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Hydrolysis and Transformation of Grape Glycosidically Bound Volatile Compounds during Fermentation with Three *Saccharomyces* Yeast Strains

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The ability of three *Saccharomyces* wine yeasts (*S. cerevisiae* AWRI 838, *S. cerevisiae* AWRI 1537, and *S. bayanus* AWRI 1375) to liberate volatile compounds from sugar-bound aroma precursors was investigated using synthetic and grape glycosides under different experimental conditions. In model systems involving the incubation of yeast cells with either synthetic or grape-derived glycosides under conditions more favorable for glycosidase activities and less favorable for acid-catalyzed hydrolysis (pH 5.0 and 30 °C), all yeast strains studied proved to be capable of hydrolyzing glycosides, with *S. bayanus* AWRI 1375 displaying greater hydrolytic activity than *S. cerevisiae* AWRI 838 and AWRI 1537. During the fermentation of a chemically defined grape juice-like medium containing glycosidic precursors extracted from *Vitis vinifera* cv. White Frontignac (synonym Muscat à Petit Grains Blanc), all yeasts promoted a significant hydrolysis of different precursors, which varied according to the chemical structures of both the sugar and the aglycon moieties, as determined by GC-MS analysis of trifluoroacetylated derivatives. Hydrolysis of the White Frontignac derived glycosidic precursors during fermentation resulted in the release of monoterepene alcohols, terpene oxides, terpene diols, and 3-oxo-α-ionol, demonstrating the significant potential of these yeast strains to contribute to wine varietal volatile composition during alcoholic fermentation.

KEYWORDS: Glycosides; yeasts; fermentation; *Saccharomyces cerevisiae*; *Saccharomyces bayanus*; wine aroma

INTRODUCTION

The aroma characteristics of wine result from the contribution of volatile compounds originating from the grapes, yeast and bacterial metabolism, winemaking practices, and oak when used. Within this highly complex array of odor-active substances, it is generally accepted that grape-derived aroma compounds play a primary role in the expression of distinctive aroma attributes which are characteristic of the grape variety employed for winemaking. The pool of grape-derived volatile compounds in wine includes potent odorants such as terpenes, C-13 norisoprenoids, and benzenoids (*1*−*3*). These compounds can be present in grapes either as free, odor-active forms or as odorless precursors, mainly sugar-bound conjugates (glycoconjugates) (*1*−*3*). Although many of these volatiles can be found in the majority of grape varieties employed for winemaking, their concentration and the possible interactions with other volatile compounds determine their overall contribution to the aroma character of each wine (*4*).

In the case of wines obtained from so-called "aromatic" grape varieties (e.g., Muscat, Gewürztraminer, Riesling), many of the volatile compounds potentially contributing to the aroma of the final wines, particularly terpene alcohols, are already present in the grapes as free forms in relatively high concentrations (*5*). For this reason, the typical floral aroma of these wines is also noted in the grapes. On the contrary, in "nonfloral" grapes (e.g., Chardonnay, Semillon, Trebbiano), odor-active forms of grape-derived volatile compounds occur at trace or subtrace concentrations, although they are present at higher concentration as odorless glycoconjugates (*1*−*3*). Consistent with these observations, juices obtained from these grapes generally lack any distinctive aroma. Nevertheless, after the completion of alcoholic fermentation, wines from nonfloral grapes very often exhibit aroma attributes that are specific for the grape variety employed for winemaking. For many of these wines, a clear connection has been established between their typical aroma attributes and those revealed by hydrolysis of the glycoconjugated fraction (*4*, *6*, *7*). These observations suggest that, during winemaking, one or more mechanisms are responsible for the hydrolysis of

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the grape glycosidic precursors and the consequent release of volatile compounds involved in the expression of wine varietal character.

Glycosidic precursors of grapes include monosaccharide glycosides, in which the sugar moiety consists of a β-D-glucose unit, and disaccharides, in which the glucose is further substituted with a second sugar unit, typically α-L-arabinofuranoside, α-L-rhamnopyranoside, or β-D-apiofuranoside (*8*). Under the mild acidic conditions of wine, spontaneous hydrolysis of the β-glucosidic linkage of glycosides results in the release of the bound volatile compounds (*1*, *3*), a relatively slow process that leads to the formation of powerful wine odorants, particularly norisoprenoids (*1*, *9*, *10*). Alternatively, enzymes with glycosidase activity, including those produced by some yeasts and filamentous fungi, are able to promote the rapid hydrolysis of grape glycosides and the consequent release of the bound odor-active fraction (*11*). The mechanism proposed for this liberation of sugar-bound aglycons involves, in the case of disaccharide glycosides, the preliminary action of an appropriate glycosidase (arabinofuranosidase, rhamnopyranosidase, or apiofuranosidase) to release the terminal sugar before the β-glucosidase is able to release the bound volatile component. For glucosidic precursors only this latter step is needed (*8*).

Wine yeasts have been investigated for the presence of enologically significant glycosidases. Several studies have shown that enzymatic extracts of *Saccharomyces cerevisiae* are able to liberate the volatile fraction of glycoconjugated precursors of grapes (*12*, *13*). The direct incubation of yeast cells with grape-extracted glycosides under favorable glycosidase activity conditions (e.g., pH 5.0 and 30 °C) has also been demonstrated to promote the release of volatile compounds from glycosidic precursors (*14*, *15*). Delfini et al. (*16*) also found that incubating yeast cells in the presence of grape skins can reveal typical aroma attributes of different red and white wines. Nevertheless, the various studies regarding the activity of yeast glycosidase enzymes during winemaking are somewhat contradictory. Several authors (*17*−*19*) found that little hydrolysis of glycosides was observed during the fermentation of Muscat grape juice, possibly because of the low activity of yeast glycosidase enzymes under winemaking conditions (*17*). However, this apparent stability of the glycosides during fermentation might, at least in part, be due to the unintentional prior exposure of the glycosides during juice preparation to glycosidic enzymes arising from grape berries, grape-associated microorganisms or enzymes added as processing additives. Considering that only a portion of the pool of grape glycosides is normally transformed during fermentation, this could result in a significant decrease in the concentration of glycosides available for yeast-driven glycoside transformations. On the other hand, other studies have reported a large decline of glycosides during alcoholic fermentation, suggesting that yeast metabolism can result in a significant degree of hydrolysis of glycosides (*20*−*23*). However, in none of these studies was the respective contribution of yeast-driven and acid-catalyzed hydrolysis of glycosides elucidated, due to the lack of appropriate nonfermented reference controls to allow a direct comparison of the extent of the two different processes.

In the present study, we compared the ability of three *Saccharomyces* yeast strains commonly used for winemaking to hydrolyze grape-derived glycosidic precursors and release the related volatile compounds under different experimental conditions. Yeasts were first tested under optimal conditions for their glycosidase activities in model systems using either synthetic or grape-derived glycosides. Subsequently, the same yeasts were used to conduct alcoholic fermentation in a chemically defined grape juice-like medium containing glycosides extracted from grape juice. The use of model grape juice media allowed us to define and control experimental conditions, which minimized the interference of native grape glycosidases as well as hydrolytic enzymes derived from other microorganisms commonly present in grape must. The aim of the investigation was to elucidate whether the yeasts studied possessed the enzymatic activities necessary for the hydrolysis of glycosides and if these activities were operating under typical winemaking conditions. Moreover, the chemical structural characteristics of the glycosides that were not subjected to hydrolysis were studied by analysis of their trifluoroacetylated derivatives to reveal the nature of glycosides which are more susceptible to hydrolysis during fermentation.

MATERIALS AND METHODS

Yeast Strains. The three yeasts used in this study were *Saccharomyces cerevisiae* strains AWRI 838 (an isolate of Lalvin EC1118, Lallemand) and AWRI 1537 (Vin 13, Anchor) and *Saccharomyces bayanus* strain AWRI 1375 (*24*). Yeasts were maintained by bimonthly serial propagation on yeast−malt extract (YM) medium (Amyl Media, Dandenong, Australia) supplemented with 1.5% agar with storage at 4 °C. All strains were obtained from The Australian Wine Research Institute Culture Collection (Adelaide, Australia).

Preparation of the Glycosidic Extract. Amberlite XAD-2 resin (Supelco, Bellefonte, PA) was used for the extraction of glycosidic precursors from grape juice (*25*). A methanol suspension of the resin, previously washed with methanol and diethyl ether, was poured into a glass column (22 × 3.5 cm i.d.) fitted with a PTFE tap and a glass wool stopper. The packed column contained ≈17 cm of resin. Two columns prepared from the same batch of resin were used in parallel. Prior to use, 50 mL of methanol followed by 50 mL of water were passed through the columns. A *Vitis vinifera* cv. White Frontignac (synonym of Muscat Blanc à Petit Grains) grape juice, prepared during the 2004 vintage season without the addition of commercial enzymes but with sulfur dioxide (80 mg/L) added to prevent microbial activity, was used to isolate glycosides. The total concentration of glycosides in this juice, expressed as glycosyl-glucose (G-G) (*26*), was 467 μmol/L. Batches of 1.5 L of juice were extracted at ≈3 mL/min. The columns were then washed with 100 mL of water to remove sugars, followed by 100 mL of methanol to recover the glycosides. The four batches of extracts were pooled together, and the solvent was then evaporated under vacuum in a rotary evaporator. The extract was then dissolved in water, washed successively with pentane and dichloromethane to remove residual volatile compounds, then treated again in a rotary evaporator under vacuum to remove possible traces of solvent, and finally sterile filtered and stored at −20 °C until used.

Determination of Yeast Glycosidase Activities with Synthetic Substrates (Experiment 1). The evaluation of glycosidase activities of the yeasts under study was carried out by determining the liberation of *p*-nitrophenol (*p*NP) from different *p*-nitrophenyl glycosides. Yeast cells were cultured in YPD medium at 30 °C with shaking, for a total of 72 h. At intervals of 24 h, 200 μL of culture was collected under aseptic conditions and assayed for enzymatic activities present in the supernatant. Samples for assay were clarified by centrifugation, and 180 μL of the supernatant was transferred to a 1.5 mL tube containing 20 μL of 50 mM sodium acetate buffer (pH 5). Two hundred microliters of 5 mM *p*NP substrate (*p*-nitrophenyl-β-D-glucoside, *p*-nitrophenyl-α-L-arabinoside, or *p*-nitrophenyl-α-L-rhamnoside, Sigma-Aldrich) was added, and the mixture was incubated for 24 h at 30 °C. The reaction was then stopped by adding 500 μL of 1 M sodium carbonate, and the absorbance was read at 400 nm against a blank containing sterile water in place of the sample. Results were given as nanomoles of *p*NP per milliliter per hour per 1×10^7 cells. All assays were performed in duplicate.

Study of the Hydrolysis of Grape Glycosides by Nonproliferating Yeast Cells (Experiment 2). The method described in ref *14*, with small modifications, was used. A loopful of yeast was inoculated into 30 mL of sterile YM medium contained in sterile 50 mL tubes and

Table 1. Composition of the Chemically Defined Grape Juice-like Medium Used for Model Fermentations

ingredient	concn
sugars (g/L)	
glucose	100
fructose	100
acids (g/L)	
potassium hydrogen tartrate	2.5
L-malic acid	3.0
citric acid	0.2
minerals (g/L)	
K_2HPO_4	1.14
$MgSO_4 \cdot 7H_2O$	1.23
$CaCl_2 \cdot 2H_2O$	0.44
nitrogen compounds (mg/L)	
γ-aminobutyric acid	69.7
alanine	74.4
arginine	98.5
asparagine	14.9
aspartic acid	24.9
cysteine	1.4
glutamic acid	75.3
glutamine	111.9
glycine	4.7
histidine	19.6
isoleucine	11
leucine	11.2
lysine	5.2
methionine	3.7
NH_3 (as NH_4Cl)	52
ornithine	1.1
proline	764.8
serine	50.8
threonine	48.6
tryptophan	10.9
tyrosine	18.7
valine	18.6
trace elements (μg/L)	
$Co(NO_3) \cdot 6\ H_2O$	30
$CuCl_2$	15
$FeCl_2$	30
H_3BO_3	5
KIO_3	10
$MnCl_2 \cdot 4H_2O$	200
$NaMoO_4 \cdot 2\ H_2O$	25
$ZnCl_2$	135
vitamins (mg/L)	
biotin	0.125
calcium pantothenate	1
folic acid	0.2
myo-inositol	100
nicotinic acid	2
PABA·K	0.2
pyridoxine·HCl	2
riboflavin	0.2
thiamin·HCl	0.5
glycosidic extract	532 μM (glycosyl-glucose)
pH	3.2 (adjusted with NaOH)

incubated at 30 °C, with shaking. When the biomass reached $\approx 4 \times 10^7$ cells/mL, determined microscopically using a hemocytometer, 25 mL of culture was centrifuged at 4000*g* for 5 min, and the pellet was washed with sterile water, centrifuged, and resuspended in 5 mL of 0.05 M phosphate−citrate buffer at pH 5.0, containing the glycosidic extract equivalent to 25 mL of White Frontignac grape juice. This cell suspension was transferred to a 10 mL glass tube and incubated at 30 °C for 72 h with shaking. A noninoculated sample and an inoculated sample without glycosidic extract were used as controls. All assays were performed in duplicate.

Study of the Hydrolysis of Grape Glycosides by Yeast during Alcoholic Fermentation (Experiment 3). The fermentation experiment was carried out in a chemically defined grape juice-like (CDGJ) medium as described by Henschke and Jiranek (*27*), but with the modifications described in **Table 1**. For treatments requiring the presence of glycosides, the glycosidic extract was added to a final concentration of 532 μmol/L of G-G, equivalent to 1.14 times the G-G concentration of the original grape juice. The medium was sterilized by filtration through a 0.2 μm sterile membrane and divided into 200 mL aliquots.

Table 2. Enzymatic Activities of *Saccharomyces* Yeast, Expressed as Nanomoles of *p*NP per Milliliter per Hour[a]

time (h)	*S. cerevisiae* AWRI 838	*S. cerevisiae* AWRI	*S. bayanus* AWRI 1375
	β-Glucosidase		
24	0.160 b	0.144 b	0.189 a
48	0.359 b	0.355 b	0.381 a
72	0.050 b	0.080 a	0.048 b
	α-Rhamnosidase		
24	–	–	–
48	0.015 a	0.016 a	0.018 a
72	–	–	–
	α-Arabinosidase		
24	–	–	
48	0.013 a	0.012 a	0.013 a
72	–	–	–

[a] Means of duplicate assays. Within each treatment, different letters denote significant differences at $p < 0.05$. –, not detected.

A loopful of yeast cells was incubated in 10 mL of YM medium with shaking for 24−48 h at 28 °C. Preadaptation of the cells to the fermentation medium was carried out by inoculating 100 μL of the cultures into 20 mL of 50% (v/v) CDGJ medium. These subcultures were grown at 28 °C until a biomass of $(1-2) \times 10^8$ cells/mL, determined microscopically using a hemocytometer, was reached, after which they were inoculated in the CDGJ medium at a final concentration of 1×10^6 cells/mL.

Fermentations were carried out at 18 °C in 250 mL Erlenmeyer flasks sealed with fermentation water locks and shaken at 180 oscillations per minute. Flasks were kept in a thermostatically controlled water bath with recirculating water acting as coolant, to ensure optimal dispersion of the heat generated during fermentation. Samples (1 mL) for monitoring the progress of alcoholic fermentation were removed daily under sterile conditions with a needle and syringe via a sample port closed with a rubber Suba seal and were analyzed enzymatically for total reducing sugar concentration (Roche Molecular Biochemicals) using an automated Cobas FARA centrifugal analyzer. For each yeast strain, triplicate fermentations of CDGJ medium with and without glycosides were performed. A noninoculated sample of CDGJ medium containing glycosides was used to evaluate acid-catalyzed hydrolysis of glycosides. Upon completion of fermentation (residual sugars < 1 g/L), samples were cold-settled for 5 days at 5 °C, with sterile nitrogen supplied at low pressure to prevent the ingress of air into the flasks. Following the addition of potassium metabisulfite at 200 mg/L, the wines were racked off gross yeast lees and submitted to extraction and analysis of free volatile compounds and remaining glycosides.

Extraction and Analysis of Volatile Compounds and Glycosides. *Hydrolysis of Grape Glycosides by Nonproliferating Yeast Cells (Experiment 2).* 2-Octanol was added as an internal standard (125 μL of a 67 mg/L solution in ethanol) to the samples, which were then centrifuged, and volatiles were extracted using a Sep-Pak Plus C_{18} solid-phase extraction cartridge (Millipore Aust. Pty. Ltd), as described by Di Stefano (*28*).

Hydrolysis of Grape Glycosides by Yeast during Alcoholic Fermentation (Experiment 3). The four main monoterpene alcohols (linalool, geraniol, nerol, and α-terpineol) were determined as described by Pedersen et al. (*29*). For the quantification of all other volatile compounds, a 25 mL sample was diluted 1:1 with water, spiked with 2-octanol in ethanol (125 μL of a 67 mg/L solution in ethanol) as internal standard, and extracted with 5 mL of dichloromethane. The emulsion was centrifuged (3500 rpm for 10 min) and the solvent recovered and dried with anhydrous Na_2SO_4 for GC-MS analyses.

For the analysis of residual glycosides at the end of alcoholic fermentation, a 25 mL sample was diluted 1:1 with water and loaded onto a Sep-Pak Plus C_{18} solid-phase extraction cartridge (Millipore Aust. Pty. Ltd) containing 900 mg of sorbent, previously activated with 10

Table 3. Volatile Compounds (Micrograms per Liter) Released by Nonproliferating *Saccharomyces* Cells during Incubation with Grape Glycosides

				S. cerevisiae AWRI 838		*S. cerevisiae* AWRI 1537		*S. bayanus* AWRI 1375	
	LRI[a]	ID[b]	control[c]	w/gly[d]	no gly	w/gly	no gly	w/gly	no gly
hexanol	1357	A	–[e]	27 b	–	23 b	–	36 a	–
linalool	1558	A	–	97 b	–	63 c	–	113 a	–
α-terpineol	1695	A	–	19 b	–	15 b	–	32 a	–
citronellol	1769	A	–	246 b	–	198 c	–	446 a	–
nerol	1796	A	–	236 b	–	194 c	–	358 a	–
geraniol	1850	A	–	71 c	–	87 b	–	242 a	–
benzyl alcohol	1865	A	–	–	–	–	–	142	–
β-phenylethanol	1896	A	–	4424 b	4295 b	3392 c	3140 c	4706 a	4481 a
3,7-dimethyl-1,5-octadiene-3,7-diol	1936	A	–	99 a	–	83 b	–	97 a	–
3,7-dimethyl-1,7-octadiene-3,6-diol	2195	A	–	41 b	–	51 a	–	41 b	–
trans-2,6-dimethyl-2,7-octadiene-2,7-diol	2277	B	–	187 b	–	121 c	–	244 a	–
8-acetoxylinalool	2362	B	–	–	–	–	–	53	–
3-hydroxy-β-damascone	2534	A	–	108 b	–	46 c	–	122 a	–
3-oxo-α-ionol	2640	A	–	269 b	–	243 b	–	292 a	–

[a] Linear retention index on a DB-Wax column. [b] A, identities confirmed by comparing mass spectra and LRI with those of pure reference standards available in the laboratory; B, identities tentatively assigned by comparing mass spectra and LRI with those available in literature or database. [c] Noninoculated sample containing glycosides. [d] w/gly, treatments with glycosides; no gly, treatments without glycosides. Means of duplicate treatments, each analyzed in duplicate. Within each treatment, different letters denote significant differences at $p < 0.05$. [e] –, not detected.

Table 4. Fermentation Rates (Grams of Sugars per Liter per Day) for the Different *Saccharomyces* Strains within Two Sugar Concentration Ranges[a]

	with glycosides			without glycosides		
sugar range (%)	*S. cerevisiae* AWRI 838	*S. cerevisiae* AWRI 1537	*S. bayanus* AWRI 1375	*S. cerevisiae* AWRI 838	*S. cerevisiae* AWRI 1537	*S. bayanus* AWRI 1375
5–50	8.9 a	9.5 a	8.2 b	14.2 a	14.7 a	9.4 b
0–99	12.2 a	10.8 a	7.5 b	13.9 a	14.0 a	7.8 b

[a] Means of triplicate treatments. Within each treatment, different letters denote significant differences between strains, at $p < 0.05$.

mL of methanol followed by 10 mL of water. Samples were extracted at ≈3 mL/min. Cartridges were rinsed with water, and the glycosides were recovered with methanol. Analysis of these glycoside extracts was carried out by GC-MS of their trifluoroacetylated derivatives (*30*). For this purpose, 1.6 mL of each methanol extract was transferred to a glass vial, dried at 40 °C under a stream of pure nitrogen, and redissolved in 50 μL of anhydrous pyridine (Sigma-Aldrich). To this were added internal standard (30 μL of 200 mg/L phenyl-β-D-glucopyranoside in anhydrous pyridine, Sigma-Aldrich) and 40 μL of derivatization reagent *N*-methylbis(trifluoroacetamide) (Sigma-Aldrich). The reaction mixture vial was capped, heated in a heater block for 20 min at 50 °C, and then cooled, diluted with pyridine to ≈200 μL, and analyzed by GC-MS.

GC-MS Conditions. Conditions for GC-MS analysis of the four monoterpene alcohols, linalool, geraniol, nerol, and α-terpineol, in samples obtained from experiment 3 have been previously described (*29*). The remaining volatile compounds in samples obtained from experiments 2 and 3 were analyzed with a Hewlett-Packard (HP) 6890 gas chromatograph coupled to a HP5973 mass spectrometer operating in scan mode and a HP 6890 series liquid injector operating in fast liquid injection mode with a 10 μL syringe (SGE, Ringwood, Australia). The gas chromatograph was fitted with a Zebron ZB-Wax column (30 m × 0.25 mm × 0.25 μm film thickness; Phenomenex, Torrance, CA). The oven was initially set at 50 °C, held at this temperature for 1 min, then increased at a rate of 4 °C/min until 220 °C, and held at this temperature for 10 min. The injector was held at 220 °C and the transfer line at 250 °C. Carrier gas was helium, with a column flow rate of 1.2 mL/min. Two microliters of extract was injected in pulsed splitless mode, with a split ratio of 42:1 and the splitter opening after 36 s. Positive electron impact spectra were recorded at 70 eV in the range *m*/*z* 50−350. Volatile compounds were identified by comparison of experimental mass spectra with those of NBS75000, Wiley 275, and The Australian Wine Research Institute spectral libraries and by co-injection with pure reference standards, where available, and quantified by normalization of their chromatographic peaks areas with those of the internal standard.

Residual glycosides at the end of fermentation (experiment 3) were analyzed as trifluoroacetylated derivatives, with the same gas chromatograph used for volatiles analysis but fitted with a DB-5 MS column (30 m × 0.25 mm × 0.25 m film thickness; J&W Scientific, Folsom, CA). The oven was initially set at 125 °C, held at this temperature for 2 min, then increased at a rate of 3 °C/min until 290 °C, and held at this temperature for 30 min. The injector was held at 220 °C and the transfer line at 250 °C. Injection conditions were the same as previously described for volatile analysis. Carrier gas was helium, with a column flow rate of 1.2 mL/min. Positive electron impact spectra were recorded at 70 eV in the range *m*/*z* 50−600. Phenyl glucoside was used as internal standard for quantification. Tentative identification of glycosides was elucidated using NBS75000, Wiley 275, and The Australian Wine Research Institute spectral libraries and by comparing experimental spectra of individual compounds with those available in the literature. Specifically, identification of glycosides of monoterepene alcohols, benzyl alcohol, and 2-phenylethanol was based on the GC data and mass spectra reported by Voirin et al. (*30*−*32*). Identification of the two furan linalyloxide β-D-glucopyranoside isomers was based on GC data and mass spectra reported in refs *33* and *34*.

Statistical Analysis. Analysis of variance and least significant difference (LSD) test were used to interpret the differences in means at the 95% confidence level. The data were processed using Statgraphics 5.0 Plus-PC (Manugistics, Inc.).

RESULTS

Study of the Hydrolysis of Glycosides by Nonproliferating Yeast Cells (Experiments 1 and 2). The ability of the three *Saccharomyces* yeast strains to hydrolyze glycosides was first investigated under conditions optimal for the glycosidase

Table 5. Volatile Compounds (Micrograms per Liter) Released by Different *Saccharomyces* Strains during Fermentation Determined at the End of Alcoholic Fermentation

				S. cerevisiae AWRI 838		*S. cerevisiae* AWRI 1537		*S. bayanus* AWRI 1375	
	LRI[a]	ID[b]	control H+ [c]	w/gly[d]	no gly	w/gly	no gly	w/gly	no gly
hexanol	1357	A	39 a	42 a	–[e]	32 a	–	33a	–
trans-furanic linalool oxide	1443	A	–	22 a	–	23 a	–	21 a	–
linalool	1558	A	100 c,x	248 a	1 y	249 b	0.5 z	239 a	0.5 z
hotrienol	1612	B	6 c	86 a	–	60 b	–	53 b	–
α-terpineol	1695	A	53 b,x	177 a	0.5 y	182 a	0.5 y	169 a	0.5 y
trans-pyranic linalool oxide	1740	B	15 b	25 a	–	25 a	–	13 b	–
citronellol	1769	A	–	62 a	–	57 a	–	47 b	–
nerol	1796	A	5 b	16 a	–	16 a	–	18 a	–
geraniol	1850	A	21 a,x	8 b	0.5 y	10 b	0.5 y	21 a	–
β-phenylethanol	1896	A	1128 c,z	3912 b	3277 x	4167 b	2662 y	5039 a	3445 x
3,7-dimethyl-1,5-octadiene-3,7-diol	1936	A	630 a	250 c	–	320 b	–	190 d	–
3,7-dimethyl-1-octene-3,7-diol	2020	B	31 c	110 b	–	123 a	–	87 c	–
3,7-dimethyl-1,7-octadiene-3,6-diol	2195	A	95 b	95 b	–	219 a	–	99 b	–
trans-2,6-dimethyl-2,7-octadiene-1,6-diol	2277	B	–	98 b	–	108 a	–	57 c	–
cis-2,6-dimethyl-2,7-octadiene-1,6-diol	2326	B	–	84 b	–	97 a	–	81 b	–
4-vinylguaiacol	2175	A	–	2690 a	–	108 c	–	1108 b	–
4-vinylphenol	2372	A	–	424 a	–	99 c	–	205 b	–
3-oxo-α-ionol	2640	A	–	36 a	–	27 a	–	19 b	–

[a] Linear retention index on DB-Wax column. [b] A, identities confirmed by comparing mass spectra and LRI with those of pure reference standards available in the laboratory; B, identities tentatively assigned by comparing mass spectra and LRI with those available in literature or database. [c] Noninoculated sample containing glycosides, kept in the same conditions as fermented samples during fermentation. The values indicate the release of volatile compounds due to acid-catalyzed hydrolysis of glycosides. [d] w/gly, treatments with glycosides; no gly, treatments without glycosides. Means of triplicate treatments, each analyzed in duplicate. Within each treatment, different letters denote significant differences at $p < 0.05$. [e] –, not detected.

enzyme activities but less favorable for acid-catalyzed hydrolysis. Assays made with model substrates (expt 1) showed that the yeasts used for this study possessed different glycosidase activities responsible for the hydrolysis of the various *p*-nitrophenyl-glycosides typically used to study the relevant glycosidase activities in wine microorganisms (**Table 2**). β-Glucosidase activity was generally higher than that of α-rhamnosidase or α-arabinosidase and reached a peak after 48 h of incubation. Under the conditions of this study, strain AWRI 1375 exhibited higher β-glucosidase activity, whereas no difference was observed between strains regarding the other enzymatic activities. Although at that stage of the study it was not clear whether these activities were also effective on grape-derived glycosides and/or under typical winemaking conditions, subsequent experiments involving incubation of yeast cells at 30 °C for 72 h at pH 5.0 in the presence of White Frontignac glycosides (expt 2) showed a significant increase in the concentration of several volatile compounds known to be liberated from glycoside hydrolysis, such as monoterpene alcohols, terpene diols, terpene oxides, and the norisoprenoids 3-hydroxy-β-damascone and 3-oxo-α-ionol (**Table 3**). This confirmed the potential of the yeasts to release volatile compounds from the precursors contained in the White Frontignac extract, indicating their suitability for the fermentation study. Also under these experimental conditions, higher concentrations of volatile compounds were generally observed with the *S. bayanus* strain, which was also characterized by the presence of benzyl alcohol and 8-acetoxylinalool, which were not observed in samples obtained with the two *S. cerevisiae* strains. Differences were also observed between the two *S. cerevisiae* strains, with the AWRI 838 strain releasing higher concentrations of 3-hydroxy-β-damascone, *trans*-2,6-dimethyl-2,7-octadiene-1,6-diol, and monoterpene alcohols except α-terpineol and geraniol. Interestingly, the final concentration of citronellol, a compound potentially deriving from the yeast-driven transformation of geraniol, was also higher in the samples obtained with the AWRI 838 strain.

Study of the Hydrolysis of Grape Glycosides by Yeast during Alcoholic Fermentation (Experiment 3). For all yeast strains, the rate of daily sugar consumption was higher when fermentations were carried out without the glycosidic extract (**Table 4**). Mean days required for the completion of alcoholic fermentation ranged from 16 for *S. cerevisiae* AWRI 838 to 26 for *S. bayanus* AWRI 1375 in fermentations with glycosides, whereas in treatments without glycosides 14 days was needed for *S. cerevisiae* AWRI 838 and *S. cerevisiae* AWRI 1537 and 25 days for *S. bayanus* AWRI 1375. Differences between fermentations with and without glycosides were greater when the proliferation phase (yeast growth) without the lag phase (5−50% of sugar consumption) was considered, particularly for the two *S. cerevisiae* yeasts. The nature of this inhibitory effect was not investigated. It should be noted that the CDGJ medium was not optimized for *S. bayanus* yeast, which may account for the lower fermentation rate when compared to that of *S. cerevisiae*.

The hydrolysis of glycosidically bound volatile compounds during fermentation with the three *Saccharomyces* yeast strains was investigated by GC-MS analysis of volatile compounds and precursors extracted from samples at the end of fermentation.

Tables 5 and **6** report the concentration of free volatile compounds and glycosidic precursors, respectively, measured in the model wines at the end of alcoholic fermentation. A total of 19 volatiles potentially resulting from the hydrolysis of glycosides were identified in samples obtained with all three yeast strains (**Table 5**). Derivatization with *N*-methylbis-(trifluoroacetamide) of the residual glycosides and subsequent GC-MS analysis allowed the identification of β-D-glucopyranosides (*10*), rhamnopyranosyl-β-D-glucopyranosides (*4*), ara-

Table 6. Residual Glycosides (Micrograms per Liter) at the End of Fermentation with Different *Saccharomyces* Yeast Strains

	LRI[a]	control[b]	control H+ [c]	*S. cerevisiae* AWRI 838	*S. cerevisiae* AWRI 1537	*S. bayanus* AWRI 1375
β-D-glucopyranosides						
benzyl β-D-glucopyranoside	1751	204 a	198 a	119 b	126 b	123 b
furan linalyloxide β-D-glucopyranoside (*E*/*Z*)	1764	114 a	98 b	88 c	90 b	95 b
furan linalyloxide β-D-glucopyranoside (*Z*/*E*)	1777	268 a	240 b	218 c	248 b	236 b
(*S*)-linalyl β-D-glucopyranoside	1799	112 a	83 b	25 c	26 c	26 c
β-D-glucopyranoside of unidentified terpene diol[d]	1815	110 a	104 a	74 b	82 b	86 b
β-phenylethyl β-D-glucopyranoside	1840	95 a	94 a	71 b	74 b	69 b
neryl β-D-glucopyranoside	1853	213 a	193 a	122 b	136 b	128 b
β-D-glucopyranoside of unidentified terpene diol[e]	1861	117 a	114 a	85 b	87 b	91 b
geranyl β-D-glucopyranoside	1881	210 a	185 b	121 c	138 c	129 c
α-terpineyl β-D-glucopyranoside	1918	121 a	85 b	52 c	48 c	46 c
total		*1563* a	*1392* b	*975* c	*1056* c	*1029* c
6-*O*-(α-L-rhamonopyranosyl)-β-D-glucopyranosides						
benzyl 6-*O*-(α-L-rhamonopyranosyl)-β-D-glucopyranoside	2095	179 a	157 b	118	122 c	165 b
(*S*)-linalyl 6-*O*-(α-L-rhamonopyranosyl)-β-D-glucopyranoside	2109	236 a	223 a	93 b	96 b	87 b
geranyl 6-*O*-(α-L-rhamonopyranosyl)-β-D-glucopyranoside	2205	188 a	166 b	77 c	73 c	74 c
6-*O*-(α-L-rhamonopyranosyl)-β-D-glucopyranoside of unidentified terpene[f]	2187	136 a	124 a	92 b	74 c	74 c
total		*739* a	*670* b	*381* c	*366* c	*400* c
6-*O*-(α-L-arabinofuranosyl)-β-D-glucopyranosides						
benzyl 6-*O*-(α-L-arabinofuranosyl)-β-D-glucopyranoside	2150	294 a	251 a	198 b	205 b	206 b
geranyl 6-*O*-(α-L-arabinofuranosyl)-β-D-glucopyranoside	2261	142 a	122 b	93 c	91 c	90 c
total		*437* a	*373* b	*291* c	*296* c	*295* c
6-*O*-(β-D-apiofuranosyl)-β-D-glucopyranosides						
furanlinalyloxide 6-*O*-(β-D-apiofuranosyl)-β-D-glucopyranoside + β-phenylethyl 6-*O*-(β-D-apiofuranosyl)-β-D-glucopyranoside	2167	786 a	778 a	763 a	750 a	765 a
neryl 6-*O*-(β-D-apiofuranosyl)-β-D-glucopyranoside	2213	199 a	156 b	144 b	160 b	139 b
total		*985* a	*933* b	*906* b	*910* b	*905* b

[a] Linear retention indices on DB-5 column. [b] Noninoculated sample containing glycosides, at the beginning of experiment. [c] Noninoculated sample containing glycosides, kept in the same conditions as fermented samples during fermentation; the values account for the decrease of glycosides due to acid-catalyzed hydrolysis. Means of triplicate treatments, each analyzed in duplicate. Different letters denote significant differences at $p < 0.05$. [d–f] Characteristic fragment ions [*m/z* (relative intensity)]: [d] Sugar moiety, 319 (*100*), 177 (8.3), 205 (7.4), 193, (*7*), 265 (2.3); aglycon moiety, 71 (*100*), 69 (*85*), 93 (*68*), 81 (*67*), 109 (*45*), 107 (*42*), 135 (*20*). [e] Sugar moiety, 319 (*100*), 217 (*19*), 193 (*15*), 177 (*11*), 281 (3.4), 265 (3.1), 504 (2.2); aglycon moiety, 69 (*100*), 93 (*95*), 81 (*66*), 109 (*32*), 111 (*22*), 135 (*20*), 119 (*20*), 153 (*19*). [f] Sugar moiety, 207 (*15*), 319 (*5*), 193 (1.6), 265 (1.2), 278 (0.9); aglycon moiety, 69 (*100*), 68 (*28*), 81 (*16*), 97 (*7*).

binofuranosyl-β-D-glucopyranosides (*2*), and apiofuranosyl-β-D-glucopyranosides (*3*) (**Table 6**).

In all samples there was a significant increase in the concentration of several volatile compounds, including monoterpene alcohols, terpene diols and oxides, and norisoprenoids. By comparing the data of the fermented samples containing glycosides with those of the unfermented reference samples, it was clear that fermentation played a major role in determining the composition of the pool of varietal volatile compounds of the model wines. The increase of volatiles due to acid-catalyzed hydrolysis of glycosides was lower than that associated with alcoholic fermentation, and several volatiles, such as *trans*-furanic linalool oxide, citronellol, *cis*- and *trans*-2,6-dimethyl-2,7-octadiene-1,6-diol, 3,7-dimethyl-1,7-octanediol, 4-vinylphenol, 4-vinylguaiacol, and 3-oxo-α-ionol, were observed only in fermented samples containing glycosides. Moreover, very small concentrations (between 0.5 and 1 μg/L) of linalool, α-terpineol, and geraniol were detected in fermented samples not containing glycosides. The only compound for which a decrease was observed in all samples during fermentation was 3,7-dimethyl-1,5-octadiene-3,7-diol, most likely due to the high hydrophilic character of this compound and the consequent poor extraction in the presence of ethanol. Geraniol also decreased during fermentation with the two *S. cerevisiae* strains.

With regard to differences between yeast strains, the *S. cerevisiae* yeasts were generally characterized by higher concentrations of volatiles released from precursors than the *S. bayanus* strain, contradictory to the trend observed in the preliminary "optimized" assay, by which higher concentrations of volatiles were observed for *S. bayanus*. *S. cerevisiae* AWRI 1537 released the highest concentrations of terpene diols and the lowest vinylphenols.

As for glycosides, fermentation caused a decrease in the concentration of these constituents ranging from 22% for *S. bayanus* AWRI 1375 to 28% for *S. cerevisiae* AWRI 838, whereas in nonfermented samples the decrease observed over the fermentation time was ≈5%. β-D-Glucosides, α-L-arabinofuranosyl-β-D-glucosides, and α-L-rhamnopyranosyl-β-D-glucosides were generally hydrolyzed to a higher degree than β-D-apiofuranosyl-β-D-glucosides by yeast. Differences between yeast strains regarding their ability to hydrolyze specific glycosides were, however, minor.

DISCUSSION

The role of yeast in the release of aroma compounds from precursors during winemaking has been widely investigated. Although it is generally accepted that *S. cerevisiae* yeast can liberate powerful sulfur odorants through enzymatic degradation of *S*-cysteinic precursors (*35*, *36*), the possible action of these micro-organisms toward glycoconjugated precursors has not been completely clarified.

Studies with model glycosides appear to indicate that enzymatic extracts of *S. cerevisiae* possess low glycosidase

activities, especially when compared to other non-*Saccharomyces* wine yeasts (*37*−*39*). It was also found that *S. cerevisiae* glycosidases possess low activity at wine pH and are strongly inhibited by high sugar and ethanol concentrations (*17*). Nevertheless, Darriet et al. (*12*) found that glycoside hydrolytic enzymes of *S. cerevisiae* are mainly contained in the periplasmic space of cells and that their activity is glucose independent. Subsequently, a detailed investigation on the β-glucosidase of different wine yeasts suggested that this enzyme is not inhibited by high glucose concentrations and can, therefore, be effective in the early stages of fermentation (*13*). The recent identification of strains of *S. cerevisiae* exhibiting high β-glucosidase activity at low pH and high sugar and ethanol concentrations has renewed interest in the actual activity of *S. cerevisiae* β-glucosidase during winemaking (*40*, *41*).

In the present study, preliminary experiments showed the existence of detectable glycosidase activities in the three yeasts under investigation (expt 1). When nonproliferating cells were incubated at pH 5.0 and 30 °C in the presence of glycosides extracted from a White Frontignac grape juice (expt 2), a significant release of volatile compounds was observed. Under these conditions the intrinsic yeast glycosidases would be expected to show near-optimal activity, providing a measure of potential enzymatic activity toward grape-derived glycosides. This result suggests, therefore, that the three yeast strains tested had the potential to hydrolyze grape glycosides, consistent with previously published studies on *S. cerevisiae* (*14*, *15*).

When the same yeasts were used to ferment a CDGJ medium containing grape glycosidic precursors (expt 3), a high release of volatile compounds arising from their hydrolyzed precursors, such as monoterpene alcohols, terpene oxides and diols, and the C-13 norisoprenoid 3-oxo-α-ionol, was observed. For the majority of these compounds, this increase due to yeast-driven hydrolysis was considerably larger than that due to acid-catalyzed hydrolysis of precursors. The very low concentrations of monoterpene alcohols in fermented samples not containing glycosides, besides confirming the recent findings of Carrau et al. (*42*) regarding the ex novo synthesis of monoterpenes by *S. cerevisiae*, indicates that, under our experimental conditions, the contribution of this pathway to the final terpene composition of wines was small when compared to that of glycosides hydrolysis. Moreover, the data in **Table 6** show that the decline of glycosides during fermentation was the result not only of the hydrolysis of β-D-glucosides but also of complex disaccharide glycosides. This implies that the yeast strains studied have enzymatic activities able to hydrolyze the various glycoconjugates commonly present in grape juice and that these activities were effective under simulated winemaking conditions. All together, these results provide evidence that yeast have the potential to hydrolyze glycosidic aroma precursor during fermentation, most likely through the expression of glycosidase activities that are effective under winemaking conditions.

Noteworthy among the β-D-glucosides of the four terpene alcohols, linalool, geraniol, nerol, and α-terpineol, the highest decrease associated with yeast fermentation was observed for linalool (up to 70%), whereas in the case of the three other terpenols the extent of hydrolysis due to yeast enzyme activity did not exceed 40%. This is interesting, as previously published characterization of β-glucosidase enzymes of enological interest, particularly those from the filamentous fungi *Aspergillus niger*, have shown that hydrolysis of glucosides of primary terpene alcohols, such as geraniol and nerol, usually takes place more easily than in the case of glucosides of tertiary terpene alcohols (linalool and α-terpineol), due to lower steric hindrance (*43*). A similar behavior was observed for the β-glucosidase extracted from grapes (*44*) and from *Candida molischiana* and *Candida wickerhamii* yeasts (*45*). It is likely that the β-glucosidase of *Saccharomyces* yeasts possesses a different pattern of substrate specificity than other microrganisms. Nonetheless, in a dynamic system such as fermenting CDGJ medium and in the presence of a highly complex pool of glycosides such as that found in grapes and used for this study, understanding the factors responsible for the behavior of specific glucosides during fermentation could be difficult. The concomitant hydrolysis of complex disaccharide glycosides associated with enzymatic activities different from β-glucosidase (e.g., α-arabinosidase, α-rhamonosidase, β-apiosidase) is expected to result in the release of glucosides, which can in part counterbalance the hydrolysis of the latter due to β-glucosidase. Moreover, Gil et al. (*46*) have shown that induced overproduction of an endogenous exoglucanase in a *S. cerevisiae* strain led to an increase in the release of glycoside-related volatile compounds in wine, suggesting the involvement of other enzymatic activities in the hydrolysis of glycosides by yeast, a hypothesis that is worthy of further investigation.

As for disaccharide glycosides, the higher decrease of α-L-arabinofuranosyl-β-D-glucopyranosides and α-L-rhamnopyranosyl-β-D-glucopyranosides compared to β-D-apiosyl-β-D-glucopyranosides indicates that production and/or activity of enzymes specific for apioside substrates might be limited in *Saccharomyces* yeast, at least in the three strains tested, during fermentation.

Whereas expt 2, which assayed intrinsic glycoside hydrolytic activities under optimal conditions, showed that *S. bayanus* AWRI 1375 exhibited a higher ability to release volatile compounds from glycosidic precursors compared to *S. cerevisiae* AWRI 838 and AWRI 1537, differences among the three yeast strains were generally less significant when the same yeast strains were used to conduct alcoholic fermentation in chemically defined grape juice medium with added grape glycosides (expt 3), either in the final concentration of volatiles released or in the residual glycosides at the end of alcoholic fermentation. Because, due to the lower rate of sugar consumption, the duration of fermentation with *S. bayanus* AWRI 1375 in the presence of glycosides was ≈10 days longer than with the two *S. cerevisiae* strains, the time of contact between yeast cells and glycosides appeared not to influence the extent of substrate hydrolysis and the consequent release of volatile compounds during fermentation. It has been suggested that the glucosidase activity of wine yeasts is restricted to the early stages of fermentation due to ethanol inhibition (*13*). This might explain the behavior observed in this study regarding the weak influence of the enzyme−substrate contact time, considering that differences in fermentation rates between the three yeast strains were minor in the proliferation phase (**Table 4**). Alternatively, it is possible that the enzyme-catalyzed reactions, including glycoside hydrolysis and reduction of geraniol to citronellol, simply reached equilibrium early during fermentation, so that prolonged contact between enzymes and substrate did not result in enhanced hydrolysis.

For all three yeast strains, the pool of volatiles released during alcoholic fermentation was characterized by a large proportion of terpenoid compounds, consistent with a Muscat variety related grape juice glycosidic extract (*5*). Among the monoterpene alcohols, linalool was the volatile with the highest concentration at the end of fermentation. This trend was consistent with the high decrease of bound linalool observed, although minor amounts of linalool can also originate from enzymatic trans-

formation of geraniol promoted by yeast (*47*, *48*). Among the other volatile compounds, *trans*-furanic linalool oxide, epoxylinalool, hotrienol, various terpene diols, and 3-oxo-α-ionol have been previously identified among volatiles released by enzymatic hydrolysis of glycosides with commercial enzyme preparations (*5*) and were observed in a preliminary characterization of the volatile composition of the enzyme hydrolysates of the White Frontignac glycosidic extract used in this study (data not shown). However, the observation that *trans*-furanic and pyranic linalool oxides and hotrienol were not detected during the experiment with nonproliferating yeast cells might indicate that they are partially formed through acid-catalyzed hydrolysis of either glycosidic precursor or aglycons released by yeast glycosidases. Mateo et al. (*49*) reported that heating at pH 3.0 of the volatile fraction obtained after enzymatic hydrolysis of Muscat glycosides resulted in an increase in the concentration of furanic linalool oxides and hotrienol. Structural rearrangements of the liberated aglycons could be important from a sensory point of view, as aglycons with high odor thresholds can give, at wine pH, other compounds with lower thresholds values. For example, 3,7-dimethyl-1-octene-3,7-diol has been shown to form linalool in mild acidic conditions (*50*), whereas (*E*)-2,6-dimethyl-2,7-octene-1,6-diol can give 3,9-epoxy-*p*-menth-1-ene (*51*), recently reported among the grape-derived potential odorants of Muscadet wine (*52*). Similarly, 3,7-dimethyl-1,5-octadiene-3,7-diol is known to generate hotrienol at wine pH (*50*).

The occurrence of citronellol in samples obtained from both experiments 2 and 3 was also interesting (**Tables 3** and **5**) and serves to illustrate the complexity of aroma compound formation during fermentation. During fermentation, citronellol is known to be formed through enzymatic isomerization of geraniol and, to a lower extent, nerol (*47*, *48*, *53*). However, the final concentrations of citronellol detected in this study were always higher than the concentrations of geraniol and nerol present in the noninoculated reference samples, even when the contribution of the acid-catalyzed hydrolysis of precursors during fermentation (**Table 5**) was taken into consideration. It is likely, therefore, that citronellol is formed by enzymatic transformation of the geraniol arising from the yeast-driven hydrolysis of glycosides, a hypothesis consistent with the decrease of free geraniol during fermentation, in spite of the decrease of its precursors.

Among the benzene derivatives observed, two vinyl phenols (4-vinylphenol and 4-vinylguaiacol) were detected in samples from expt 2. Glycosidic precursors of these compounds were indirectly observed during a preliminary characterization of the extract through GC-MS analysis of the enzyme hydrolysates (data not shown). However, the GC-MS conditions adopted for the analysis of glycosides did not allow for the identification and quantification of these precursors. This does not permit any conclusion about the origin of the two vinyl phenols to be drawn, as these compounds can be also formed by yeast through enzymatic decarboxylation of ferulic and *p*-coumaric acid (*54*). The presence of these precursors was determined by HPLC analysis of the glycosidic extract (data not shown).

In conclusion, this study has provided further evidence to confirm the highly significant contribution of *Saccharomyces* yeast glycoside hydrolytic activities to the composition of the pool of grape-derived volatile compounds of wine. In a chemically defined grape juice-like medium containing low concentrations of free volatile compounds but a high concentration of Frontignac/Muscat glycosidic precursors, fermentation mediated by *Saccharomyces* species promoted a major increase in the concentration of grape-derived volatiles, due in a large part to the enzymatic hydrolysis of glycosides. Under the fermentation conditions of this study, differences between yeast strains with respect to their ability to release volatile compounds from glycosides were minor, although the number of strains tested was too small to draw a general conclusion regarding the possible existence of yeast strains with enhanced hydrolytic activity. The extent of the hydrolysis of different glycosides appears to be dependent on the chemical structures of both the aglycon and the sugar moieties of glycosides. Glucosides of linalool were more reactive than those of other monoterpene alcohols, whereas β-D-apiosyl-β-D-glucopyranosides were less reactive than other disaccharide glycosides. Considering the relationship between the hydrolysis of glycoconjugated precursors and the expression of wine varietal aroma characteristics, these preliminary results in chemically defined grape juice indicate the need for further detailed investigations on the factors influencing the behavior of glycosides during alcoholic fermentation of real grape juice, also in light of the findings of Günata et al. (*18*, *19*), who reported that alcoholic fermentation had little effect on terpene glycosidic precursors during the processing of Muscat juice.

Among the volatile compounds released by yeast during the present study, monoterpene alcohols, particularly geraniol, linalool, and citronellol, have low sensory thresholds, suggesting that their release from odorless precursors can play an important role in the development of wine varietal flavor during winemaking. For example, linalool was released in concentrations that were up to 10 times higher than its odor threshold (15 μg/L; *55*). In this sense, however, the findings of this study are merely indicative, as the composition of the pool of glycosidically bound volatile compounds can vary significantly with grape variety, vintage, and geographical region (*1*−*4*, *56*). Different cases should therefore be analyzed individually, also considering the substrate specificity observed in this study for the glycosidases of *Saccharomyces* yeasts. Nevertheless, the hydrolysis of a large array of different substrates observed here suggests that fermentation can actively contribute to the liberation of glycosidically bound volatiles of different origins.

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LITERATURE CITED

(1) Sefton, M. A.; Francis, I. L.; Williams, P. J. The volatile composition of Chardonnay juices. A study by flavor precursors analysis. *Am. J. Enol. Vitic.* **1993**, *44*, 359−370.

(2) Wirth, J.; Guo, W.; Baumes, R.; Günata, Z. Volatile compounds released by enzymatic hydrolysis of leaves and grape berries from *Vitis vinifera* Muscat of Alexandria and Shiraz cultivars. *J. Agric. Food Chem.* **2001**, *49*, 2917−2923.

(3) Sefton, M. A. Hydrolytically-released volatile secondary metabolites from a juice sample of *Vitis vinifera* cvs Merlot and Cabernet Sauvignon. *Aust. J. Grape Wine Res.* **1998**, *4*, 30−38.

(4) Francis, I. L.; Kassara, S.; Noble, A. C.; Williams, P. J. The contribution of glycoside precursors to Cabernet Sauvignon and Merlot aroma: sensory and compositional studies. In *Chemistry of Wine Flavour*; Waterhouse, A. L., Ebeler, S. E., Eds.; American Chemical Society: Washington, DC, 1999; pp 13−30.

(5) Strauss, C. R.; Wilson, B.; Gooley, P. R.; Williams, P. J. Role of monoterpenes in grape and wine flavor. In *Biogeneration of Aromas*, a symposium sponsored by the Division of Agricultural and Food Chemistry at the 190th National Meeting of the American Chemical Society, Sept 8−13, 1985, Chicago, IL; ACS Symposium Series 317; Parliment, T. H., Croteau, R., Eds.; American Chemical Society: Washington, DC, 1986; pp 222−242.

(6) Abbott, N. A.; Coombe, B. G.; Williams, P. J. The contribution of hydrolyzed flavour precursors to quality differences in Shiraz juice and wines: an investigation by sensory descriptive analysis. *Am. J. Enol. Vitic.* **1991**, *42*, 167−174.

(7) Francis, I. L.; Sefton, M. A.; Williams, P. J. Sensory descriptive analysis of the aroma of hydrolysed flavour precursor fractions from Semillon, Chardonnay, and Sauvignon blanc grape juices. *J. Sci. Food Agric.* **1992**, *59*, 511−520.

(8) Gunata, Y. Z.; Bitteur, S.; Brillouet, J.-M.; Bayonove, C. L.; Cordonnier, R. E. Sequential enzymic hydrolysis of potentially aromatic glycosides from grapes. *Carbohydr. Res.* **1988**, *184*, 139−149.

(9) Winterhalter, P.; Sefton, M. A.; Williams, P. J. Volatile C_{13} norisoprenoids in Riesling wine are generated from multiple precursors. *Am. J. Enol. Vitic.* **1990**, *41*, 277−283.

(10) Cox, A.; Skouorumounis, G. K.; Elsey, G. M.; Perkins, M. V.; Sefton, M. A. Generation of (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene from C_{13}-norisoprenoid precursors. *J. Agric. Food Chem.* **2005**, *53*, 6777−6783.

(11) Sarry, J.-E.; Günata, Y. Z. Plant and microbial glycoside hydrolases: volatiles release from glycosidc aroma precursors. *Food Chem.* **2004**, *87*, 509−521.

(12) Darriet, P.; Boidron, J. N.; Dubourdieu, D. L'hydrolyse des hétérosides terpéniques du Muscat à petits grains par les enzymes périplasmatiques de *Saccharomyces cerevisiae*. *Connaiss. Vigne Vin* **1988**, *22*, 89−195.

(13) Mateo, J. J.; Di Stefano, R. Description of the β-glucosidase activity of wine yeasts. *Food Microbiol.* **1997**, *14*, 583−591.

(14) Fernàndez-Gonzàlez, M.; Di Stefano, R.; Briones, A. Hydrolysis and transformation of terpene glycosides from muscat must by different yeast species. *Food Microbiol.* **2003**, *20*, 35−41.

(15) Fernàndez-Gonzàlez, M.; Di Stefano, R. Fractionation of glycoside aroma precursors in neutral grapes. Hydrolysis and conversion by *Saccharomyces cerevisiae*. *Lebensm.-Wiss. Technol.* **2004**, *37*, 467−473.

(16) Delfini, C.; Cocito, C.; Bonino, M.; Schellino, R.; Gaia, P.; Baiocchi, C. Definitive evidence for the actual contribution of yeast in the transformation of neutral precursors of grape aromas. *J. Agric. Food Chem.* **2001**, *49*, 5397−5408.

(17) Delcroix, A.; Günata, Z.; Sapis, J. C.; Salmon, J. M.; Bayonove, C. Glycosidase activities of three enological yeast strains during winemaking: effect on terpenols content of Muscat wine. *Am. J. Enol. Vitic.* **1994**, *45*, 291−296.

(18) Gunata, Y. Z.; Bayonove, C.; Baumes, R.; Cordonnier, R. Stability of free and bound fractions of some aroma compounds of grapes *cv.* Muscat during wine processing: preliminary results. *Am. J. Enol. Vitic.* **1986**, *37*, 112−114.

(19) Gunata, Y. Z.; Dugelay, I.; Sapis, J.-C.; Baumes, R.; Bayonove, C. Action des glycosidases exogènes au cours de la vinification: libération de l'arôme a partir de précurseurs glycosidiques. *J. Int. Sci. Vigne Vin* **1990**, *24*, 133−144.

(20) Williams, P. J.; Francis, I. L.; Black, S. Changes in concentration of juice and must glycosides, including flavour precursors, during primary fermentation. In *Proceedings of the IVth International Symposium on Cool Climate Viticulture and Enology*; Henick-Kling, T., Wolf, T., Eds.; Communications Services, New York State Agricultural Experiment Station: Rochester, NY, 1996; pp VI-5−VI-9.

(21) Zoecklein, B. W.; Marcy, J. E.; Jasinsky, Y. Effect of fermentation, storage *sur lie* or post-fermentation thermal processing on White Riesling (*Vitis vinifera* L.) glycoconjugates. *Am. J. Enol. Vitic.* **1997**, *48*, 397−402.

(22) Zoecklein, B. W.; Marcy, J. E.; Williams, J. M.; Jasinsky, Y. Effect of native strain of *Saccharomyces cerevisiae* on glycosyl-glucose, potential volatile terpenes, and selected aglycones of White Riesling (*Vitis vinifera* L.) wines. *J. Food Compos. Anal.* **1997**, *10*, 55−65.

(23) Moio, L.; Ugliano, M.; Gambuti, A.; Genovese, A.; Piombino, P. Influence of different clarification treatments on the concentration of selected free varietal aroma compounds and glycoconjugates of Falanghina (*Vitis vinifera* L.) must and wine. *Am. J. Enol. Vitic.* **2004**, *51*, 7−12.

(24) Eglinton, J. M.; McWilliam, S. J.; Fogarty, M. W.; Francis, I. L.; Kwiatkowski, M. J.; Høj, P. B.; Henschke, P. A. The effect of *Saccharomyces bayanus*-mediated fermentation on the chemical composition and aroma profile of Chardonnay wine. *Aust. J. Grape Wine Res.* **2000**, *6*, 190−196.

(25) Gunata, Y. Z.; Bayonove, C.; Baumes, R. L.; Cordonnier, R. The aroma of grapes 1. Extraction and determination of free and glycosidically bound fractions of some grape aroma components. *J. Chromatogr.* **1985**, *331*, 83−90.

(26) Williams, P. J.; Cynkar, W.; Francis, I. L.; Gray, J. D.; Iland, P.; Coombe, B. G. Quantification of glycosides in grapes, juices and wine through a determination of the glycosyl-glucose (G-G). *J. Agric. Food Chem.* **1995**, *43*, 212−128.

(27) Henschke, P.; Jiranek, V. Yeasts−metabolism of nitrogen compounds. In *Wine Microbiology and Biotechnology*; Fleet, G. H., Ed.; Harwood Academic Publishers: Chur, Switzerland, 1993; pp 77−164.

(28) Di Stefano, R. Proposal for a method of sample preparation for the determination of free and glycoside terpenes of grapes and wines. *Bull. O. I. V.* **1991**, *721−722*, 219−223.

(29) Pedersen, D. S.; Capone, D. L.; Skouroumounis, G. K.; Pollnitz, A. P.; Sefton, M. A. Quantitative analysis of geraniol, nerol, linalool, and α-terpineol in wine. *Anal. Bioanal. Chem.* **2003**, *375*, 517−522.

(30) Voirin, S. G.; Baumes, R.; Gunata, Z. Y.; Bitteur, S. M.; Bayonove, C.; Tapiero, C. Analytical methods for monoterpene glycosides in grape and wine I. XAD-2 extraction and gas chromatographic-mass spectrometric determination of synthetic glycosides. *J. Chromatogr.* **1992**, *522*, 313−328.

(31) Voirin, S. G.; Baumes, R.; Bitteur, S. M.; Günata, Z. Y.; Bayonove, C. Novel monoterpene disaccharide glycosides in *Vitis vinifera* grapes. *J. Agric. Food. Chem.* **1990**, *38*, 1373−1378.

(32) Voirin, S. G.; Baumes, R.; Sapis, C. L.; Bayonove, C. Analytical methods for monoterpene glycosides in grape and wine II. Qualitative and quantitative determination of monoterpene glycosides in grape. *J. Chromatogr.* **1992**, *595*, 269−281.

(33) Wang, D.; Yoshimura, T.; Kubota, K.; Kobayashi, A. Analysis of glycosidically bound aroma precursors in tea leaves. 1. Qualitative and quantitative analyses of glycosides with aglycons as aroma compounds. *J. Agric. Food Chem.* **2000**, *48*, 5411−5418.

(34) Sakho, M.; Chassagne, D.; Coruzet, J. African mango glycosidically bound volatile compounds. *J. Agric. Food Chem.* **1997**, *45*, 883−888.

(35) Dubourdieu, D.; Tominaga, T.; Masneuf, I.; Peyrot des Gachons, C.; Murat, M. L. The role of yeasts in grape flavor development: the example of Sauvignon Blanc. *Proceedings of the ASEV 50th Anniversay Annual Meeting*, Seattle, WA, June 19−23, 2000; American Society for Enology and Viticulture: Davis, CA, 2000.

(36) Howell, K. S.; Klein, M.; Swiegers, J. H.; Hayasaka, Y.; Elsey, G. M.; Fleet, G. H.; Høj, P. B.; Pretorius, I. S.; de Barros Lopes, M. A. Genetic determinants of volatile-thiol release by *Saccharomyces cerevisiae* during wine fermentation. *Appl. Environ. Microbiol.* **2005**, *71*, 5420−5426.

(37) Rosi, I.; Vinnella, M.; Domizio, P. Characterization of β-glucosidase activity in yeast of enological origin. *J. Appl. Bacteriol.* **1994**, *77*, 519−527.

(38) Charoenchai, C.; Fleet, G. H.; Henschke, P. A.; Todd, B. E. N. Screening of non-*Saccharomyces* wine yeasts for the presence of extacellular hydrolytic enzymes. *Aust. J. Grape Wine Res.* **1997**, *3*, 2−8.

(39) McMahon, H.; Zoecklein, B. W.; Fugelsang, K.; Jasinsky, Y. Quantification of glycosidase activities in selected yeasts and lactic acid bacteria. *J. Ind. Microb. Biotechnol.* **1999**, *23*, 198−203.

(40) Spagna, G.; Barbagallo, R. N.; Palmeri, R.; Restuccia, C.; Giudici, P. Properties of endogenous β-glucosidase of a *Saccharomyces cerevisiae* strain isolated from Sicilian musts and wines. *Enzyme Microb. Technol.* **2002**, *31*, 1030−1035.

(41) Hernàndez, L. F.; Espinosa, J. C.; Fernàndez-Gonzàlez, M.; Briones, A. β-Glucosidase activity in a *Saccharomyces cerevisiae* wine strain. *Int. J. Food Microbiol.* **2003**, *80*, 171−176.

(42) Carrau, F. M.; Medina, K.; Boido, E.; Farina, L.; Gaggero, C.; Dellacassa, E.; Versini, G.; Henschke, P. A. De novo synthesis of monoterpenes by *Saccharomyces cerevisiae* wine yeasts, *FEMS Microbiol. Lett.* **2005**, *243*, 107−115.

(43) Günata, Y. Z.; Bayonove, C. L.; Tapiero, C.; Cordonnier, R. E. Hydrolysis of grape monoterpenyl β-D-glucosides by various β-glucosidases. *J. Agric. Food Chem.* **1990**, *38*, 1232−1236.

(44) Aryan, A. P.; Wilson, B.; Strauss, C.; Williams, P. J. The properties of glycosidases of *Vitis vinifera* and a comparison of their β-glucosidase activity with that of exogenous enzymes. An assessment of possible application in enology. *Am. J. Enol. Vitic.* **1987**, *38*, 182−188.

(45) Gunata, Y. Z.; Bayonove, C. L.; Cordonnier, R. E.; Arnaud, A.; Glazy, P. Hydrolysis of grape monoterpenyl glycosides by *Candida molischiana* and *Candida wickerhamii* β-glucosidases. *J. Sci. Food Agric.* **1990**, *50*, 499−506.

(46) Gil, J. V.; Manzanares, P.; Genovés, S.; Vallés, S.; Gonzalez-Candelas, L. Over-production of the major exoglucanase of *Saccharomyces cerevisiae* led to an increase in the aroma of wine. *Int. J. Food Microbiol.* **2005**, *103*, 57−68.

(47) Di Stefano, R.; Maggiorotto, G.; Giannotti, S. Trasformazioni di nerolo e geraniolo indotte dai lieviti. *Riv. Vitic. Enol.* **1992**, *45*, 43−49.

(48) Dugelay, I.; Gunata, Y. Z.; Sapis, J. C.; Baumes, R.; Bayonove, C. Etude de l'origine du citronellol dans les vins. *J. Int. Sci. Vigne Vin* **1992**, *26*, 177−184.

(49) Mateo, J. J.; Gentilini, N.; Huerta, T.; Jiménez, M.; Di Stefano, R. Fractionation of glycoside precursors of aroma in grapes and wines. *J. Chromatogr. A* **1997**, *778*, 219−224.

(50) Williams, P. J.; Strauss, C. R.; Wilson, B. Hydroxylated linalool derivatives as precursors of volatile monoterpenes of Muscat grapes. *J. Agric. Food Chem.* **1980**, *28*, 766−771.

(51) Strauss, C. R.; Wilson, B.; Williams, P. J. Novel monoterpene diols and diol glycosides in *Vitis vinifera* grapes. *J. Agric. Food Chem.* **1988**, *36*, 569−573.

(52) Schneider, R.; Razungles, A.; Augier, C.; Baumes, R. Monoterpenic and norisoprenoidic glycoconjugates of *Vitis vinifera* L. cv. Melon B. as precursors of odorants in Muscadet wine. *J. Chromatogr. A* **2001**, *936*, 145−157.

(53) Grammatica, P.; Mannitto, P.; Ranzi, B.; Del Bianco, A.; Francavilla, M. Stereospecific reduction of geraniol to (*R*)-(+)-citronellol by *Saccharomyces cerevisiae. Experientia* **1982**, *38*, 775−776.

(54) Chatonnet, P.; Dubourdieu, D.; Boidron, J. N.; Lavigne, V. Synthesis of volatile phenols by *S. cerevisiae* in wines. *J. Sci. Food Agric.* **1993**, *62*, 191−202.

(55) Guth, H. Quantitation and sensory studies of character impact odorants of different white wines varieties. *J. Agric. Food Chem.* **1997**, *45*, 3027−3032.

(56) López-Tamames, E.; Carro-Mariño, N.; Gunata, Y. Z.; Sapis, C.; Baumes, R.; Bayonove, C. Potential aroma in several varieties of Spanish grapes. *J. Agric. Food Chem.* **1997**, *45*, 1729−1735.

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