The Effect of Endozym β-split, a Commercial Enzyme Preparation Used for Aroma Release, on Tannat Wine Glycosides

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Commercial preparations with glycosidase activities are used to enhance wine aroma, but they are not extensively characterized. The aim of this work was to study the effect of three enzymes on aroma improvement in Tannat red wine. After the selection of the most appropriate enzyme for further testing, its effectiveness on the hydrolysis of the total glycosides present in Tannat wine was measured. The three preparations showed high β-glucosidase and α-arabinosidase activities (in the range of 10 to 174 U/mL or U/g), but very low levels of α-rhamnosidase (less than 1 U/mL). The β-glucosidases studied remained active in the presence of Tannat wine. The selected enzyme, Endozym β-split, supplemented with α-rhamnosidase, resulted in almost 30% hydrolysis of the glycosides in Tannat wine, when added at a concentration higher than that recommended by the manufacturers. A sensory evaluation showed that the enzyme-treated wines were significantly different from the controls, suggesting that at least a part of the hydrolyzed glycosides in the Tannat wine were aroma precursors. However, it cannot be assumed that all commercial enzymes would be effective in hydrolyzing aroma precursors just because they show glycosidase activities. Higher concentrations of Endozym β-split than that recommended by the manufacturer are necessary to reach an appreciable level of glycoside hydrolysis. Supplementation of Endozym β-split with α-rhamnosidase is recommended in this enzyme – wine system for the greater release of the aroma from the glycosidically-bound precursors.

INTRODUCTION

Musts and fruit juices contain small quantities of a wide range of glycosides, which remain non-hydrolyzed after vinification (Gunata *et al.*, 1988). Some of these glycosides are aroma precursors because their aglycones are aromatic compounds. They are mainly the *O*-β-D-glucosides and the *O*-diglycosides: α-L-arabino-β-D-glucosides, α-L-rhamno-β-D-glucosides and β-D-apio-β-D-glucosides. Aglycones, which are always directly bound to glucose, are mainly monoterpenes, C13-norisoprenoids and benzene derivatives (Williams *et al.*, 1982; Gunata *et al.*, 1985; Sarry & Gunata, 2004). Since only partial hydrolysis occurs during vinification, some of these glycosides remain in the wine as a potential source of aroma to be exploited. Thus, hydrolysis of these compounds using exogenous glycosidases may result in an improvement of wine aroma (Cabaroglu *et al.*, 2003; Sánchez Palomo *et al.*, 2005). Most of the enzymes hydrolyzing diglycosides act sequentially; in the first step α-L-rhamnosidase (EC 3.2.1.40), α-L-arabinosidase (EC 3.2.1.55) or β-D-apiosidase liberate the corresponding sugar and a glucoside, which is subsequently hydrolyzed by β-D-glucosidase (EC 3.2.1.21) (Gunata *et al.*, 1988).

Commercial enzymes employed in winemaking (Martino *et al.*, 1996; Guo *et al.*, 1999; Cabaroglu *et al.*, 2003; Tamborra *et al.*, 2004) and other glycosidases produced by bacteria (Michlmayr *et al.*, 2010), yeast (Arévalo Villena *et al.*, 2007) or fungi (Le Traon-Masson & Pellerin, 1998) were tested for their glycosidase activity, for aroma enhancement in white and red wines produced from several cultivars.

Even though there are numerous published studies related to the use of exogenous glycosidases to enhance wine aroma, there were no commercially available enzymes for this purpose until a few years ago. Only preparations with pectolytic, hemicellulolytic and cellulolytic activities to improve extraction and clarification steps were being promoted to hydrolyze aroma precursors, due to their secondary glycosidase activities. Although these older commercial enzymes showed high glucosidase and arabinosidase activities, they had low levels of rhamnosidase and apiosidase (Dupin *et al.*, 1992) and their glycosidase activities were not extensively characterized. In recent years specific enzymes to enhance wine aroma have appeared on the market, but there is little or no information about

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their glycosidase activity, stability or inhibition by wine compounds.

Endozym Rouge is an enzymatic preparation suitable for color and aroma extraction from red grapes, due to its pectinolytic, cellulolytic and hemicellulolytic activities. Its additional value for aroma liberation is also recommended by its manufacturer (www.aeb-group.com). Guérin *et al*. (2009) found that among several preparations for color extraction, Endozym Rouge had considerable β-glucosidase and α-arabinosidase activities, but low levels of α-rhamnosidase and β-apiosidase. However, we found that its β-glucosidase was highly inhibited by wine compounds and could hardly act efficiently in the hydrolysis of aroma precursors (Cid & Ellenrieder, 2009).

Although Endozym β-split, Endozym ICS 10 Arome and Endozym cultivar are pectolytic enzymes, they are promoted to extract aroma precursors or to liberate aroma compounds from these glycosidic precursors. The presence of β-glucosidase activity in all of these preparations is emphasized by the manufacturers (www.aeb-group.com), but the actual activity values are not mentioned. There are only a few studies of these commercial preparations, especially with regard to their capacity to hydrolyze glycosidic precursors. Armada *et al*. (2010) studied the effect of Endozym cultivar on the extraction of aromatic compounds during maceration, and Guérin *et al*. (2009) quantified different activities in the same preparation, including glycosidases, but there is no published data that we know of that address the inhibition or stability of these enzymes in the presence of wine, or about their effect on wine glycosides.

Red wines made from *Vitis vinifera* cv. Tannat are produced in Uruguay, and are the wines most representative of this country. Even though *Vitis vinifera* cv. Tannat has been cultivated for several years in Salta, Argentina, until a few years ago there were no red wines produced in Argentina from this grape variety. Little information is available about wines made from this varietal. Boido *et al.* (2003) characterized the aroma composition of *Vitis vinifera* cv. Tannat and studied the effect of a β-glucosidase from *Oenococcus oeni* on glycosidic precursors during malolactic fermentation (Boido *et al.*, 2002). In a previous study we tested some glycosidase activities on synthetic substrates in Tannat wine (Cid & Ellenrieder, 2009).

In order to predict the behavior of these newer enzymes on the hydrolysis of natural glycosides in wines, three commercial preparations for aroma enhancement, Endozym β-split, Endozym ICS 10 Arome and Endozym cultivar, were studied and compared with an enzyme used to improve color extraction from grapes, Endozym Rouge. The main aroma releasing glycosidases, their optimum pH for activity and the inhibition produced by Tannat wine were determined using synthetic substrates of the *p*-nitrophenylglycoside type. Based on the observed results, one preparation was selected for a study of its effect on the glycoside concentration in Tannat wine. The effectiveness on glycoside hydrolysis in wine was compared to the concentrations recommended by the manufacturers and by other studies of wine aroma enhancement. Finally, samples of untreated and treated wine were submitted to a sensory analysis using a triangle test.

MATERIALS AND METHODS

Materials

Tannat red wine was produced by a local winery in Cafayate, Salta, Argentina. In addition *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), *p*-nitrophenyl-α-L-rhamnopyranoside (*p*NPR) and *p*-nitrophenyl-α-L-arabinofuranoside (*p*NPA) were supplied by Sigma (St. Louis, MO, USA), *p*-nitrophenol (*p*NP) by Merck (Darmstadt, Germany), Amberlite XAD-2 by Supelco (Bellefonte, PA, USA) and acetonitrile HPLC grade by Carlo Erba (Rodano, Italy). The enzymatic kit for glucose determination was purchased from Wiener Laboratorios (Rosario, Argentina).

Endozym cultivar, Endozym ICS 10 Arome, Endozym β-split and Endozym Rouge were commercial enzymes from Pascal Biotech (Paris, France) that are used in the winemaking industry. The α-rhamnosidase HPS 11518 was from Tanabe (Japan). All enzymes were produced by *Aspergillus niger*. All the other reagents were analytical grade.

Enzyme assays

Spectrophotometric assays

The activity of β-D-glucopyranosidase (β-glu), α-L-rhamnopyranosidase (α-rham) and α-L-arabinofuranosidase (α-ara) was determined by taking out samples from the reaction medium at different time intervals during 10 minutes and measuring the absorbance of the produced *p*-nitrophenol in basic medium at its absorbance maximum, 400 nm, using the corresponding synthetic substrate, 1.9 mM *p*NPG, 2.4 mM *p*NPR or 1 mM *p*NPA, respectively (Cid & Ellenrieder, 2009). Activity units (U) expressed as μmol/min were calculated from the slope, taking a molar absorptivity, ε, of 16.44 mM.

Chromatographic assays

When *p*NP could not be measured spectrophotometrically due to the presence of colored substances absorbing at 400 nm, it was quantified by HPLC in an acidic medium as previously described (Cid & Ellenrieder, 2009).

Glycosidases in commercial preparations

The activity of β-D-glucopyranosidase, α-L-rhamnopyranosidase and α-L-arabinofuranosidase at 50°C was measured spectrophotometrically in the commercial enzymes Endozym β-split, Endozym ICS 10 Arome, Endozym cultivar and Endozym Rouge using the appropriate synthetic substrates. Optimum pH of β-D-glucopyranosidases and α-L-arabinofuranosidases was determined at 50°C by measuring these activities in 50 mM citric acid/Na citrate buffer in a range of pH from 3.0 up to 5.0 as described above.

Glycosidase inhibition by Tannat wine

Endozym β-split, Endozym ICS 10 Arome, Endozym cultivar and Endozym Rouge β-glucosidase activity on 2 mM *p*NPG at 50°C in 60 mM tartaric acid/Na tartrate buffer pH3.6 with or without 50% Tannat red wine in the reaction medium was measured chromatographically. Endozym β-split and Endozym Rouge α-arabinosidase on 1 mM *p*NPA at 50°C was also determined by HPLC in the same buffer with or without 50% Tannat wine.

Glycoside hydrolysis in Tannat wine at different enzyme concentrations

According to the concentration recommended by the manufacturer (www.aeb-group.com), 12.5 µL Endozym β-split and 1.76 µL HPS 11518 were added to 250 mL of Tannat red wine. In another experiment 172 µL Endozym β-split and 24 µL HPS 11518 were added to the same volume of wine to reach concentrations similar to those used by other studies (Gunata *et al.*, 2000; Martino *et al.*, 2000). Samples and a control without enzymes were incubated at 20°C and the remaining glycoside concentration measured after 12 days.

Glycoside extraction and quantification

Wine glycosides were extracted following a procedure described by Gunata *et al*. (1985) and Williams *et al*. (1995) by adsorbing them selectively on Amberlite XAD-2. The column (80 mm × 0.6 mm i.d.) was pre-treated six times with 10 mL methanol and six times with 10 mL diethyl ether, and then washed six times with 10 mL water. After 5 mL of wine was loaded the resin was washed with 10 mL water and eluted with 7 mL methanol.

Glycoside fractions, after solvent evaporation, were re-dissolved in 0.5 mL of 27% ethanol in water and glycosides were determined according to Arévalo Villena *et al*. (2006). Also, 0.5 mL 2.25 M H_2SO_4 was added to 0.25 mL of samples to give solutions for hydrolysis containing 1.5 M H_2SO_4. A control solution for determination of free glucose concentration was prepared by adding water instead of H_2SO_4. The samples were boiled for 1 h, while controls were held at room temperature. Released glucose was measured using a glucose oxidase assay kit after neutralizing the acid with an equal volume of 3 M NaOH.

Sensory analysis

Samples of wine, incubated with enzymes (688 µL/L Endozym β-split and 96 µL/L HPS 11518) for 12 days, were submitted to an expanded triangle test (ASTM, 1977). The panel consisted of 16 judges, who had previous experience of wine sensory analysis. During the assessment, one session of 2 hours, each assessor performed two triangles. The 'believed' samples were selected by the assessors, who then described the perceived aroma and taste attributes.

Physico-chemical determination

Total and volatile acidities, pH, alcohol level, reducing sugars and dry extract were determined in the enzyme-treated wines and controls according to the methods of the Association of Official Analytical Chemists (1990).

Statistical analyses

At least two replicates were measured in each determination. Statistical analyses were carried out using PRISM software (GraphPad, San Diego, CA, USA). Binomial distribution was used to calculate the significance level in the triangle test based on the number of correct answers. ANOVA analysis of the physico-chemical determinations was performed using Infostat software (Córdoba, Argentina).

RESULTS AND DISCUSSION

Glycosidases in commercial preparations

The commercial preparations Endozym Rouge, Endozym β-split, Endozym ICS 10 Arome and Endozym cultivar were tested for β-glucosidase, α-arabinosidase and α-rhamnosidase activities (Table 1). The first two activities were determined in buffer at the optimum pH (4.5 for the β-glucosidases and 4 for the α-arabinosidases) and 50°C and the α-rhamnosidase activity at pH 4. Values of the expected activities in wines are also shown.

All of the preparations presented high values of β-glucosidase and α-arabinosidase, but much lower levels of α-rhamnosidase, which is consistent with data published on glycosidase activities in commercial preparations used in winemaking (Gunata *et al.*, 1997; Sarry & Gunata, 2004; Barbagallo *et al.*, 2004; Pogorzelski & Wilkowska, 2007). This result suggests that these enzymes would allow hydrolysis of only some of the glycosidic precursors, since those containing rhamnose linked to the *O*-D-glucoside would remain unchanged in wines.

The expected glycosidase activities in wine were calculated taking into account the activity at optimum pH and 50°C and the amount of enzyme per hL of wine recommended by the manufacturers: 2 to 5 ml/hL of Endozym β-split, 0.4 to 0.8 mL/hL of Endozym ICS 10 Arome, 2 to 3 g/hL of Endozym cultivar and 2 to 4 g/hL of Endozym Rouge. The activities present in the wines would probably be insufficient to hydrolyze the glycosidic precursors, especially if we consider that these activities will be even

TABLE 1
Glycosidase activities in commercial preparations.

Enzyme	Activity ([a]U/mL or [b]U/g) [1]			Maximum expected activity in wines (U/mL) (x 10^4) [2]		
	β-glu	α-ara	α-rham	β-glu	α-ara	α-rham
E. β-split	174.1±1.6[a]	42.6±2.5[a]	0.77±0.15[a]	87.0	21.3	0.39
E. ICS 10 Arome	67.4±0.7[a]	165.0±3.0[a]	0.80±0.05[a]	5.4	13.2	0.06
E. cultivar	10.6±0.3[b]	15.1±0.6[b]	n.d.	3.2	4.5	
E. Rouge	77.0±0.1[b]	80.0±0.1[b]	0.94±0.04[b]	15.4	16.0	0.19

[1] Activity at 50°C in 50 mM citric acid / Na citrate buffer at optimum pH for β-glucosidase (β-glu) and α-arabinosidase (α-ara) and at pH 4 for α-rhamnosidase (α-rham)
n.d.: not detected
[2] Maximum expected activity in wines: Activity (at 50°C and optimum pH for β-glu and α-ara and at pH 4 for α-rham) x recommended dose
[a] Activity expressed as U/mL for liquid preparations
[b] Activity expressed as U/g for powder preparations

lower once the enzymes are added to the wines, where pH and temperature conditions are different from those shown in Table 1 and where there are several compounds that would act as inhibitors, such as ethanol, glucose and gluconolactone (Sarry & Gunata, 2004; Cid & Ellenrieder, 2009). In addition the activities of the glycosidases are usually higher on the synthetic than the natural substrates.

β-Glucosidase and α-arabinosidase were assayed at 50°C in buffer at a pH range from 3 to 5. Figures 1 and 2 show the relative activity as a percentage of the maximum value of activity observed for these enzymes. α-Rhamnosidase was not tested because its presence was much lower than the other two activities.

It could be observed that the pH of maximum activity for the four β-glucosidases was 4.5, while for the α-arabinosidases it was 4.0. These values are similar to those found in other studies of glycosidases produced by fungi (Le Clinche *et al.*, 1997; Le Traon-Masson & Pellerin, 1998; Saha, 2000). Among the different enzyme preparations, the β-glucosidase and the α-arabinosidase from Endozym β-split had a higher relative activity when the pH was in the range of that found in wines (3 to 4), maintaining 50% and 92% of their activities at pH 3, respectively.

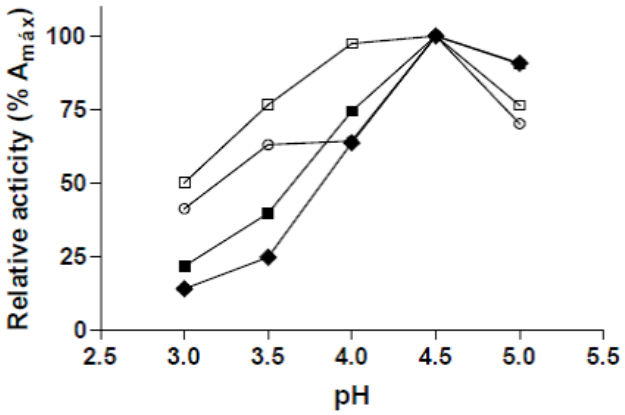

FIGURE 1
Influence of the pH on β-glu activities
50 mM citric acid/citrate buffer, 50°C
□ Endozym β-split; ○ Endozym ICS 10 Arome; ■ Endozym cultivar; ♦ Endozym Rouge.

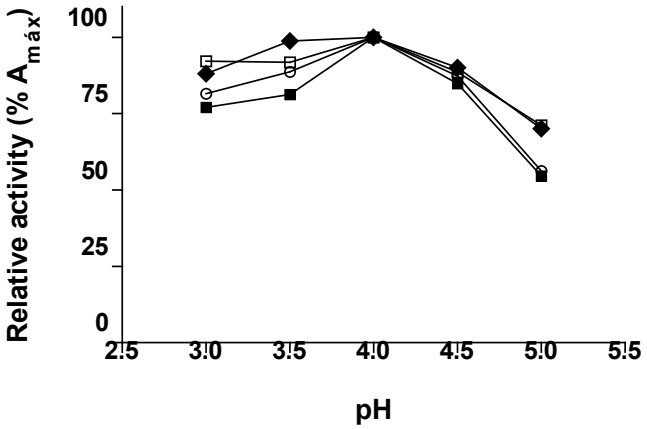

FIGURE 2
Influence of the pH on α-ara activities
50 mM citric acid/citrate buffer, 50°C
□ Endozym β-split; ○ Endozym ICS 10 Arome; ■ Endozym cultivar; ♦ Endozym Rouge.

Glycosidase inhibition by Tannat wine

Taking into account the sequential mechanism for the hydrolysis of the glycosidic precursors (Gunata *et al.*, 1988), the action of β-glucosidase is essential for the complete hydrolysis of all the glycosides and the consequent liberation of aroma compounds. However, among glycosidases to enhance wine aroma, β-glucosidase usually suffers the greatest inhibition and is the least stable (Sarry & Gunata, 2004). For this reason, many studies about glycosidases focus on this activity.

Although testing the inhibition produced by ethanol, glucose and gluconolactone on β-glucosidase for *p*NPG hydrolysis is a common practice, this information is not enough to predict its behavior in wine, since there are other compounds in this medium that can act as inhibitors of its activity (Cid & Ellenrieder, 2009). Thus, when many enzymes are being compared, it should be appropriate to determine β-glucosidase activity on *p*NPG in the presence of wine and to express the results as the relative activity to that in buffer solution under the same conditions to select the most appropriate enzyme preparation to choose.

Table 2 shows the values of β-glucosidase activity at 50°C on 2 mM *p*NPG for the four preparations in buffer and with 50% Tannat wine added to the reaction medium.

This study was performed with only 50% wine added because the technique for measuring enzyme inhibition in 100% wine is prone to more errors, since it involves lyophilization and redissolution of wine; furthermore, 50% wine usually produces most of the inhibition found in 100% wine as observed using β-glucosidase from Endozym Rouge in Torrontés and Tannat wine (Cid & Ellenrieder, 2009).

The β-glucosidases from the three preparations sold for aroma enhancement exhibited a higher relative activity in wine than the β-glucosidase from Endozym Rouge. The activity from Endozym β-split was the least inhibited.

Although all of the preparations showed similar quantities of glycosidase activities, with relatively low levels of α-rhamnosidase, this result suggests that at least the β-glucosidase from the specific enzymes for aroma improvement would probably have a better activity when added to wine than the preparations used for other purposes, as was expected.

Since the presence of α-arabinosidase is necessary for the hydrolysis of glycosides containing arabinose in the sugar moiety, the effect of 50% of Tannat wine in the reaction medium on Endozym β-split and Endozym Rouge α-arabinosidase using *p*NPA was also tested.

Table 3 shows that both enzymes maintained a significant percentage of their activity observed in buffer under the same conditions when 50% of wine was added to the medium. Even though the behavior of the α-arabinosidase of Endozym β-split was slightly better than that of Endozym Rouge, both presented values comparable to those of the β-glucosidases from the preparations to enhance wine aroma.

TABLE 2
β-Glucosidase activity from commercial enzymes at 50°C on 2 mM *p*-NPG in buffer at pH 3.6 and in the presence of 50% of Tannat wine.

Enzyme β-glu	Activity (U/mL)		Relative activity (%)
	Buffer pH 3,6	Tannat wine (50%)	
Endozym cultivar	0.058±0.012	0.018±0.003	35.6±7.4
Endozym ICS 10 Arome	0.250±0.025	0.086±0.002	36.6±6.0
Endozym β-split	0.303±0.007	0.129±0.009	42.6±2.3
Endozym Rouge	0.148±0.003	0.013±0.003	8.8±1.7

Relative activity is (activity in wine/activity in buffer under the same conditions) × 100. Buffer: 60 mM tartaric acid/Na tartrate

TABLE 3
α-Arabinosidase activity from Endozym β-split and Endozym Rouge on 1 mM *p*NPA at 50°C in buffer at pH 3.6 and in presence of 50% of Tannat wine.

Enzyme	Activity (U/mL)		Relative activity (%)
	Buffer pH 3.6	Tannat wine (50%)	
Endozym β-split	0.23±0.06	0.11±0.01	47.5±9.3
Endozym Rouge	0.23±0.01	0.084±0.003	35.9±0.1

Relative activity is (activity in wine/activity in buffer under the same conditions) × 100. Buffer: 60 mM tartaric acid/Na tartrate

Glycoside hydrolysis in Tannat wine with different enzyme concentrations

A preliminary assessment of glycosidase activity, inhibition and stability could be done using *p*-nitrophenylglycosides to select the enzymes displaying better performance. However, in order to evaluate the characteristics and kinetics of the hydrolysis of the glycosidic precursors produced by the selected exogenous glycosidases, complex procedures for the extraction and quantification of glycosides and aromas, including liquid and gas chromatography, are necessary (Cabaroglu *et al.*, 2003; Rocha *et al.*, 2005; Valcárcel & Palacios, 2008). The use of these procedures on a routine basis, especially when a lot of enzyme preparations are being tested, is not practical. For this reason, the proposed method by Arévalo Villena *et al*. (2006) based on the determination of the whole glycosides, offers results appropriate to estimate the effectiveness of an enzyme treatment on the hydrolysis of aroma precursors in wines, allowing one to discard the enzymes displaying a negative or a very low level of glycoside hydrolysis.

The enzyme activity on their natural substrates was evaluated by measuring total glycoside concentration as a function of time. Glycosides were extracted from wine and then quantified by the determination of the glucose released after acid hydrolysis.

The effectiveness of the enzymatic hydrolysis of wine precursors depends on enzyme characteristics (rapid inhibition and long term stability) and the type of wine to be treated (inhibitor content and quantity); for this reason, the optimum concentration of enzyme may also be very variable. When a dose is recommended, it would provide enough glycosidase activity for the most unfavorable cases. However, according to the data shown in Table 1, manufacturers advise doses for the enzymatic preparations used to enhance wine aroma that are about five to ten times lower than those used in earlier studies of this subject (Gunata *et al.*, 2000; Martino *et al.*, 2000).

Endozym β-split was selected to study its effect on the glycosides of Tannat wine because it presents two of the glycosidase activities necessary for wine aroma enhancement, β-glucosidase and α-arabinosidase, with low inhibition. However, α-rhamnosidase activity was negligible in this commercial preparation and for this reason it was supplemented with the enzyme HPS 11518, which contains an α-rhamnosidase that shows low inhibition and high stability in Tannat wine (Cid & Ellenrieder, 2009). The presence of α-rhamnosidase is necessary for the release of the aglycone from glycosides with rhamnose in their structure. In a preliminary study, an expert panel in a local winery found that Tannat wines treated with α-rhamnosidase presented a better bouquet that those treated in the absence of this activity (data not shown), suggesting that its presence is important for aroma release.

The hydrolysis of Tannat wine glycosides was compared using two different concentrations of enzymes: the first as recommended by the manufacturers of Endozym β-split and the second based upon data published previously (Gunata *et al.*, 2000; Martino *et al.*, 2000). The glycoside concentration was measured before the treatment and after 12 days of incubation in a control sample of wine without added enzymes and in both samples treated with different amount of enzymes. The total glycoside concentration in the wine before the treatment was 313 ± 27 nmol/mL, and it was 308 ± 1 nmol/mL in the control sample without enzyme added after 12 days, showing that glycosides did not break down by acid hydrolysis or hydrolysis by endogenous enzymes during this incubation period. On the other hand, the total glycoside concentrations after incubation of the wines treated with the enzymes were 320 ± 35 nmol/mL and 221 ± 1 nmol/mL when the dose was that recommended by the manufacturers and by published data, respectively. This result indicates that the amount recommended in the enzyme brochures was not enough to hydrolyze the glycosides present in Tannat wine and so the aromatic precursors, while using a significantly higher amount of the enzyme preparation achieved the desired effect. Rocha *et al.* (2005) studied the effect of adding an enzyme preparation to Maria Gomez and Bical wines during bottling at the dose

TABLE 4
Physico-chemical parameters in samples of wine with and without enzymatic treatment.

Sample	Volatile acidity (g/L)	Total acidity (g/L)	pH	Alcohol level (%)	Reducing sugars (g/L)	Dry extract (g/L)
Wine control	0.73±0.01	5.05±0.07	3.68±0.04	13.4±0.1	4.75±0.06	38.6±0.2
Enzyme-treated wine	0.72±0.01	5.81±0.13	3.64±0.05	13.4±0.2	5.15±0.21	38.0±0.1

recommended by the manufacturer. They measured the total amount of volatile compounds one year later in both wines and found that it increased by 9% in Maria Gomez wine but remained unchanged in Bical wine. This result could be explained by the fact that the amount of enzyme was not enough, as this study observed. Arévalo Villena *et al.* (2006) obtained similar results when testing a commercial preparation in three wines using the recommended doses. They measured the concentration of glycosidic precursors after the enzymatic treatment and it was slightly lower in two wines and no difference was observed in the third. One year later, Arévalo Villena *et al.* (2007) studied the hydrolysis of glycosides in two wines produced by a purified yeast extract showing β-glucosidase activity and found that 59 and 63% of the total glycosides were hydrolyzed, while the levels ranged from 21.5 to 32.6% when the wines were treated with a commercial enzyme for 12 days.

Sensory analysis

Even though the determination of the total glycoside concentration after the enzymatic hydrolysis informs whether the enzymes have acted upon them, the assay is not specific and all of the glycosides present are quantified by this assay, not only the aroma precursors. For this reason, a preliminary sensory analysis was performed to determine whether wine aroma was modified after the treatment with exogenous glycosidases, which would indicate if some of the glycosides hydrolyzed by the enzymes were aroma precursors.

Of the possible discriminative analyses, the triangle test was selected because it is commonly used to detect if perceptible changes are produced in wines after introducing some modification in the process (Cabaroglu *et al.*, 2003; Zocca *et al.*, 2008; Ruiz *et al.*, 2010).

A total of 32 triangles were performed. The results indicated that there was a significant difference between the controls and the enzyme-treated wines ($P = 0.01$). Taking into account only the correct answers, an analysis of frequency of mention of attributes was carried out. The assessors found differences in sourness, astringency and aroma intensity between the wines with and without enzyme treatment. Nine of them described the wines without enzyme treatment sourer and six more astringent, while six mentioned that the enzyme-treated wines had higher aroma intensity. According to these results, it can be assumed that at least a part of the hydrolyzed glycosides were aroma precursors.

Physico-chemical determination

Some physico-chemical assays were carried out on a treated wine sample and on a wine control after 12 days of incubation to observe if the enzymatic treatment produced any modification. The results are shown in Table 4.

The ANOVA tests indicated that there were no significant differences in the measured parameters except in the total acidity ($P < 0.05$), which was higher in the enzyme-treated wine. However, the increase in the total acidity was not enough to produce a modification of the pH of the wine or be detected during sensory testing by the assessors. It is important to note that the total acidity in both samples was in the order of the values reported in many studies (Izquierdo Cañas *et al.*, 2008; Todaro *et al.*, 2008).

CONCLUSIONS

The use of the easy and quick methods described showed that three enzymatic preparations studied in this work presented high β-glucosidase and α-arabinosidase activities and low levels of α-rhamnosidase. Taking into account the recommended doses for use of these preparations, the total glycosidase activity that would be added to the wine does not seem to be enough to produce a detectable hydrolysis. All of the preparations should be supplemented with α-rhamnosidase in order to release all the precursors, especially those containing rhamnose in their structure.

The presence of glycosidase activity as determined with synthetic substrates is not enough to state that a commercial enzyme would produce an aroma improvement. However, the β-glucosidases from three preparations studied retained their activity in the presence of Tannat wine.

The selected enzyme Endozym β-split, supplemented with α-rhamnosidase activity from HPS 11518, produced an appreciable hydrolysis of the glycosidic precursors present in Tannat red wine. However, considerably higher amounts were necessary than the dose recommended by the manufacturers to produce glycoside hydrolysis to achieve the desired effect. At least a part of the hydrolyzed glycosides were aroma precursors, since the enzymatic treatment produced a detectable modification in the wine, which was described by some assessors as an increase in aroma intensity.

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