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ABSTRACT

The impact of mepanipyrim (Mep) and its corresponding commercial formulation (Mep Form) on *Saccharomyces cerevisiae* metabolites was assessed, separately, by using laboratory-scale wine fermentation assays on pasteurized red must. The presence of Mep did not alter the fermentation course. With regard to volatiles formed at intracellular level by fermenting yeast cells, Mep residues affected mainly the acetate and ethyl ester biochemical pathways. In particular, the target acetates showed a notorious increment, more than 90 %, in presence of commercial Mep Form at the higher dose assayed. The addition of Mep and Mep Form, at both tested levels, highly increased ethyl caprylate (between 42-63 %) and ethyl caprate (between 36-60 %) contents as the same as their respective fatty acid precursors. No important effects were observed on colour and non-volatile pyranoanthocyanins, probably due to the low anthocyanin content characteristic of pasteurized musts.

***Keywords*:** fungicide; yeasts; aroma profile; red wine

1. Introduction

In traditional wine cellars occur spontaneous alcoholic fermentations associated with endogenous yeasts, present on the surface of grapes but also associated with the surfaces of winery equipment and the winery environment, being *S. cerevisiae* the dominant active yeasts when the fermentation proceeds (Belda et al., 2017; Fleet, 2003). Nowadays, most wineries inoculate selected dry yeasts to increase the initial population of *S. cerevisiae* in order to guarantee alcoholic fermentations without any deviation. Selected dry yeasts are able to resist high ethanol and sugar concentrations, wide range of temperatures, low pH levels, oxidative and osmotic stress, high sulphur dioxide concentrations and fungicides at trace levels (Gamero, 2011; Sipiczki, 2008).

The anaerobic fermentation of sugars by *Saccharomyces* wine yeasts contributes to the wine quality through several mechanisms: (i) using grape juice constituents; (ii) producing ethanol and other alcohols which are able to extract flavour components from grape solids; (iii) producing enzymes which transform neutral grape compounds into flavour active compounds; and (iv) producing secondary fermentation-derived compounds such as volatile and non-volatile compounds which contribute to the wine character (Bell & Henschke, 2005; Fleet, 2003).

Several factors might affect wine yeasts during the alcoholic fermentation including fungicide residues applied to vineyard which persist at trace levels in grapes and could be transferred to musts and finally detected in wines (Cabras et al., 1998; Cabras & Conte, 2001; Fernández, Oliva, Barba, & Cámara, 2005; González-Rodríguez, Cancho-Grande, & Simal-Gándara, 2009, 2011; González-Rodríguez, Cancho-Grande, Torrado-Agrasar, Simal-Gándara, &

Mazaira-Pérez, 2009). The mode of action of fungicides is focused on target-organisms but residual bioavailable levels may reach and affect non-target organisms such as wine yeast population. As a result, changes in the secondary metabolism of yeasts can be produced and, as a consequence, changes affecting both aroma and wine colour (Briz-Cid et al., 2014, 2015; Cabras, Farris, Fiori, & Pusino, 2003; Mulero et al., 2015; Noguerol-Pato et al., 2016; Noguerol-Pato, González-Rodríguez, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2011; Noguerol-Pato, Sieiro-Sampedro, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2015; Noguerol-Pato, Sieiro-Sampredro, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2014; Oliva, Navarro, Barba, Navarro, & Salinas, 1999). Nevertheless, it remains unknown how fungicide residues can affect the complex network of biochemical pathways that constitute the secondary metabolism of yeasts. Up to date, only three papers published by Gil and co-workers analysed the potential mechanisms underlying the effects of relevant pesticides (carbofuran, pyrimethanil, alachlor, S-metolachlor, diuron and methyl(4-chloro-2-methylphenoxy)acetate) in the aroma biosynthesis through gene expression profiling in *S. cerevisiae* (Gil, Becker, & Viegas, 2014; Gil, Bellí, & Viegas, 2017; Gil, Gonçalves, Becker, & Viegas, 2018; Viegas et al., 2005).

Mepanipyrim (Mep), a fungicide belonging to the aniline-pyrimidines group, is widely used in Spanish vineyards. Maximum residue level (MRL) for this fungicide set by the European Commission for wine grapes is 2.0 mg/kg (Part A of Annex I to Regulation 396/2005 and the later amendment, the Commission Regulation (EU) 2016/486). The present study intends to make a contribution for better understanding the impact and toxicity of Mep residues on the secondary metabolism of *S. cerevisiae* yeasts, including the synthesis of volatile and non-volatile fermentation-derived compounds, by using laboratory-scale fermentation assays. The novelty of this work is the assessment, for first time, of the effect of both Mep and its

corresponding commercial formulation (considering other ingredients besides fungicide) on the yeast metabolism. At the same time, two levels of fungicide contamination: MRL, equivalent to Good Agricultural Practices and twice MRL (2MRL) in a scenario of abusive use of this fungicide are evaluated.

2. Materials and methods

2.1. Fungicides

Mepanipyrim active substance (Mep) and mepanipyrim commercial formulation (Mep Form) were added, separately, on commercial Garnacha pasteurized red must. The active substance was purchased as Pestanal standard grade, of certified purity > 99 %, from Sigma-Aldrich (Steinheim, Germany). Its corresponding commercial vineyard protection product, a wettable powder that contains the active substance Mep (50 %, w/w) mixed with inert material (kaolin 10 - < 25%), diluents (sodium salt of polynaphthalene sulphonic acid < 5%) and surfactants (sodium dodecyl sulphate < 5%), was purchased from Sipcam Iberia. Individual stock solutions (1000 mg/L) and working solutions (10 mg/L) were prepared in methanol and acetone, respectively.

2.2. Chemicals

Ingredients and culture media (glucose, peptone, yeast extract and agar) were obtained from Panreac (Barcelona, Spain). D(+)-glucose anhydrous and D(-)-fructose extrapure Pharmpur® were from Scharlab S.L. (Barcelona, Spain).

The solvents used such as dichloromethane, methanol, acetone, acetonitrile and water (HPLC quality) were purchased from Sigma-Aldrich, whilst ethanol absolute, ethyl acetate, hexane

and toluene (HPLC grade) were acquired from Scharlau (Barcelona, Spain); ethanol 96 % PA-ACS, glycerol PA-ACS-ISO and sulphuric acid 95-98 % PRS-CODEX were obtained from Panreac (Barcelona, Spain). Tartaric, acetic, formic and trifluoroacetic acids were obtained from Sigma-Aldrich, meanwhile anhydrous sodium sulphate and sodium chloride for residue analysis were obtained from Panreac (Barcelona, Spain).

The sorbent materials used for solid-phase extraction (SPE) were Supelclean Envi-Carb II/PSA dual layer tube (500 mg/500 mg, 6 mL size) from Supelco (Bellefonte, PA, USA) for the extraction of fungicides; Strata-X, 33 µm polymeric reversed phase (500 mg, 6 mL size) from Phenomenex (Torrance, CA, USA) for the extraction of volatiles; and Strata C18-E (2g, 12 mL size) from Phenomenex for the extraction of anthocyanins and anthocyanin-derived pigments.

The chemical standards used to determine volatile compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA) except tyrosol, which was purchased from Extrasynthese (Genay Cedex, France). Individual standard solutions of appropriate concentrations were prepared in ethanol according to Noguerol-Pato, González-Barreiro, Cancho-Grande, & Simal-Gándara (2009), meanwhile tyrosol was prepared in methanol. Secondary standard solutions were also prepared by dilution in ethanol from the individual standard solutions. All of them were stored in the darkness at -20 ˚C. Both 2-octanol (used as internal standard) and 4-nonanol (used as surrogate standard) were also purchased from Sigma-Aldrich.

The chemical standard used to quantify anthocyanins and anthocyanin-derived pigments was malvidin-3-*O*-glucoside chloride purchased from Extrasynthese. Individual stock solution was

prepared in methanol. Different working standards solutions were prepared by appropriate dilution in 12 % ethanol and then stored in dark vials at -20 ˚C.

2.3. Laboratory-scale assays

2.3.1. Preparation of yeast inoculum

The commercial dry yeast used was the *S. cerevisiae* var. *bayanus* Lalvin T73™ strain from Lallemand Bio (Montreal, Canada). The yeast was rehydrated, as described by the manufacturer, and inoculated on GPY-solid medium (20 g/L glucose, 20 g/L peptone, 15 g/L yeast extract and 15 g/L agar), which were incubated without agitation at 30 ºC for 48 h in a refrigerated incubator Unimax 2010 (Heidolh, Schwabach, Germany) and kept at 5 ºC until use. Inoculum was prepared by transferring cells from a sole colony in the plates to GPY-liquid medium (without agar) placed in Erlenmeyer flasks, and incubated in a thermostatised orbital shaker (Optic Ivymen System, Barcelona, Spain) at 150 rpm, 30 °C during 24 h. The exponentially growing cells were separated from the supernatant by centrifugation (5000 rpm during 15 min) in a Rotina 35R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), washed twice and suspended in the fungicide enriched musts, in an amount enough to reach an initial concentration of $2x10^6$ cell/mL.

2.3.2. Alcoholic fermentation assays

Five fermentation experiments (control, A, B, C and D) were performed by triplicate. The first experiment, considered as a control, was done with uncontaminated commercial Garnacha pasteurized musts (glucose and fructose content 123±7 g/L of each one, no presence of glycerol and ethanol was detected). For experiments A and B, the commercial musts were fortified with Mep at concentration levels corresponding to its MRL (2 mg/kg, experiment A)

and 2MRL (4 mg/kg, experiment B), respectively. For experiments C and D, the commercial musts were spiked with Mep Form at the same concentration levels described above: MRL (2 mg/kg, experiment C) and 2MRL (4 mg/kg, experiment D).

Fermentation assays were done in semi-aerobical conditions in semi-covered 500 mL glass bottles filled with 400 mL of fungicide enriched musts (control, A, B, C and D), inoculated and incubated for 10 days with orbital agitation (120 rpm), in a thermostatically controlled chamber at 21 ºC.

2.3.3. Control of the fermentation course

Aliquots (5 mL) were aseptically collected during the fermentation process at different times after inoculation (0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days) to monitor the fermentation course. Yeast cells were washed twice and re-suspended in the same volume of water to measure the biomass concentration (as turbidity in a UV/VIS spectrophotometer at 650 nm (Jenway, Essex, England). The remaining volume was centrifuged at 4000 rpm for 10 min and the supernatant was stored at -20 ºC for glucose, fructose, glycerol and ethanol analyses. Samples pH was also measured at sampling using a Basic 20 pH-meter with a 50 10T pH-electrode from Crison Instruments (Barcelona, Spain).

Samples were filtered throughout Chromafil PET (0.45 µm) filters from Macherey-Nagel (Düren, Germany). Glucose, fructose, glycerol and ethanol contents were measured by injection of 5 µL of sample in a Agilent 1200 high-performance liquid chromatograph equipped with a refractive index detector (Santa Clara, CA, USA) and an ICSep Corregel-87H1 column (300 x 7.8 mm i.d.) of Transgenomic (San Jose, CA, USA). Compounds were eluted with 0.0085 M H_2SO_4 at 35 ºC with a constant flow-rate of 0.4 mL/min during 40 min

(González-Rodríguez, González-Barreiro, et al., 2011). Under these conditions, the retention times were 16.50 min for glucose, 17.85 min for fructose, 23.40 min for glycerol and 34.11 min for ethanol.

In order to control the fermentation evolution several parameters were evaluated. The maximum specific growth rate (μ_{max}), sugar consumption rate (r_s) and the ethanol production rate (r_e) were calculated by linear regression, representing the ln(biomass), the concentration of sugars and ethanol *versus* time during the exponential growth phase, respectively. The sugar to ethanol yield ($Y_{E/S}$) was calculated as the ratio between the ethanol concentration and the sugars consumption at the stationary growth phase.

2.4. Instrumental analysis of wines

2.4.1. Mepanipyrim residues

Fungicide determination was performed according to González-Rodríguez, Cancho-Grande, & Simal-Gándara, (2009). Briefly, ethanol of final wine samples was evaporated under a stream of nitrogen in a Turbo Vap LV evaporator (Caliper Life Sciences, Hopkinton, MA, USA). After that, a liquid-liquid extraction followed by a SPE clean-up step with a dual layer Envi-Carb II/PSA cartridge was carried out for the extraction of Mep residues in all samples.

Gas chromatographic analyses were carried out on a Trace GC Thermo Finnigan gas chromatograph (Rodano, Italy) equipped with a PolarisQ ion trap mass selective (ITMS) detector, interfaced to a computer running the software program Xcalibur 2.2 from Thermo Electron Corporation. To compensate the effect known as matrix-induced response enhancement and to ensure quantification accuracy, analyte protectants (3-ethoxy-1,2-propanediol at 10 g/L; D-sorbitol at 1 g/L and L-gulonic acid γ-lactone at 1 g/L) were used.

Lindane (0.5 mg/L) was used as internal standard. Chromatographic separations were done on a SPB-5 fused-silica capillary column (30 m, 0.25 mm ID, 0.25 µm film thickness) from Supelco following the chromatographic conditions described by González-Rodríguez, Cancho-Grande, & Simal-Gándara (2009). For quantification purposes, selected ions (m/z) 222 and 223 were monitored for Mep in the selected ion monitoring (SIM) mode. Mep concentration was determined by the internal standard method; calibration curves with eleven levels were used and calculated recovery (>99 %) was applied to guarantee reliable results.

2.4.2. Volatile aroma compounds

Volatile derived compounds from *S. cerevisiae* metabolism were determined according to González-Álvarez, Noguerol-Pato, González-Barreiro, Cancho-Grande, & Simal-Gándara, (2012). A SPE system was used for the extraction of volatile compounds in red wines. Briefly, wine samples were loaded in a Strata-X cartridge previously conditioned. A cleaning step with water was performed after the sample loading. Subsequently, the sorbent was dried and the volatile compounds were eluted with dichloromethane. The eluate was dried over anhydrous sodium sulphate and concentrated to a volume <1 mL under a N_2 stream. Afterwards, internal standard was added (20 µL of the ethanolic solution of 2-octanol 50 mg/L) and made up to 1 mL with dichloromethane prior to GC-ITMS analysis.

Volatile compounds were also separated and identified on the same GC-ITMS equipment described above. Chromatographic separations were done on a ZB-WAX Zebron Phenomenex polyethylene glycol capillary column (60 m x 0.25 mm i.d., 0.25 µm film thickness) following the chromatographic conditions established by González-Álvarez, González-Barreiro, Cancho-Grande, & Simal-Gándara (2012). Identification and confirmation of the volatile compounds were achieved by comparing the GC retention times and mass

spectra over the mass range 35-300 amu for the samples with those for pure standards analysed under the same conditions. Quantification was performed by choosing specific m/z values of each volatile compound from the full-scan mode (Noguerol-Pato et al., 2009). The concentrations of these compounds were determined by the internal standard method; calibration curves with twelve concentration levels were used and synthetic wine recoveries were applied to guarantee reliable results, considering the absolute recovery (%) of each compound.

2.4.3. Tyrosol

Analyses were carried out without any prior purification step of the sample according to La Torre, Saitta, Vilasi, Pellicanò, & Dugo, (2006). Briefly, an aliquot of centrifuged wine was injected into the HPLC Thermo Separation-Products (TSP) equipped with an analytical column Phenomenex C18 Luna (150 x 4.6 mm i.d., 5 µm) and a diode array UV6000LP DAD detector. Tyrosol eluted at 30 ºC, at a constant flow rate of 0.6 mL, under the following gradient conditions for the mobile phase (formic acid in water - pH 3, solvent A - and formic acid in acetonitrile - pH 3, solvent B-) A:B (95:5) for 10 min; changed to A:B (83:17) for 15 min; changed to A:B (20:80) for 5 min and held for 5 min; and finally, changed to A:B (95:5) for 2 min and held for 10 min. Detection was carried out at wavelength of 278 nm.

2.4.4. Native anthocyanins and vitisins

These compounds were extracted according to the procedure described by Figueiredo-González, Regueiro, Cancho-Grande, & Simal-Gándara (2014). Briefly, ethanol of wine samples was previously evaporated under a stream of nitrogen and reconstituted with water. The reconstituted wine was loaded into a Strata C18 cartridge. The sorbent was dried and after washing with ethyl acetate, the anthocyanin fraction was eluted with 0.1% trifluoroacetic

acid in methanol. The eluate was evaporated down and substituted with EtOH 12% in water. The ethanolic extract was passed through a filter of 0.45 µm pore size prior to HPLC/DAD–ESI/MS analysis, following the instrumental conditions described by Briz-Cid et al., (2014). Identification was based on MS/MS data reported by Blanco-Vega, Gómez-Alonso, & Hermosín-Gutiérrez (2014). Quantification was performed using DAD-chromatograms extracted at 520 nm and malvidin-3-*O*-glucoside calibration curve.

2.4.5. Characterisation of the colour

Chromatic characteristics were determined in the studied wines by spectrophotometric methods using a Beckman Coulter DU730 Life Science UV/Vis spectrophotometer (California, USA). Spectrophotometric parameters were taken after the centrifugation of the wines for 15 min at 3000 rpm in a Rotina 35R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) and using quartz cells from Hellma (Müllheim, Germany) of 1 mm and 1 cm path length for undiluted and diluted samples, respectively. All measurements were carried out in triplicate. A hydroalcoholic solution (12 % ethanol) was used as blank in all measures.

In addition, wine colour was determined from CIELab parameters. Data processing for CIELab parameters (lightness (L^*), colour channels (a^* and b^*), chroma (C_{ab}^*), and hue angle (h_{ab})) were performed with the original software MSCV® (Copyright 2001-2012 Grupo de Color, Universidad de La Rioja) (Ayala, Echávarri, & Negueruela, 2001).

3. Results and discussion

3.1. Mepanipyrim residues in wines

Regarding to Mep residues at the end of the fermentation process (10 days after inoculation), contents determined in experiments A and B were practically coincident with MRL (1.8 ± 0.2 mg/kg) and 2MRL (3.8 ± 0.3 mg/kg), respectively. By the other hand, neither yeasts induce Mep degradation nor yeasts were able to reduce Mep concentration by adsorption on their cell walls.

When Mep Form is added, the active substance is present in the medium together with inert material, adjuvants and other additives. In this case, Mep content was lower than MRL and 2MRL in wines corresponding to experiments C (0.9 ± 0.03 mg/kg) and D (1.6 ± 0.07 mg/kg) at the end of fermentation. These results seem to indicate that the presence of the other ingredients of the commercial formulation exert an influence on Mep solubility and/or Mep adsorption to the residual solid particles of pasteurized musts.

3.2. Alcoholic fermentation course

Once the *S. cerevisiae* yeasts have been adapted to the environmental conditions, the yeast development on commercial red must was evaluated in the exponential growth phase, by the determination of sugar consumption and ethanol production; and in the quasi-stationary growth phase, by the determination of sugar to ethanol yield. On the basis of the obtained results, no influence of Mep and Mep Form on *S. cerevisiae* viability and strain growth was observed (**Table 1**). In addition, pH and glycerol production were similar in all experiments (data not shown). With regard to the evaluated fermentation parameters, the main results are discussed as follow.

Glucose and fructose consumption. The consumption of sugar by *S. cerevisiae* displayed a stronger specificity towards glucose consumption with respect to fructose. At the end of the fermentation process, glucose was practically consumed in all experiments while 13-23 % of the initial fructose content remain in the fermentation media, although no significant differences were observed among treatments (data not shown). For evaluating the substrate uptake, the sugar consumption rate (r_s) was estimated during the exponential growth phase in all experiments. As can be seen in **Table 1**, all experiments showed comparable sugar uptake rates, regardless of the addition of Mep and Mep Form to the growth medium. Such behaviour was described previously by other authors (Saaris, Kotseridis, Linga, Galiotou-Panayotou, & Papanikolaou, 2009) and by our research group in previous works (González-Rodríguez, González-Barreiro, et al., 2011; Noguerol-Pato, Torrado-Agrasar, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2014).

Ethanol production. Ethanol production rate (r_e) was assessed during the exponential growth phase. **Table 1** shows that there were not significant effects derived from the addition of fungicides in terms of ethanol production. The same behaviour was observed by (González-Rodríguez, González-Barreiro, et al., 2011) for metiram and pyraclostrobin. However, the level of ethanol reached at the end of the fermentation process in supplemented musts with Mep Form at 2MRL was statistically higher than control samples.

Sugars to ethanol yield ($Y_{E/S}$). This parameter is defined as the ratio between ethanol concentration and consumed sugar in the quasi-stationary phase. The same behaviour described for ethanol was observed (see **Table 1**). A slight negative effect was registered in

those experiments carried out in presence of Mep Form but the differences were not statistically significant.

3.3. Volatile fermentation derived compounds

The anaerobic fermentation of sugars by *Saccharomyces* yeasts generates a variety of volatile metabolites that contribute to the sensory profile of wine. Volatile compounds released from non-volatile grape flavour precursor compounds by yeast enzymes (monoterpenes, C_{13}-norisoprenoids, C_6-alcohols and benzene derivatives) are shown in **Table 2**. As expected, minimal variations on these compounds were observed with the exception of a reduction of *trans* 2-hexen-1-ol content and increments on vainillin and ethyl vanillate concentrations. The pasteurized process applied to the must could explain this behaviour, since several enzymes involved on their release from precursors are inhibited.

Volatile compounds derived from yeast metabolism including higher alcohols, acetates, volatile fatty acids, ethyl esters, volatile phenols and lactones are shown in **Table 3**. The assessment of the effect of fungicides was obtained by comparing the volatile concentration levels of the resulting wines with respect to the control. Statistical significant differences can be established by ANOVA ($p < 0.05$), but only those that exceed a percentage of variation greater than 20 % with respect to the control wine were considered as remarkable. Changes in concentration values lower this percentage could be accepted as variations linked to the fermentation process and not to the fungicide.

Higher alcohols and their acetates

Higher alcohols (or fusel alcohols), the acetate ester precursors, are the most important group of the volatile fraction of wines from a quantitative point of view. 2-phenylethanol and

isoamyl alcohol levels are around 10.5 mg/L and 34.5 mg/L in control wines, respectively. They can be synthesized from amino acids catabolism mainly via the Ehrlich pathway (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008) and/or via the central carbon metabolism (Lambrechts & Pretorius, 2000). Although there are several factors influencing higher alcohols biosynthesis, it is known that exists a synergistic relationship between total production of higher alcohols and its amino acid precursors (Fleet, 2003).

Methionol is an alcohol synthesized from amino acid methionine via the Ehrlich pathway. Due to Mep-mode of action inhibits methionine biosynthesis (FRAC, 2018), methionol content could be affected in treated wines with respect to the control wine (316.9 µg/L); nevertheless, methionol content was identical in wines where Mep or Mep Form was added previously in musts (from 314.5 to 359.2 µg/L), as can be seen in **Table 3**. However, an overexpression of some proteins involved in methionine biosynthesis and sulphate assimilation (sulphite reductase [NADPH] subunit beta, sulphite reductase [NADPH] flavoprotein component and phosphoadenosine phosphosulphate reductase coded by *MET5*, *MET10* and *MET16* genes, respectively) was observed in Mep and Mep Form supplemented musts (Briz-Cid et al., unpublished results). In the same way, Gil et al. (2018) emphasized the significant up-regulating of some genes involved in the same processes in presence of pyrimethanil (Pyr), another anilinopyrimidine fungicide with the same mode of action than Mep.

In relation with other target alcohols determined in wines, statistical significant differences were observed for isoamyl alcohol coming from isoleucine (Alves et al., 2015; Styger, Jacobson, & Bauer, 2011). The content was reduced from 34.5 mg/L in control wines to 26.2-26.8 mg/L in treated wines obtained from must where Mep was added at MRL and 2MRL

levels. In particular, Gil et al. (2014) confirmed that Pyr overexpressed *AAD10* gene affecting the levels of isobutanol and 2-phenylethanol. In our case, any influence of Mep was observed on the 2-phenylethanol content.

Acetates constituted the most significant class of esters which are formed in yeast cells through the condensation of higher alcohols with acetyl-CoA by alcohol acetyltransferase enzymes (Mason & Dufour, 2000; Rollero et al., 2017), as it can be seen in **Figure 1**. The obtained results confirmed that acetate concentrations does not always depend on the availability of their corresponding higher alcohols. Mep residues did not seem to produce any change in acetate content with respect to control wines. Nevertheless, three target acetates showed a notorious increment (between 92-135 %) in presence of commercial Mep Form at the higher dose assayed; the isobutyl acetate content was also higher in wines where Mep Form was added at MRL and 2MRL levels. This effect could be related to the other ingredients of the commercial formulation which could be produce changes in the activity of Atf1p and Atf2p enzymes (Saerens et al., 2010; Verstrepen et al., 2003).

Fatty acids and their ethyl esters

With regard to ethyl fatty acid ester precursors, MCFAs are by-products of long-chain saturated fatty acids and not by their degradation (Marchesini & Poirier, 2003; Saerens et al., 2010), which are required for cell membrane phospholipid biosynthesis (Torija et al., 2003). Caproic acid (2.3 mg/L in control wines), caprylic acid (3.6 mg/L) and capric acid (1.5 mg/L) were the three MCFAs determined in all fermentation experiments. Although no statistical significant differences were observed for capric acid with respect to the control wine, the three target MCFAs increased their contents (between 8-41 %) in presence of Mep and Mep Form at both concentration levels. There is no doubt that the greater synthesis of these acids is

a consequence of changes that take place in the yeast's environment. Pesticide molecules may be accumulate in the lipid bilayers perturbing membrane function as selective permeability barrier and interfering with lipid bilayers and transmembrane enzymes, and/or may diffuse through the plasma membrane and reach target sites in different locations inside the yeast cell (Cascorbi et al., 1993; Gil et al., 2018). Therefore, according to these authors, changes related with remodelling of membrane lipid composition and limitation of membrane damage could be produced. In order to adapt the fluidity of their plasmatic membranes to this aggressive new environment produced by the presence of fungicides, yeasts could incorporate MCFAs within the phospholipids of the membrane (Hirst & Richter, 2016; Rozès, 1992; Zamora, 2004). Nevertheless, some of these MCFA could be released into the medium. Gil et al., (2014) confirmed that Pyr overexpressed *AAD10* gen (gen that influences the levels of propionic, valeric, hexanoic and decanoic acids) and *PLB2* gen (with influence on the concentration of octanoic and decanoic acids). In order to confirm this explanation, additional transcriptomic responses to Mep in functional categories like lipid/fatty acid metabolism and/or ergosterol metabolism and/or lipid/fatty acid transport in the cells should be done. In the case of the content of isovaleric acid, derived from the oxidation of the aldehydes formed from α-keto acids during amino acid metabolism, it was not affected by Mep residues (ca. 400 µg/L).

Biosynthesis of ethyl esters of medium-chain fatty acids (MCFA) is due to the esterification of ethanol with activated medium-chain fatty acyl-CoA during the early stages of lipid biosynthesis by alcohol acyltransferase enzymes Eeb1p and Eht1p (Suomalainen, 1981) (see **Figure 1**). As a difference of acetates, ethyl fatty ester formation is closely linked to the availability of their corresponding fatty acid precursors (Loviso & Libkind, 2018; Moreno-Arribas & Polo, 2009; Saerens et al., 2008) while the enzyme activity appears not to be the

limiting factor (Saerens et al., 2010). Therefore, higher content of MCFA precursors into the fermentation medium resulted in higher ethyl ester production. It is necessary to highlight that ethyl caproate (C_6, 424.2 µg/L) > caprate (C_{10}, 297.5 µg/L) > caprylate (C_8, 192.8 µg/L) contents in control wine were not strongly positively correlated to concentrations of their corresponding precursors capryplic (C_8, 3.6 mg/L), caproic (C_6, 2.3 mg/L) and capric (C_{10}, 1.5 mg/L) acids, respectively. The addition of Mep and Mep Form, at both tested levels, highly increased ethyl caprylate (between 42-63 %) and ethyl caprate (between 36-60 %) contents. Other volatiles from this group presented only statistically significant increments when Mep Form was added at 2MRL: ethyl butyrate (66 %), ethyl-2-methylbutyrate (43 %) and ethyl caproate (43 %). On the contrary, ethyl laurate content was identical in all fermentation experiments.

Several hypothesis about the synthesis of ethyl fatty esters formation are postulated (Swiegers & Pretorius, 2005). The first hypothesis is based on ethyl fatty esters are much less toxic than their related acids (especially from C_8 to C_{14}) and therefore their synthesis has been proposed as a protection mechanism for the yeast cells against accumulation of their toxic precursors; ethyl esters can also diffuse more easily through the plasma membrane and leak into the medium, further reducing the risk of toxic acid accumulation by shifting the equilibrium towards ester synthesis (Saerens et al., 2010). The second hypothesis is based on the yeasts might synthetize esters to redress any imbalance in the coA-SH / acetyl-CoA ratio caused by the cessation of the lipid pathway through fermentation (Rosi & Bertuccioli, 1992; Thurston, Taylor, & Ahvenainen, 1981).

Volatile phenols

4-vinylphenol derived from *p*-coumaric is usually biosynthetized during fermentation by the action of Pad1 and Fgc1 enzymes against the toxic effect of its precursor (Gerbaux, Vincent, & Bertrand, 2002). In fact, 4-vinylphenol content was 39.2 µg/L in the control wine; later, this compound can be reduced in minor extension to 4-ethylphenol (0.75 µg/L) due to *Saccharomyces* generally lacks vinylphenol reductase activity (Dzialo et al., 2017). While Mep residues did not alter 4-vinylphenol concentration with respect to control wines, Mep Form residues seemed to stimulate the conversion of *p*-coumaric to 4-vinylphenol (with an increment around 90 %). Other ingredients of the commercial formulation different than Mep possibly increased the activity of Pad1p and Fdc1p enzymes, encoded by *PAD1* and *FDC1* genes, responsible for decarboxylation of hydroxycinnamic acids (Mukai, Masaki, Fujii, Kawamukai, & Iefuji, 2010).

Lactones

Mechanisms regulating the formation of lactones in wine are not well known. They are carbonyl compounds produced by yeasts during alcoholic fermentation (Wurz, Kepner, & Webb, 1988) but its origin is also lies in grapes (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). Four lactones were identified in control wines in the following order according their content: γ-butyrolactone (81.2 µg/L) > γ-decalactone (24.3 µg/L) > γ-(R)-(-)-pantolactone (16.0 µg/L) > γ-nonalactone (12.86 µg/L). γ-butyrolactone, formed from glutamic acid during fermentation by deamination and descaboxylation through the Ehrlich pathway (Ribéreau-Gayon et al., 2006), increased in presence of Mep Form residues in musts (around 26 %). On the contrary, there was a downward trend in levels of γ-decalactone in presence of Mep and Mep Form (decreased between 16-31 %); although the origin of decalactone is varietal (Ferreira, López, & Cacho, 2000).

As described above, the main changes were produced in volatile aroma compounds generated at intracellular level by fermenting yeasts cells. Statistical significant effects were mainly observed in esters - acetates derived from higher alcohols and ethyl esters derived from medium chain fatty acids (MCFA). These compounds can be released to the fermentation medium through plasmatic membrane of yeasts since they are lipid soluble (Loviso & Libkind, 2018; Saerens, Delvaux, Verstrepen, & Thevelein, 2010) resulting in a significant enhancement of fruity and floral notes of the young wines. It is important to remark that this increment is mainly related with those volatile compounds with contents higher than their odour perception threshold. In this sense, banana nuances in wines could be increased as a consequence of the notorious increment of isoamyl acetate content (135 %) in presence of Mep Form 2MRL.

3.4. Non-volatile fermentation-derived compounds

The colouring matter of red wines derives from the presence of anthocyanins, flavonoid pigments formed in the berry from veraison onwards via the phenylpropanoid pathway. Although native anthocyanins are extracted from the grape skins during maceration/fermentation and are diffused into the wine, these compounds are involved in the formation of a new non volatile anthocyanin derived pigments - pyranoanthocyanins - by condensation with metabolites (e.g. pyruvic acid, acetaldehyde) produced in the cytoplasm of *S. cerevisiae* during the catabolism of sugars and diffused out of the cell (Morata, Gómez-Cordovés, Colomo, & Suárez, 2003). Pyruvic acid takes part in the formation of vitisins A, meanwhile acetaldehyde is a precursor of vitisins B; both types of vitisins are low weight anthocyanin-derived pigments and they are more stable than other anthocyanins (Blanco-Vega et al., 2014; Morata et al., 2003). Other secondary metabolites from yeasts such as

acetone, acetoin, oxalacetic acid and diacetyl are also likely to react with native anthocyanins to form similar pyranoanthocyanins.

In **Table S1** (supplementary material), concentrations of native anthocyanins, vitisins A and vitisins B identified and confirmed in all wines by HPLC- MS/MS are shown. Six native anthocyanins, derived mainly from malvidin, delphinidin and peonidin, were determined in all wines; malvidin-3-*O*-glucoside was the main compound identified at levels around 2.6 mg/L. This low anthocyanin content can be explained taking into account that fermentation experiments were done on pasteurized grape must (absence of maceration). Vitisins A (determined at levels between 0.50-0.58 mg/L) and B (determined at levels between 0.08-0.09 mg/L) were the main anthocyanin-derived pigments and they were not affected by fungicide residues. Other minor derived anthocyanin-derived pigments like cm-vitisin A and cm-vitisin B were only affected by Mep residues, meanwhile 10-carboxy-pypn-3-glc, 10-carboxy-pypn-3-cmglc and ac-vitisin B were affected by Mep and Mep Form residues. However, the concentrations and differences found were minimal and it is difficult to make proper assumptions related to the impact of fungicides over these compounds.

Due to the colour is a part of the overall perceived organoleptic impression and it is closely related to the content of anthocyanins and their derivatives, its characterisation was carried out by determining colorimetric indexes and/or CIELab parameters for control wine as well as for treated wines (see **Table S2** of supplementary material). Among these parameters colour intensity (CI), red-green colour contribution (a*), chroma (C*) and lightness (L*) are related with polyphenolic composition of the red wine and they are considered as wine quality indicators (Esparza, Santamaría, Calvo, & Fernández, 2009). As expected, in general, the presence of Mep and Mep Form residues, at MRL and 2MRL levels, had no effect on the

chromatic characteristics of the final wines, with the exception of a slight increment of a* and C* for Mep Form 2MRL (**Table S2**).

4. Conclusions

The experimental results obtained by using laboratory-scale fermentation assays showed a clear effect of Mep on volatile derived compounds from *S. cerevisiae* metabolism. Mep residues affect in a more extension the aroma biosynthesis pathways into the yeast cell, particularly with regard to esters, rather than the release of varietal aroma compounds from their grape precursors. In particular, the observed effect for acetates derived from higher alcohols was basically produced when Mep Form was present at 2MRL level, indicating the important role of the other components of the commercial formula. In the case of ethyl esters, ethyl caproate, caprylate and caprate increased in all experiments. Complementary studies involving proteomic and transcriptomic techniques are necessary to understand in depth the influence of fungicides on yeast metabolism.

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Table 1. Parameters used to describe *S. cerevisiae* fermentation on pasteurized must in presence of Mep residues (n = 3).

	Exponential growth phase			Quasi-stationary growth phase
	Maximum specific growth rate (μ_{max})	**Sugar consumption (r_s)**	**Ethanol production (r_e)**	**Sugar to ethanol yield ($Y_{E/S}$)**
Control	0.47 ± 0.03[a]	48.92 ± 2.73[a]	18.32 ± 1.87[a]	0.53 ± 0.03[ab]
Mep MRL	0.48 ± 0.12[a]	50.51 ± 6.74[a]	18.17 ± 2.56[a]	0.56 ± 0.04[b]
Mep 2MRL	0.55 ± 0.04[a]	50.41 ± 7.10[a]	17.52 ± 2.26[a]	0.55 ± 0.07[b]
Mep Form MRL	0.44 ± 0.07[a]	50.28 ± 3.27[a]	18.37 ± 2.69[a]	0.47 ± 0.05[a]
Mep Form 2MRL	0.51 ± 0.04[a]	50.85 ± 4.38[a]	21.41 ± 1.63[a]	0.47 ± 0.05[a]

Different letters means statistical significant differences according to the *ANOVA* test ($p < 0.05$).

Table 2. Volatile compounds (expressed as µg/L ± SD) resulting from grape precursor biotransformation in Garnacha fermented musts after fungicides supplementation.

	Control			Mep MRL			Mep 2MRL			Mep Form MRL			Mep Form 2MRL		
Monoterpenes															
linalool	5.84	±	0.72 a	5.58	±	0.44 a	5.65	±	0.46 a	5.99	±	0.20 a	6.11	±	0.58 a
α-terpineol	29.38	±	3.27 a	28.44	±	1.22 a	28.48	±	1.11 a	31.46	±	29.15 a	30.64	±	28.38 a
β-citronellol	5.58	±	0.60 a	4.96	±	0.31 a	5.01	±	0.25 a	5.21	±	4.82 a	5.90	±	4.22 a
cis-nerolidol	21.45	±	3.44 a	19.34	±	3.01 a	20.57	±	1.83 a	24.00	±	21.36 a	19.45	±	16.87 a
trans-trans farnesol	32.70	±	5.48 a	33.54	±	4.16 a	31.23	±	3.85 a	32.37	±	30.74 a	42.22	±	23.64 a
terpinen-4-ol	1.39	±	0.18 a	1.31	±	0.25 a	1.33	±	0.12 a	1.40	±	1.25 a	1.48	±	1.19 a
cis- linalool oxide	22.16	±	2.57 a	21.40	±	1.25 a	21.99	±	1.52 a	25.60	±	2.27 a	24.37	±	1.00 a
trans-linalool oxide	16.38	±	2.01 a	15.13	±	1.20 a	14.78	±	0.80 a	16.05	±	1.26 a	15.99	±	1.02 a
C_{13}-Norisoprenoids															
β-damascenone	6.04	±	0.63 a	6.52	±	0.64 a	6.08	±	0.29 a	6.43	±	0.6 a	6.70	±	0.35 a
C_6-Alcohols															
1-hexanol	161.17	±	19.42 a	148.55	±	5.48 a	146.99	±	7.35 a	162.30	±	5.86 a	159.29	±	8.77 a
trans 3-hexen-1-ol	6.18	±	1.27 a	6.08	±	1.08 a	4.97	±	0.34 a	5.73	±	0.42 a	5.80	±	0.28 a
cis 3-hexen-1-ol	37.82	±	4.87 a	34.27	±	2.52 a	33.75	±	1.74 a	36.78	±	2.03 a	34.44	±	1.28 a
trans 2-hexen-1-ol	3.02	±	0.22 b	1.92	±	0.45 a	1.72	±	0.28 a	1.74	±	0.15 a	2.11	±	0.27 a
cis 2-hexen-1-ol	2.28	±	0.49 a	2.31	±	0.22 a	2.13	±	0.19 a	1.90	±	0.37 a	2.07	±	0.24 a
Benzene derivatives															
benzyl alcohol	31.08	±	4.12 b	27.01	±	3.04 a	25.14	±	2.70 a	28.11	±	1.66 a	26.71	±	1.32 a
benzaldehyde	45.58	±	7.42 a	42.79	±	4.76 a	39.56	±	2.88 a	39.05	±	1.58 a	40.77	±	1.91 a
guaiacol	11.04	±	1.04 ab	10.68	±	0.62 a	10.49	±	0.82 a	11.57	±	0.52 ab	12.03	±	0.61 b
methyl vanillate	44.93	±	3.64 a	45.76	±	2.09 a	46.54	±	4.17 a	44.79	±	6.20 a	49.68	±	1.55 a
vanillin	19.26	±	1.46 a	21.76	±	1.77 a	20.84	±	1.61 a	21.86	±	1.27 ab	24.30	±	0.92 b
ethyl vanillate	6.25	±	1.01 a	7.25	±	0.68 ab	6.45	±	0.68 a	7.45	±	1.22 ab	8.25	±	0.95 b
acetovainillone	188.49	±	12.99 a	188.31	±	8.51 a	189.25	±	10.71 a	178.70	±	19.51 a	196.54	±	6.84 a a
eugenol	1.00	±	0.13 ab	1.06	±	0.11 b	0.86	±	0.03 a	0.95	±	0.09 ab	0.97	±	0.04 b
syringol	112.06	±	8.96 a	116.70	±	9.00 a	111.31	±	7.01 a	105.58	±	11.62 a	111.79	±	3.94 a
methyl salicylate	2.90	±	0.38 ab	2.71	±	0.22 a	2.54	±	0.16 a	3.20	±	0.12 b	3.13	±	0.10 b
4-methyl guaiacol	0.74	±	0.07 a	0.68	±	0.17 a	0.81	±	0.10 a	0.78	±	0.08 a	0.68	±	0.05 a

Different letters (a, b, c, d) mean statistical significant differences according to the *ANOVA* test ($p < 0.05$).

Table 3. Volatile aroma compound levels (expressed as µg/L ± SD) in wines obtained after Mep and Mep Form supplementation of grape musts.

	Control			Mep MRL			Mep 2MRL			Mep Form MRL			Mep Form 2MRL		
Higher alcohols and their acetates															
2-phenylethanol	10529.56	±	1326.31 ^{a}	10223.41	±	1900.78 ^{a}	10012.74	±	859.31 ^{a}	10168.09	±	1836.50 ^{a}	11377.87	±	2185.43 ^{a}
isoamyl alcohol	34553.39	±	2906.53 ^{b}	26180.15	±	4618.58 ^{a}	26776.28	±	2969.48 ^{a}	28674.45	±	2642.02 ^{ab}	30529.98	±	6094.44 ^{ab}
3-methyl-1-pentanol	131.39	±	7.32 ^{bc}	114.75	±	16.75 ^{ab}	84.38	±	17.05 ^{a}	114.12	±	24.43 ^{ab}	165.22	±	24.42 ^{c}
tyrosol	4.74	±	0.05 ^{c}	4.82	±	0.04 ^{c}	4.53	±	0.04 ^{b}	4.36	±	0.04 ^{a}	5.75	±	0.09 ^{d}
methionol	316.86	±	56.07 ^{a}	314.54	±	36.14 ^{a}	359.18	±	43.49 ^{a}	391.95	±	73.00 ^{a}	323.27	±	20.58 ^{a}
2-phenylethyl acetate	85.34	±	13.24 ^{a}	90.23	±	6.91 ^{a}	82.32	±	14.89 ^{a}	91.36	±	5.20 ^{a}	163.76	±	11.15 ^{b}
isoamyl acetate	790.51	±	76.33 ^{ab}	748.72	±	115.21 ^{a}	1038.49	±	212.61 ^{b}	852.96	±	87.59 ^{ab}	1771.75	±	146.19 ^{c}
isobutyl acetate	22.33	±	1.30 ^{a}	26.12	±	2.73 ^{a}	28.41	±	6.84 ^{ab}	40.63	±	15.65 ^{bc}	52.58	±	3.97 ^{c}
Fatty acids and their ethyl esters															
caproic acid	2258.26	±	268.04 ^{a}	2761.29	±	477.16 ^{b}	2772.34	±	266.34 ^{b}	2765.77	±	115.78 ^{b}	2898.78	±	154.57 ^{b}
caprylic acid	3583.72	±	435.85 ^{a}	4681.79	±	677.86 ^{b}	4511.19	±	627.88 ^{b}	4600.71	±	419.95 ^{b}	5063.36	±	176.02 ^{b}
capric acid	1477.16	±	207.23 ^{a}	1738.25	±	436.55 ^{a}	1704.60	±	311.65 ^{a}	1757.26	±	166.90 ^{a}	1590.62	±	163.85 ^{a}
isovaleric acid	418.26	±	80.69 ^{a}	365.96	±	46.60 ^{a}	400.09	±	46.80 ^{a}	424.56	±	43.56 ^{a}	428.33	±	40.89 ^{a}
ethyl caproate	424.24	±	41.15 ^{a}	478.69	±	88.22 ^{a}	464.55	±	53.91 ^{a}	492.62	±	63.88 ^{a}	608.58	±	53.73 ^{b}
ethyl caprylate	192.79	±	31.02 ^{a}	313.55	±	83.32 ^{b}	308.53	±	54.30 ^{b}	273.81	±	47.94 ^{b}	275.18	±	17.13 ^{b}
ethyl caprate	297.51	±	23.61 ^{a}	406.00	±	73.21 ^{b}	476.31	±	29.99 ^{b}	468.60	±	76.84 ^{b}	474.80	±	51.74 ^{b}
ethyl laurate	241.79	±	4.36 ^{b}	214.47	±	18.61 ^{b}	241.21	±	35.13 ^{b}	204.35	±	82.92 ^{a}	220.43	±	12.50 ^{b}
ethyl butyrate	225.38	±	19.46 ^{a}	260.51	±	53.11 ^{a}	257.54	±	40.15 ^{a}	249.99	±	25.25 ^{a}	373.33	±	14.10 ^{b}
ethyl-2-methylbutyrate	1.24	±	0.12 ^{a}	1.19	±	0.07 ^{a}	1.20	±	0.12 ^{a}	1.47	±	0.39 ^{ab}	1.77	±	0.20 ^{b}
Volatile phenols															
4-vinylphenol	39.20	±	6.21 ^{a}	40.73	±	6.61 ^{a}	47.52	±	5.01 ^{a}	74.93	±	13.46 ^{b}	73.73	±	14.14 ^{b}
4-ethylphenol	0.75	±	0.09 ^{a}	0.76	±	0.03 ^{a}	0.75	±	0.09 ^{a}	0.89	±	0.13 ^{b}	0.84	±	0.06 ^{ab}
Lactones															
γ-butyrolactone	81.23	±	7.53 ^{a}	86.69	±	8.91 ^{ab}	89.66	±	8.71 ^{ab}	103.16	±	15.87 ^{b}	101.81	±	11.54 ^{b}
γ-nonalactone	12.86	±	1.25 ^{a}	13.17	±	1.00 ^{a}	13.05	±	0.77 ^{a}	13.91	±	1.66 ^{a}	14.20	±	0.43 ^{a}
(R)-(-)-pantolactone	16.00	±	2.20 ^{a}	14.74	±	2.84 ^{a}	14.93	±	2.10 ^{a}	15.98	±	2.46 ^{a}	13.02	±	1.98 ^{a}
γ-decalactone	24.35	±	1.42 ^{c}	17.01	±	1.26 ^{ab}	16.78	±	1.25 ^{a}	20.53	±	3.16 ^{b}	17.58	±	2.22 ^{ab}

Different letters (a, b, c, d) mean statistical significant differences according to the *ANOVA* test ($p < 0.05$).

Conflict of Interest and Authorship Conformation Form

Please check the following as appropriate:

- ☒ All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.
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HIGHLIGHTS

- The presence of Mep did not alter the fermentation course
- Mep Form at 2MRL increased the acetate content more than 90 %
- Ethyl caprylate and ethyl caprate were the more affected ethyl esters