1 **Effect of refermentation conditions and MO on the reduction of volatile acidity by**

2 **commercial *S. cerevisiae* strains and their impact on the aromatic profile of wines**

3 Vilela-Moura A. [1], Schuller D. [2], Falco V [3], Mendes-Faia A. [1] and Côrte-Real M. [2*]

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5 (1) Institute for Biotechnology and Bioengineering, Centre of Genetics and Biotechnology,

6 (IBB/CGB-UTAD), Universidade de Trás-os-Montes e Alto Douro, 5001-801 Vila Real,

7 Portugal

8 (2) Centre of Molecular and Environmental Biology (CBMA) / Department of Biology /

9 University of Minho, Campus de Gualtar, 4710-057 Braga Portugal

10 (3) Department of Food Science, Universidade de Trás-os-Montes e Alto Douro, Portugal

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12 Running title: Biological reduction of excessive volatile acidity in wines

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14   *For correspondence:

15

16   Manuela Côrte-Real

17   e-mail: mcortereal@bio.uminho.pt

18   Tel. : (+351) 253 604 314

19   Fax : (+351) 253 678 980.

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21   **ABSTRACT**

22   Herein, we evaluate the applicability of previously characterized commercial and indigenous

23   *Saccharomyces cerevisiae* strains and non-*S. cerevisiae* species for the deacidification of

24   white and red wines at a pilot scale. The effect of the refermentation process (mixture of

25   acidic wine with musts from freshly crushed grapes or with residual marc) as well as MO

26   (MO) on acetic acid removal efficiency and wine aromatic composition was also assessed in a

27   red wine. The commercial strains S26 and S29 efficiently reduced both acetic acid (43 and 47

28   %, respectively) and sugar (100 %) after 264 hours of refermentation of an acidic white wine

29   that was supplemented with grape must. Similar results (60-66 % of acetic acid removal) were

30   observed for red wine deacidification using grape must, independently of MO. When residual

31   marc was used for deacidification, strain S26 removed 40% of acetic acid, whereas strain S29

32   did not initiate refermentation with or without MO. Wines obtained by refermentation with

33   the must had significantly lower acetic acid and a higher total SO_2 concentration in

34   comparison to the wines deacidified by the grape marcs. The volatile aroma compound´s

35   composition of deacidified red wines was dependent on the refermentation process used,

36   rather than on MO. The marc-deacidified wine obtained by the use of strain S26 and without

37   MO achieved the best sensory classification. When data from all analytical and sensory

38   evaluation were combined, Principal Component Analysis (PCA) separated the wines into

39   three distinct groups according to the strain and the refermentation process independently of

40   MO. We successfully established an efficient and cheap enological solution for the

41   rectification of volatile acidity of wines.

42   Keywords: Biological deacidification of wines, volatile acidity, MO

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INTRODUCTION

Volatile acidity corresponds essentially to acetic acid and is an important factor for wine quality. Consequently, its production is carefully monitored and controlled throughout the wine production process. Currently, few processing options are available to winemakers for the removal of sensorial objectionable levels of volatile acidity. Nanofiltration and reverse osmosis are complex and expensive physical methods that may be applied presently (Han and Cheryan 1995; Massot et al. 2008). Bioreduction methods using yeasts have been known for a long time but have not been sufficiently well characterized for commercial application. Actually, winemakers have been using an empirical biological deacidification process to lower acetic acid contents of wines with volatile acidity above 0.8 g/L that consists in a refermentation associated with acetic acid consumption by yeasts. This enological practice is performed by mixing the acidic wine with freshly crushed grapes, musts or marcs (remaining pulp, after draining the newly made wine) from finished fermentations, in a proportion of no more than 20-30% (v/v) (Ribéreau-Gayon et al., 2000a). The added wine should be microbiologically stable before incorporation to avoid bacterial growth. In our previous studies, we found that the *S. cerevisiae* autochthonous strains 43C and 45C and the commercial strains S26, S29 and S30, as well as the non-*Saccharomyces* strains (*Lachancea thermotolerans* 44C and *Zygosaccharomyces bailii* ISA 1307) have distinctive capacity to consume acetic acid from a mixture containing two-thirds of a synthetic medium and one third of an acidic white wine. However, the reduction of acetic acid by these strains was shown to require low amounts of oxygen as observed under limited-aerobic conditions (Vilela-Moura et al., 2008). This constraint might compromise the application of the above mentioned strains in refermention processes for the deacidification of acidic wines.

Oxygen is known to play an important role in the winemaking process (Sablayrolles et al., 1996; Salmon, 2006). Before fermentation the grape juice may be saturated with oxygen,

69 causing browning of the juice due to enzymatic and non-enzymatic reactions (Traverso-Rueda

70 and Kunkee, 1982). At the beginning of fermentation, a fine balance between oxygen

71 concentration and sulfur dioxide (SO_2) addition must be taken into account due to the

72 possibility of reductive flavors (rotten eggs) formation (Mendes-Ferreira et al., 2002). Close

73 to the end of fermentation, the presence of ethanol, oxygen, and acetic acid bacteria can

74 promote spoilage and wine oxidation to vinegar (Bartowsky and Henschke, 2008; Du Toit et

75 al., 2006; Traverso-Rueda and Kunkee, 1982). Moreover, oxygen can alter significantly the

76 wine's chemical composition, causing loss of organoleptical fruitiness and the appearance of

77 sherry-like and aldehydic flaws (Ribéreau-Gayon et al., 2000b). The oxidation of phenolic

78 compounds leads to H_2O_2 formation, which oxidizes ethanol to acetaldehyde (Shadyro et al.,

79 2008), with a grass- or apple-like aroma (Henschke and Jiranek, 1993).

80 However, yeast performance improves when oxygen is delivered in a controlled manner

81 during fermentation (Zoecklein et al., 1995). Yeast require oxygen for the synthesis of lipids

82 such as sterols and unsaturated fatty acids, which are indispensable for plasma membrane

83 integrity (Andreasen and Stier, 1953; Andreasen and Stier, 1954; Traverso-Rueda and

84 Kunkee, 1982; Zoecklein et al., 1995). Ergosterol represents about 50% of the total sterol

85 content in yeast (Bourot, 1995). A recent study showed that lipid synthesis and optimal

86 growth of *S. cerevisiae* during alcoholic fermentation requires about 5.0 – 7.5 mg of oxygen/L

87 (Rosenfeld et al., 2003). The absorption rate of the oxygen in the must is variable and has an

88 average of 2 mg/L/min. (Macheix et al., 1991).

89 Controlled wine oxygenation is currently achieved through MO. By this technique small

90 amounts of oxygen are delivered along fermentation. Oxygen is usually added by a stainless

91 steel sparger that produces small bubbles, promoting the dissolution of oxygen. The aim of

92 MO is to provide oxygen at a rate equal to or slightly less than the wine's oxygen

93 consumption rate to avoid too much oxygen build up in the wine (Llaudy et al., 2006; Parish

94 et al., 2000; Tao et al., 2007). This procedure has an impact on multiple aspects of wine

95 production such as: (i) increased production of sterols and other fatty acids by yeast

96 (Traverso-Rueda and Kunkee, 1982; Zoecklein et al., 1995), (ii) enhanced color stabilization

97 in red wines (Sánchez-Iglesias et al., 2009; Zironi et al., 2010), (iii) removal of unwanted

98 reductive flavors (Paul, 2002) and reduced vegetative aromas (McCord, 2003) (iv) accelerated

99 aging process (McCord, 2003; Llaudy et al., 2006; Zironi et al., 2010). However, MO can

100 promote the growth of acetic acid bacteria (Bartowsky and Henschke, 2008; Du Toit et al.,

101 2006) and the formation of unwanted off-flavors by *Brettanomyces* sp., depending on the SO_2

102 concentrations (Snowdon, 2006).

103 To evaluate the applicability of previously characterized commercial strains S26 and S29

104 (Vilela-Moura et al., 2008; Vilela-Moura et al., in press) in refermentation processes for the

105 removal of volatile acidity from too acidic wines, we herein assess acetic acid reduction of an

106 white wine by refermentation with grape must at a pilot scale (10 L). We also evaluate the

107 effect of refermentation conditions (mixtures of acidic wines with must or residual marc) and

108 of MO at a pilot scale (30 L) on the volatile acidity reduction of an acidic red wine. The

109 influence of MO on the aromatic composition of wines, and other enological parameters was

110 also determined.

111 This study adds new information on the applicability of two commercial *S. cerevisiae* strains

112 on the biological reduction of volatile acidity of acidic wines, and on the effect of

113 refermentation conditions and MO on the removal efficiency of acetic acid from a red wine.

114

MATERIALS AND METHODS

Microorganisms

The strains used for deacidification of wines were previously selected and described. *S. cerevisiae* strains 43C, 45C and *Lachancea thermotolerans* 44C were natural isolates (Vilela-Moura et al. 2008); *Zygosaccharomyces bailii* ISA 1307 was obtained from the Instituto Superior de Agronomia (Lisbon, Portugal); strains S26, S29 and S30 were kindly provided by Lallemand and Laffort Oenologie, respectively. Strains used were kept at -80ºC in micro tubes containing YPD broth (glucose 2%, w/v; peptone 1%, w/v; yeast extract 0.5%, w/v) supplemented with glycerol (30%, v/v).

Refermentation conditions

Fresh grape must from *V. vinifera* cv. Viosinho was pasteurized (60ºC during 20 min.) and used for the deacidification assays of an acidic white wine. Refermentations were performed in vapor-sterilized 10 L vessels, and consisted of 6.6 L of must and 3.3 L of acidic wine. The physico-chemical characteristics of the must, acidic wines and the respective mixtures are summarized in Table 1. Aliquots of the frozen strains were streaked onto YPD plates (glucose 2%, w/v; peptone 1%, w/v; yeast extract 0.5%, w/v and agar 2%, w/v) and incubated for 48 h at 25ºC. An overnight culture was then prepared by inoculation of 500 ml of the grape juice used in the mixture (white must plus acidic white wine) and incubated at 25 ºC, 100 rpm, until attaining a sufficiently high cell density to achieve $\cong 10^6$ CFU/mL after transfer to 10 L vessels, as referred above. Refermentations were carried at 20-23 ºC for 264 hours.

Deacidification assays of red wines were performed by refermentation with fresh must or by using marcs (remaining pulp, after draining the newly made wine) from *V. vinifera* cv Touriga Nacional. The must used for the refermentation process included the grape skins and was

140 supplemented with 40 mg/L of sulfur dioxide (SO_2). Ten L of acidic red wine was then added

141 to 20 L of must and refermentations were performed in stainless steel tanks (30 L capacity).

142 The physico-chemical characteristics of the must, acidic red wine and the mixture are

143 mentioned in Table 1. The inoculation of the commercial *S. cerevisiae* strains S26 and S29

144 was performed as described above.

145 The remaining pulp (residual marc), was obtained after draining the newly made wine at the

146 end (96 hours) of fermentation. At this stage, the marc, prepared from *V. vinifera* cv Touriga

147 Nacional contained 30 - 35 g/L of sugar. Ten L of acidic red wine were then added to 20 L of

148 residual marcs and refermentation was performed in 30 L stainless steel tanks at a temperature

149 between 18 and 20ºC.

150 Refermentation assays with acidic red wine were conducted with or without MO during one

151 hour per day (20 mg/L/h of oxygen applied with a MicroSafeO$_2$ - AEB device). Two daily

152 pump overs, of one minute each, were performed in each tank to homogenize the

153 refermenting wine. Yeast cell concentration was evaluated by spreading diluted must samples

154 (10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) onto YPD plates (glucose 2%, w/v; peptone 1%, w/v; yeast extract

155 0.5%, w/v and agar 2%, w/v). At the end of refermentations, (264 and 192 hours for the musts

156 and the marcs, respectively), the wines were analyzed and SO_2 – supplemented to a final

157 concentration of 50 mgL^{-1}. After two months, the wines were bottled and organoleptical

158 evaluation was performed. All experiments were performed in triplicate.

159

160 **Analytical determinations**

161 Sugar consumption was monitored daily by the DNS method (Miller, 1959). Acetic acid

162 consumption was monitored by the Cazenave-Ferré method, followed by titration with

163 phenolphthalein. Analysis of the density, pH, alcohol concentration, volatile acidity, sulfur

164 dioxide, titratable acidity, estimated alcohol content and residual sugar were performed

165 according to standard methods (Office International de la Vigne et du Vin, 1990), as outlined

166 in Table 1.

167 A solid-phase microextraction (SPME) methodology described by Mendes-Ferreira et al.

168 (2009) was used for the isolation of aroma compounds determined by gas chromatography

169 and mass spectroscopy (GC-MS) analysis. Compounds were adsorbed onto a fiber (100 μm

170 polydimethylsiloxane – PDMS -, 85 μm Carboxen – polydimethylsiloxane – CAR/PDMS -

171 and 50/30 μm Divinylbenzene/Carboxen/PDMS -DVB/CAR/PDMS) by solid-phase micro-

172 extraction (SPME). Ten ml of sample, 10 ml of internal standard solution and 4 g NaCl were

173 transferred to 40 ml vials (Supelco P/N 27181), containing a 10 mm magnetic stirring bar and

174 then capped with PTFE-faced silicone seals. Extractions in headspace mode were carried out

175 at $20\pm1^\circ$C with magnetic stirring (1300 rpm). The sample solution was equilibrated for 10

176 minutes; the fiber was then introduced into the vial headspace and held for 60 minutes at

177 constant temperature.

178 Chromatographic analysis was performed using an Agilent 6890 N gas chromatograph

179 equipped with a 5973N mass spectrometer. The volatile compounds were thermally desorbed

180 in the GC injector port for 10 minutes, where a 0.75 mm liner was used. Analysis was

181 performed in the splitless mode. Volatile compounds were then separated using an Innovax

182 capillary column, 30 m x 0.25 mm, with 0.5 μm film thickness (Agilent, Santa Clara, CA,

183 USA). The desorption temperature was 270°C during 10 min. The column was maintained at

184 40°C for 5 minutes after desorption, ramped at 4°C per minute up to 200°C, and then ramped

185 at 10°C per minute up to 240°C, where it was held for 15 minutes. Helium was used as the

186 carrier gas at 34 cm/s average linear velocity. All mass spectra were acquired in electron

187 impact (EI) mode at 70 eV, using full scan with a scan range of 26–250 atomic mass units, at

188 a rate of 6.12 scans/s. The Wiley database (Wiley/NBS Registry of Mass Spectral Data 1989

189 – McLafferty and Stauffer 1989) was used for compounds spectra identification. Whenever

190 possible, identification was confirmed by comparing mass spectra and retention indices with

191 those of authentic standards.

192 The compounds were quantified in selected ion monitoring (SIM) mode. 2-octanol was used

193 as the internal standard to eliminate variations in extraction efficiency caused by some

194 divergences in the sample matrix such as ethanol.

195

196 **Wine sensory analysis**

197 The sensory analysis was performed by a trained panel of 5 judges that have an extensive

198 wine tasting experience and participate on a regular basis in Wine Awards. Fourteen attributes

199 were selected: appearance (limpidity, tone and intensity), aroma (limpidity, intensity, vinegar,

200 acetaldehyde, ethyl acetate), oral perception (mouth intensity, body, harmony, persistence,

201 mouth feel, and acidic taste) according to reference standards (Noble et al., 1987). The

202 attributes were quantified using a six-point intensity scale (ISO 4121, 2003). A total sensory

203 score was calculated for each wine as the sum of average scores of appearance, aroma, taste

204 and mouth feel attributes. The judges were also requested to describe the global impression of

205 each wine. Each judge evaluated six wines in one session. All evaluations were conducted

206 from 10:00 to 12.00 A.M. in individual booths (ISO 8589, 2007) and according standardized

207 procedures (ISO 3591, 1977).

208

209 **Statistical analysis**

210 Both sensory and chemical data were submitted to variance analysis (ANOVA) using the

211 STATISTICA 7.0 software (StatSoft Inc., 2004). Tukey honestly significant difference (HSD)

212 test was applied to chemical and sensory data to determine significant differences between the

213 samples; the model was statistically significant with a P value less than 0.05.

214   A Principal Component Analysis (PCA) of the combined data from chemical and sensory

215   analysis was performed using the STATISTICA 7.0 software (StatSoft Inc., 2004).

216

RESULTS

Deacidification of an acidic white wine

In our previous studies, the *S. cerevisiae* autochthonous strains 43C and 45C and the commercial strains S26, S29 and S30, as well as the non-*Saccharomyces* strains (*L. thermotolerans* 44C and *Z. bailii* ISA 1307) have demonstrated capacity to remove acetic acid during a refermentation process using synthetic media, under aerobic and limited-aerobic conditions. The commercial strains S26 and S29 had the best acetic acid removal efficiency. We further showed that strain S26 had a higher tolerance to the combined stress factors imposed by acidic wines (Vilela-Moura et al., 2008, Vilela-Moura et al., in press).

Within this study, we aimed to evaluate the capacity of the above mentioned strains to remove acetic acid from an acidic white wine using a refermentation process with grape must of the Viosinho variety at a pilot scale (10 L). The sugar concentration of the mixture of the acidic white wine with the must was 157 g/L, whereas the concentration of ethanol and acetic acid were 4.3 % (v/v) and 1.15 g/L, respectively (Table 1). The chemical characterization of the deacidified wines obtained after 264 h of incubation is shown in Table 2. With the exception of strain *L. thermotolerans* 44C, all strains produced refermented wines with similar ethanol concentration, pH, acetic acid, titrable acidity, total SO_2, and free SO_2 *(P<0.001)*. The *S. cerevisiae* strains (S26, S29, S30, 43C and 45C) were more efficient acetic-acid consuming strains compared to the non-*Saccharomyces* strains *Z. bailii* ISA1307 and *L. thermotolerans* 44C. Acidic white wine that was refermented with the latter strain had a lower pH and a much reduced total SO_2 content, about six to eight times lower than the remaining strains (Table 2). This strain also showed an increase in volatile acidity, probably due to the oxidation of ethanol to acetaldehyde and acetic acid. The commercial strains S26 and S29 initiated sugar consumption most rapidly (18 and 16%, respectively, after 48 h) under the unfavourable conditions imposed by the acidic environment, whereas the other strains did not even start to

242   consume sugar at this time point (data not shown). After 264 hours, both commercial strains

243   exhausted the sugars (Table 2, statistical class "a").

244   Taking all data in consideration, strains S26 and S29 revealed as the most promising strains

245   and were used for subsequent refermentation experiments with acidic red grape must or

246   residual marc prepared from the Touriga Nacional variety.

247

248   **Deacidification of an acidic red wine**

249   We evaluated the capacity of strains S26 and S29 to remove acetic acid from an acidic red

250   wine at a pilot scale (30 L) using two alternative refermentation processes involving (i) grape

251   must (fresh grape juice with grape skins) (ii) a residual marc from a finished fermentation

252   (residual sugars 30-35 g/L), obtained from Touriga Nacional grapes.

253   In the first process, involving must addition, the initial sugar, ethanol and acetic acid

254   concentrations were 160 g/L, 4.2 % (v/v) and 1.12 g/L, respectively (Table 1). As shown in

255   Table 2, both strains produced wines with a similar final concentration of ethanol, acetic acid

256   and total SO_2, independent of MO. Wines obtained by refermentation with the must had

257   significantly lower acetic acid and a higher total SO_2 concentration in comparison to the

258   wines deacidified by the grape marcs.

259   Besides, both strains consumed simultaneously sugar and acetic acid, independent of MO

260   (Table 3). The highest acetic acid consumption of 66% was achieved at the end of

261   refermentation by the strains S29 and S26 with and without MO, respectively. There were no

262   statistical significant differences detected between strains or MO conditions. Oxygen

263   availability in this process has, however, increased the biomass of both strains during

264   refermentation (from 10^7 cells/mL without MO to 10^8 cells/mL with MO).

265   When the refermentation was carried out with the residual marc from an almost finished

266   fermentation of Touriga Nacional grape variety, the initial sugar concentration in the marc

was 30-35 g/L and dropped to 10-15 g/L after wine addition (Table 1). The ethanol and acetic acid concentrations of the wine-marc mixture were of 9.5-10 % (v/v) and 1.14 g/L, respectively. These experiments were only performed with strain S26 since strain S29 did not initiate fermentation under the experimental conditions used. The acidic red wine was added to a residual marc obtained after 96 hours of fermentation, when the marc had a volatile acidity of 0.4 g/L, increasing its concentration to 1.14 g/L. After 96 hours, the consumption of the sugars (10-15 g/L) was accompanied by a decrease of 40.4% and 39.5% of the volatile acidity, with or without MO conditions, respectively. By the use of marc for refermentation, we observed complete sugar depletion after 72 hours, significantly higher than the concentrations determined after 48 hours of refermentation with grape must.

As shown in Table 3, there were no significant differences regarding acetic acid consumption at early refermentation stages (48 and 72 hours) for both methods. However, strain S26 decreased acetic acid more efficiently in a longer refermentation process with grape must (62 – 66 %, 264 hours), than in a shorter process involving the marc (40 %, 96 hours). It seems that the consumption of the sugars was faster in refermentations conducted with marcs (96 h), possibly due to its lower initial sugar concentration (10-15 g/L). Oxygen availability also increased the biomass of this strain from 10^7 to 10^8 cells/mL, similar to the refermentation with grape must.

Aromatic characterization of the deacidified wines

The volatile aroma compound´s concentration of the six wines, deacidified by strains S26 and S29, obtained through the use of must or marc of the Touriga Nacional variety, and using or not MO, were determined by GC-MS analysis. Table 4 shows the concentrations of 22 aromatic compounds of the deacidified wines under the different conditions tested. The wines obtained from the refermentation processes with must and using strains S26 and S29 showed

292 very similar patterns of aromatic compounds. The MO conditions had no significant impact

293 on the volatile compounds in the respective deacidified wines. Contrarily, when residual marc

294 from fermentation and strain S26 was used, a significant change occurred in the aromatic

295 profile, affecting mainly the concentration of esters, which are well-known for their positive

296 contribution to the wine`s bouquet with strong, fruity aromatic notes. Independently of MO,

297 esters such as ethyl propionate (rum-like), ethyl isobutyrate (strawberry, ethereal, buttery,

298 ripe), ethyl 2-methylbutyrate (sweet, floral, fruity, apple) and ethyl isovalerate (fruity) were

299 found in significantly higher concentrations. Other esters such as ethyl butyrate (buttery, ripe

300 fruit), isoamyl acetate (banana) and 2-phenylethyl acetate (rose-like) decreased significantly

301 in comparison to the must-deacidified wines.

302 The composition of the fatty acid fraction was also analyzed. Small amounts (depending on

303 the odour threshold) of these volatile compounds contribute positively to the wine quality,

304 while excessive concentrations have detrimental effects. Octanoic acid (grass, acid like odour)

305 occurred in high concentrations in all wines. Isovaleric and hexanoic acids have rancid and

306 cheese sensory descriptors and were the ones occurring at significantly higher concentration

307 in the wines made with marcs, whereas decanoic acid (natural soap odor) was present in

308 higher concentrations in must-deacidified wines; however, the differences were not

309 significant.

310 Acetaldehyde confers a grass or apple-like aroma when found in concentrations higher than

311 100 mg/L (Carlton et al., 2007). The concentrations of this compound were rather low in

312 acidic red wine/must and red wine/marc mixtures (14.3 and 20.0 µg/L, respectively, Table 1)

313 and increased during deacidification, but not above the detection limit. When the wine/must

314 mixture was used, strain S29 showed a lower acetaldehyde concentration than strain S26.

315 Interestingly, strain S26 showed a lower concentration when the wine/marc mixture was used.

316   Under these conditions, and in combination with MO, the acetaldehyde concentration did not

317   change during refermentation.

318   The concentration of fusel alcohols such as 2-phenylethanol and isoamyl alcohol were similar

319   for all deacidified wines and remained below the detection threshold of 10 and 30 mg/L,

320   respectively. Linalool, the only terpene determined, which has pleasant lavender notes,

321   appeared in very similar concentrations in all deacidified wines.

322

323   **Wine sensory analysis**

324   The sensory analysis was performed by a trained panel of 5 judges. As mentioned in the

325   Materials and Methods section, fourteen attributes were quantified using a six-point intensity

326   scale. A total score was calculated for each wine and was expressed as the sum of average

327   scores of appearance, aroma, taste and mouth feel attributes. As shown in Table 2, strain S26

328   was better classified than strain S29 when refermentations were performed with acidic red

329   wine / must mixtures, whereas the MO had no influence. Interestingly, when the acidic wine /

330   marc mixture was used for refermentation, strain S26 achieved the highest and second lowest

331   quotes (statistically most different, $P<0.05$), without and with MO, respectively. Aroma and

332   oral perception were the attributes that mostly distinguished the six wines, while the attributes

333   grouped under the appearance criterion had no contribution for their distinction (not shown).

334   Oxygen availability neither improved nor worsened the wine sensory characteristics in these

335   kinds of fermentations.

336   All deacidified wines were analyzed by PCA, by combining data from chemical analysis and

337   sensorial evaluation (Table 2), as well as volatile compounds concentration (Table 4). Figure

338   1A represents the bi-dimensional projection of the data according to the used parameters and

339   shows that the first (factor 1) principal component (PC) explained 55.47% of the total

340   variability between the wines. This factor was mainly associated with analytical parameters

341 such as volatile acidity, pH, SO$_2$, ethyl acetate, linalool, but also other chemical compounds.

342 The second PC (factor 2) explained 26.04% of the total variability and was more associated

343 with aromatic compounds such as acetaldehyde, octanoic acid, ethyl octanoate, 2-

344 phenyletanol, diethyl succinate and others. Both principal component explained 81.51% of the

345 variability between the six wines.

346 PCA analysis also showed that the global characteristics of the six wines could separate them

347 into three well-defined groups according to the strains and the deacidification process (Figure

348 1B). These results confirm the previously described score values showing that MO had no

349 influence on the formation of these groups. Wines deacidified with strain S26 using must or

350 marc were more characterized by factor 2 and 1, respectively. Contrarily, wines obtained with

351 strain S29 were equally characterized by both factor 1 and 2. There was no clear correlation

352 between the sensorial evaluation by the panel of judges and the global PCA analysis. The

353 most preferred wine (sensory score 59.0, fermentation of acidic red wine using marc and

354 strain S26) was apart from the least preferred wine (sensory score 43.6, fermentation of acidic

355 red wine using must, MO and strain S29). On the other hand, the most preferred wine was

356 close to the second least preferred wine (sensory score 44.4, fermentation of acidic red wine

357 using marc, MO and strain S26).

358

359 **DISCUSSION**

360

361 This study shows the applicability of *S. cerevisiae* commercial strains S26 and S29 to remove

362 volatile acidity from acidic white and red wines through refermentation processes with grape

363 must or residual marc at pilot scale. Besides, data are provided and discussed regarding the

364 effect of the refermentation process and the application of MO on acetic acid removal and the

365 aromatic composition of the resulting wines. Among the different yeast species tested, the

commercial *S. cerevisiae* strains S26 and S29 were most interesting because they efficiently reduced both acetic acid (43 and 47 %, respectively) and sugar (100 %), after 264 hours of refermentation of an acidic white wine that was supplemented with grape must. Moreover, they initiated sugar consumption much earlier than the other strains, and were therefore used for subsequent experiments with acidic red wines. Their better tolerance to the combined stressful conditions caused by sugar, ethanol and acetic acid is not surprising because commercial strains are selected and improved for a very high robustness. The degree of acetic acid reduction in the refermentations carried out with grape must was not dependent on the yeast strain, but rather on the wine style. With red wine and after 264 h, the decrease was more pronounced than with white wine (2/3 and 1/3 of the initial acetic acid value, respectively). This might be due to a better adaptation of both strains to red wine or a more favourable composition in the red wine / must mixture for acetic acid consumption. Another explanation might be the vinification process itself - red wines are usually produced with some aeration of the grape must during pump overs, that can transfer an amount of 5 mg/L of oxygen (Silva and Lambri, 2006), stimulating yeast growth, and leading also to the formation of tannin-anthocyanin bonds and color stabilization (McCord, 2003; Sánchez-Iglesias et al., 2009; Zironi et al., 2010). However, 40% of removal in white wine was sufficient to attain acetic acid concentrations that correspond to the usual concentration in wines (0.6 – 0.8 g/L). We then assessed the deacidification performance of both strains using a red acidic wine applying or not MO. Oxygen supplementation improves synthesis of sterols and other unsaturated fatty acids that are necessary for plasma membrane integrity (Rosenfeld et al., 2003; Traverso-Rueda and Kunkee, 1982) and increases cell biomass (Sablayrolles and Barre, 1987). Consistently, we could observe that additional oxygen supplementation increased the final cell number. However, acetic acid consumption during must-mediated refermentation was not affected by MO. This behaviour indicates that the oxygen availability provided

391   during pumpovers is sufficient for acetic acid removal by strain S26 from a red acidic wine.

392   Strain S26 conducted both refermentation processes, with grape must or with residual marc,

393   showing higher tolerance than strain S29 to the combined effects of various stress factors,

394   such as high concentration of acetic acid and ethanol.

395   Both deacidification processes of the acidic red wine mixtures conferred distinct aromatic

396   characteristics to the final wines. This was most notable for the fraction of ester compounds,

397   (e.g. ethyl propionate, ethyl isobutyrate, ethyl-2-methylbutyrate, ethyl isovalerate), but also

398   for the isovaleric and hexanoic fatty acids, that were significantly higher in wines prepared

399   from the marc/wine mixture than from must/wine mixture. Slight and (in most cases)

400   statistically not significant differences were observed between micro-oxygenated or not

401   micro-oxygenated wines, independently of the deacidification process and strain. Nitrogen

402   source limitation (Mendes-Ferreira et al., 2004), high ethanol concentrations (Boulton et al.,

403   1996), or a combination of both may have favored the expression of enzymes like ATF1- and

404   ATF2-encoded alcohol acetyltransferases of *S. cerevisiae,* responsible for the synthesis of

405   ethyl acetate and isoamyl acetate esters**,** that improve the floral and fruity aroma of wine

406   (Lilly et al., 2006). In fact, these conditions occur in the deacidification process with the

407   marcs and are most probably the cause of the aromatic characteristics achieved by the wine at

408   the end of refermentation. The aromatic profiles of wines prepared from must/wine mixtures

409   tend to be richer in esters like isoamyl acetate and 2-phenylethyl acetate. The lack of MO

410   conferred more floral notes to the respective wines. González-Sanjosé et al. (2009) found

411   significant lower intensity of vegetal and reduction odor notes, and slightly more intense

412   fruity notes in micro-oxygenated wines.

413   PCA clearly showed that MO had no significant impact on the final aromatic composition of

414   the wines that are grouped according to the strain and deacidification process used. These

415   findings were, partially, confirmed by a panel of 5 judges. Their order of preference did not

distinguish wines that were micro-oxygenated or not and that were prepared from acidic wine/must mixtures. PCA analysis (and the judges' scores, to some degree) distinguished the wines obtained with either strain. This is in agreement with a plethora of publications showing the effect of different *S. cerevisiae* strains on the concentration of aromatic volatile compounds (Mauriello et al., 2009, Callejon et al., 2010, and references cited therein). Wines obtained from marc/wine mixture that were refermented by strain S26 without MO were most preferred and obtained clearly the highest rating by the evaluation panel. In general, neither the projection of a wine on the PCA factor plane was correlated with the judge's order of preference, nor with single compounds, such as acetic acid or acetaldehyde. These results are expected and explained by the very high complexity of hundreds of compounds that occur in a wine. Their relative concentrations and interactions that are perceived by a trained wine taster is certainly not reflected by the comparatively very low number of 27 compounds that were evaluated within the present study.

In summary, we successfully established an efficient enological solution for the biological reduction of volatile acidity of acidic wines based on the refermentation with residual marcs and the use of the commercial yeast strain S26. About 40% of acetic acid reduction was achieved after 72 hours. Besides, MO had no impact on both the acetic acid removal efficiency and the global composition of volatile compounds. The judges clearly preferred the wine produced without MO, using marc and strain S26. The proposed procedure was achieved by a very careful evaluation of both the yeast physiology and the process used for refermentation. We propose our approach can be an efficient and cheap alternative for the biological rectification of too acidic wines, using marc as a fermentation end product.

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578

579   Figure 1.

580   Bi-dimensional PCA by the combination of data from chemical analysis and sensorial

581   evaluation (Table 2), and volatile compounds concentration (Table 4). (A) projection of the

582   data according to the chemical parameters (B) projection of the data according to the strains

583   and the deacidification process.

1 Table 1.

2 Oenological parameters of the acidic wines, musts and mixtures of acidic wines with musts or marcs used in the deacidification assays carried

3 out at a pilot scale.

Chemical characteristics	Acidic white wine	Viosinho must	Viosinho must plus acidic white wine	Acidic red wine	T. Nacional must	T. Nacional must plus acidic red wine	T. Nacional marc plus acidic red wine	Analytical Methods (CEE N.º 2676/90)[1]
Density at 20ºC	0.9906	n.d.	n.d.	0.9908	n.d.	n.d.	n.d.	Densitometry
Free SO_2 (mg/L)	0.0	n.d.	0.0	0.0	n.d.	40.0	0.0	Ripper Method
Total SO_2 (mg/L)	23.0	n.d.	7.7	25.0	n.d.	49.0	41.6	Ripper Method
Volatile acidity (g/L acetic acid)	3.30	0.13	1.15	2.80	0.21	1.12	1.14	Destillation (Cazenave-Ferré, followed by titration with phenolphthalein)
Sugar (g/L)	1.00	224	157	1.12	230	160	10-15	Lane-Eynon Method
Titratable acidity (g/L tartaric acid)	7.10	9.83	8.90	7.05	10.73	8.90	8.03	Titration with bromothymol blue
pH	2.88	3.23	3.00	2.98	3.25	3.02	3.15	Potentiometer
Alcoholic degree %, Ethanol (v/v)	12.0	n.d.	4.3	12.5	n.d.	4.2	9.5 - 10	Distillation
Estimated alcohol content (%, v/v)	n.d.	13.0	n.d.	n.d.	13.0	n.d.	n.d.	Refractometry
Acetaldehyde (µg/L)	n.d.	n.d	n.d.	42.9	n.d	14.3	20.0	SPME /GC-MS[2]

4 (1) CEE N.º 2676/90 – Official Journal of the European Communities, 33, 3.10.1990. (ISSN 0257 – 7771)
5 (2) (SPME/GC-MS – As described in Mendes-Ferreira et al. (2009))
6 n.d. – not determined.

7 Table 2

8 Chemical characterization of the mixtures of white and red acidic wines with must or marcs before and after refermentation with grape must of

9 Viosinho grapes (without MO, after 264 h) and with grape must or marc of Touriga Nacional grapes (with or without MO, after 96 h).

Conditions	Yeast Strains	Ethanol % (v/v)*	pH*	Acetic acid (g/L)*	Titratable acidity (g/L)*	Total SO$_2$ (mg/L)*	Free SO$_2$ (mg/L)*	Sugars (g/L)*	Sensory analysis[#]** (total score)
Acidic white wine/must mixture		4.3	3.00	1.15	8.90	7.7	0.0	157	
Deacidified white wines	S26	12.1±0.04 [a]	3.19±0.01 [b]	0.61±0.02 [a, b]	6.62±0.19 [a]	33.3±1.08 [b]	0.48±0.23 [a]	0.0±0.0 [a]	n.d.
	S29	11.9±0.04 [a]	3.19±0.01 [b]	0.66±0.08 [a, b]	6.62±0.04 [a]	34.3±2.54 [b]	0.33±0.23 [a]	0.0±0.0 [a]	n.d.
	S30	11.8±0.04 [a]	3.16±0.01 [b]	0.73±0.02 [a, b]	7.11±0.18 [a]	37.9±3.26 [b]	0.64±0.45 [a]	3.13±1.03 [a]	n.d.
	43C	11.9±0.14 [a]	3.16±0.01 [b]	0.72±0.08 [a, b]	7.13±0.95 [a]	36.1±3.61 [b]	0.80±0.68 [a]	0.79±1.30 [a]	n.d.
	45C	11.9±0.11 [a]	3.14±0.01 [a, b]	0.67±0.02 [a, b]	6.73±0.24 [a]	39.4±2.54 [b]	0.91±0.98 [a]	0.0±0.0 [a]	n.d.
	44C	8.0±5.59 [a]	3.08±0.03 [a]	1.40±0.20 [b]	8.63±1.59 [a]	4.6±3.53 [a]	1.14±1.56 [a]	101.89±2.15 [b]	n.d.
	ISA 1307	11.4±0.11 [a]	3.17±0.01 [b]	0.83±0.02 [a, b]	6.75±0.08 [a]	38.4±3.26 [b]	0.32±0.98 [a]	32.97±2.51 [b]	n.d.
Acidic red wine/must mixture		4.2	3.02	1.12	8.90	49.00	40.0	160	
Deacidified red wines with MO	S26	11.8±0.20 [a]	3.29±0.02 [c, d]	0.42±0.06 [a]	8.35±0.61 [a]	115.01±4.86 [c]	0.0±0.0 [a]	0.0±0.0 [a]	50.2 ± 5.5 [a, b, c]
	S29	11.3±0.10 [a]	3.31±0.03 [c, d]	0.38±0.11 [a]	7.80±0.27 [a]	130.71±17.05 [d]	0.0±0.0 [a]	0.0±0.0 [a]	43.6 ± 9.3 [a]
Deacidified red wines without MO	S26	11.1±0.30 [a]	3.29±0.05 [c]	0.38±0.07 [a]	8.35±0.17 [a]	105.22±15.57 [c]	0.0±0.0 [a]	0.0±0.0 [a]	55.4 ± 7.9 [b, c]
	S29	11.0±0.30 [a]	3.32±0.05 [d]	0.45±0.08 [a]	8.03±0.40 [a]	102.57±2.24 [c]	0.0±0.0 [a]	0.0±0.0 [a]	45.2 ± 10.3 [a, b]
Acidic red wine/marc mixture		9.5-10	3.15	1.14	8.03	41.60	0.0	10-15	
Deacidified red wines with MO	S26	12.1±0.60 [a]	3.44±0.03 [e]	0.69±0.09 [a, b]	7.46±0.16 [a]	46.93±2.96 [b]	0.0±0.0 [a]	0.0±0.0 [a]	44.4 ± 8.4 [a, b]
Deacidified red wines without MO	S26	11.9±0.30 [a]	3.50±0.03 [e]	0.68±0.05 [a, b]	7.56±0.49 [a]	56.32±7.13 [b]	0.0±0.0 [a]	0.0±0.0 [a]	59.0 ± 7.2 [c]

10
11 * Results obtained for strains/wines marked with the same superscript letters are not significantly different (*P*<0.001) for the same analytical parameter.
12 [#] Total score (sum of average scores for appearance, aroma, taste and mouth feel attributes)
13 ** Results obtained for strains/wines marked with the same superscript letters are not significantly different (*P*<0.05).
14

15 Table 3

16 Percentage of acetic acid (**bold letters**) and sugar consumption after refermentation of red wine with must or marc by *S. cerevisiae* strains S26

17 and S29, after 48 and 264 hours (refermentation with the must) or 72 and 96 hours (refermentation with the marcs) with and without MO. Results

18 obtained for strains and culture conditions with the same superscript letter are not significantly different (*P*<0.001).

19

	Red wine refermentation with grape must				Red wine refermentation with marc			
	48 h		264 h		72 h		96 h	
	With MO	Without MO	With MO	Without MO	With MO	Without MO	With MO	Without MO
Yeast	**Acetic acid**	**Acetic acid**	**Acetic acid**	**Acetic acid**	**Acetic acid**	**Acetic acid**	**Acetic acid**	**Acetic acid**
strain	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar	Sugar
	35.7 ± 1.57 [a]	**37.5 ± 4.72** [a]	**66.1 ± 6.30** [b]	**62.5 ± 5.45** [b]	**38.6 ± 3.15** [a]	**41.2 ± 6.86** [a]	**40.4 ± 4.17** [a]	**39.5 ± 8.18** [a]
S26	20.6 ± 4.33 [a]	14.6 ± 6.51 [a]	100.0 ± 0.0 [b]	100.0 ± 0.0 [b]	100.0 ± 0.0 [b]	100.0 ± 0.0 [b]	100.0 ± 0.0 [b]	100.0 ± 0.0 [b]
	42.9 ± 5.68 [a]	**44.6 ± 12.30** [a]	**59.8 ± 7.22** [b]	**66.1 ± 9.58** [b]	no refermen-tation	no refermen-tation	no refermen-tation	no refermen-tation
S29	25.8 ± 10.10 [a]	23.8 ± 4.10 [a]	100.0 ± 0.0 [b]	100.0 ± 0.0 [b]				

20

21

30
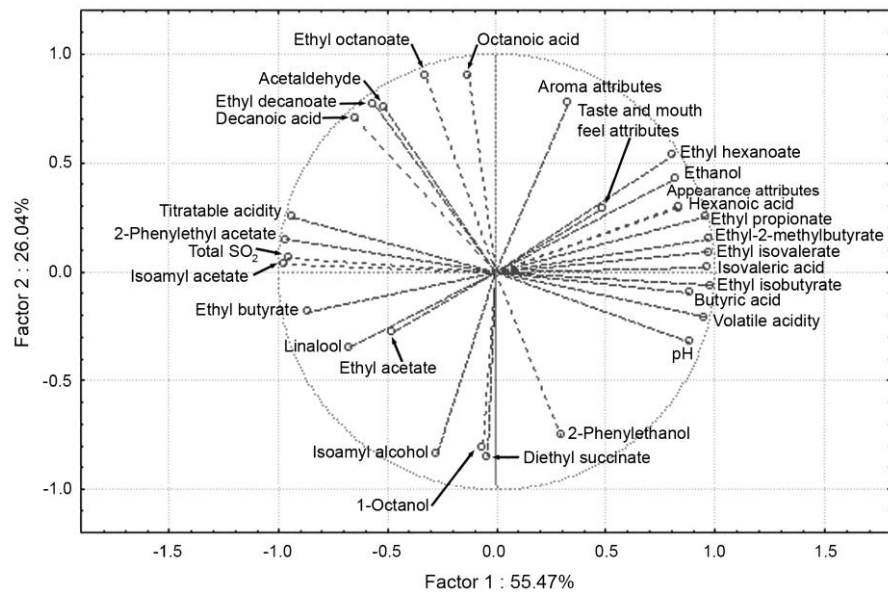
22   Table 4

23   Average volatile compounds concentration (µg/L) determined by GC-MS. Results refer to the six deacidified wines through refermentation by *S.*

24   *cerevisiae* strains S26 and S29, with grape must or marc of the Touriga Nacional grape variety, after 96 h and applying or not MO. Values with

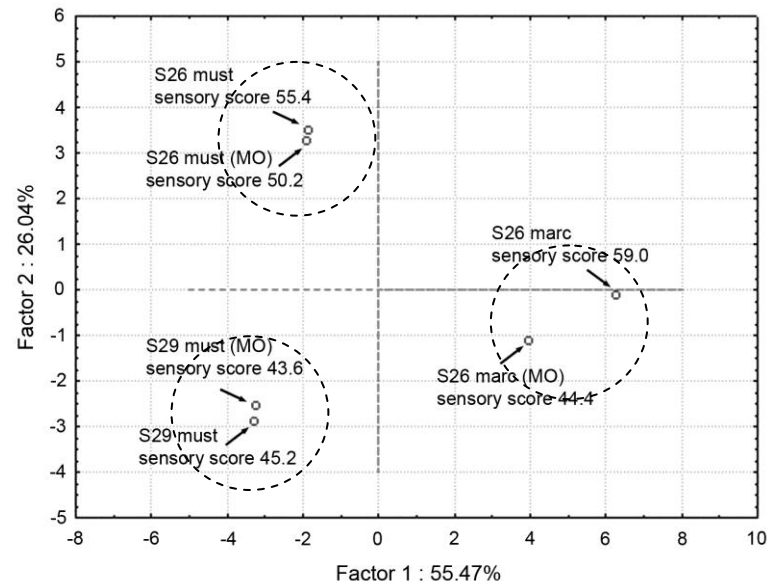25   the same superscript letter, for the same aromatic compound, are not significantly different (*P*<0.05).

Compounds		Yeast strains and deacidification processes					
		S26 must	S26 must MO	S29 must	S29 must MO	S26 marc	S26 marc MO)
Ethyl acetate	Esters	59.90 ± 0.98 [a]	47.50 ± 2.28 [a]	58.16 ± 8.26 [a]	60.14 ± 0.26 [a]	54.07 ± 6.19 [a]	47.95 ± 3.85 [a]
Ethyl propionate		90.26 ± 1.57 [a, b,c]	80.76 ± 7.57 [a, b]	64.77 ± 10.66 [a]	68.07 ± 1.91 [a]	117.96 ± 12.14 [c]	107.48 ± 4.96 [b, c]
Ethyl isobutyrate		58.36 ± 0.72 [a]	52.71 ± 7.06 [a]	53.01 ± 2.96 [a]	52.74 ± 4.50 [a]	93.51 ± 12.35 [c]	77.19 ± 2.68 [b, c]
Ethyl butyrate		179.50 ± 7.04 [a]	153.60 ± 6.41 [c]	188.23 ± 2.91 [a]	191.30 ± 1.63 [a]	139.46 ± 5.44 [b]	122.56 ± 7.05 [a,b]
Ethyl 2-methylbutyrate		10.23 ± 0.37 [a, b]	10.09 ± 1.14 [a, b]	8.96 ± 0.62 [a, b]	8.16 ± 0.40 [a]	15.22 ± 1.69 [c]	12.60 ± 0.71 [b, c]
Ethyl isovalerate		10.23 ± 0.45 [a, b]	9.82 ± 0.54 [a, b]	9.19 ± 0.16 [a]	8.91 ± 0.43 [a]	14.37 ± 0.93 [c]	11.98 ± 0.71 [b]
Isoamyl acetate		4.30 ± 0.22 [a]	3.59 ± 0.50 [a]	4.65 ± 0.22 [a]	4.57 ± 0.56 [a]	0.57 ± 0.05 [b]	0.42 ± 0.04 [b]
Ethyl hexanoate		128.96 ± 33.59 [a]	132.93 ± 5.42 [a]	110.41 ± 3.89 [a]	109.73 ± 2.38 [a]	149.80 ± 7.46 [a]	129.38 ± 35.07 [a]
Ethyl octanoate		56.60 ± 23.26 [a]	54.26 ± 2.83 [a]	44.14 ± 7.26 [a]	47.34 ± 8.95 [a]	45.45 ± 10.13 [a]	46.74 ± 9.02 [a]
Ethyl decanoate		39.33 ± 31.76 [a]	49.43 ± 12.95 [a]	25.90 ± 24.04 [a]	20.29 ± 2.49 [a]	13.12 ± 8.22 [a]	18.39 ± 5.06 [a]
Diethyl succinate		2.22 ± 0.19 [a]	1.94 ± 0.12 [a]	3.23 ± 1.84 [a]	4.91 ± 0.76 [a]	3.41 ± 0.40 [a]	3.04 ± 0.09 [a]
2-Phenylethyl acetate		192.63 ± 9.33 [a]	155.43 ± 8.56 [a]	176.12 ± 34.82 [a]	183.17 ± 23.53 [a]	15.66 ± 0.36 [b]	12.09 ± 0.63 [b]
Butyric acid	Fatty acids	129.21 ± 20.72 [a]	103.84 ± 7.31 [a]	121.81 ± 5.57 [a]	111.85 ± 24.32 [a]	166.69 ± 33.91 [a]	139.82 ± 0.79 [a]
Isovaleric acid		232.50 ± 19.20 [a, b]	210.00 ± 8.85 [a]	191.18 ± 16.89 [a]	215.68 ± 1.43 [a]	336.11 ± 15.57 [c]	278.22 ± 17.99 [b, c]
Hexanoic acid		758.49 ± 27.45 [a, b]	660.63 ± 16.35 [a]	560.38 ± 36.62 [a]	653.00 ± 16.05 [a]	918.75 ± 59.92 [b]	734.80 ± 121.04 [a, b]
Octanoic acid		1551.41 ± 127.82 [a]	1361.09 ± 58.87 [a]	1084.24 ± 285.47 [a]	1187.44 ± 65.61 [a]	1243.41 ± 110.88 [a]	1154.40 ± 211.97 [a]
Decanoic acid		729.48 ± 349.61 [a]	780.90 ± 113.21 [a]	617.81 ± 419.42 [a]	472.01 ± 104.18 [a]	394.15 ± 167.38 [a]	454.90 ± 42.42 [a]
Acetaldehyde	Others	86.09 ± 0.91 [b]	65.17 ± 14.77 [a, b]	45.20 ± 21.32 [a, b]	45.20 ± 25.75 [a, b]	41.23 ± 6.34 [a, b]	20.96 ± 1.91 [a]
Linalool		17.99 ± 1.06 [a]	18.97 ± 3.79 [a]	18.98 ± 1.97 [a]	22.52 ± 0.03 [a]	17.62 ± 0.47 [a]	16.15 ± 0.05 [a]
1-octanol		13.14 ± 1.65 [a]	12.61 ± 1.16 [a]	18.55 ± 0.83 [b]	14.91 ± 1.29 [a, b]	14.71 ± 0.59 [a, b]	15.62 ± 0.13 [a, b]
2-phenylethanol		24.05 ± 2.21 [a,b]	23.14 ± 3.95 [a]	26.37 ± 0.00 [a,b]	31.33 ± 1.66 [b]	30.20 ± 1.19 [a]	26.79 ± 0.69 [a,b]
Isoamyl alcohol		194.34 ± 20.03 [a]	174.50 ± 12.62 [a]	227.86 ± 29.74 [a]	244.89 ± 13.27 [a]	209.86 ± 19.23 [a]	193.43 ± 1.50 [a]

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A

B

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30   Figure 1

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