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ORIGINAL PAPER

Aromatic compounds released from natural precursors by selected *Oenococcus oeni* strains during malolactic fermentation

Fátima Pérez-Martín · Pedro Miguel Izquierdo-Cañas · Susana Seseña · Esteban García-Romero · María Llanos Palop

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Abstract The changes produced at the volatile fraction of a Tempranillo wine added with a natural glycosidic extract and commercial esters by the action of inoculated *Oenococcus oeni* strains during malolactic fermentation were assessed. The glycosidic extract was obtained from must of Muscat grape variety, and strains used had been selected in a previous work by their glycosidase and esterase activities against synthetic substrates. Fermentation assays were maintained in incubation for 22 days; after that, wines were analysed for their chemical parameters and the volatile compounds. Significant differences in the concentration of some volatile compounds in wines obtained with the different strains were observed, which could affect the sensory characteristics of the wines. The assayed strains were able to release terpenes, norisoprenoids and C6 alcohols, and to hydrolyse esters such as butyl acetate and 2-phenylethyl acetate. These results revealed that strains presented both glycosidase and esterase activities although in a strain-dependent manner. Strains Da32 and Ab11 are good candidates to be used when floral wines are desired, while strain 93 is the best preserving the fruity aroma of wines.

F. Pérez-Martín · S. Seseña (✉) · M. L. Palop
Department of Analytical Chemistry and Food Technology, Environmental Sciences and Biochemistry Faculty, University of Castilla-La Mancha, Avda. Carlos III, s/n, 45071 Toledo, Spain
e-mail: susana.sprieto@uclm.es

P. M. Izquierdo-Cañas · E. García-Romero
Institute of Vine and Wine of Castilla-La Mancha, Crta. Toledo-Albacete, s/n, 13700 Tomelloso, Ciudad Real, Spain

P. M. Izquierdo-Cañas
Scientific and Technologic Park of Albacete, Paseo de la Innovación 1, 02006 Albacete, Spain

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Introduction

Malolactic fermentation (MLF) is a biochemical process that typically occurs in wine after alcoholic fermentation converting L-malic acid into L-lactic acid and CO_2, resulting in deacidification and enhanced microbiological stability of the wine. In addition, changes in the aroma profile of wine, associated with the metabolic side activities of lactic acid bacteria (LAB) participating (i.e. *Lactobacillus*, *Pediococcus* and *Oenococcus* species), have also been reported [1–3]. The ability of these bacteria to produce a wide range of potentially important enzymes, including those capable of hydrolysing glycosides and ester substrates, would be responsible of these changes [4–9] since a significant fraction of the aroma compounds present in grapes and wine occurs as non-volatile odourless glycosides [10]. Monoterpenes, C_{13}-norisoprenoids, benzene derivatives and aliphatic compounds are frequently found as D-glucopyranosides being the volatile aglycone linked, by β-glycoside bond, to a single D-glucopyranose, or to a disaccharide, in which the glucose can further be conjugated to apiose, arabinose, rhamnose or xylose [10, 11]. For it, and to improve the wine aroma, some authors have assessed the possibility of carrying out sequential enzymatic hydrolysis of these aroma precursors by adding commercial fungal glycosidases (glucosidase, arabinosidase, rhamnosidase, apiosidase). However, these preparations contain side activities and often unpredictable effects [12, 13].

The glycosidase activity in LAB has been proven in several studies using synthetic substrates, such as *p*-nitrophenyl-glycosides, under controlled conditions [8, 9, 14–17].

However, Gagné et al. [9] report that the glycosidase potential is not accurately estimated in assays conducted with this kind of substrates indicating that this activity should be assayed using natural substrates. The ability of *Oenococcus* (*O.*) *oeni* to release diverse aglycons from natural glycosylated compounds extracted from grapes [18, 19] and oak wood [9, 20, 21] has been displayed.

Esters are, coupled to glycosides, an important group of aroma-active compounds being responsible for the fruity aroma of wine. The main esters at wines are C_4 to C_{10} ethyl esters of organic acids, ethyl esters of fatty acids and acetates of higher alcohols [22]. However, the ester-associated aroma profile in wines depends not only on the esters involved, but also on the compounds liberated by the esterases, such as fatty acids and higher alcohols [23]. Esterolytic activity during wine production has the potential to either increase or decrease the amount of esters present, influencing therefore the perceived quality.

LAB have a variable ability to hydrolyse a range of artificial ester substrates [5–7, 24, 25], and production of esterases for many LAB from dairy industry has been reported [26, 27]. On the contrary, the esterase activity of wine-related LAB is still not well-documented, although evidences exist that some LAB from wine possess a strain-dependent esterase activity, as observed from changes in concentration of individual esters during MLF [28–30]. Sumby et al. [31] compared the activity of purified *O. oeni* esterases with that from whole cells towards natural substrates in a wine environment, demonstrating the ability of these esterases to both synthesise and hydrolyse esters depending on the strain and the wine variety used. Therefore, it is important to evaluate the behaviour of the strains directly in the wine where they will be used.

The aim of the present study was to assess the changes produced at the volatile fraction of a wine added with a natural glycosidic extract and commercial esters by the action of inoculated *O. oeni* strains during MLF. Strains used had been selected in a previous work by their glycosidase and esterase activities against synthetic substrates [16, 25], and now, their behaviour using natural precursors is assayed.

Materials and methods

Bacterial strains and growth conditions

Five *O. oeni* strains were used in this study: four of them (Da32, Ab11, 93 and 23) had been selected in a previous work by their glycosidase and/or esterase activities [16, 25] and the strain *O. oeni* CECT 7621 had been previously selected in our laboratory to be used as a starter culture for MLF [32]. Strain Da32 was used as a positive control for glycosidase activity and *O. oeni* CECT 7621 was the negative control. For esterase activity, positive and negative controls were strain 23 and 93, respectively. Strain Ab11 had shown an intermediate value for both glycosidase and esterase activities.

Before use, pure cultures of these strains, kept as frozen stocks at −80 °C, were revitalised by culturing on MLO (*Leuconostoc oenos* Medium, Scharlab, Barcelona, Spain) agar and anaerobic incubation (Gas Pack System, Oxoid, Ltd, Basingstoke, Hampshire, UK) for 5 days at 30 °C.

Preparation of the extract

An extract from must of Muscat grape variety, one of the most aromatic and rich in glycoside compounds' varieties, was obtained following the procedure described by Hernández-Orte et al. [4] with slight modifications. 500 mL of must obtained in the experimental winery of the Institute of Vine and Wine of Castilla-La Mancha (IVICAM) from grapes of its own vineyards were filtered through filter paper and percolated through a LiChrolut EN (1,300 mg, Merck, Darmstadt, Germany) resin bed previously pre-conditioned with 32 mL of dichloromethane, 32 mL of methanol and 65 mL of water. Then, the column was washed with 250 mL of water, dried by letting air pass through and washed again with 40 mL of a pentane: dichloromethane (2:1 v/v) mixture. Finally, the retained compounds were eluted with 100 mL of an ethyl acetate: methanol (9:1 v/v) mixture. This extract was evaporated to dryness in a rotary evaporator at 150 mbar and 40 °C, and the dry glycosidic extract was reconstituted in 5 mL of a 10 % (v/v) ethanol solution and stored at −20 °C.

Fermentation assays

A Tempranillo wine after the completion of alcoholic fermentation was used. It was filtered through a 0.2-μm pore size module (Millipore, Billerica, MA, USA) for sterilisation.

The chemical composition of this wine was as follows: 13.02 % (v/v) alcohol content, 4.84 g/L total acidity, 0.29 g/L volatile acidity, pH 3.53, 2.00 g/L malic acid, 0.55 g/L citric acid and 16.00 mg/L free SO_2 content.

For fermentations, 2 L of the Tempranillo wine was added with the glycosidic extract obtained as described above, and with the commercial esters 2-phenylethyl acetate (400 μg/L) and butyl acetate (4 mg/L) (Sigma, Madrid, Spain). Flasks containing a volume of 110 mL of this modified Tempranillo wine were used for each fermentation assay.

Cultures in MLO from each *O. oeni* strain were used to inoculate (1 % v/v) the modified wine to reach an initial population of 10^6 cells/mL. For each strain, fermentations in triplicate at a constant temperature of 25 °C were carried out.

The evolution of MLF was monitored by measuring the L-malic acid content using the kit L-malic acid (Megazyme, Ireland), and when this content was lower than 0.2 g/L, MLF was considered as finished. All the fermentation assays were maintained in incubation for 22 days, independently of when MLF had finished, to avoid differences due to a different time of contact wine bacteria. After the 22 days, SO_2 was added to reach a concentration of 25 mg/L free SO_2.

Microbiological analysis

Samples were taken under aseptic conditions immediately after inoculation and after 22 days of incubation. Counts were carried out by plating on MLO Agar plates, which were incubated under anaerobic conditions (Gas Pack System, Oxoid Ltd., Basingstoke, UK) at 30 °C for 5 days. Counts were performed in duplicate and expressed as colony forming units (CFU) per mL of wine.

In order to confirm the presence of the inoculated *O. oeni* strain, a 10 % from the total number of colonies from countable MLOA plates was picked at random and purified by successive streaking on the same medium prior to typing by RAPD-PCR (Randomly Amplified Polymorphic DNA analysis) as described by Ruiz et al. [33].

A comparison between genetic profiles of the isolates and the profile of the inoculated strain was performed, and the implantation rate was calculated as the number of colonies with the specific RAPD-PCR profile divided by the total number of colonies picked, expressed as a percentage [34].

Chemical analysis of wines

Wines after 22 days of fermentation were chemically characterised by determining total acidity, pH, volatile acidity, L-lactic acid and citric acid, following the Official Analytical Methods established by the International Organisation of Vine and Wine [35].

Volatile compound analysis

Minor volatile compounds were extracted by solid phase extraction (SPE) following the procedure described by Ibarz et al. [36]. 25 mL of wine was passed through columns filled with 0.2 g of LiChrolut EN (40–120 µm, Merck) using 4-nonanol as internal standard. Then, the columns were washed with 25 mL of water to remove sugars, acids and other polar substances. The minority fraction of volatile compounds (free flavour) was eluted with 15 mL of pentane: dichloromethane (2:1 v/v). The extracts were concentrated by distillation at 40 °C with a Vigreux column and subsequently under a stream of N_2 to a volume of 150 µL and stored at −20 °C until analysis.

The volatile compounds were analysed by GC/MS using a gas chromatograph TraceGC Ultra coupled to a mass spectrometer DSQ II with electron impact ionisation source and quadrupole analyser, equipped with an autosampler TriPlus all from ThermoQuest. The conditions of the detector were as follows: electron multiplier voltage, 1,592 V; impact energy, 70 eV; ion source temperature, 250 °C; and mass scanning range, 39–400 amu.

The separated compounds were identified by their mass spectra and chromatographic retention time using commercial products as standard. Quantification was performed by analysing the characteristic m/z fragments for each compound using the internal standard method. The results for the unavailable products were expressed as ratio of the area of each compound and internal standard.

Statistical analysis

The ANOVA and the Student–Newman–Keuls *t* test were used to determine whether there were significant differences between the results from chemical and volatile compounds analysis. For statistical analysis, the SPSS 12.0 software was used.

Results

Microbiological and chemical analysis

For all the strains, counts on MLOA plates were on the order of 10^7–10^8 CFU/mL at day 22 of incubation. The implantation values for the assayed strains at this time ranged from 73.3 % for strain Da32 to 100 % for strains *O. oeni* CECT 7621, 93 and Ab11, which confirmed the presence and the involvement in the MLF of the inoculated strain.

Table 1 summarises the mean values ± standard deviation of the chemical parameters analysed in the wines after 22 days of incubation. A similar degradation of L-malic acid was observed for all the strains being slower during the first days following the inoculation. Values at Table 1 show that MLF was completed in all wines.

As expected, a decrease in the total acidity and the citric acid content and the subsequent increases in pH and lactic acid content were observed. Strain Da32 produced the highest quantity of lactic acid and almost a complete degradation of citric acid, and consequently, the volatile acidity was the highest. On the contrary, the strain CECT 7621 showed the lowest lactic acid content and volatile acidity.

The remaining strains showed a similar behaviour with values for lactic acid content and volatile acidity between those of the strains Da32 and CECT 7621.

Table 1 Chemical composition of wines from different *O. oeni* strains after 22 days of incubation

	Initial wine	*O. oeni* CECT 7621 Negative control for glycosidase activity	Da32 Positive control for glycosidase activity	93 Negative control for esterase activity	23 Positive control for esterase activity	Ab11
Total acidity (g/L)	4.84	4.22 ± 0.05^{bc}	4.27 ± 0.13^{c}	3.95 ± 0.05^{a}	4.01 ± 0.16^{ab}	4.19 ± 0.01^{bc}
pH	3.53	3.64 ± 0.01^{b}	3.67 ± 0.02^{c}	3.70 ± 0.01^{d}	3.70 ± 0.02^{d}	3.62 ± 0.01^{a}
Volatile acidity (g/L)	0.29	0.42 ± 0.01^{a}	0.75 ± 0.05^{d}	0.58 ± 0.05^{bc}	0.64 ± 0.06^{c}	0.53 ± 0.01^{b}
L-lactic acid (g/L)	0.03	1.00 ± 0.02^{a}	1.33 ± 0.04^{c}	1.19 ± 0.02^{b}	1.25 ± 0.02^{b}	1.25 ± 0.05^{b}
Citric acid (g/L)	0.55	0.54 ± 0.03^{d}	0.03 ± 0.01^{a}	0.11 ± 0.04^{b}	0.08 ± 0.03^{b}	0.45 ± 0.01^{c}
Malic acid (g/L)	2.00	0.20 ± 0.03^{b}	0.10 ± 0.01^{a}	0.15 ± 0.05^{a}	0.12 ± 0.02^{a}	0.19 ± 0.02^{b}

Different superscripts (a, b, c) indicate significant differences between strains for $\alpha = 0.05$ according to the Student–Newman–Keuls test

Volatile compounds

Table 2 shows the mean values and the standard deviation for the volatile compounds analysed in the wines after 22 days of incubation. Statistically significant differences between strains were observed for some of the volatile compounds analysed, being some of them important aroma-active compounds.

For all the strains, increases in the concentration for the majority of terpenes and for *t*-2-hexenol and damascenone were observed. Benzyl alcohol concentration increased at two of the five strains. Decreases in the concentration were observed for the majority of remaining compounds.

Statistically significant differences between the five strains were observed for *t*-2-hexenol content, showing the strains Ab11 and Da32 the highest concentrations of this compound, with a 58 and 103 % more concentration, respectively, than the strain *O. oeni* CECT 7621, which showed the lowest one.

Strains Ab11 and Da32 also presented significantly higher concentrations of some terpenes if compared with *O. oeni* CECT 7621. So, nerol concentrations were 75 and 77 % higher, respectively; *c*-linalool furanic oxide concentrations were 66 and 144 % higher; and *t*-linalool piranic oxide concentrations were 76 and 150 % higher. Likewise, they produced a 40 and 82 % more quantity of *t*-linalool furanic oxide, respectively, compared with the strain 93, which showed the lowest concentration of this compound.

For benzyl alcohol and eugenol, the same behaviour was observed, and concentrations for strain Ab11 were 36 and 57 % higher, respectively, than those for *O. oeni* CECT 7621, while concentrations for strain Da32 were 48 and 99 % higher.

The highest concentrations for *t*-isoeugenol were from strains Da32 and 23, being three- and fivefold, respectively, that of *O. oeni* CECT 7621. In addition, strain 23 showed ninefold more concentration of zingerone than strain Da32.

With respect to the two esters, butyl acetate and 2-phenylethyl acetate, added to the Tempranillo wine, a different behaviour of the strains was observed, although significant differences in the concentrations for these compounds were not observed between them. It is worth noting that strains 23, Ab11 and Da32 degraded around a 55 % more butyl acetate than *O. oeni* CECT 7621, while this last degraded a 16 % more 2-phenylethyl acetate than the remaining strains. Wines obtained from the strain 93 showed higher concentrations of the different acetates followed by those from the strains Ab11 and Da32.

For the ethyl esters group, it is important to highlight that strain *O. oeni* CECT 7621 presented, contrarily to what expected, higher esterase activity than the remaining strains since the wines obtained from it presented a lower concentration for the majority of the ethyl esters analysed. Concentrations of ethyl hexanoate and 2-hydroxy-ethyl caproate were 23 and 32 % lower than those for strain Da32, respectively. Only the ethyl butyrate, 4-hydroxy-ethyl butyrate and 3-hydroxy-ethyl butyrate concentrations were lower at wines from the strain 23.

Concentrations of some esters varied significantly between strains. Thus, the ethyl hexadecanoate concentration was a 55 % higher at strain Ab11 and that of *c*-3-hexenyl acetate was a 144 % higher at strain 93, when compared with the strains *O. oeni* CECT 7621 and Da32, producing the lowest concentrations of these compounds, respectively. Ethyl octanoate concentration was a 100 % higher at wines from strains 23, Ab11 and Da32 and, on the contrary, that of ethyl butyrate was around a 270 % higher at wines from strains *O. oeni* CECT 7621 and 93. Finally, concentrations for isoamyl acetate in wines from the strains Ab11, Da32, 93 and 23 were also higher than that from *O. oeni* CECT 7621 with increases ranging between 75 and 280 % depending on the strain.

Discussion

The ability of five selected *O. oeni* strains to hydrolyse glycosides and esters in wine during MLF has been assessed

Table 2 Volatile compounds of wines from different *O. oeni* strains after 22 days of incubation

	Initial wine	*O. oeni* CECT 7621 Negative control glycosidase	Da32 Positive control glycosidase	93 Negative control esterase	23 Positive control esterase	Ab11
Terpenes						
Linalool[2]	3.22	49.6 ± 3.73	52.3 ± 3.16	49.3 ± 11.6	46.9 ± 2.41	55.9 ± 4.49
α-terpineol[2]	2.64	29.3 ± 2.63	35.1 ± 1.81	28.5 ± 8.10	28.4 ± 0.89	36.4 ± 3.25
Citronellol[2]	14.7	8.84 ± 2.15	10.5 ± 1.52	10.8 ± 3.98	10.2 ± 0.60	10.9 ± 1.35
Hydroxycitronellol[4]	16.1	8.35 ± 4.41	13.7 ± 3.86	9.91 ± 5.71	11.6 ± 1.76	12.0 ± 1.54
Nerol[2]	2.67	6.47 ± 0.91[a]	11.5 ± 1.06[b]	9.33 ± 2.75[ab]	8.06 ± 0.42[ab]	11.3 ± 1.44[b]
Geraniol[2]	15.6	20.3 ± 2.05	28.07 ± 1.78	25.7 ± 9.90	24.1 ± 1.38	28.0 ± 3.12
3,7-Dimethyl-1,5-octadien-3,7-diol[4]	9.45	134 ± 46.2	173 ± 25.6	164 ± 63.3	167 ± 18.7	183 ± 27.9
t-linalool furanic oxide[4]	0.24	2.49 ± 0.17[a]	4.33 ± 0.08[c]	2.39 ± 0.66[ab]	2.51 ± 0.19[ab]	3.34 ± 0.30[b]
c-linalool furanic oxide[4]	0.16	1.30 ± 0.31[a]	3.17 ± 0.22[c]	1.34 ± 0.35[a]	1.39 ± 0.29[a]	2.16 ± 0.17[b]
c-linalool piranic oxide[4]	0.55	1.30 ± 0.11	1.88 ± 0.12	1.44 ± 0.81	1.45 ± 0.09	1.84 ± 0.24
t-linalool piranic oxide[4]	1.81	2.30 ± 0.08[a]	5.76 ± 0.18[b]	3.04 ± 1.23[a]	2.83 ± 0.18[a]	4.05 ± 0.34[a]
3,7-Dimethyl-1,5,7-octatrien-3-ol[4]	0.79	2.52 ± 0.43	2.48 ± 0.38	2.42 ± 0.70	2.38 ± 0.29	2.51 ± 0.43
3,7-Dimethyl-oct-1-en-3,7-diol[4]	4.09	6.87 ± 2.52	10.9 ± 1.43	7.30 ± 3.06	7.47 ± 1.01	10.6 ± 1.78
3,7-Dimethyl-octa-1,7-dien-3,6-diol[4]	1.48	3.20 ± 0.57	4.49 ± 0.91	3.39 ± 1.50	4.01 ± 0.15	4.21 ± 0.86
C6 alcohols						
1-hexanol[1]	1.81	1.96 ± 0.27	1,723 ± 103	1.98 ± 0.21	1.72 ± 0.01	1.83 ± 0.01
c-2-hexenol[2]	5.28	4.78 ± 0.19	4.69 ± 0.58	5.00 ± 0.69	4.77 ± 0.43	4.68 ± 0.20
t-2-hexenol[2]	9.95	10.4 ± 0.55[a]	21.1 ± 1.34[c]	14.7 ± 3.65[ab]	12.4 ± 0.91[ab]	16.5 ± 1.77[b]
t-3-hexenol[2]	64.8	67.6 ± 6.68	62.7 ± 4.27	68.7 ± 8.65	58.7 ± 2.97	63.7 ± 2.57
c-3-hexenol[2]	252	269 ± 23.5	251 ± 12.46	272 ± 37.4	243 ± 16.8	260 ± 10.6
Bencenic alcohols						
Benzyl alcohol[2]	355	288 ± 12.4[a]	427 ± 16.9[b]	336 ± 94.4[ab]	310 ± 16.4[a]	392 ± 32.5[ab]
2-Phenylethanol[1]	48.3	35.7 ± 2.52	38.88 ± 1.85	40.8 ± 10.8	38.5 ± 1.70	40.7 ± 3.35
Methoxyphenols						
Vanillin[2]	92.9	42.9 ± 30.8	66.5 ± 18.7	44.9 ± 27.9	54.1 ± 5.68	70.1 ± 11.6
Homovainillyl alcohol[2]	46.0	18.6 ± 16.9	37.0 ± 8.68	24.2 ± 19.1	32.5 ± 5.92	35.5 ± 5.35
Acetovanillone[2]	53.2	23.7 ± 16.1	39.7 ± 9.48	28.9 ± 18.8	37.1 ± 5.62	38.4 ± 5.32
Propiovanillone[2]	6.67	2.71 ± 2.12	4.87 ± 1.17	3.96 ± 3.16	4.71 ± 0.85	4.81 ± 0.99
Eugenol[2]	2.91	2.10 ± 0.39[a]	4,19 ± 0.24[b]	2.61 ± 0.86[a]	2.83 ± 0.19[a]	3.31 ± 0.46[ab]
t-isoeugenol[2]	0.92	0.34 ± 0.08[a]	1.57 ± 0.36[b]	0.43 ± 0.23[a]	0.99 ± 0.25[b]	0.72 ± 0.20[ab]
Methyl vanillate[2]	5.80	3.33 ± 1.12	4.50 ± 0.94	3.02 ± 1.50	4.08 ± 0.59	4.71 ± 0.37
Ethyl vanillate[2]	154	64.7 ± 48.3	104 ± 24.2	80.9 ± 54.0	103 ± 15.1	103 ± 16.0
Syringaldehyde[2]	446	175 ± 24.1	340 ± 132	193 ± 19.3	292 ± 54.7	369 ± 61.3
Acetosyringone[2]	28.1	9.95 ± 12.9	19.7 ± 8.22	12.3 ± 11.2	20.6 ± 6.37	19.0 ± 2.93
Methoxyeugenol[2]	1.87	1.00 ± 0.71	2.77 ± 0.73	1.13 ± 0.75	1.92 ± 0.81	2.13 ± 0.31
Zingerone[2]	13.4	0.45 ± 0.06[a]	0.36 ± 0.16[a]	0.83 ± 0.10[a]	3.22 ± 0.94[b]	0.38 ± 0.16[a]
Syringol[2]	1,013	82.9 ± 12.3	53.7 ± 18.0	19.2 ± 9.29	21.6 ± 0.68	193 ± 19.1
Norisoprenoids						
β-ionone[2]	0.36	0.25 ± 0.02	0.24 ± 0.05	0.23 ± 0.15	0.27 ± 0.10	0.22 ± 0.03
3-Oxo-α-ionol[4]	115	41.8 ± 45.7	89.4 ± 23.3	56.6 ± 50.8	80.5 ± 14.0	84.7 ± 12.9
Damascenone[2]	2.33	2.71 ± 0.64	3.34 ± 0.23	3.25 ± 0.83	3.50 ± 0.53	3.54 ± 0.35
β-ionol[4]	5.60	2.79 ± 1.17	5.89 ± 2.57	3.05 ± 2.00	3.83 ± 0.51	4.27 ± 0.75
3-Hydroxy-7,8-dihydro-β-ionol[4]	2.31	0.93 ± 0.93	2.34 ± 0.75	1.46 ± 1.16	1.96 ± 0.43	2.12 ± 0.14
Acetates						
Butyl acetate[1]	0.67	2.59 ± 3.28	1.11 ± 0.55	2.48 ± 1.15	1.16 ± 0.60	1.16 ± 0.88
Hexyl acetate[1]	0.05	0.10 ± 0.05	0.08 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.05 ± 0.02

Table 2 continued

	Initial wine	*O. oeni* CECT 7621 Negative control glycosidase	Da32 Positive control glycosidase	93 Negative control esterase	23 Positive control esterase	Ab11
Isoamyl acetate[1]	1.32	1.05 ± 0.41	1.91 ± 0.09	2.78 ± 0.80	1.84 ± 0.27	3.93 ± 4.17
Benzyl acetate[2]	0.56	0.57 ± 0.04	0.63 ± 0.05	0.59 ± 0.13	0.54 ± 0.06	0.59 ± 0.08
t-3-hexenyl acetate[4]	0.77	1.10 ± 0.21	0.91 ± 0.14	0.92 ± 0.35	0.71 ± 0.05	1.01 ± 0.13
c-3-hexenyl acetate[2]	0.48	1.93 ± 0.25^{a}	1.63 ± 0.50^{a}	3.99 ± 0.22^{b}	3.40 ± 0.84^{b}	1.82 ± 0.24^{a}
1,3-Propanediol acetate[4]	46.0	52.0 ± 29.4	40.4 ± 3.47	46.1 ± 28.7	33.7 ± 2.88	43.5 ± 4.27
2-Phenylethyl acetate[2]	36.8	164 ± 16.9	191 ± 11.7	193 ± 55.4	190 ± 5.72	196 ± 23.6
Ethyl esters						
Ethyl butyrate[1]	1.05	1.64 ± 1.28	1.00 ± 0.31	1.72 ± 0.84	0.45 ± 0.23	1.04 ± 0.29
4-Hydroxy-ethyl-butyrate[3]	4.38	3.25 ± 0.73	3.38 ± 0.24	3.68 ± 1.66	3.09 ± 0.19	3.56 ± 0.44
Ethyl octanoate[2]	54.6	36.6 ± 3.95	76.9 ± 9.65	57.2 ± 32.1	71.8 ± 4.62	74.0 ± 14.1
Ethyl hexanoate[2]	16.1	21.0 ± 4.28^{a}	27.3 ± 2.71^{b}	22.4 ± 3.55^{b}	23.1 ± 1.23^{b}	23.2 ± 3.16^{b}
Ethyl decanoate[2]	34.7	18.7 ± 3.20	42.6 ± 7.07	34.7 ± 31.8	41.0 ± 4.45	40.7 ± 9.43
Ethyl dodecanoate[2]	18.9	9.59 ± 5.93	20.3 ± 3.64	16.4 ± 17.7	17.9 ± 2.48	18.0 ± 5.39
9-Ethyl decenoate[4]	1.28	0.59 ± 0.10	1.41 ± 0.26	1.05 ± 0.86	1.37 ± 0.18	1.45 ± 0.33
Ethyl hexadodecanoate[2]	33.6	94.4 ± 86.0	131 ± 57.2	110 ± 10.9	118 ± 22.0	147 ± 41.4
Ethyl monosuccinate[3]	22.5	10.0 ± 9.99	18.07 ± 4.77	13.1 ± 10.2	14.63 ± 1.72	20.6 ± 2.80
Diethyl malate[3]	172	97.2 ± 34.3	127 ± 19.1	105 ± 44.8	112 ± 5.79	124 ± 15.2
2-Phenylethyl lactate[4]	6.79	12.7 ± 5.91^{ab}	33.4 ± 6.00^{c}	20.4 ± 11.7^{abc}	22.4 ± 1.21^{bc}	5.88 ± 1.24^{a}
3-Hydroxyethyl decanoate[4]	11.6	5.10 ± 2.81	8.15 ± 1.92	6.23 ± 3.68	7.49 ± 1.06	7.43 ± 1.08
3-Hydroxyethyl butyrate[2]	236	234 ± 45.6	198 ± 5.91	225 ± 98.7	176 ± 3.64	215 ± 5.42
2-Hydroxyethyl caproate[4]	28.8	28.4 ± 2.07^{a}	41.8 ± 0.95^{b}	33.2 ± 8.84^{ab}	32.6 ± 2.25^{ab}	28.9 ± 1.70^{a}

Values are the mean of triplicates. [1] mg/L; [2] μg/L; [3] area compound/area IS; [4] area compound/area IS*1000. Different superscripts (a, b, c) indicate significant differences between strains for $\alpha = 0.05$ according to the Student–Newman–Keuls test

in the present study. MLF evolution and counts on MLOA plates at the end of the process were the usual [32, 33, 37]. The slow degradation of malic acid during the first days following the inoculation has already been reported by other authors [30] and has been attributed to the characteristics of wine, such as the pH and the alcohol and SO_2 contents, which make wine a very harsh environment for bacterial growth [38]. The implantation values obtained for each strain after 22 days of incubation although different confirmed their presence in this process.

Respect to the chemical composition of wines, the behaviour of strains was similar to that described by other authors when a starter culture is inoculated to carry out MLF [39, 40]. Significant differences between the strains were found for all the parameters analysed. Strains 93, 23 and Da32 degraded almost totally the citric acid although the final values for volatile acidity were not excessive except for the strain Da32. As described by some authors [41], concentrations higher than 0.7 g/L can produce sour and pungent aroma and a vinegar-like character to wine. On the contrary, concentrations between 0.2 and 0.7 g/L are beneficial contributing to the complexity of wine aroma [42].

Changes in volatile compounds composition at wines occurred during MLF suggests an active contribution of *O. oeni* strains through the hydrolysis of glycosylated compounds, although part of the detected changes could also be due to the chemical hydrolysis of glycosides and esters caused by the pH and acidity of the wine [4].

Glycosidase and esterase activities from LAB at wine have been studied using both wine and synthetic substrates [4–7, 16, 17, 25, 30, 43, 44], and both activities have been described to be strain dependent. In concordance with those studies, the results from volatile compounds' analysis at wine obtained in this study have displayed differences, some of them statistically significant, in the content for some compounds, which would be attributable to differences in the glycosidase and esterase activities of the *O. oeni* strains inoculated. It is well known that LAB have the potential to alter the aroma profile of wine by the production of volatile secondary metabolites or the modification of grape- and yeast-derived metabolites [4, 45–47].

However, differences between strains observed in this study were not as clear as those observed when the same strains were assayed using synthetic substrates such as *p*-nitrophenyl-β-D-glucopyranoside and

p-nitrophenyl-xylopyranoside [16]. The complex composition of wine could mask the release of aglycones by LAB. Similar results have been reported by other authors [4, 9].

As expected, the compounds showing the highest increases were the terpenes which are the most abundant glycosides in the Muscat variety from which the glycosidic extract added to wine was obtained. The ability of *O. oeni* strains to release terpenes from glycosidic precursors has been described by some authors [4, 48]. Typical aroma descriptions of some important terpenes are floral, rose-like (geraniol, nerol, rose oxides), coriander (linalool), camphoraceous (linalool oxides), green (nerol oxide) and herbaceous [49].

It is important to highlight that concentrations of nerol, *c*-linalool furanic oxide, *t*-linalool furanic oxide and *t*-linalool piranic oxide were significantly higher in wine inoculated with strains Da32 and Ab11, which had been selected in a previous work by their high glycosidase activity [16], and significantly lower in wine inoculated with the strain *O. oeni* CECT 7621, used as a negative control for this activity.

In addition, strains Ab11 and Da32 showed significantly higher concentrations of other compounds such as *t*-2-hexenol, benzyl alcohol, damascenone and eugenol. Hernández-Orte et al. [4] also reported increases of damascenone and *c*-3-hexenol in wines whose MLF had been performed by the strain *O. oeni* 5106, and Boido et al. [50] reported increases of benzyl alcohol in wines inoculated with three *O. oeni* commercial strains.

All these compounds contribute substantially to the aroma of wine. Thus, the norisoprenoids provide fruity, floral or spicy notes, and hence, their presence has very beneficial effects for the sensory quality of wines. Damascenone, a norisoprenoid compound, is a potent odorant produced from the hydrolytic cleavage of some C_{13}-norisoprenoidic polyols present in wines in free form or as glycosides [51–53]. Ugliano and Moio [54] found a significant increase of the concentration of β-damascenone after MLF and suggested that the formation of this compound could be related with the hydrolytic activity of the bacteria participating. On the other hand, Antalick et al. [55] found low variations in concentrations of some compounds measured, such as linalool, norisoprenoids and damascenone, indicating that their sensory impact would be unlikely, due to their perception threshold, which in addition, could be altered by the matrix.

Eugenol and zingerone, compounds belonging to the methoxyphenols group, give spicy and smoked characteristics [56] to wines. The benzyl alcohol provides notes to blackberry, floral and sweet aroma [57, 58].

Concentrations for other compounds, such as citronellol, vanillin, acetovanillone, syringol and 3-oxo-α-ionol, were lower than expected which, as described by some authors [4, 50, 55], could be due to an effect of adsorption onto polysaccharides and peptidoglycans produced by *O. oeni*.

As observed by Pérez-Martín et al. [25] using synthetic substrates, results for esters showed differences not only between strains but also between substrates. Thus, the strain Da32 presented the highest hydrolysis of butyl acetate and one of the lowest of 2-phenylethyl acetate, and the strain *O. oeni* CECT 7621 presented the highest hydrolysis of 2-phenylethyl acetate and the lowest of butyl acetate. The increase in the concentrations of other esters such as ethyl hexadecanoate, *c*-3-hexenyl acetate and ethyl octanoate, which contribute to pleasant fruity notes, was also observed to be strain dependent. On the contrary, some authors [39] indicated that concentration of some of these compounds did not depend on MLF and the strain used, probably due to that they inoculated a strain with low esterase activity.

Some authors [31, 44] reported modifications of ester concentrations depending on the strain conducting the MLF, and other authors [39, 59, 60] also reported the influence of the wine composition (pH, ethanol content, substrates released by the yeast) and the cultivar on such modifications. The influence of these factors could explain that the correlation between results obtained in the present study for strains 23 (positive control for esterase activity) and 93 (negative control for esterase activity) and those obtained by Pérez-Martín et al. [25] in the previous study for characterisation of these strains was not as clear as for glycosidase activity, since for the majority of ester compounds analysed the behaviour of both strains was not the expected. As an example, the 2-phenylethyl acetate concentration was identical for both strains.

Differences between strains observed in the concentration of 2-phenylethyl lactate can be related with the concentration of lactic acid in the wine, since this compound is produced during MLF as result of the esterification of the lactic acid with the 2-phenylethanol. Thus, wines from Da32 strain, which showed highest lactic acid production, exhibited concentrations of this ester higher than those from *O. oeni* CECT 7621 with the lowest lactic acid production. An exception occurred with strain Ab11.

Esters are important in determining wine aroma, and the presence of some short-chain esters, such as ethyl acetate, isobutyl acetate, isoamyl acetate and hexyl acetate, provides fruity flavours. However, there is a great disagreement regarding the influence of MLF on the final ester content in wine and the convenience of using LAB showing esterase activity for MLF in order to improve sensory characteristics of wines. Thus, whereas some authors state that the metabolism of LAB may induce significant increases in the concentrations of different esters originating from alcoholic fermentation [28, 61], others affirm that their concentrations decrease significantly during MLF with the subsequent reduction in fruity attributes [62].

Conclusions

The results obtained in this study reveal that although differences in the concentration of some volatile compounds in wines obtained with the different *O. oeni* strains have been quantitatively not significant, they could affect the sensory characteristics of the wines, because some of them contribute either positively or negatively to their aroma.

It has been confirmed that both glycosidase and esterase activities are strain dependent making necessary a previous assay of the strains to know their behaviour before the selection to be used as a starter culture. In addition, it has been observed that sometimes correlation between results obtained using synthetic substrates, such as *p*-nitrophenol-linked substrates, and those from wines is not coincident, and therefore, those previous studies should be carried out using the particular wine on which the strain will act.

From the results reported, it can be concluded that the strain 93, presenting the lowest esterase activity, is the best to preserve the fruity aroma of wines, while strains Da32 and Ab11, showing the highest glycosidase activity, are good candidates to produce wines with higher floral aromatic profile. Future researches using each of these strains will be carried out to know their influence in the sensory characteristics of the wines.

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Conflict of interest None.

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