionine during malolactic fermentation, forming volatile sulfur compounds (Inês & Falco, 2018). In this sense, it is essential to remember that a secondary malolactic fermentation happened spontaneously in the presence of the studied fungicide residues. Sieiro-Sampedro and coworkers attributed the promotion of methionol content in Mencía wines spiked with high doses (2MRL) of Tetra to an increment of the abundance of two proteins (aspartokinase and homoserine dehydrogenase encoded by *HOM3* and *HOM6* genes, respectively) involved in the methionine biosynthesis pathway from L-aspartate, another metabolic pathway of methionine supplying with the participation of glucose as a precursor (Sieiro-Sampedro, Figueiredo-González, et al., 2019). Furthermore, taking into account that methionol is considered a *quorum-sensing* molecule, yeasts collectively could secrete this compound to adapt their metabolism to exogenous changes, as the fungicide residues are.

Increments in the content of some higher alcohols (including 2-methylpropanol, 3-metyhylbutanol, and 1-octen-3-ol) were also reported after treating vineyards with commercial formulations incorporating flusilazole (Aubert, Baumes, Günata, Lepoutre, Cooper, & Bayonove, 1997), fenarimol, penconazole (Oliva et al., 1999), fenhexamid, and flunquinonazole (Oliva et al., 2008) as active substances. All these fungicides share the same mode of action as Tetra (FRAC, 2021).

3.2.2.2. Fatty acids

Volatile medium straight-chain fatty acids can contribute to the flavour and aroma of wine, although, at high concentrations, they are toxic to the yeast cells (Styger, Jacobson, & Bauer, 2011). They are by-products of saturated fatty acids metabolism. This complex process is catalyzed by the multienzymatic complex (fatty acid synthetase) using as substrates acetyl-CoA and malonyl-CoA to produce palmitic acid (C_{16}). Afterward, it can be used to produce other fatty acids with shorter chains (Moreno-Arribas & Polo, 2009).

In general, adding any of the three active substances at critical levels seemed not to affect the concentration of C_6-, C_8-, and C_{10}- acids (**Table 1**). Similar results were obtained in Mencía wines after the addition of Mepa and Tetra (**Table 2**). A similar outcome was previously found in wines from Graciano and Tempranillo grapes treated under GAPs with a commercial formulation of Mepa (Noguerol-Pato et al., 2015; Noguerol-Pato et al., 2014). No effects on the concentration of fatty acids were also found when Monastrell grapes were treated in the field under CAPs with commercial products containing famoxadone, fluquinconazole, kresoxim-methyl, quinoxyfen, fenhexamid, and trifloxystrobin as active substances (Oliva et al., 2015).

3.2.2.3. Esters

Esters comprise the most crucial set of yeast-derived aroma-active compounds. Due to their low odour thresholds, they are responsible for highly desired fruity and flowery-like aroma character of wines (Saerens, Delvaux, Verstrepen, & Thevelein, 2010). Esters are mainly synthesized in the cytoplasm of yeasts during the alcoholic fermentation by enzymatic chemical condensation of organic acids and alcohols when the stationary growth phase is reached, but also during the malolactic fermentation and aging of wines (Belda et al., 2017).

Acetates

Acetate esters result from the reaction of acetyl-CoA with higher alcohols (Styger et al., 2011). This reaction is catalyzed by alcohol acetyltransferases (Atf1p and Atf2p, encoded by *ATF1* and *ATF2* genes). Two acetates, of the major importance as aromatic constituents, were overproduced compared to the control wine with all treatments (**Table 1**). That is isoamyl acetate (increasing its concentration between 26 % and 43 %) and 2-phenylethyl acetate (between 20 % and 36 %).

Yeasts, under stressful conditions, can respond producing some esters to maintain plasma membrane fluidity (Dzialo et al., 2017; Saerens et al., 2010). Although the substrate concentration is essential to their formation, several studies have demonstrated that the expression levels of the alcohol acetyltransferases are the most significant factor determining the acetate ester levels during fermentation (Pires, Teixeira, Brányik, & Vicente, 2014; Robinson et al., 2014; Saerens et al., 2010). As previously stated, no effects derived from target fungicides were observed in the content of their precursors (isoamyl alcohols and 2-phenylethanol). Therefore, the increment of the acetate levels could be attributed to enhance the activity of Atf1p and/or Atf2p enzymes. In fact, overexpression of the *ATF2* gene of *S. cerevisiae* T73™ strain was observed after 48 h of must fermentation in the presence of Tetra commercial formulation (Sieiro-Sampedro et al., 2020).

An increment in the content of acetates was also found after adding the aniline-pyrimidine active substances cyprodinil and pyrimethanil to Airen grapes (García et al., 2004). Similar results were also observed in wines from Monastrell grapes treated in the vineyard with fenarimol and fenhexamid commercial formulations (Oliva et al., 1999; Oliva et al., 2008) or Mencía grapes treated with a tebuconazole commercial formulation (Noguerol-Pato et al., 2011). Nevertheless, no changes in acetates were observed either in Mencía wines after the application of Mepa and Tetra active substances (Sieiro-Sampedro, Figueiredo-González, et al., 2019) or in wines of other grape varieties treated with flusilazole (Aubert, Baumes, Günata, Lepoutre, Cooper, & Bayonove, 1997), penconazole (Dzedze et al., 2019; Oliva et al., 1999), and flunquinconazole (Oliva et al., 2015) commercial formulations. On the other hand, their content decreased after adding Mepa to Tempranillo pasteurized must (Noguerol-Pato et al., 2014). Consequently, it could be hypothesized that grape variety, type and concentration of fungicide, and also the winemaking process are the limiting factors in the biosynthesis of acetates.

Ethyl esters

Ethyl esters are formed from the ethanolysis of acyl-CoA, which is an intermediate metabolite of fatty acid metabolism. The ethanol radical is derived from ethanol and the acid group from a medium-chain fatty acid. The formation of ethyl esters has been attributed to two acyl-CoA/ethanol *O*-acyltransferases (Eeb1p and Eht1p) (Styger et al., 2011). Nevertheless, it has been observed that fatty acid precursor levels are the primary factor limiting their production, rather than the activity of the biosynthetic enzymes (Saerens et al., 2008; Saerens et al., 2010). This could cause the statistically significant increase (between 31 and 112 %) experienced by those esters formed from branched-chain fatty acids (ethyl-2-methylbutyrate and ethyl isovalerate) with all fungicide treatments (**Table 1**). Thus, the concentration of one of the detected precursors, isovaleric acid, showed an uptrend with all treatments, being statistically significant for Mepa (5MRL). Increments in the content of both esters were also reported in Mencía wines treated with Tetra (**Table 2**).

The production of esters derived from linear fatty acids was also enhanced (between 12 and 68 %) by the action of fungicide residues, especially for ethyl caprylate with Mepa and Tetra and ethyl caprate at the highest dose assayed for both fungicides (**Table 1**). Since the concentration of their fatty acid precursors remained invariable, it could be assumed that target fungicides could also regulate the activity of acyltransferase enzymes. Increments of ethyl caprylate and ethyl caprate were also observed in a laboratory-scale assay using a pasteurized Garnacha grape must fortified with Mepa at MRL and 2MRL. However, in this case, these increments were correlated with a higher concentration of fatty acids compared to the control wine (**Table 2**). An opposite trend was observed by (Noguerol-Pato et al., 2016, 2014) in Graciano and Tempranillo red wines after the addition of a commercial formulation of Mepa. The application of this formulation on vineyards provoked a general decrease in the content of esters. Besides, an increment of the ethyl lactate content (between 26 % and 45 %) was found with the addition of Ipro and Mepa, being statistically significant only for Ipro 5MRL. This increment could be related to the higher lactic acid concentration registered in these wines due to the malolactic fermentation (**Table 2S** of Supplementary Material).

Finally, some ethyl esters of organic acids also suffered statistically significant modifications (**Table 1**). Thus, all levels of fungicide residues increased the concentration of diethyl succinate (between 58 % and 87 %) and decreased the diethyl malate content (between 18 % and 52 %) with respect to the control wine. (Sieiro-Sampedro, Figueiredo-González, et al., 2019) also observed an increase in the diethyl succinate content in Mencía wines caused by adding a commercial product of Tetra and a decreasing trend (although not statistically significant) in the concentration of diethyl malate. While, with the active substance, no effects were observed (**Table 2**). Nevertheless, (Noguerol-Pato et al., 2011) observed that tebuconazole (a triazolic fungicide belonging to the same chemical family as Tetra) promoted a decrease in the content of diethyl succinate in Mencía wines at concentrations higher than MRL.

3.2.2.4. Lactones

A cyclic ester group characterizes lactones. Many lactones have been identified in wine and are thought to arise from a range of sources, including the metabolism of amino and keto acids by yeasts, the presence of *Botrytis cinerea* on grapes, the aerobic metabolism of flor yeasts on wines, the release from precursors extracted from oak wood during aging, and as by-products from the metabolism of pantothenic acid. In a particular way, long-chain fatty acids are precursor compounds in the biosynthesis of γ-lactones. Thus, *S. cerevisiae* has been shown to produce γ-nonalactone from linoleic acid by two biosynthetic pathways (Brown, 2007). All treated Monastrell-based wines exhibited nearly identical concentrations of γ-nonalactone irrespective of the fungicide treatment applied, but lower than the control wine by 30-36 %. These results are consistent with those obtained in Mencía-based wines supplemented with critical doses of Mepa (**Table 2**).

3.3. Impact of fungicides on the odorant profile of Monastrell based-wines

To make a tentative approximation to the organoleptic profile of wines from the quantitative data provided by the chromatographic analysis, volatile compounds with similar odour descriptors were grouped into seven odorant series characterized by a generic descriptor (**Table 2S** of Supplementary Material). The total OAV of each odorant series was calculated by summing the single OAV of the volatile compounds belonging to a particular series ($OAV = c/t$, where c is the total concentration of the compound concerned in the wine and t its odour threshold value).

The changes previously described in the concentrations of most of the analysed volatile compounds have resulted in a statistically significant increase in the global odorant intensity of those wines obtained under the presence of fungicides (from a value of 328 for the control wine to 379-397 for treated wines) (**Figure 1**). This difference is mainly due to the increase of the fresh fruit series, which involves compounds whose concentrations were significantly higher in the fortified wines, especially ethyl esters derived from fatty acids. These compounds, also at low concentrations, have a notorious impact on the aroma profile due to their low olfactory perception threshold. The increment registered for the sulfur compound methionol at the highest dose assayed (5MRL) also significantly increased herbaceous nuances. The remaining odorant series had levels comparable to those of the control wine but together helped to increase the overall odour activity value.

3.4. Multivariate analysis

PCA was chosen as a multivariate unsupervised method to identify general trends by grouping samples with certain similarities. A standardized matrix data was constructed with the measured variables (in this case, those 21 volatile compounds depicted in **Table 1,** which have variations higher than 30 % concerning the control wine for any treatment) and the wine samples (38 analyses in total). The purpose of PCA was to reduce the dimensionality of the original data with scarce loss of information.

PCA composition resulted in 4 principal components (PCs) with eigenvalues > 1 (PC1 = 10.67; PC2 = 3.89; PC3 = 1.76; PC4 = 1.09) that accounted for 82.94% of the total variance of the original data matrix. PC1 explained 50.80 % of the variance and PC2 explained 18.54 % of the variance (which together accounted for 69.34 % of the variance). The loadings (**Table 3**) express how well the new PCs correlate with the old variables (loading values >+0.20 and <-0.20 are marked in boldface type). From the loadings of the variables (**Table 3**), mainly ethyl 2-methylbutyrate, 3-methyl-1-pentanol, and 4-methyl-1-pentanol are the dominant features in PC1, whereas benzyl alcohol and *trans*-3-hexen-1-ol dominate in PC2.

Figure 2 shows the biplot of the first two principal components (PC1 *vs*. PC2). As expected, samples from control wines and all fungicide treatments are clearly separated along PC1. The figure shows that control samples are inversely correlated with most of the volatile compounds, except diethyl malate, syringol, citronellol, and γ-nonalactone, whose concentrations decreased in all treated wines (**Table 1**). Besides, it was possible to identify different groups between treatments:

- ***Grouping by type of fungicide:*** using PC2, those wines treated with Tetra (PC2 >1.5) can be separated from those treated with Mepa and Ipro (PC2 < 1.5). However, Mepa and Ipro produce more similar wines, especially at the lowest dose assayed (2MRL).
- ***Grouping by fungicide concentration:*** according to PC1, it is also possible to separate those samples fortified with fungicides at 5MRL (PC1 <-1.15) from those treated at 2MRL

(PC1 >-1.15). Tetra samples at the highest dose are correlated with variables associated with negative values of PC1 and positive values of PC2 (benzyl alcohol, *trans*-3-hexen-1-ol, ethyl caprylate, and ethyl caprate). Ethyl lactate is a characteristic variable for Ipro and Mepa 5MRL (negative values of PC1 and PC2).

4. CONCLUSIONS

The winemaking process contributed to decrease fungicide residues in wines, making the final wines a safe product for consumers (reductions of 97 % (in mass units) for Mepa, 91–92 % for Tetra, and 72–74 % for Ipro, at the end of the process). Moreover, the concentration of volatile compounds in Monastrell-based wines obtained after the addition of Ipro, Mepa, and Tetra at critical concentrations (2MRL and 5MRL) to crushed grapes (*i.e.*, avoiding the fungicide influence during grapes growth) showed significant variations in relation to the control wines, above all acetate and ethyl esters.

A comprehensive data exploration by PCA was also applied. The PCA model working with the refined set indicated that circa 68 % of the information was captured with two PCs, giving an extraordinary differentiation between control wines and the rest of the treated samples. Besides, it was possible to separate those wines treated with Tetra from those treated with Mepa and Ipro. Wines with different concentrations for the same fungicide treatment were also clearly separated.

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FIGURE CAPTIONS:

Figure 1. A. Global odorant intensity of the studied wines obtained in absence and presence of fungicides. Different letters (a and b) refer to statistically significant differences according to the ANOVA and Tukey's HSD tests ($p < 0.05$). **B.** Zoom for a better visualization of vinous, spicy and herbaceous series.

Figure 2. Distribution of the studied wines (control and treated wines) in a biplot system defined by the first two principal components (Component 1 *vs.* Component 2).

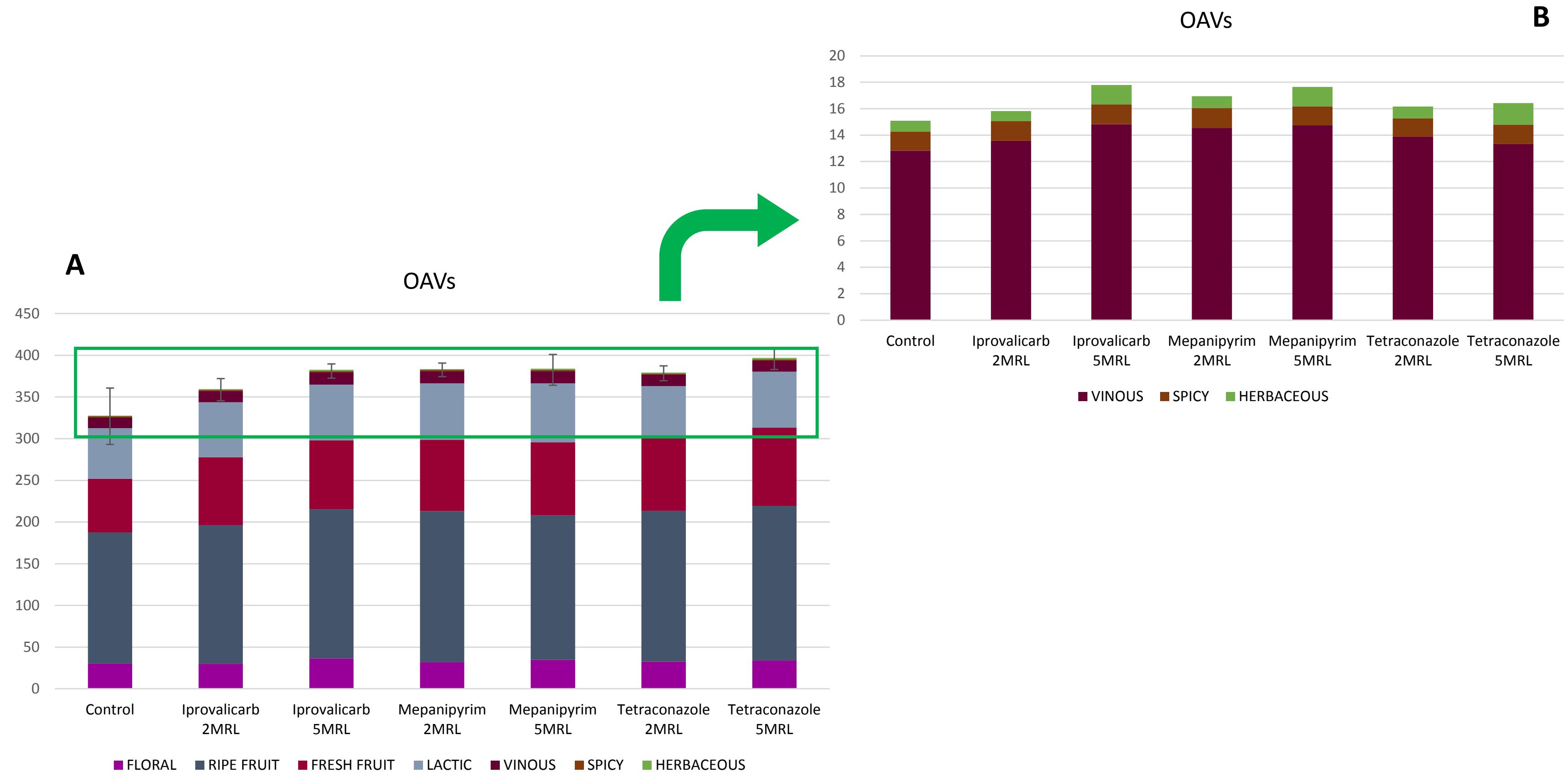

Figure 1. A. Global odorant intensity of the studied wines obtained in absence and presence of fungicides. Different letters (a and b) refer to statistically significant differences according to the ANOVA and Tukey's HSD tests ($p < 0.05$). **B.** Zoom for a better visualization of Vinous, Spicy and Herbaceous Series.

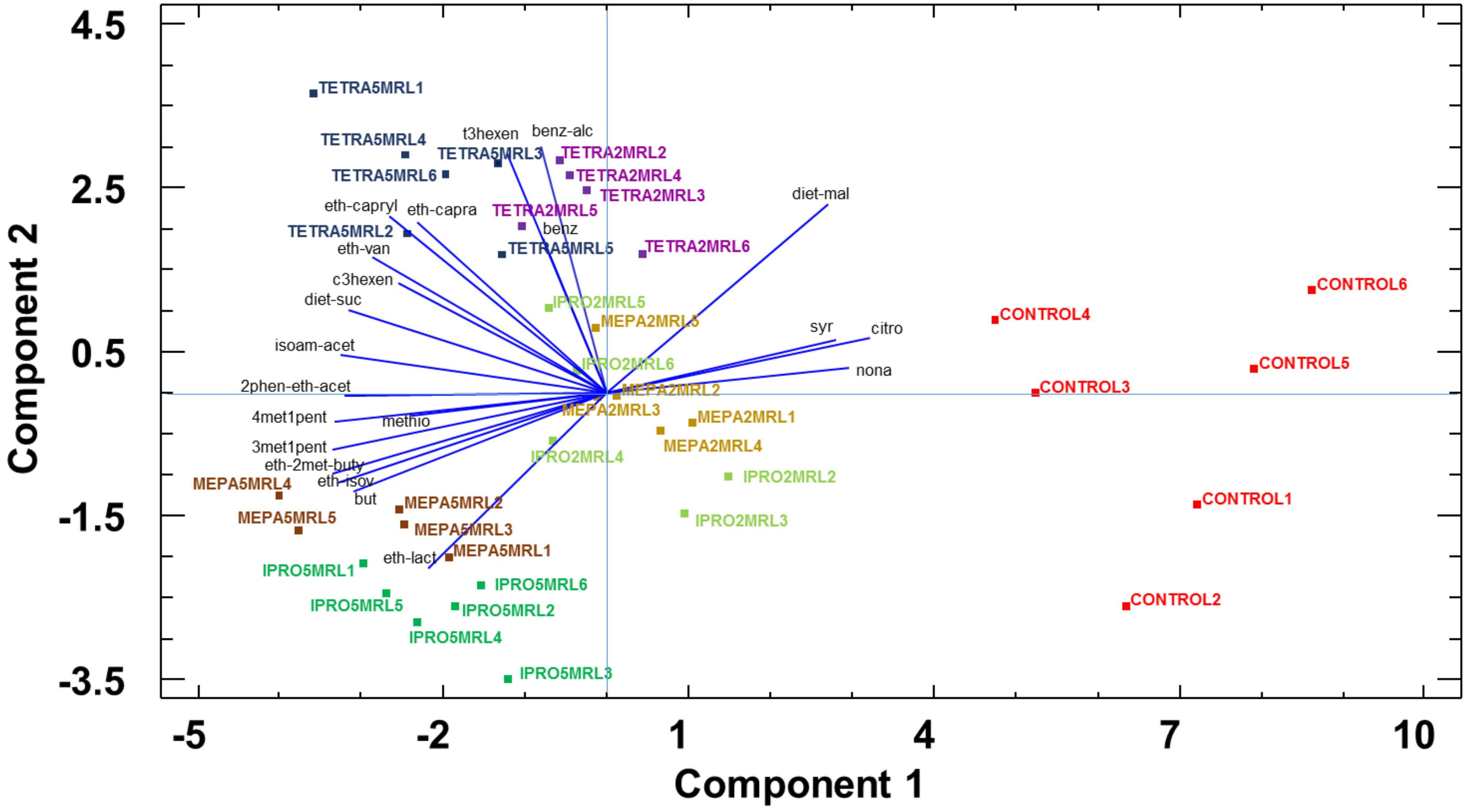

Figure 2. Distribution of the studied wines (control and treated wines) in a biplot system defined by the first two principal components (Component 1 *vs*. Component 2).

Acronyms: **citro**: β-citronellol; **t3hexen**: trans-3-hexen-1-ol; **c3hexen**: cis-3-hexen-1-ol; **benz-alc**: benzyl alcohol; **benz**: benzaldehyde; **eth-van**: ethyl vanillate; **syr**: syringol; **but**: 1-butanol; **3met1pent**:3-methyl-1-pentanol; **4met1pent**: 4-methyl-1-pentanol; **methio**: methionol; **isoam-acet**: isoamyl acetate; **2phen-eth-acet**: 2-phenylethyl acetate; **eth-2met-buty**: ethyl 2-methylbutyrate; **eth-isov**: ethyl isovalerate; **eth-lact**: ethyl lactate; **eth-capryl**: ethyl caprylate; **eth-capra**: ethyl caprate; **diet-suc**: diethyl succinate; **diet-mal**: diethyl malate; **nona**: γ-nonalactone.

Table 1. Volatile compounds in Monastrell-based wines obtained after iprovalicarb, mepanipyrim and tetraconazole supplementation. Values are expressed as average ± standard deviation (mg/L).

	CONTROL	IPROVALICARB 2MRL	IPROVALICARB 5MRL	MEPANIPYRIM 2MRL	MEPANIPYRIM 5MRL	TETRACONAZOLE 2MRL	TETRACONAZOLE 5MRL
VARIETAL COMPOUNDS RESULTING FROM GRAPE PRECURSOR BIOTRANSFORMATION							
Monoterpenes							
linalool	4.54^{a} ± 0.16	4.90^{a} ± 0.38	4.70^{a} ± 0.48	4.97^{a} ± 0.18	4.64^{a} ± 0.34	4.70^{a} ± 0.29	4.76^{a} ± 0.41
α-terpineol	7.95^{a} ± 1.35	7.73^{a} ± 0.53	7.81^{a} ± 0.68	7.55^{a} ± 0.21	8.31^{a} ± 0.88	7.65^{a} ± 0.45	9.20^{a} ± 0.39
β-citronellol	9.70^{b} ± 1.96	4.36^{a} ± 0.45	4.45^{a} ± 0.72	4.65^{a} ± 0.63	4.24^{a} ± 0.53	5.19^{a} ± 0.57	4.73^{a} ± 0.41
C_{13}-Norisoprenoids							
β-damascenone	4.50^{a} ± 0.92	4.37^{a} ± 0.25	4.97^{a} ± 0.54	4.91^{a} ± 0.24	4.78^{a} ± 0.16	4.67^{a} ± 0.41	4.71^{a} ± 0.24
β-ionone	1.89^{a} ± 0.28	1.84^{a} ± 0.21	2.21^{a} ± 0.24	2.18^{a} ± 0.21	2.14^{a} ± 0.31	1.99^{a} ± 0.12	2.15^{a} ± 0.15
C_{6}-Alcohols							
1-hexanol*	2.69^{a} ± 0.47	2.69^{a} ± 0.13	2.50^{a} ± 0.12	2.58^{a} ± 0.30	2.50^{a} ± 0.16	2.94^{a} ± 0.30	2.97^{a} ± 0.11
2-ethyl-1-hexanol	10.63^{a} ± 1.32	10.11^{a} ± 0.97	12.19^{a} ± 1.52	11.84^{a} ± 1.22	11.51^{a} ± 1.64	11.48^{a} ± 1.00	11.44^{a} ± 1.37
cis-2-hexen-1-ol	11.68^{b} ± 1.53	10.54^{ab} ± 0.90	10.40^{ab} ± 0.56	10.05^{ab} ± 1.02	10.80^{ab} ± 1.46	9.67^{a} ± 1.16	10.19^{ab} ± 0.70
trans-3-hexen-1-ol	75.10^{a} ± 5.80	83.58^{ab} ± 7.14	73.72^{a} ± 8.24	82.88^{ab} ± 3.08	77.02^{a} ± 6.85	94.92^{bc} ± 7.84	99.26^{c} ± 13.55
cis-3-hexen-1-ol	22.54^{a} ± 3.17	26.44^{ab} ± 4.47	25.85^{ab} ± 2.45	26.57^{ab} ± 4.41	29.76^{b} ± 2.54	28.45^{ab} ± 4.69	28.69^{ab} ± 1.73
Benzene derivatives							
benzyl alcohol	230.45^{a} ± 29.10	257.24^{ab} ± 17.78	219.55^{a} ± 12.30	245.16^{ab} ± 26.91	238.18^{a} ± 13.91	281.77^{bc} ± 20.83	300.85^{c} ± 25.79
benzaldehyde	21.87^{a} ± 3.52	27.60^{bcd} ± 1.53	23.93^{ab} ± 1.82	29.82^{cd} ± 2.21	24.07^{ab} ± 4.44	31.61^{d} ± 1.68	25.16^{abc} ± 1.77
guaiacol	3.66^{abc} ± 0.52	4.41^{c} ± 0.42	4.03^{bc} ± 0.78	4.34^{c} ± 0.59	3.11^{a} ± 0.41	3.15^{ab} ± 0.18	2.86^{a} ± 0.28
methyl vanillate	23.50^{b} ± 4.59	18.38^{a} ± 1.02	18.57^{a} ± 1.45	20.16^{ab} ± 1.13	20.01^{ab} ± 1.26	20.85^{ab} ± 1.23	20.96^{ab} ± 1.05
vanillin	35.96^{a} ± 6.82	29.97^{a} ± 2.16	35.92^{a} ± 4.42	34.77^{a} ± 5.48	34.03^{a} ± 5.71	29.36^{a} ± 2.41	34.43^{a} ± 5.25
ethyl vanillate	161.56^{a} ± 11.33	225.20^{b} ± 5.78	220.74^{b} ± 14.48	236.64^{bc} ± 26.66	255.11^{cd} ± 10.04	271.83^{d} ± 13.88	273.95^{d} ± 23.13
acetovainillone	64.42^{b} ± 10.74	51.84^{a} ± 8.06	53.44^{a} ± 3.54	57.31^{ab} ± 1.89	57.49^{ab} ± 4.93	58.39^{ab} ± 4.09	58.89^{ab} ± 5.04
syringol	44.83^{b} ± 11.52	39.74^{b} ± 3.71	19.24^{a} ± 1.13	42.46^{b} ± 6.54	26.04^{a} ± 1.86	28.30^{a} ± 2.76	24.08^{a} ± 2.69
methyl salicylate	6.97^{a} ± 0.75	7.95^{a} ± 0.52	7.76^{a} ± 1.05	7.84^{a} ± 0.74	7.46^{a} ± 0.42	7.61^{a} ±0.33	8.17^{a} ± 0.55
FERMENTATION DERIVED VOLATILE AROMA COMPOUNDS							
Aldehydes, Fusel Alcohols, and Acids							
phenylacetaldehyde	4.42^{a} ± 0.39	4.90^{a} ± 0.29	4.97^{a} ± 0.87	4.96^{a} ± 0.87	4.25^{a} ± 0.62	4.59^{a} ± 0.33	3.97^{a} ± 0.30

2-phenylethanol*	62.19^{a} ± 5.21	66.41^{ab} ± 10.06	80.08^{b} ± 0.77	71.47^{ab} ± 3.26	76.00^{ab} ± 3.01	71.37^{ab} ± 10.55	66.45^{ab} ± 2.43
isoamyl alcohols*	383.79^{a} ± 38.37	405.90^{a} ± 23.90	443.90^{a} ± 21.58	434.96^{a} ± 46.00	441.31^{a} ± 23.43	415.29^{a} ± 23.78	398.89^{a} ± 16.56
1-butanol	138.08^{a} ± 24.51	378.20^{bc} ± 54.19	456.31^{c} ± 53.29	362.47^{bc} ± 51.68	490.92^{c} ± 83.92	306.68^{b} ± 59.91	310.82^{b} ± 58.39
1-octanol	22.49^{ab} ± 4.34	22.78^{ab} ± 1.96	24.60^{b} ± 2.01	20.64^{ab} ± 1.48	21.56^{ab} ± 1.44	19.87^{a} ± 2.19	22.67^{ab} ± 1.18
3-methyl-1-pentanol*	0.71^{a} ± 0.15	1.12^{bcd} ± 0.11	1.21^{cd} ± 0.08	1.02^{bc} ± 0.18	1.31^{cd} ± 0.12	0.93^{ab} ± 0.12	1.13^{bcd} ± 0.08
4-methyl-1-pentanol	70.04^{a} ± 11.52	91.30^{bc} ± 8.46	98.66^{bc} ± 5.91	89.63^{abc} ± 16.33	107.35^{c} ± 12.06	86.81^{ab} ± 8.87	95.45^{bc} ± 10.11
3-methyl-3-buten-1-ol	21.03^{ab} ± 2.97	22.11^{ab} ± 2.58	18.57^{a} ± 1.57	23.14^{ab} ± 2.53	20.13^{a} ± 3.54	25.34^{b} ± 3.25	22.54^{ab} ± 1.64
methionol	328.78^{a} ± 58.55	228.20^{a} ± 12.88	948.86^{b} ± 147.11	240.83^{a} ± 33.15	918.41^{b} ± 117.21	321.23^{a} ± 54.25	1053.71^{b} ± 136.68
isovaleric acid*	1.69^{a} ± 0.30	1.88^{ab} ± 0.11	1.93^{ab} ± 0.08	1.95^{ab} ± 0.25	2.04^{b} ± 0.15	1.77^{ab} ± 0.13	1.91^{ab} ± 0.10
Fatty Acids							
caproic acid*	2.98^{a} ± 0.32	2.74^{a} ± 0.13	2.69^{a} ± 0.16	2.70^{a} ± 0.25	2.75^{a} ± 0.15	2.77^{a} ± 0.17	2.84^{a} ± 0.15
caprylic acid*	1.11^{b} ± 0.09	1.04^{ab} ± 0.04	0.95^{a} ± 0.04	1.02^{ab} ± 0.06	1.02^{ab} ± 0.06	1.09^{b} ± 0.07	1.11^{b} ± 0.07
capric acid	85.65^{a} ± 14.25	75.73^{a} ± 6.08	79.57^{a} ± 8.46	79.13^{a} ± 5.23	77.08^{a} ± 4.60	75.33^{a} ± 5.61	78.95^{a} ± 5.00
Acetate Esters							
isoamyl acetate*	1.08^{a} ± 0.21	1.36^{b} ± 0.08	1.53^{b} ± 0.11	1.44^{b} ± 0.09	1.45^{b} ± 0.09	1.41^{b} ± 0.14	1.55^{b} ± 0.08
hexyl acetate	7.53^{a} ± 1.52	7.02^{a} ± 1.06	7.08^{a} ± 0.59	8.09^{a} ± 1.34	7.10^{a} ± 0.83	8.05^{a} ± 1.02	9.29^{a} ± 1.75
2-phenylethyl acetate	89.13^{a} ± 8.58	106.77^{b} ± 4.37	120.39^{b} ± 11.69	114.96^{b} ± 8.46	121.56^{b} ± 7.95	106.82^{b} ± 8.11	116.39^{b} ± 10.59
Ethyl Esters							
ethyl 2-methylbutyrate	19.11^{a} ± 4.17	29.93^{bc} ± 2.67	36.79^{cd} ± 2.61	35.80^{cd} ± 4.74	40.49^{d} ± 4.81	28.74^{b} ± 3.71	32.44^{bc} ± 3.56
ethyl isovalerate	26.96^{a} ± 5.07	41.94^{bc} ± 5.72	46.37^{c} ± 3.59	44.18^{c} ± 4.65	48.42^{c} ± 3.48	35.45^{b} ± 2.30	41.71^{bc} ± 4.30
ethyl lactate*	8.76^{a} ± 1.06	11.47^{ab} ± 1.07	12.68^{b} ± 1.91	11.01^{ab} ± 1.36	11.65^{ab} ± 1.38	9.90^{ab} ± 1.60	9.62^{ab} ± 0.66
ethyl caproate	403.75^{a} ± 46.87	496.23^{b} ± 26.28	478.70^{b} ± 30.05	498.96^{b} ± 17.00	492.85^{b} ± 9.20	510.41^{b} ± 33.65	518.59^{b} ± 33.30
ethyl caprylate	116.09^{a} ± 23.70	145.13^{b} ± 8.09	143.79^{b} ± 8.46	155.51^{bc} ± 15.39	159.67^{bc} ± 11.64	175.69^{cd} ± 11.50	195.43^{d} ± 10.88
ethyl caprate	5.59^{a} ± 0.82	6.40^{ab} ± 0.52	6.26^{ab} ± 0.61	6.63^{ab} ± 0.64	7.36^{b} ± 0.43	6.71^{ab} ± 0.22	8.58^{c} ± 0.82
ethyl laurate	836.34^{a} ± 99.41	793.18^{a} ± 42.26	757.34^{a} ± 35.45	793.88^{a} ± 74.37	808.12^{a} ± 44.42	832.07^{a} ± 50.05	842.45^{a} ± 51.53
ethyl monosuccinate*	55.40^{a} ± 6.13	61.54^{a} ± 10.55	73.82^{a} ± 0.42	56.54^{a} ± 2.47	59.70^{a} ± 2.38	55.02^{a} ± 4.86	60.73^{a} ± 8.13
diethyl succinate*	1.25^{a} ± 0.24	2.07^{bc} ± 0.10	2.18^{bc} ± 0.09	2.03^{bc} ± 0.30	1.98^{b} ± 0.12	2.05^{bc} ± 0.16	2.34^{c} ± 0.13
diethyl malate	238.36^{d} ± 48.71	167.54^{bc} ± 5.05	113.87^{a} ± 9.77	163.19^{bc} ± 10.68	140.35^{ab} ± 21.79	183.65^{c} ± 15.08	195.13^{c} ± 11.90
Lactones							
γ-nonalactone	36.48^{b} ± 6.16	24.71^{a} ± 1.75	25.33^{a} ± 1.83	25.52^{a} ± 1.53	23.86^{a} ± 1.40	23.45^{a} ± 1.28	24.86^{a} ± 1.94

* Different letters (a, b, c, d, e) refer to statistically significant differences according to the ANOVA and Tukey's HSD tests ($p < 0.05$).

Table 2. Effect of mepanipyrim and tetraconazole active substances on the volatile profile of different wines obtained under specific conditions. Colour code: **white:** compound not determined; **grey:** no effect observed; **green:** increment in its content concerning the control wine; and **red:** decrement in its content concerning the control wine.

	MEPANIPYRIM						**TETRACONAZOLE**					
	Garnacha [a]		**Mencía** [b]		**Monastrell** [c]		**Garnacha** [d]		**Mencía** [b]		**Monastrell** [c]	
	No MLF		***Inoculated MLF***		***Spontaneous MLF***		***No MLF***		***Inoculated MLF***		***Spontaneous MLF***	
	MRL	**2MRL**	**MRL**	**2MRL**	**2MRL**	**5MRL**	**MRL**	**2MRL**	**MRL**	**2MRL**	**2MRL**	**5MRL**
Monoterpenes												
linalool	▨	▨	▨	▨	▨	▨	▨	▨	🟩	▨	▨	▨
α-terpineol	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
β-citronellol	▨	▨	▨	▨	🟥	🟥	▨	▨	▨	▨	🟥	🟥
geraniol			▨	🟩					▨	▨		
p-cimene			▨	🟥					▨	▨		
C_{13}-Norisoprenoides												
β-damascenone	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
β-ionone			▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
C6-Alcohols												
1-hexanol	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
cis-2-hexen-1-ol	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
trans-3-hexen-1-ol	▨	▨			▨	▨	▨	▨			▨	🟩
cis-3-hexen-1-ol	▨	▨	▨	▨	▨	🟩	▨	▨	▨	▨	▨	▨
Benzene derivatives												
benzyl alcohol	▨	▨	🟩	🟩	▨	▨	▨	▨	▨	▨	▨	🟩
benzaldehyde	▨	▨	▨	🟩	🟩	▨	▨	▨	▨	▨	🟩	▨
guaiacol	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
methyl vanillate	▨	▨			▨	▨	▨	▨			▨	▨
vanillin	▨	▨			▨	▨	▨	▨			▨	▨
ethyl vanillate	▨	▨	▨	▨	🟩	🟩	▨	▨	▨	🟩	🟩	🟩
acetovainillone	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
syringol	▨	▨	▨	▨	▨	🟥	▨	▨	▨	▨	🟥	🟥
methyl salicylate	▨	▨			▨	▨					▨	▨
eugenol	▨	▨	▨	▨					▨	▨		
Higher alcohols, aldehydes and acids												
phenylacetaldehyde			🟥	▨	▨	▨			▨	🟥	▨	▨
2-phenylethanol	▨	▨	▨	▨	▨	▨	▨	▨	▨	🟩	▨	▨
isoamyl alcohols	🟥	🟥	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
1-butanol					🟩	🟩					🟩	🟩
1-octanol			▨	▨	▨	▨			▨	▨	▨	▨

3-methyl-1-pentanol	▨	🟧			🟩	🟩	▨	▨			▨	🟩
4-methyl-1-pentanol			▨	▨	▨	🟩			▨	▨	▨	🟩
methionol	▨	▨	▨	▨	▨	🟩	▨	▨	▨	🟩	▨	🟩
isovaleric acid	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Fatty acids												
caproic acid	🟩	🟩	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
caprylic acid	🟩	🟩	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
capric acid	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
Acetate esters												
isoamyl acetate	▨	▨	▨	▨	🟩	🟩	▨	▨	▨	▨	🟩	🟩
hexyl acetate					▨	▨					▨	▨
2-phenylethyl acetate	▨	▨	▨	▨	▨	🟩	▨	▨	▨	▨	▨	🟩
Ethyl esters												
ethyl butyrate	▨	▨	▨	▨			▨	▨	▨	▨		
ethyl 2-methylbutyrate	▨	▨	▨	▨	🟩	🟩	▨	▨	🟩	🟩	🟩	🟩
ethyl isovalerate			▨	▨	🟩	🟩			🟩	▨	🟩	🟩
ethyl lactate					▨	▨	▨	▨			▨	▨
ethyl caproate	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨	▨
ethyl caprylate	🟩	🟩	▨	▨	🟩	🟩	▨	▨	▨	▨	🟩	🟩
ethyl caprate	🟩	🟩	▨	🟧	▨	🟩	▨	▨	▨	▨	▨	🟩
ethyl laurate	▨	▨			▨	▨	▨	▨			▨	▨
ethyl monosuccinate			▨	▨	▨	▨			▨	▨	▨	▨
diethyl succinate			▨	▨	🟩	🟩			▨	▨	🟩	🟩
diethyl malate			▨	▨	🟧	🟧			▨	▨	▨	▨
Lactones												
γ-nonalactone	▨	▨	▨	🟧	🟧	🟧	▨	▨	▨	▨	🟧	🟧
γ-butyrolactone	▨	▨	▨	▨					▨	▨		

[a] Laboratory fermentation assays inoculating *S. cerevisiae* T73 strain in Garnacha pasteurised must. Malolactic fermentation was not performed. Sieiro-Sampedro et al. (2019). Food Research International, 126, 108566.

[b] Winery fermentation assays inoculating *S. cerevisiae* T73 strain in destemmed and crunched Mencía grapes. Malolactic fermentation was performed by inoculating *Oenococcus oeni* bacteria. Sieiro-Sampedro et al. (2019). Food Chemistry, 300, 125223.

[c] Winery fermentation assays inoculating *S. cerevisiae* T73 strain in destemmed and crunched Monastrell grapes. Malolactic fermentation was performed by endogenous bacteria. This study.

[d] Laboratory fermentation assays inoculating *S. cerevisiae* T73 strain in Garnacha pasteurised must. Malolactic fermentation was not performed. Sieiro-Sampedro et al. (2020). Food Research International, 130, 108930.

Table 3. Loadings of the volatile compounds in the first four principal components.

Compounds	**Component 1**	**Component 2**	**Component 3**	**Component 4**
β-citronellol	**0.254848**	0.0938292	**-0.259097**	**0.238701**
trans-3-hexen-1-ol	-0.0970736	**0.407852**	-0.000435899	0.150009
cis-3-hexen-1-ol	**-0.202106**	0.187617	-0.110178	**0.364944**
benzyl alcohol	-0.0631483	**0.421254**	0.0844378	**-0.284644**
1-butanol	**-0.244626**	-0.16834	0.138773	0.0600479
3-methyl-1-pentanol	**-0.265629**	-0.0973123	-0.0869002	0.113997
4-methyl-1-pentanol	**-0.263791**	-0.0499861	-0.187816	**0.323461**
isoamyl acetate	**-0.258168**	0.0641179	0.0283668	-0.0617908
2-phenylethyl acetate	**-0.253598**	-0.00738981	-0.106767	**0.30985**
ethyl lactate	-0.173561	**-0.300656**	**0.218171**	0.13813
ethyl caprylate	**-0.209987**	**0.302417**	-0.0648962	-0.0557137
ethyl caprate	-0.183739	**0.291705**	**-0.253602**	0.00467175
diethyl succinate	**-0.24989**	0.141075	0.15587	-0.134882
ethyl 2-methylbutyrate	**-0.265854**	-0.13852	-0.02313	0.130058
ethyl isovalerate	**-0.259663**	-0.154587	0.0533505	0.0192544
diethyl malate	**0.214877**	**0.321398**	-0.118454	0.0783737
ethyl vanillate	**-0.226838**	**0.231098**	0.117079	**-0.264316**
syringol	**0.221319**	0.0908266	**0.259069**	**0.283588**
γ-nonalactone	**0.234303**	0.0432211	**-0.255525**	**0.331665**
benzaldehyde	-0.0625149	**0.263478**	**0.502083**	**0.37416**
methionol	-0.189482	-0.0394366	**-0.532235**	-0.145429

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Supplementary Material

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Table 1S. CAS numbers, m/z, odour thresholds, odour descriptors and odorant series of the selected volatile compounds.

Volatile compounds	CAS	m/z	Odour threshold (µg/L)	Odour descriptors	Odorant series[a]
Monoterpenes					
linalool	78-70-6	91, 93	15[b]	Orange flowers, citrus	1, 3
α-terpineol	10482-56-1	93, 121	250[c]	Lilac	3
β-citronellol	106-22-9	67, 81	100[d]	Rose, citrus	1, 3
C_{13}-Norisoprenoids					
β-damascenone	23726-91-2	105, 121	0.05[b]	Dry plum	2
β-ionone	79-77-6	177	0.09[c]	Violets	3
C_6-Alcohols					
1-hexanol	111-27-3	41, 69	8,000[b]	Grass	4
2-ethyl-1-hexanol	104-76-7	57, 70	8,000[e]	Floral, sweet	4
cis-2-hexen-1-ol	928-94-9	39, 67	400[b]	Grass	4
trans-3-hexen-1-ol	928-97-2	41, 67	1,000[f]	Green	4
cis-3-hexen-1-ol	928-96-1	39, 67	400[b]	Grass	4
Benzene derivatives					
benzyl alcohol	100-51-6	77, 108	200,000[d]	Walnut, fruity	1
benzaldehyde	100-52-7	77, 105	5,000[g]	Cherry	1, 2
guaiacol	90-05-1	109, 124	10[c]	Sweet, smoky	6
methyl vanillate	3943-74-6	151, 182	3,000[h]	Vanilla	6
vanillin	121-33-5	151, 152	60[d]	Vanilla	6
ethyl vanillate	617-05-0	196	990[d]	Honey, vanillin	
acetovainillone	498-02-2	151, 166	1,000[i]	Vanilla, clove	6
syringol	91-10-1	139, 154	570[i]	Smoky	6
methyl salicylate	119-36-8	92, 120	40[j]	Medicine	6
Aldehydes, Fusel Alcohols, and Acids					
phenylacetaldehyde	122-78-1	91, 92	5[k]	Rose	2, 3
2-phenylethanol	60-12-8	91, 92	10,000[b]	Rose	3
isoamyl alcohols	123-51-3	41, 55	30,000[b]	Alcohol	5
1-butanol	71-36-3	39, 41	150,000[d]	Alcohol	5
1-octanol	111-87-5	41, 56	10,000[g]	Rose, jasmine, citrus	1, 3
3-methyl-1-pentanol	589-35-5	41, 69	50,000[g]	Vinous, grass	4, 5
4-methyl-1-pentanol	626-89-1	41, 69	50,000[g]	Almond, toasted	2, 6
3-methyl-3-buten-1-ol	763-32-6	67, 68	600[e]	Fruity	1
methionol	505-10-2	47, 106	1,000[c]	Cooked potato, cabbage	4
isovaleric acid	503-74-2	60, 87	33[c]	Acid, rancid	7
Fatty Acids					
caproic acid	334-48-5	60	420[d]	Rancid fat	7
caprylic acid	124-07-2	60	500[c]	Sweat, cheese	7
capric acid	142-62-1	87, 129	1,000[d]	Sweat, rancid fat	7
Acetate Esters					
isoamyl acetate	123-92-2	43, 55	30[c]	Banana	2
hexyl acetate	142-92-7	43, 69	1,500[c]	Apple, pear, banana	3
2-phenylethyl acetate	103-45-7	104	250[b]	Rose	3
Ethyl Esters					
ethyl 2-methylbutyrate	7452-79-1	74, 102	18[c]	Strawberry, green apple	1
ethyl isovalerate	108-64-5	88, 101	3[c]	Forest fruits, blackberry	1
ethyl lactate	97-64-3	43, 45	154, 636[c]	Strawberry, raspberry, buttery	1, 7
ethyl caproate	123-66-0	88, 101	14[d]	Green apple, banana	1, 2
ethyl caprylate	106-32-1	55, 129	5[d]	Pineapple, strawberry	1, 2
ethyl caprate	110-38-3	157	200[c]	Sweet, fruity	2
ethyl laurate	106-33-2	55, 157	500[f]	Fruity, floral	2, 3
ethyl monosuccinate	1070-34-4	101, 128	1,000,000[h]	Caramel, coffee	2
diethyl succinate	123-25-1	101, 129	200,000[d]	Wine-like	5
diethyl malate	7554-12-3	117	760,000[d]	Over-ripe, peach	2
Lactones					
γ-nonalactone	104-61-0	85	30[c]	Coconut	2
Internal Standards					
2-octanol	123-96-6	45, 55			
4-hydroxy-4-methyl-2-pentanone	123-42-2	43, 59			
4-methyl-2-pentanol	108-11-2	41, 69			

[a] 1=Fresh fruit; 2=Ripe fruit; 3=Floral; 4=Herbaceous; 5=Vinous; 6=Spicy; 7=Lactic.

[b]Guth (1997); [c]Ferreira, López & Cacho (2000); [d]Etiévant (1991); [e]Tao & Zhang (2010); [f]Moyano, Zea, Moreno & Medina (2002); [g]Moreno, Zea, Moyano & Medina (2005); [h]García-Carpintero, Sánchez-Palomo & González-Viñas, (2011); [i]López, Aznar, Cacho & Ferreira (2002).; [j]Buttery, Seifert, Guadagni, & Ling, (1969); [k]Aznar, López, Cacho, & Ferreira, (2003).

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Table 2S. Oenological parameters of the wines obtained in absence and presence of the studied fungicides (average ± standard deviation).

PARAMETERS	CONTROL	IPROVALICARB 2MRL	IPROVALICARB 5MRL	MEPANIPYRIM 2MRL	MEPANIPYRIM 5MRL	TETRACONAZOLE 2MRL	TETRACONAZOLE 5MRL
Alcoholic degree (% vol)	13.73 ^{a} ± 0.63	13.95 ^{ab} ± 0.07	13.83 ^{a} ± 0.05	14.03 ^{ab} ± 0.03	14.04 ^{ab} ± 0.03	14.27 ^{b} ± 0.07	14.34 ^{b} ± 0.04
Acidity (g/L tartaric acid)	6.17 ^{a} ± 0.22	6.48 ^{b} ± 0.06	6.85 ^{c} ± 0.05	6.61 ^{b} ± 0.08	6.53 ^{b} ± 0.07	6.15 ^{a} ± 0.03	6.18 ^{a} ± 0.05
Volatile acidity (g/L acetic acid)	0.42 ^{a} ± 0.05	1.66 ^{d} ± 0.03	3.62 ^{e} ± 0.06	1.67 ^{d} ± 0.04	3.63 ^{e} ± 0.14	0.66 ^{b} ± 0.02	1.10 ^{c} ± 0.01
pH	3.43 ^{a} ± 0.02	3.46 ^{b} ± 0.01	3.49 ^{c} ± 0.01	3.45 ^{b} ± 0.01	3.46 ^{b} ± 0.01	3.42 ^{a} ± 0.01	3.45 ^{b} ± 0.01
Malic acid (g/L)	1.96 ^{d} ± 0.16	0.00 ^{a} ± 0.00	0.00 ^{a} ± 0.00	0.08 ^{a} ± 0.09	0.00 ^{a} ± 0.00	1.57 ^{c} ± 0.06	0.85 ^{b} ± 0.10
Lactic acid (g/L)	0.34 ^{a} ± 0.04	3.03 ^{d} ± 0.10	6.98 ^{e} ± 0.18	2.93 ^{d} ± 0.15	6.91 ^{e} ± 0.23	0.79 ^{b} ± 0.03	1.90 ^{c} ± 0.08
Glucose/fructose ratio	0.18 ^{a} ± 0.16	0.00 ^{b} ± 0.00	0.00 ^{b} ± 0.00	0.00 ^{b} ± 0.00	0.00 ^{b} ± 0.00	0.01 ^{b} ± 0.01	0.00 ^{b} ± 0.00
Dry extract (g/L)	24.05 ^{a} ± 0.58	27.45 ^{c} ± 0.35	32.70 ^{d} ± 0.54	27.07 ^{bc} ± 0.55	33.52 ^{e} ± 0.29	24.55 ^{a} ± 0.37	26.30 ^{b} ± 0.21

Different letters (a, b, c, d, and e) refer to statistically significant differences according to ANOVA and Tukey's HSD tests ($p < 0.05$).