JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effect of β-Glycosidase Activity of *Oenococcus oeni* on the Glycosylated Flavor Precursors of Tannat Wine during Malolactic Fermentation

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Under traditional wine-making conditions, this work examines the β-glycosidic activity of *Oenococcus oeni* on glycosylated aroma compounds of Tannat wines during malolactic fermentation (MLF) by comparing the changes on slected aglycones liberated. MLF diminished the content of all the glycosylated compounds. The level of the free aroma components was slightly modified by the action of the malolactic fermentation so that the cleavage of the glycosidic linkage by the β-glycosidic activity of *O. oeni* did not appear to increase significatively the aglycone contents. The consequences of further chemical rearrangements of the algycones under wine conditions were explored using synthesized glycoconjugates on synthetic medium. Bacteria could also be responsible for the cleavage of aroma glycosylated compounds, being the aglycone adsorbed on polysaccharides or peptidoglycans and was released into the external medium. This hypothesis was studied through the evaluation of a stable arrangement of aroma compounds with polysaccharides produced by lactic acid bacteria. A possible retaining of free-made aroma compounds into the whole cells of *O. oeni* was also investigated through cell culture analysis. Through the results obtained, we assume stable linkage of aroma compounds with bacterial polysaccharides.

KEYWORDS: Tannat wine; malolactic fermentation; β-glycosidase activity; glycoconjugate; benzyl and geranyl glycosides; aroma

INTRODUCTION

Several attractive flavor compounds, like monoterpenes, C_{13}-norisoprenoids, and shikimate-derived compounds, are typically formed as odorless β-D-glucoside and diglycoside conjugates in grape and other fruit juices (*1*−*3*). In general, the aroma compounds are conjugated in the first instance to glucose as β-D-glucopyranosides or form more complex disaccharides with glucose being further conjugated with a second sugar unit of α-L-arabinofuranose, α-L-rhamnopyranose, β-D-xylopyranose, or β-D-apiofuranose (*1, 3, 4*).

Liberation of glycosylated compounds, like monoterpenes, is important, contributing to the aroma of certain varieties, in particular Muscats (*5, 6*). Sensory analysis of hydrolyzed products also demostrates the contribution of other glycosylated compounds on varieties such as Semillon (*7*).

Heat and acid hydrolysis of grape glycosides has been studied as a method for release of bound compounds with a view to enhancing aroma of grape juice through formation of free volatiles; however, this hydrolysis procedure can also promote the rearrangement of aglycones (*8*). As an alternative, enzymatic methods have been reported to transform these flavor precursors into active aroma compounds with minimal change of aglycones and appear to be more practical in wine-making (*1, 2, 9*).

The hydrolysis of monoglucosides requires the action of a β-glucosidase, while hydrolysis of disaccharide glycosides requires sequentially an appropriate *exo*-glycosidase to remove the outermost sugar molecule followed by a β-glucosidase to remove the remaining glucose (*10*). It was also shown that an *endo*-glycosidase alone is capable of hydrolyzing this linkage, thus liberating disaccharide and aglycon (*11*). β-Glycosidase activity of grape juice is virtually absent because of the low pH of the medium and the presence of glucose and, later, ethanol that inihibits the enzymatic activity (*2, 11*−*15*). The glycosidic activity related to *Saccharomyces cerevisiae* is weakly sensitive to the presence of sugar, but its action is very reduced because of the low pH of must and wine (*2, 16, 17*). On the other hand, several non-*Saccharomyces* yeasts have glycosidic activities but are inhibited severely by glucose concentration (*18*−*20*). More promising findings come from work with *Debaryomyces* and *Candida* strains (*21*−*23*).

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10.1021/jf0109367 CCC: $22.00 © 2002 American Chemical Society
Published on Web 03/09/2002

Many commercial fungal β-glycosidases have been reported to carry out the hydrolysis of glycosylated compounds, but in practical wine-making they also present problems because of glucose inhibition (*2, 12, 24, 25*). They have been also reported to present undesirable side activities that can transform free aglycones into flavorless compounds or produce unwanted artifacts in the resulting aglycones (*26, 27*).

In vitro β-glycosidase of one strain of *Oenoccocus oeni* performing malolactic fermentation (MLF) was found to be active at must pH (3.5), retaining 78% of the maximum activity value (*28*). In a recent work examining performances of cultures derived from 11 commercial preparations of *Oenococcus oeni*, responses of β-glucosidase activity to enological pH values, glucose/fructose ratio, and ethanol concentration were determined with synthetic substrates (*29*). While these activities were readily detectable under the synthetic medium conditions used in this study, changes under enological conditions remain to be investigated. This study evaluates the β-glycosidase activity of LAB, during MLF, on glycosylated aroma compounds under the enological conditions of red wine (cv. Tannat) production by comparing the changes in the free compounds obtained from selected aglycones.

Vitis vinifera L. cv. Tannat is the major variety in Uruguay, being introduced there in 1870 from France and very well acclimatized to produce red premium wines. Uruguay is the only country in the Americas where this variety is commonly grown.

Strategies to improve Tannat wine quality using advanced viticultural and wine-making technology (*30*) started with the analytical characterization of the complex flavor profile of Tannat (*31*−*34*), which is described as raspberry, plum, quince, and small-berry-like scents. In aged wines it changes to wild animal-like, smoked, and liquorice character (*34, 35*). Possible peculiarities of the free forms and also the heteroside fraction may explain some of these aroma descriptors as previously reported (*31*).

Furthermore, the richness of the flavor and quality improvement can be reached through MLF. Possible rearrangements or disappearance of free-made compounds were also investigated, considering synthesized glycoconjugates similarly reacted in synthetic medium. Finally, the production of a stable arrangement of aroma compounds with polysaccharides produced by LAB was also carried out.

MATERIALS AND METHODS

Strains, Media, and Cultivation. *Oenococcus oeni* isolates used in this study were isolate 1 (DSM 7008, Viniflora, Chr. Hansen), isolate 2 (D-11, Malolactine O, Groupe Oeno-France), and isolate 3 (Lalvin 31, Lallemand). Dried bacterial preparations were inoculated into 5 mL of *m*alo*l*actic *b*asal (MLB) medium (*37*) and incubated for four to five days at 25 °C. Culture growth was monitored spectrophotometrically (600 nm) and expressed as dry weight using a calibration curve relating the two parameters. Such cultures formed the inoculum for subsequent experiments.

Measurement of β-Glucosidase Activity. The β-glucosidase activity for the three bacteria isolates was determinated according to Grimaldi et al. (*29*), using *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) as substrate. All the isolates showed activity against *p*-NPG, as previously reported for the same strains (*28, 29*).

Wine-Making. Fresh grapes (*Vitis vinifera* L. cv. Tannat) were sourced from a local vineyard (Santa Lucía, Canelones Province, Uruguay) and delivered in good condition to our wine-making facilities. Four samples (s1, s2, s3, s4), 30 kg each, were microvinified during the 1998 vintage. The grapes were destemmed and crushed, and a subsample was analyzed for sugar content (g/L), total acidity (g/L, expressed as H_2SO_4), and pH. Sugar contents for the four samples were 193, 195, 188, and 203 g/L. The total acidity was 6.9, 5.4, 5.0, and 6.5 g/L, and the corresponding pH values were 3.37, 3.19, 3.15, and 3.22, respectively. SO_2 was added to the must (50 mg/L), which was then inoculated with reactivated dry yeast (*Saccharomyces cerevisiae*, strain CIVC 8130; Gist Brocades, Chile). Fermentation was carried out at 22−25 °C. At a density of 1000 g/L, the wine was run off from the fermentor, pomace was pressed, and pressed wine was added to runoff wine. Upon completion of alcoholic fermentation, each batch was divided into three equal portions. Two portions were taken for MLF by inoculation, in duplicate, with pure cultures of *Oenoccocus oeni*, isolates 1 and 2. The third portion was treated with 50 mg/L SO_2 to control MLF (control) and then held under conditions similar to those applied to the other two portions. A 200 mL aliquot of wine was kept as a control (with no addition of SO_2) to monitor if MLF took place spontaneously. In sum, the experimental design was as follow: 4 wines × 3 treatments × 2 duplicates to give 24 wines samples, plus 4 wines held for evidence of spontaneus MLF. All samples were held at 18 °C, and MLF was followed by determining the concentrations of malic and lactic acid by thin-layer chromatography (TLC) (*38*). Upon completion of MLF, the wines were treated with 50 mg/L SO_2. All samples were stabilized at 4 °C for 20 days and sterile-filtered (0.45 μm membrane), and free SO_2 content was then adjusted to 35 mg/L. After bottling, the wines were held for 3 months at 10 °C before analysis.

Synthesis of Glycoconjugates. The glycoconjugates to be synthesized were selected by their content in Tannat wines, as in the case of benzyl alcohol, and in order to verify chemical rearrangements produced as a consequence of its eventual degradation in the medium, as in the case of geraniol. Benzyl β-D-glucopyranoside was synthesized following the modified Köenigs and Knorr method (*39*). Acetylated geranyl 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside supplied by Versini (Istituto Agrario di San Michele all'Adige, Italia) was deacetylated at room temperature with diluted $NaOCH_3$ in methanol according to Voirin et al. (*40*). The structure of the free glycosides was confirmed by ^1H NMR and ^{13}C NMR spectroscopy according to Agrawal (*41*) on a Bruker Avance DPX400 spectrometer.

Synthetic Media. Actively growing cultures of the three isolates of *Oenoccocus oeni* were inoculated in duplicate at a rate of 0.1% (v/v) into 75 mL of MLB medium at pH 4.5, then benzyl β-D-glucopyranoside and geranyl 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside were added. The media was incubated at 22 °C for 10−14 days. After the growing of culture, the media was centrifuged and filtered through a cellulose nitrate membrane (Sartorius, Germany, 0.45 μm). Free and bound compounds were analyzed in the supernatant. Results for isolate 1 were not considered because one of the duplicates failed to produce a growing culture.

Wine Analysis. All samples were analyzed at the same time after completion of MLF (malic acid content below 0.1 g/L). Total acidity and pH were measured with the usual method (*42*). The malic and lactic acids were analyzed by reversed-phase HPLC (*43*), using a Shimadzu equipment (Shimadzu Corp., Kyoto, Japan) composed of a model LC-10AT pump and a SPD-6AV UV−vis detector. Peak integration and quantitative calculations were performed on a C-R3A integrator using a calibration curve obtained for each standard acid. The column was a Beckman Ultrasphere ODS C_{18} column (150 mm × 4.6 mm i.d., particle size 5 μm) with the following parameters: mobile phase, 0.005 M sulfuric acid; flow rate, 0.6 mL/min; injection volume, 20 μL; column temperature, 20 °C. Detection was by UV absorbance at $\lambda = 214$ nm.

Extraction and Determination of Free and Bound Aroma Compounds. *Extraction.* The free and bound aroma components were eluted as previously reported (*44*). The bound fractions were evaporated to dryness and then dissolved in 3 mL of citrate buffer at pH 5, and Cytolase PCL5 (DSM Gist-brocades Food Specialities, Seclin, France) was added. After the mixture was stirred, the tube was sealed and placed in a water bath at 40 °C for 14 h. After the addition of 0.1 mL of the same internal standard, aglycones were extracted three times with 3 mL of pentane/dichloromethane, 2:1, v/v, and the organic phase was dried with sodium sulfate and concentrated to 0.2 mL before GC and GC/MS analysis.

High-Resolution Gas Chromatography (HRGC)/MS Analysis. HRGC/MS or SIM/MS analyses were conducted using a Shimadzu QP 5050

Table 1. Chemical Analysis of Wines, Showing Mean Values of Two Fermentations for Each Treatment (TA, Total Acidity (g of H_2SO_4/L)) for Malic and Lactic Acids (g/L)

sample	treatment[a]	TA	pH	malic acid	lactic acid
s1	control	6.3	3.45	4.6	0.8
	MLF 1	4.1	3.65	*b*	3.9
	MLF 2	4.1	3.68	*b*	4.0
s2	control	4.6	3.34	3.0	0.7
	MLF 1	3.7	3.45	*b*	2.9
	MLF 2	3.4	3.47	*b*	2.5
s3	control	5.2	3.28	2.0	*b*
	MLF 1	4.4	3.38	*b*	1.5
	MLF 2	4.3	3.37	*b*	1.2
s4	control	5.5	3.42	2.7	*b*
	MLF 1	4.6	3.58	*b*	2.1
	MLF 2	4.5	3.52	*b*	1.8

[a] Control, wine sample without MLF performed; MLF 1 and MLF 2, wines with MLF performed using strain 1 or 2. [b] Below detection limit (0.1 g/L)

(Shimadzu Corporation, Kyoto, Japan) equipped with reference libraries (Adams, 1995; McLafferty and Stauffer, 1991) using a BP 20 (SGE, Ringwood, Australia) bonded fused-silica capillary column (25 m × 0.25 mm i.d.) coated with poly(ethylene glycol) (0.25 μm phase thickness); the column temperature varied from 40 °C (8 min) to 180 °C at 3 °C/min to 230 °C at 20 °C/min. The following additional parameters were used: injector temperature, 250 °C; injection mode, split; split ratio, 1:40; volume injected, 1.0 μL; carrier gas, He, 92.6 kPa (55.9 cm/s); interface temperature, 250 °C; acquisition mass range, 40−400 amu.

The components of the wine aroma were identified by comparison of their linear retention indices, determined in relation to a homologous series of *n*-alkanes, with those from pure standards or those reported in the literature. Comparison of fragmentation patterns in the mass spectra with those stored in databases (*45, 46*) was also performed.

HRGC Analysis. For quantitative results each sample was analyzed by HRGC on a Carlo Erba Fractovap series 2900 (Carlo Erba Strumentaziones, Milan, Italy) gas chromatograph equipped with a Shimadzu EZ-Chrom data processor; a MegaAcid (Mega, Legnano, Italy) fused-silica capillary column (25 m × 0.32 mm i.d., 0.40−0.45 μm film thickness) was used. The following parameters were used: column temperature, 40 °C (8 min) to 180 °C at 3 °C/min to 230 °C at 20 °C/min; injection mode, split; split ratio, 1:30; detector, FID; injector temperature, 250 °C; detector temperature, 280 °C; volume injected, 0.5 μL; carrier gas, H_2, 55 kPa. The quantification of the components was performed on the basis of their GC peak areas, considering as "total terpenes" the sum of linalool, α-terpineol, nerol, and geraniol and as "total norisoprenoids" the sum of 3-hidroxy-β-damascone and 3-oxo-α-ionol.

Statistical Analysis. For the free and bound components, variance analyses were performed between the control wine (without MLF) and each treatment (MLF with different strains). Significative differences of the means were calculated according to the least-significant differences test (LSDs). All statistical analyses were performed using the Statistica 5.1 software (StatSoft, Inc., 1998).

RESULTS AND DISCUSSION

Wine Analysis. Table 1 gives the chemical analyses of the wine samples for the different treatments. Microbial degradation of malic acid into lactic acid was carried out duly by both strains. Total acidity and pH showed the same behavior for the different strains tested, with final values depending on the initial malic acid content in each sample.

Effect of MLF on Free and Bound Aroma Compounds. The effect of MLF on glycosylated aroma compounds in the wine samples is reported in **Table 2**. MLF diminished the level of all the glycosylated compounds ($p < 0.05$), but the extension of the changes was found to be strain-related ($p < 0.05$). Significant differences between strains were found for 2-phenylethanol and the sum of the two C_{13}-norisoprenoids measured.

The level of the aroma components present in free form was slightly modified by the action of MLF, as shown in **Table 3**. Significant differences were observed only for 2-phenylethanol and the sum of terpenols (linalool, α-terpineol, nerol, and geraniol). The cleavage of the glycosidic linkage did not appear to increase significantly the aglycone contents. The significant increment detected for 2-phenylethanol in the treatment with isolate 2 cannot be explained by the changing of its glycosylated level (not highr enough) but by bacterial metabolism as in

Table 2. Effect of MLF on Glycosylated Wine Aroma Compounds, Showing Mean Concentration (±Standard Deviation) (mg/L) of Two Replications of the Same Treatment for the Four Wines Analyzed

	control[a]	MLF 1[a]	MLF 2[×bb]	F^b	p^b
geraniol	0.015 ± 0.005 a	0.009 ± 0.003 ab	0.0052 ± 0.0003 b	7.62	0.0225
benzyl alcohol	0.311 ± 0.167 a	0.226 ± 0.167 b	0.199 ± 0.093 b	6.58	0.0307
2-phenylethanol	0.367 ± 0.098 a	0.225 ± 0.052 b	0.140 ± 0.024 c	26.37	0.0011
terpenols[c]	0.037 ± 0.014 a	0.010 ± 0.009 b	0.021 ± 0.008 b	10.11	0.0120
C13-norisoprenoids[d]	0.420 ± 0.023 a	0.275 ± 0.060 b	0.199 ± 0.034 c	31.15	0.0007
C6 compounds[e]	0.103 ± 0.027 a	0.061 ± 0.024 b	0.043 ± 0.009 b	13.66	0.0058

[a] Control, wine sample without MLF; MLF 1 and MLF 2, wine sample with MLF (strain 1 or 2) [b] *F* and *p*, values from the variance analysis; significant differences ($p < 0.05$) according to the LSD test are indicated by using different letters (a, b, c). [c] Linalool + α-terpineol + nerol + geraniol. [d] 3-Hydroxy-β-damascone + 3-oxo-α-ionol. [e] 1-Hexanol + *cis*-3-hexen-1-ol + *trans* 3-hexen-1-ol.

Table 3. Effect of MLF on the Free Aroma Compounds, Showing Mean Concentration (±Standard Deviation) (mg/L) of Two Replications of the Same Treatment for the Four Wines Analyzed

	control[a]	MLF 1[a]	MLF 2[a]	F^b	p^b
geraniol	0.025 ± 0.013	0.026 ± 0.011	0.024 ± 0.008	0.37	0.706
benzyl alcohol	0.073 ± 0.018	0.115 ± 0.040	0.102 ± 0.012	1.83	0.239
2-phenylethanol	33.7 ± 14.2 b	34.9 ± 17.2 b	42.8 ± 19.5 a	6.16	0.035
C6 compounds[c]	1.56 ± 0.79	1.41 ± 0.64	1.54 ± 0.39	0.51	0.624
terpenols[d]	0.046 ± 0.013 ab	0.051 ± 0.005 a	0.038 ± 0.010 b	7.81	0.021

[a] Control, wine sample without MLF; MLF 1 and MLF 2, wine sample with MLF (strain 1 or 2) [b] *F* and *p*, values from the variance analysis; significant differences ($p < 0.05$) according to the LSD test are indicated by using different letters (a, b, c). [c] 1-Hexanol + *cis*-3-hexen-1-ol + *trans*-3-hexen-1-ol. [d] Linalool + α-terpineol + nerol + geraniol.

Table 4. Effect of Bacterial Activity on the Amount (mg/L) of Aroma Compounds in Glycosidic and Free Forms, Measured in the MLB Medium, Showing Mean Concentration of Two Replications of the Same Treatment

	control	isolate 2	isolate 3
glycosylated compounds			
geraniol	3.703	2.336	1.315
benzyl alcohol	2.119	1.576	0.416
free compounds			
linalool	*a*	0.067	0.025
α-terpineol	0.005	0.042	0.024
geraniol	0.022	0.206	0.140
benzyl alcohol	0.099	0.218	0.285

[a] Below detection limit.

agreement with previously reported data for wine and synthetic media in the presence of LAB (*47−49*).

Effect of Chemical Modifications on the Aglycones in Wine. The differences between the free and bound fraction amounts could be explained by further chemical transformation of the aglycones under the mild acidity conditions of the wine. To evaluate the liberation of volatile compounds from the glycoconjugate alcohols in a medium simpler than wine, the experiments were repeated on MLB medium with added benzyl and geranyl glycosides as substrates of the enzimatic activity, at concentrations similar to those found in wines of different varieties.

As in the wine samples, the level of glycosylated components decreased (**Table 4**) after 9 days of fermentation without a quantitative increase of the corresponding alcohols. The increased amount of linalool and α-terpineol detected (**Table 4**), both produced from geraniol by acidic rearrangement (*8, 50*), was still not enough to explain the expected differences of the free geraniol content resulting from the cleavage of its glycosylated form.

Effect of Bacterial Polysaccharides on the Fixation of Aroma Compounds. The polysaccharide fraction, precipitated by adding ethanol to the substrate according to Vivas et al. (*36*), was separated by centrifugation, then 4 mL of dichloromethane was added, and the mixture was submitted to ultrasound for 10 min. The supernatant was separated, concentrated, and analyzed by GC/MS/SIM (*m/z* 69 and *m/z* 108), as described above, for detecting the presence of geraniol and benzyl alcohol at relevant retention times (**Figure 1**).

Bacteria might have cleaved the aroma glycosylated compound and used glucose as the carbohydrate source, being that the aglycone was adsorbed on polysaccharides or peptidoglycans and was released into the external medium. This is consistent with what happened with other glycoconjugates like anthocyanines, whose decrease is due to the β-glycosidase activity of *O. oeni* during the MLF, with coupled production of a stable arrangement of polysaccharide-malvidin (*36*). Besides, strong interaction of aroma compounds and polysaccharides has also been demostrated in in vitro experiments (*51*).

A possible retaining of free-made aroma compounds into the whole cells of *O. oeni* was also investigated through the cell culture analysis. Cells were separated by centrifugation (20 min, 6500*g* at 5 °C) at the end of the growing phase, then submitted to ultrasound for 3 min according to Guilloux-Benatier et al. (*28*) and extracted twice with 3 mL of dichloromethane. The extract obtained was then concentrated at 0.2 mL and analyzed by HRGC/MS/SIM as described above. The absence of geraniol, benzyl alcohol, or any of the byproducts described confirmed the only parietal location of the glycolitic enzymes (*28*).

In sum, the β-glycosidic activity of *O. oeni* on glycosylated aroma compounds was proved in oenological conditions too.

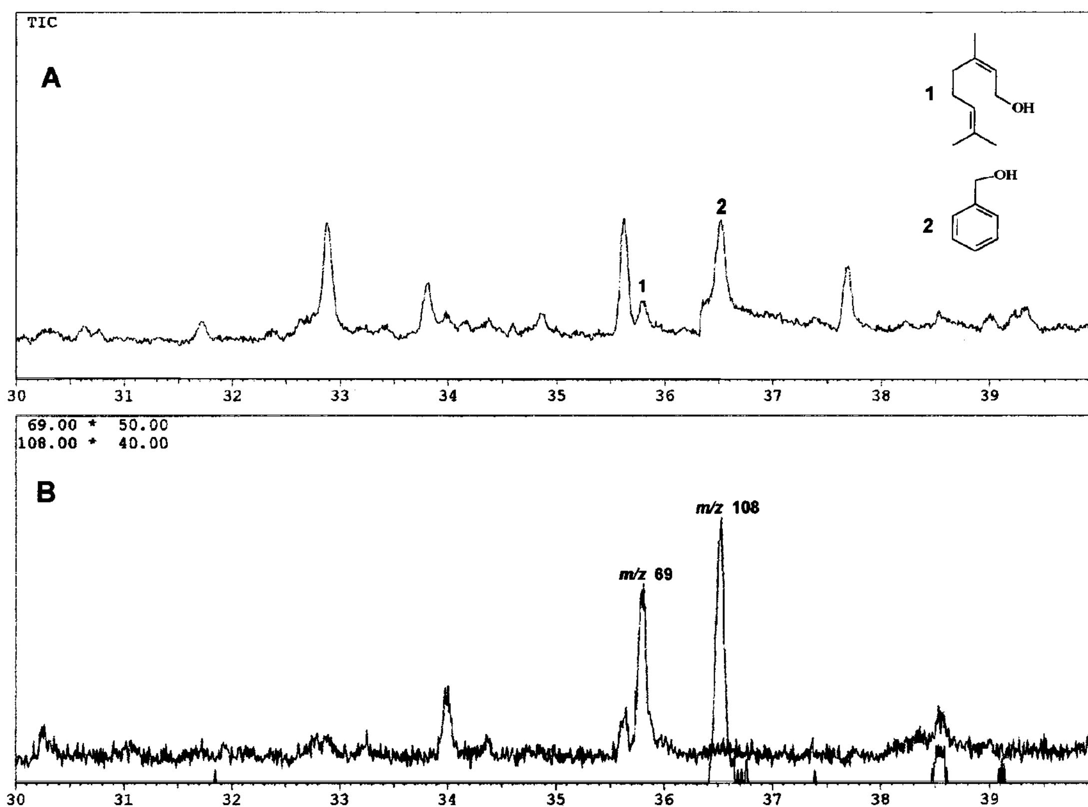

Figure 1. Analysis by coupled GC/MS of volatile compounds extracted from polysaccharides produced by LAB: (A) TIC; (B) GC/MS/SIM for geraniol (**1**, *m/z* 69) and benzyl alcohol (**2**, *m/z* 108).

The level of the free aroma components was, however, only slightly modified by the action of the MLF, so the cleavage of the glycosidic linkage did not appear to increase significatively the aglycone contents. This behavior could be explained by assuming stable linkage of aroma compounds with the polysaccharide macromolecules usually produced by *O. oeni* during MLF.

Similar trends were observed by Dufour and Bayonove (*51*), indicating that the production of polysaccharides by the malolactic bacteria could also account for, at least in part, the reduction of glycosides through binding interactions as adsorption and occlusion phenomena. This model contributes to support the hypothesized role of the polysaccharides in binding flavor aglycones.

Therefore, to enhance the aroma release in Tannat wines, future work should be focused on the selection of bacterial strains showing low capacity in the polysaccharide biosynthesis during malolactic fermentation. Because of difficulties in purifying wine polysaccharides, further studies of the polymers are also required to clarify the type of binding involved in intermolecular interactions.

ACKNOWLEDGMENT

The assistance of Dr. Versini (Istituto Agrario di San Michele all'Adige, Italy) and M. Galli (Mega, Legnano, Italy) is kindly thanked.

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Received for review July 19, 2001. Revised manuscript received December 11, 2001. Accepted January 3, 2002. The authors thank the Instituto Nacional de Investigaciones Agropecuarias (INIA, Grant No. 125) and the Consejo Nacional de Investigación Científica y Tecnlógica (CONICYT, Project 5058) for financial support that made their work possible.

JF0109367