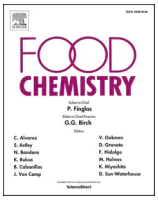
Food Chemistry 403 (2023) 134324

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Contribution of critical doses of iprovalicarb, mepanipyrim and tetraconazole to the generation of volatile compounds from Monastrell-based wines

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ARTICLE INFO

Keywords:
Fungicides
Critical conditions
Aromatic profile
Oenological parameters
Multivariate analysis

ABSTRACT

The individual effects of iprovalicarb, mepanipyrim, and tetraconazole on the volatile composition and aromatic profile of Monastrell-based wines were evaluated. To date, no studies about the effect of these fungicides on Monastrell-based wines are available, and the effect on other grape varieties is also unknown. Fungicides were added separately in the cellar to the grape must at two concentration levels (4 and 10 mg/kg for iprovalicarb and mepanipyrim and 1 and 2.5 mg/kg for tetraconazole). The aromatic composition of the final wines was analysed by gas chromatography using flame ionisation and ion trap mass selective detectors.

In the presence of fungicides, the most significant variations were observed for isoamyl acetate and 2-phenylethyl acetate (increasing between 20 and 43% compared with the control wine) and ethyl caprate and caprylate (increasing between 12 and 68%). Consequently, treated wines showed a higher global odourant intensity, with increased fresh fruit notes.

1. Introduction

The proper protection of wine grapes is the most critical factor in obtaining an excellent wine. Fungal diseases remain one of the main problems for the wine sector, and the application of different fungicides (such as iprovalicarb, mepanipyrim, and tetraconazole) is a commonly adopted measure to fight against them. However, fungicides are also modulators of the biochemical activity of yeasts. 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, et al., 2014), and alter the secondary metabolism of yeasts (Dzedze et al., 2019). These changes can occur even when the doses of fungicides and the safety periods are 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, et al., 2009; González-Rodríguez, González-Barreiro, et al., 2011; González-Rodríguez, Noguerol-Pato, et al., 2011).

The effect of mepanipyrim and tetraconazole, applied as active substances or commercial formulations, on the volatile composition of wines from different cultivars (*Vitis vinifera* var. Mencía, Tempranillo and Graciano) was previously evaluated at a laboratory and at a medium scale in an experimental cellar (Noguerol-Pato, Sieiro-Sampedro, et al., 2014; Noguerol-Pato et al., 2015; Noguerol-Pato et al., 2016; Sieiro-Sampedro, Figueiredo-González, et al., 2019; Sieiro-Sampedro, Pose-Juan, et al., 2019; Sieiro-Sampedro, Briz-Cid, et al., 2020). However, to the best of our knowledge, the effect of iprovalicarb was only evaluated in a Godello vineyard under good agricultural practices (GAPs) using a commercial formulation containing fosetyl-Al and mancozeb as other active substances (González-Rodríguez, Noguerol-Pato, et al., 2011). To date, the effect of several commercial formulations containing exclusively fenarimol, mancozeb, vinclozolin, metalaxyl, fenhexamide, fluquiconazole, quinoxyfen, kresoxim-methyl, and trifloxystrobin as active substances was evaluated on *Vitis vinifera* var. Monastrell—one of the leading Spanish grape varieties and most representative of the

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https://doi.org/10.1016/j.foodchem.2022.134324
Received 2 February 2022; Received in revised form 13 September 2022; Accepted 15 September 2022
Available online 19 September 2022

Designation of Origin Jumilla (Oliva et al., 2015; Oliva et al., 2008; Oliva et al., 1999).

According to the studies mentioned above, the variations in the content of both varietal and fermentative volatile compounds are dependent on multiple factors (type of fungicide, fungicide concentration, grape variety, yeast strains, and winemaking conditions). Therefore, we can hypothesise that the effects observed for one grape variety could not be extrapolated to other varieties, and different active substances could produce noncomparable effects.

This work aims to assess the independent effect of iprovalicarb, mepanipyrim, and tetraconazole on the volatile composition and aromatic profile of Monastrell-based wines throughout the winemaking process to control the quality of these wines. In addition, the changes produced by these substances on the aromatic profile of the final wines are explained, using our experience in previous assays as a basis. In the present study, we focus only on two variables: the 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 the Monastrell cultivar were separately fortified with the target fungicides in the cellar before alcoholic fermentation. Two critical concentration levels were evaluated, corresponding to twice and five times their maximum residual levels (MRLs) in grapes as set by Regulation (EC) No 396/2005 and later amendments (2, 2 and 0.5 mg/kg for iprovalicarb, mepanipyrim, and tetraconazole, respectively).

2. Materials AND METHODS

2.1. Monastrell grape samples

Red grapes of the *Vitis vinifera* Monastrell variety were harvested in October 2016 in a vineyard (38° 33′ 19.7″ N, 1° 21′56.6″ W) located in Jumilla (Murcia, Spain). The climatic conditions for the 2015/2016 season were a total rainfall of 250.4 L/m^2 and maximum and minimum temperatures of 40.0 and −0.8 °C, respectively.

The amino acid content of the grape must (Table 1S of the Supplementary material) was determined through liquid chromatography after sample derivatisation following the method described by Oliva et al. (2011). Derivatization of amino acids was conducted by a reaction of 1.75 mL of borate buffer 1 M (pH = 9), 750 μL of methanol (Merck, Darmstadt, Germany), 1 mL of sample (previously filtered), 20 μL of internal standard (1 g/L of 2-aminoadipic acid, Sigma-Aldrich, Gillingham, England) and 30 μL of the derivatisation reagent diethyl ethoxymethylenemalonate (DEEMM, Sigma-Aldrich). The derivatisation reaction was conducted in a screw-cap test tube over 30 min in an ultrasound bath. The sample was then heated at 70–80 °C for 2 h to allow complete degradation of excess DEEMM and reagent byproducts. Chromatographic analyses were performed on an Agilent 1100 HPLC (Palo Alto, USA) equipped with a photodiode array detector and an ACE C18-HL column (250 mm × 4.6 mm; 5 μm) from AVANTOR (Aberdeen, Scotland). The chromatographic conditions are described in Oliva et al. (2011).

In addition, grape characterisation (sugar content, pH, total acidity, malic acid and gluconic acid content) was performed using an Enological Multiparametric Analyzer Bacchus FTIR-Vis-UV MultiSpec (Tecnología Difusión Ibérica, Barcelona, Spain). MultiSpec is an automatic grape must and wine analyser based on chemometric techniques that incorporates two different spectrophotometric modules: one for the Fourier transform medium infrared spectrum (Thermo Nicolet Avatar 380) and the other for the UV–Vis spectrum (Avantes AvaSpec). The grape must (10 mL) was placed in the autosampler, filtered through an inner filter (30–50 μm) and thermostated at 25 °C before analysis. The grape characterisation results are shown in Table 2S of the Supplementary material.

2.2. Fungicide experiments and winemaking process

The individual effects of three fungicide-active substances were evaluated: iprovalicarb (abbreviated hereafter as Ipro), mepanipyrim (abbreviated as Mepa) and tetraconazole (abbreviated as Tetra). The analytical standards of the fungicides Ipro, Mepa, and Tetra (pestanal grade, purity ≥ 99%) were purchased from Supelco (Bellefonte, PA, USA). Information about the target fungicides (chemical family, CAS number, structure, mode of action, and MRLs in grapes) can be found in Table S3 of the supplementary material.

In triplicate, different microvinification assays (control, A, B, C, D, E, and F) were performed in the experimental cellar. The winemaking process was developed under the same conditions for all experiments. Briefly, approximately 200 kg of Monastrell grape samples were jointly destemmed and crushed in the cellar, and then 8 kg of the obtained grape must was transferred into 21 ((6 fungicides treatments + control) × 3 replicates) metallic fermentation vessels (15 L). The grape must was supplied with SO_2 at 80 mg/L and spiked with the fungicide solutions in ethanol. For Experiments A, C, and E, the grape must was separately fortified with Ipro, Mepa, and Tetra at concentrations of 4, 4, and 1 mg/kg, respectively (concentrations equivalent to twice their MRLs, 2MRL). For Experiments B, D, and F, grape must was separately spiked with the same fungicides at 10, 10, and 2.5 mg/kg, respectively (five times their MRLs, 5MRL). In addition, a control experiment without fungicides was performed for comparative purposes.

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 occurred at temperatures below 18 ± 2 °C (controlled by recirculating water) for ten days, the mixtures were homogenised once a day. Temperature and density (sugar percentage) were measured to control the fermentation evolution and possible stoppages or delays in the fermentation process (Table 4S of the Supplementary material). After this period, the wines were strained off, and grape residues were 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 gelatine (8 g/hL) for six days, and then the wines were filtered (0.45 μm). To stabilise the obtained wines, SO_2 (30 mg/L) was added before bottling.

Oenological parameters of the final wines (alcohol content, total and volatile acidity, pH, malic and lactic acid content, glucose/fructose ratio and dry extract) were determined in an Enological Multiparametric Analyser Bacchus FTIR-Vis-UV MultiSpec using 10 mL of the clarified wine, as previously described in Section 2.1.

2.3. Volatile determination

Individual stock solutions (approximately 30,000 mg/L) of the target volatile compounds were prepared by weighing the chemical standards (Table 4S of the Supplementary material) and dissolving them in absolute ethanol (Scharlau, Barcelona, Spain). Mixed dilutions (ca. 1000, 10, 5 and 1 mg/L) were prepared by diluting in ethanol and grouping the volatile compounds by chemical family. Three internal standards (IS) were used for quantification: 4-methyl-2-pentanol and 4-hydroxy-4-methyl-2-pentanone for major compounds and 2-octanol for minor compounds. Individual stock solutions (approximately 30,000 mg/L) of the IS were prepared in absolute ethanol. A mixed solution of the three ISs was also prepared by dilution in ethanol (50 mg/L of 2-octanol, 1,000 mg/L of 4-methyl-2-pentanol, and 10,000 mg/L of 4-hydroxy-4-methyl-2-pentanone). All solutions were stored in the dark at −20 °C.

The analysis of the volatile compounds was performed within the first six months after the winemaking process was completed, before June 2017. Major compounds were determined by direct injection of red wines in a gas chromatograph equipped with a flame ionisation detector (GC-FID) from Thermo Fisher Scientific (Waltham, MA, USA) and an HP-

INNOWAX (60 m × 0.25 mm i.d., 0.25 μm) analytical column from Agilent Technologies (Santa Clara, CA, USA) following the method described by Peinado et al. (2004). Briefly, red wines (10 mL) containing 1 mL of the internal standard dilution (1000 mg/L of 4-methyl-2-pentanol) were sonicated for 15 s with $CaCO_3$ (0.2 g) and then centrifuged at 3500 rpm for 5 min. Chromatographic conditions and the oven temperature programme were previously described by González-Álvarez et al. (2012).

Minor compounds were extracted from wines by a solid-phase extraction procedure described in González-Álvarez et al. (2012). Briefly, wine samples (50 mL) containing 25 μL of the surrogate (100 mg/L of 4-nonanol) were loaded in Strata-X 33 μm cartridges 500 mg/6 mL (Phenomenex, Torrance, CA, USA) previously conditioned with methanol and water. After drying the cartridge, volatile compounds were eluted with dichloromethane (10 mL). The eluate was dried over anhydrous sodium sulfate, concentrated to < 1 mL under a N_2 stream, enriched with 20 μL of an internal standard mixture and brought to 1 mL with dichloromethane prior to gas chromatographic analysis. 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 × 0.25 mm i.d., 0.25 μm). Chromatographic conditions and the oven temperature programme were previously described by 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 6S of Supplementary material).

2.4. Statistical analyses

One-way ANOVA and Tukey's HSD test were performed 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 performed using Statgraphics Centurion XVI software 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 visualisation of the dataset 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 component (PC) 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. Dissipation of the fungicide residues throughout the steps of this winemaking process was previously described in Briz-Cid et al. (2019). Briefly, the final fungicide concentrations in wine were 2.60 ± 0.05 mg/kg and 5.99 ± 0.05 mg/kg for Ipro at 2MRL and 5MRL, respectively (72–74% reduction in mass units), 0.25 ± 0.02 mg/kg and 0.68 ± 0.04 mg/kg for Mepa (approximately 97%), and 0.21 ± 0.03 mg/kg and 0.47 ± 0.04 mg/kg for Tetra (91–92%). As expected, fungicide 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, significant differences ($p < 0.05$) were observed in some oenological parameters (Table 7S of Supplementary Material). The volatile acidity increased in all wines (between 4.0 and 8.6 times for Ipro and Mepa and approximately 1.6–2.6 times for Tetra). Moreover, the concentration of malic acid was reduced with all fungicide treatments, mainly with Ipro and Mepa (values lower than 0.08 g/L) compared with the control wine (1.96 g/L). In contrast, the lactic acid concentration increased in a dose-dependent manner (approximately 2 and 7 g/L for both fungicides at 2MRL and 5MRL, respectively) compared with the control (0.34 g/L). The malic acid content increased, and the lactic acid content also decreased in the presence of Tetra, although to a lesser extent. In light of these results, it can be concluded that spontaneous malolactic fermentation occurred in wines made with grapes supplemented with fungicides in a dose-dependent manner, especially Ipro and Mepa. This fact could be related to the critical doses of fungicides extending the lag phase period for *S. cerevisiae* T73™ and allowing the development of other opportunistic microbiota (such as lactic and acetic acid bacteria).

Indeed, effects on the viability and metabolism of yeasts induced by the presence of Mepa and Tetra residues were previously described in the bibliography at a laboratory scale using synthetic must and pasteurised grape must (Cabras et al., 1999; Noguerol-Pato, Torrado-Agrasar, et al., 2014; Sieiro-Sampedro, Briz-Cid, et al., 2020; Sieiro-Sampedro, Alonso-del-Real et al., 2020). Moreover, effects were reported for other fungicides (pyrimethanil, tebucanozole, bentiavalicarb-isoporopil) belonging to the same chemical families and with the same mode of action (Cabras et al., 1999; Čuš and Raspor, 2008; González-Rodríguez, Cancho-Grande, Torrado-Agrasar, et al., 2009; Gil et al., 2014; Noguerol-Pato, Torrado-Agrasar, et al., 2014).

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

The average concentration values of 46 volatile compounds resulting from the transformation of volatile grape precursors or the metabolism of yeasts are listed in Table 1. One-way ANOVA and Tukey's HSD test were chosen as the statistical techniques to find similarities and differences between the aroma profiles of treated and control wines. Although many significant differences were observed between treated and control wines, only the variations in 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, participating as intermediates of 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 performed by dioxygenases that cleave double bonds in carotenoids (Rambla et al., 2016). In general, the concentrations of monoterpenes and C_{13}-norisoprenoids detected in Monastrell-based wines did not change after fungicide supplementation (Table 1), except for β-citronellol, which had decreased contents (approximately 50%) in all experiments regardless of the type and concentration of fungicide added.

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 the lipoxygenase pathway (Mozzon et al., 2016). In this work, 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).

Although the complete metabolic pathways of volatile benzenoids are still not totally understood, benzyl alcohol is formed in plants during phenylpropanoid synthesis by phenylalanine ammonia-lyase (Martin

Table 1
Volatile compounds in Monastrell-based wines obtained after iprovalicarb, mepanipyrim and tetraconazole supplementation. Values are expressed as the average ± standard deviation (μg/L). Remarked values referred to variations higher than 30% in the volatile content concerning the control wine.

Volatile Compounds	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, higher 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

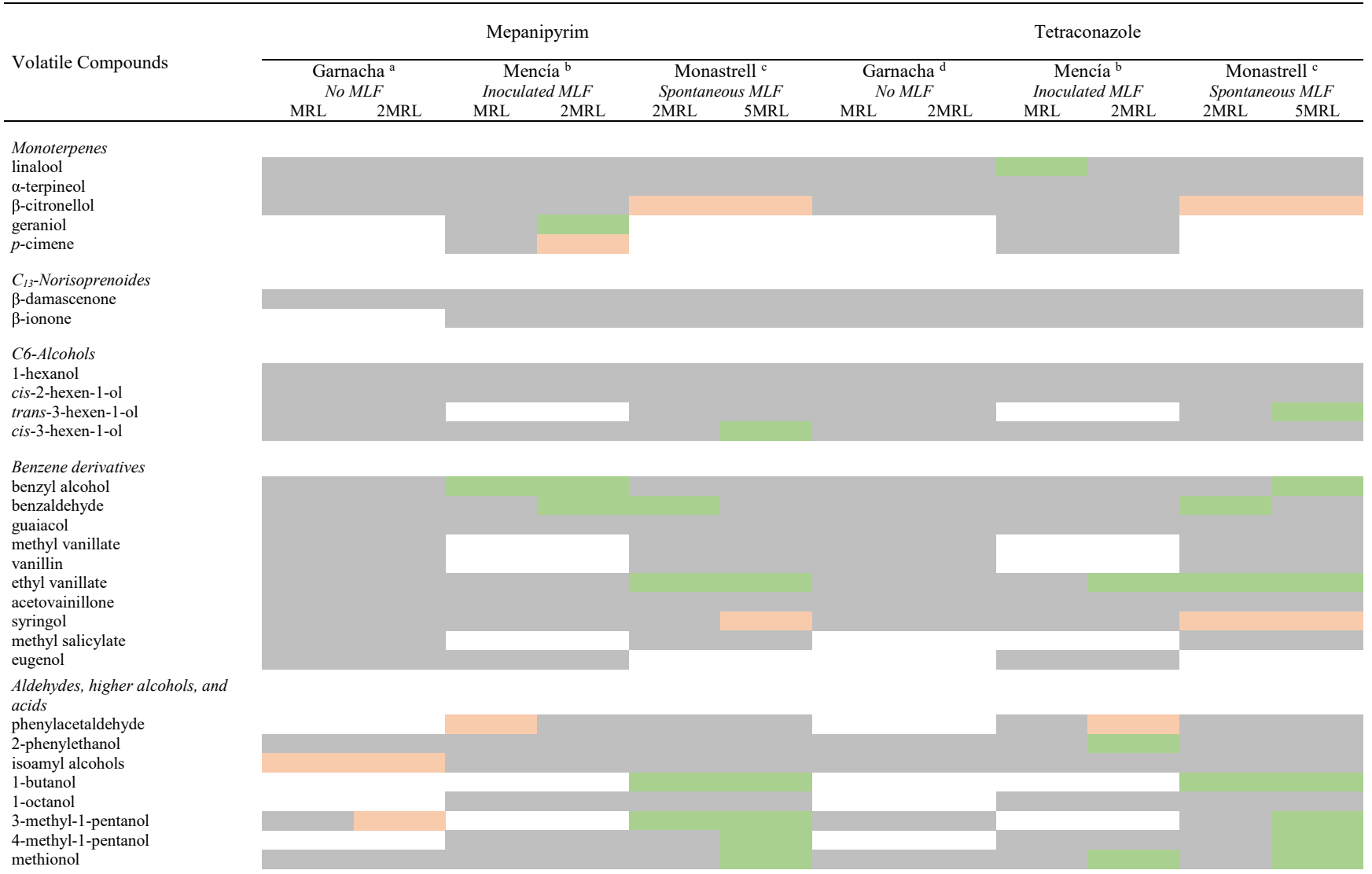
(*continued on next page*)

Table 1 (*continued*)

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

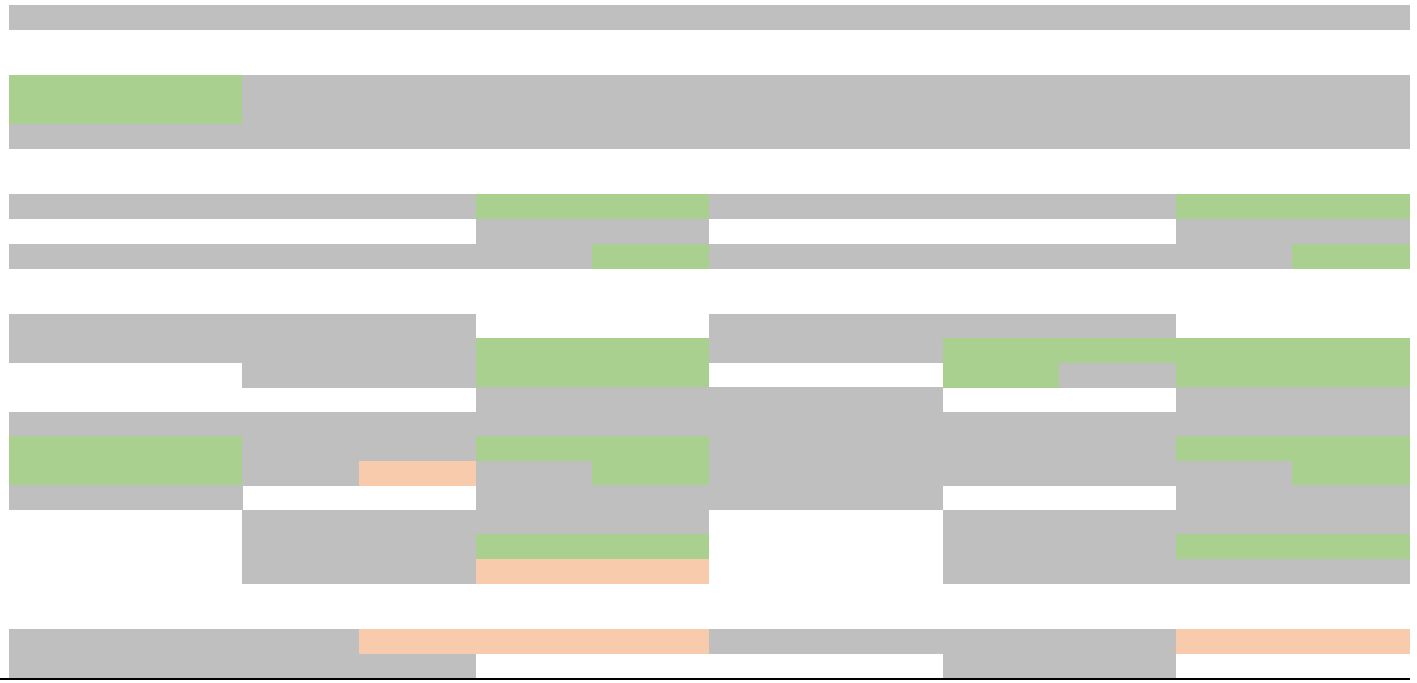
* Values are expressed as average ± standard deviation (mg/L).
Different letters (a, b, c, d, e) refer to significant differences according to the ANOVA and Tukey's HSD tests ($p < 0.05$).

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:** increase in content compared with the control wine; **orange:** decrease in content compared with the control wine.



(*continued on next page*)

Table 2 (*continued*)



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

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

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

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

et al., 2016). This enzyme catalyses the conversion of phenylalanine to *trans*-cinnamic acid, which is subsequently converted into benzyl alcohol and other derived compounds. In this group, four compounds underwent important changes. The concentration of benzyl alcohol increased between 22 and 31% after the addition of Tetra. However, the benzaldehyde content increased with all antifungal treatments, and these increases were significant ($p < 0.05$) (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 the wines treated with both levels of Tetra and the highest concentration of Ipro and Mepa (5MRL). Additionally, a slight decrease in the concentrations 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 (Noguerol-Pato et al., 2015; Noguerol-Pato et al., 2016; Sieiro-Sampedro, Briz-Cid, et al., 2020; Sieiro-Sampedro, Figueiredo-González et al., 2019; Sieiro-Sampedro, Pose-Juan et al., 2019). For comparative purposes, Table 2 summarises 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 to 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. In contrast, the effect of both fungicides on the concentration of some benzene derivatives was previously registered in medium-scale assays using Mencía (Sieiro-Sampedro, Figueiredo-González et al., 2019).

Varietal compounds are secondary products of plant metabolism that are 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). Aglycone forms can be released from precursors by hydrolysis catalysed by mild acid or glycosidase enzymatic activity (Williams, 1993; Belda et al., 2017). As fungicides were added in the cellar to the crushed and destemmed grapes, fungicides could not alter the biosynthesis of aroma precursors in grapes. Moreover, as the pH values were similar in all wines (between 3.42 and 3.49), those changes observed in the varietal fraction (Table 1) could be attributed to the effect of fungicides on the activity of endogenous grape-derived glycosidases, exogenous yeast-derived glycosidases, and bacterial glycosidases during the fermentation process. However, enzymatic activity assays should be performed to confirm this hypothesis.

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 discussed. The flavour metabolites produced by yeast during fermentation 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 lies in being the most abundant volatile components produced during fermentation; thus, they significantly impact 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). Yeasts also produce higher alcohols from amino acid catabolism via the *Ehrlich pathway* (Dzialo et al., 2017; Hirst & Richter, 2016).

As 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 >30% in the most important volatiles of this family (*i.e.,* isoamyl alcohols, 2-phenylethanol, and isovaleric acid). In addition, three minor compounds, 1-octanol, 3-methyl-3-buten-1-ol, and phenylacetaldehyde, were not modified either.

In our previous studies, isoamyl alcohols remained unchanged when different grape varieties (and consequently different microbial ecosystems and medium compositions) were contaminated after harvesting with Mepa and Tetra active substances (Table 2). However, in laboratory-scale assays with pasteurised must, the addition of Mepa decreased the content of isoamyl alcohols (Noguerol-Pato, Torrado-Agrasar, et al., 2014; Sieiro-Sampedro, Pose-Juan et al., 2019). In contrast, García and coworkers observed an increase in their content in laboratory-scale assays performed in the presence of cyprodinil and fludioxonil (two fungicides with the same mode of action as Mepa) (García et al., 2004). Moreover, increases 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 GAPs (Dzedze et al., 2019; González-Rodríguez, González-Barreiro, et al., 2011; Noguerol-Pato et al., 2015; Noguerol-Pato et al., 2016) or Monastrell grapes treated under critical agricultural practices (CAPs) (Oliva et al., 2015).

As reflected in Table 2, the effect of fungicides on the 2-phenylethanol content was dependent on the grape variety and type of fungicide. 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 increase 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 increase 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 with the control wine. In part, this effect could be related to differences in grape composition, especially in the content of the amino acids.

In contrast, 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 increases 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. As methionine is found in relatively low concentrations in Monastrell grape must (<0.09% w/w), yeasts are required to assimilate inorganic sulfur via the sulfate reduction pathway, where methionine is remetabolised to produce methionol via the Ehrlich pathway (through transamination to form α-keto-γ-(methylthio)-butyrate and the subsequent production of methional and finally methionol) (Dzialo et al., 2017). Lactic acid bacteria can also metabolise methionine during malolactic fermentation, forming volatile sulfur compounds (Inês & Falco, 2018). In this sense, it is essential to remember that a secondary malolactic fermentation occurred spontaneously in the presence of the studied fungicide residues. Previous works attributed the promotion of methionol content in Mencía wines spiked with high doses (2MRL) of Tetra to an increase in 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 supply with the participation of glucose as a precursor (Sieiro-Sampedro, Figueiredo-

González et al., 2019). Furthermore, considering 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.

Increases in the content of some higher alcohols (including 2-methylpropanol, 3-methylbutanol, and 1-octen-3-ol) were also reported after treating vineyards with commercial formulations incorporating flusilazole (Aubert et al., 1997), fenarimol, penconazole (Oliva et al., 1999), fenhexamid, and flunquinonazole (Oliva et al., 2008) as active substances. All of 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 yeast cells (Styger et al., 2011). They are byproducts of saturated fatty acid metabolism. This complex process is catalysed by the multienzymatic complex (fatty acid synthetase) using acetyl-CoA and malonyl-CoA as substrates to produce palmitic acid (C_{16}). Afterwards, this acid can be used to produce other fatty acids with shorter chains (Moreno-Arribas & Polo, 2009).

In general, the addition of any of the three active substances at critical levels did not affect the concentration of C_6-, C_8-, and C_{10}- acids (Table 1). Similar results were obtained in Mencía wines after adding 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, Sieiro-Sampedro, et al., 2014). No effects on the concentration of fatty acids were 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 the highly desired fruity and flowery-like aroma of wines (Saerens et al., 2010). Esters are mainly synthesized in the cytoplasm of yeasts during 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 ageing of wines (Belda et al., 2017).

3.2.2.4. Acetates. Acetate esters result from the reaction of acetyl-CoA with higher alcohols (Styger et al., 2011). This reaction is catalysed by alcohol acetyltransferases (Atf1p and Atf2p, encoded by the *ATF1* and *ATF2* genes). Two acetates, of major importance as aromatic constituents, were overproduced compared with the control wine in all treatments (Table 1): isoamyl acetate (between 26% and 43% increase in concentration) and 2-phenylethyl acetate (between 20% and 36% increase in concentration). Under stressful conditions, yeasts can respond by producing 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 alcohol acetyltransferases are the most significant factor determining the acetate ester levels during fermentation (Pires et al., 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 increase in acetate levels could be attributed to enhancing the activity of Atf1p and/or Atf2p enzymes. In fact, overexpression of the *ATF2* gene of the *S. cerevisiae* T73™ strain was observed after 48 h of must fermentation in the presence of a commercial Tetra formulation (Sampedro, Alonso-del-Real et al., 2020).

An increase 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 et al., 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 pasteurised must (Noguerol-Pato et al., 2014). Consequently, the grape variety, type and concentration of fungicide, and winemaking process could be the limiting factors in the biosynthesis of acetates.

3.2.2.5. Ethyl esters. Ethyl esters are formed from the ethanolysis of acyl-CoA, an intermediate metabolite of fatty acid metabolism. The ethanol radical is derived from ethanol, and the acid group is 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, fatty acid precursor levels rather than the activity of biosynthetic enzymes are the primary factor limiting their production (Saerens et al., 2008; Saerens et al., 2010). This could be the cause of the significant increase ($p < 0.05$) (between 31 and 112%) experienced by 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 and was significantly increased ($p < 0.05$) for Mepa (5MRL). Increases 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, Tetra and ethyl caprate at the highest dose assayed for both fungicides (Table 1). As the concentration of their fatty acid precursors remained invariable, target fungicides could be assumed to also regulate the activity of acyltransferase enzymes. Increases in 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 increases were correlated with a higher concentration of fatty acids compared with the control wine (Table 2). The opposite trend was observed by Noguerol-Pato et al. (2016); Noguerol-Pato, Sieiro-Sampedro, et al. (2014) in Graciano and Tempranillo red wines after adding a commercial formulation of Mepa. Applying this formulation to vineyards provoked a general decrease in the content of esters. In addition, an increase in the ethyl lactate content (between 26% and 45%) was found with the addition of Ipro and Mepa, which was significant ($p < 0.05$) only for Ipro 5MRL. This increase could be related to a higher lactic acid concentration registered in these wines due to malolactic fermentation (Table 6S of Supplementary Material).

Finally, some ethyl esters of organic acids also suffered significant modifications ($p < 0.05$) (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 significant, $p > 0.05$) in the concentration of diethyl malate. 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 the MRL.

3.2.2.6. Lactones. A cyclic ester group characterises 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 ageing, and as byproducts from the metabolism of pantothenic acid. In particular, 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 they were lower than those of 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).

3.2.2.7. Multivariate analysis. PCA was chosen as a multivariate unsupervised method to identify general trends by grouping samples with certain similarities. A standardised matrix data was constructed with the measured variables (in this case, the 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. Using a factor loadings analysis (Table 3), PC1 retained 50.80% of the data variation and differentiated the wine samples according to the contents of the following compounds with factor loadings higher than |0.21|: ethyl 2-methylbutyrate, 3-methyl-1-pentanol, 4-methyl-1-pentanol, ethyl isovalerate, isoamyl acetate, β-citronellol, 2-phenylethyl acetate, diethyl succinate, 1-butanol, γ-nonalactone, syringol, and diethyl malate. Similarly, PC2 explained another 18.54% of the variability in the original responses and separated the Monastrell-based wines based on benzyl alcohol, *trans*-3-hexen-1-ol, diethyl malate, ethyl caprylate, ethyl lactate, ethyl caprate, benzaldehyde, and ethyl vanillate. PC3 and PC4 explain only 13.6% of the data variance.

Fig. 1 shows the biplot of the first two principal components (PC1 *vs* PC2). As expected, samples from control wines and all fungicide treatments were clearly separated along PC1. Control wines located on the right side of PC1 were positively correlated with higher mean values of those volatile compounds with positive factor loadings, as shown in Table 3 (diethyl malate, syringol, β-citronellol, and γ-nonalactone). However, the treated wines located on the left side of PC1 were positively correlated with the volatile compounds with negative factor loadings (ethyl 2-methylbutyrate, 3-methyl-1-pentanol, 4-methyl-1-pentanol, ethyl isovalerate, isoamyl acetate, 2-phenylethyl acetate, diethyl succinate and 1-butanol). In addition, different groups could be identified between treatments:

- *Grouping by type of fungicide:* using PC2, the 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 the 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).

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

To make a tentative approximation of the organoleptic profile of wines from the quantitative data provided by the chromatographic analysis, volatile compounds with similar odour descriptors were grouped into seven odourant series characterised by a generic descriptor (Table 5S of Supplementary Material). The total OAV of each odourant 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 is its odour threshold value).

The changes previously described in the concentrations of most of the analysed volatile compounds resulted in a significant increase ($p < 0.05$) in the global odorant intensity of the wines obtained in the presence of fungicides (from a value of 328 for the control wine to 379–397 for treated wines) (Fig. 2). This difference is mainly due to the increase in the fresh fruit series, which involves compounds whose concentrations were significantly higher in fortified wines, especially ethyl esters

Table 3
Factor loadings of the volatile compounds for illustrating the interpretation of Fig. 1.

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

Note: Bold numbers are factor loadings higher than |0.21|.

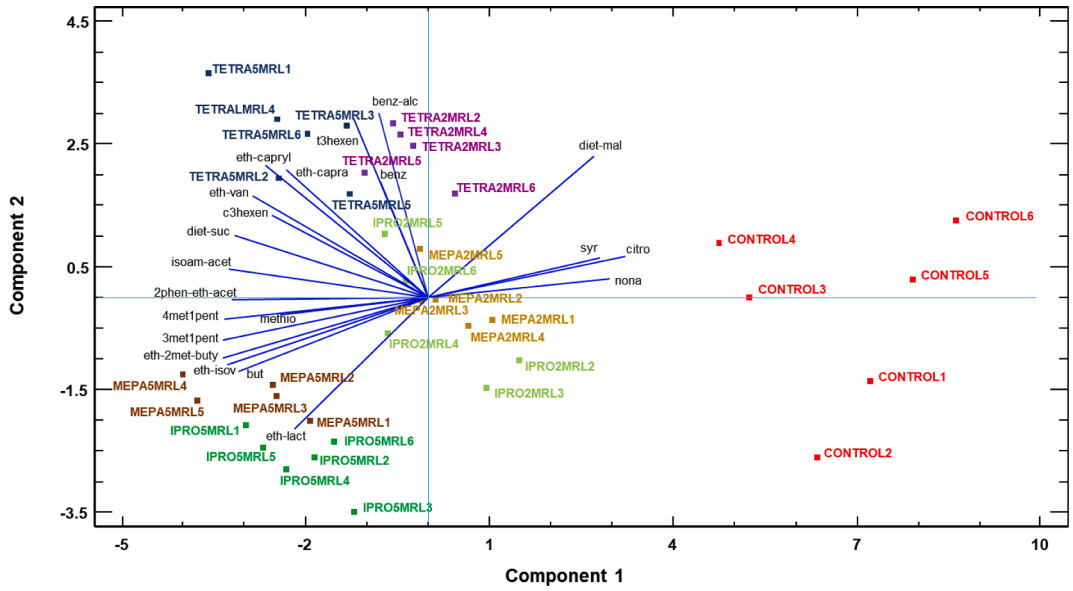


Fig. 1. Distribution of the studied wines (control and treated wines) in a biplot system defined by the first two principal components (Component 1 *vs* Component 2).

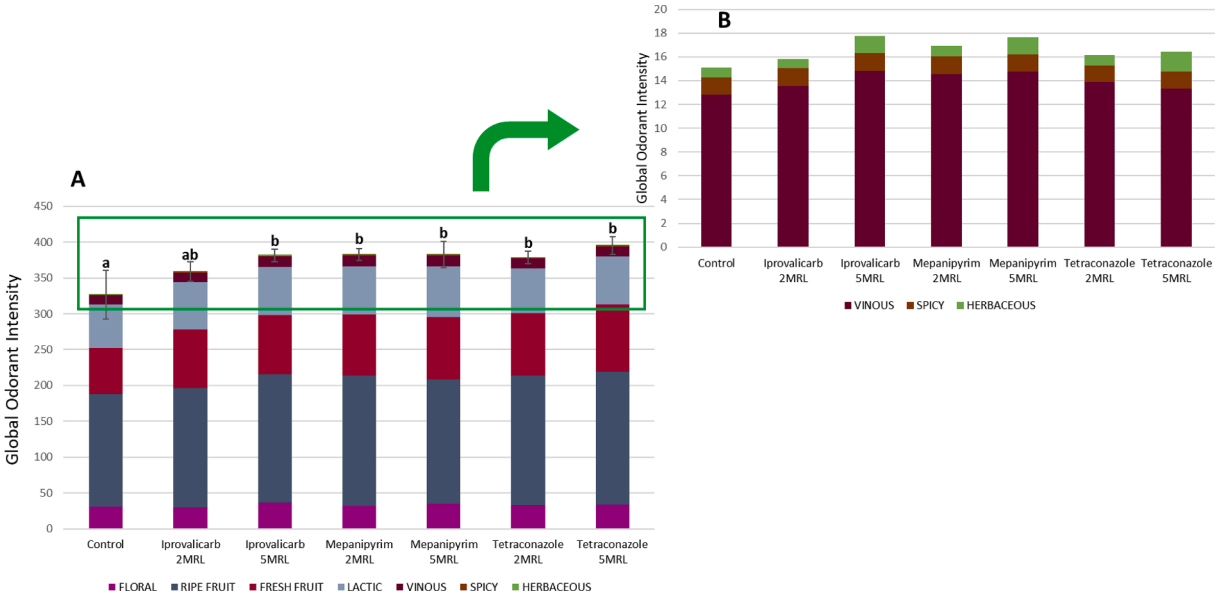


Fig. 2. A. Global odorant intensity of the studied wines obtained in the absence and presence of fungicides. Different letters (a and b) refer to significant differences according to ANOVA and Tukey's HSD tests ($p < 0.05$). B. Zoom for better visualisation of vinous, spicy and herbaceous series.

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 increase registered for the sulfur compound methionol at the highest dose assayed (5MRL) also significantly increased herbaceous nuances. The remaining odourant series had levels comparable to those of the control wine but together helped to increase the overall odour activity value.

4. Conclusions

The individual effects of Ipro, Mepa and Tetra on Monastrell-based wines were evaluated for the first time. Although the supplementation of Monastrell grape must with the target fungicides at critical doses (2MRL and 5MRL) did not promote fermentative stoppages, some oenological parameters were altered (volatile acidity and the malic acid and lactic acid content), especially in the presence of Ipro and Tetra. Moreover, the volatile profile of Monastrell-based wines obtained after fungicide supplementation showed significant variations concerning the control wines. Major changes were observed in the fermentation-derived volatile compounds. Higher alcohols (1-butanol, 3-methyl-1pentanol, 4-methyl-1-pentanol, and methionol), acetate esters (isoamyl acetate and 2-phenylethyl acetate), and ethyl esters (ethyl 2-methylbutyrate, ethyl isovalerate, ethyl caprylate, ethyl caprate, and diethyl succinate) showed increased content mainly in the presence of Mepa > Ipro > Tetra, with this increase being higher at the highest dose assayed (5MRL). In contrast, varietal compounds resulting from grape precursor biotransformations were affected mainly by Tetra > Mepa > Ipro in a dose-dependent manner. Comparing the obtained results with those published previously, the hypothesis was confirmed; the effect of fungicides on the sensorial properties of wine depends on the grape variety, the active substance considered, and the concentration used.

A comprehensive data exploration by PCA was also applied. The PCA

model working with the refined set indicated that approximately 68% of the information was captured with two PCs, giving an extraordinary differentiation between control wines and the rest of the treated samples. In addition, it was possible to separate the wines treated with Tetra from those treated with Mepa and Ipro. Wines with different concentrations for the same fungicide treatment were also clearly separated.

Funding

This work received financial support from the European Union FEDER fund and the Spanish national projects: AGL2015-66491-C2-1-R and PID2019-105061RB-C21.

CRediT authorship contribution statement

Thais Sieiro-Sampedro: Methodology, Validation. **María Figueiredo-González:** Methodology, Validation. **Raúl Garzón-Vidueira:** Methodology, Writing – review & editing. **Beatriz Cancho-Grande:** Supervision, Formal analysis. **Carmen González-Barreiro:** Conceptualization, Formal analysis, Writing – original draft. **Miguel A. Cámara:** Resources, Visualization. **José Oliva:** Resources, Visualization. **Raquel Rial-Otero:** Conceptualization, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

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.

Data availability

No data was used for the research described in the article.

Acknowledgments

M. Figueiredo-González would like to thank the University of Vigo for her postdoctoral contract. Funding for open access charge was provided by the University of Vigo/CISUG".

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134324.

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