Content of Free Terpenic Compounds in Cells and Musts during Vinification with Three *Saccharomyces cerevisiae* Races

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Intracellular changes in terpenic compounds during alcoholic fermentations of musts from grapes of cv. Pedro Ximenez carried out by three *Saccharomyces cerevisiae* races, as well as during veil formation, were studied. The intracellular accumulation of geraniol, linalool, α-terpineol, (*E*)-nerolidol, and (*Z*)-nerolidol throughout the period studied may be the result of inhibited sterol biosynthesis from squalene by anaerobic conditions. This intracellular accumulation of intermediate terpenic precursors for squalene may favor cyclizations, isomerizations, and enzymatic conversions among terpenes and their excretion, increasing the contents of these compounds in the wines.

Keywords: *Wine; yeasts; terpenes*

INTRODUCTION

The presence and changes of terpenic compounds in wines have been the subject of much research in the past few years. β-Glucosidase enzymes have been found to occur in grapes (Williams et al., 1982b; Aryan et al., 1987; Gunata et al., 1988, 1989; Biron et al., 1988; Williams, 1992) and *Saccharomyces cerevisiae* yeasts (Dubordieu et al., 1988; Darriet et al., 1988a,b; Darriet, 1992). These enzymes have mainly been investigated in relation to the fermentation of musts of aromatic cultivars and are known to hydrolyze glycosidic combinations of terpenes, which occur in significant amounts in Muscat grape varieties. They are responsible for "revealing the hidden aroma of grapes".

Sterol synthetic pathways in yeasts start from acetyl-CoA, which yields squalene, a triterpene, via mevalonic acid and several terpenic compound intermediates such as geranyl pyrophosphate and farnesyl pyrophosphate (Parks, 1978). While this pathway proceeds anaerobically, the epoxidation of squalene and the demethylation and dehydrogenation of lanosterol—two essential steps in the formation of ergosterol—both require the presence of molecular oxygen. Therefore, oxygen availability decisively influences the synthesis of sterols and other terpenic compounds and also possibly affects the formation of intermediate products (Ratledge and Evans, 1989).

Yeasts may increase the content of free terpenic compounds in the must during fermentation. The experiments performed by Fagan et al. (1981) using *Saccharomyces fermentati*, a veil yeast growing over synthetic media and with ethanol as the only source of carbon, showed linalool, (*Z*)- and (*E*)-nerolidol, and (*E,E*)-farnesol to be produced, which suggests that some yeasts can alter the contents of such aroma compounds in wines.

In this work, we studied the relationship between the contents of some free monoterpenes and sesquiterpenes in yeast cells and musts subject to vinification for 134 days. Three *S. cerevisiae* varieties, a typically fermentative one and two "flor" veil forming ones, were used.

MATERIALS AND METHODS

Must and Fermentation Conditions. Must from *Vitis vinifera* cv. Pedro Ximenez was sterilized by filtration through a Supra EK filter (Seitz, Bad Kreuznach, Germany), adjusted to pH 3.2 with tartaric acid, and potassium metabisulfite was added to an SO_2 concentration of 75 mg/L. The initial reducing sugar concentration in the must was 231 g/L (AOAC, 1970).

Three batches of the must were inoculated with pure cultures of *S. cerevisiae* races *cerevisiae, bayanus*, and *capensis*, respectively (Kreger-Van Rij, 1984). The yeast races used are predominant in the Montilla-Moriles region (southern Spain) (Guijo et al., 1986). Race *cerevisiae* is a typically fermentative yeast, while races *bayanus* and *capensis* are flor film yeasts. The inocula were added in the proportions required to obtain a final concentration of 10^6 cells/mL in the musts. Total cell numbers was measured by counting in a Thoma chamber under a light microscope.

Fermentations were carried out in triplicate at 25 °C in presterilized 10 L stainless steel vessels. Samples were withdrawn for analysis after 0, 1, 2, 3, 10, 31, and 134 days of fermentation.

Analyses. The cells were removed from the musts by filtering through Millipore sieves of 1.2 μm, and *ca.* 10^{10} cells were suspended in 2–3 mL of 12% (v/v) ethanol. The yeasts were broken by stirring with an identical volume of glass beads of 0.5 mm diameter. The cell breaking was confirmed by microscopic observation. The extracts were centrifuged at 5000*g* for 5 min (Beckman, TJ-6 centrifuge), and the cell remains were washed with 50 mL of 12% ethanol. The supernatant fluid was then adjusted to pH 3.5; 2-octanol was added as an internal standard (484 μg/L) and extracted with Freon 11 in a continuous extractor for 24 h. Must and wine samples were also extracted with Freon 11 under the same conditions as the yeast extracts. Ethanol was quantified according to the Crowell and Ough (1979) method.

The terpenic compounds were determined by GLC (Perkin-Elmer, Sigma 3 Model) in an SP 1000 capillary column of 60 m × 0.32 mm i.d. (Supelco Inc.) after concentration of the freon extracts to 0.2 mL. Five microliters was injected into the chromatograph equipped with a flame ionization detector. The oven temperature program was as follows: 10 min at 50 °C, 1.5 °C per minute up to 180 °C, and 60 min. at 180 °C. Injector and detector temperatures were 275 °C. The carrier gas was helium at 18 psi and split 1:100.

RESULTS

Table 1 lists the yeast populations and ethanol contents in musts and wines. The *cerevisiae* race grew more rapidly than the *bayanus* and *capensis* races, and

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Table 1. Yeast Populations and Ethanol Contents in the Musts and Wines

		days						
	yeast	0	1	2	3	10	31	134
population ($\times 10^6$ cells/mL)	*S. cerevisiae*	1	68.4 ± 8.51	114 ± 8.54	124 ± 5.06	124 ± 10.5	115 ± 6.33	no veil
	S. bayanus	1	47.8 ± 2.36	86.3 ± 3.33	98.0 ± 6.50	141 ± 3.63	141 ± 21.1	thin veil
	S. capensis	1	55.0 ± 2.64	91.3 ± 4.85	104 ± 4.01	151 ± 7.90	154 ± 11.0	thick veil
ethanol (% v/v)	*S. cerevisiae*	0.6 ± 0.15	1.4 ± 0.25	4.5 ± 0.21	6.3 ± 0.36	13.3 ± 0.39	13.6 ± 0.09	12.8 ± 0.25
	S. bayanus	0.6 ± 0.15	0.7 ± 0.07	3.5 ± 0.22	6.4 ± 0.13	14.3 ± 0.34	13.7 ± 0.41	12.5 ± 0.31
	S. capensis	0.6 ± 0.15	0.9 ± 0.14	2.5 ± 0.11	4.9 ± 0.05	13.5 ± 0.13	14.5 ± 0.15	13.6 ± 0.11

Table 2. Mean and Standard Deviation of the Cellular Contents in Terpenic Compounds during the Vinification[a]

		days					
compound	yeast	1	2	3	10	31	134
geraniol	*S. cerevisiae*	ND[b]	ND	ND	ND	ND	ND
	S. bayanus	0.18 ± 0.01	0.24 ± 0.05	0.36 ± 0.03	0.26 ± 0.02	0.15 ± 0.01	0.61 ± 0.03
	S. capensis	0.16 ± 0.01	0.11 ± 0.01	0.15 ± 0.01	0.23 ± 0.03	0.15 ± 0.01	0.11 ± 0.01
linalool	*S. cerevisiae*	7.5 ± 0.80	8.7 ± 1.62	0.23 ± 0.03	1.70 ± 0.10	0.72 ± 0.10	0.59 ± 0.18
	S. bayanus	0.85 ± 0.16	0.41 ± 0.07	0.46 ± 0.13	0.49 ± 0.10	0.09 ± 0.01	1.4 ± 0.03
	S. capensis	ND	ND	ND	0.26 ± 0.05	0.33 ± 0.05	0.26 ± 0.04
α-terpineol	*S. cerevisiae*	ND	ND	7.2 ± 1.13	6.4 ± 0.80	28.0 ± 1.19	9.7 ± 1.65
	S. bayanus	2.7 ± 0.42	3.6 ± 1.01	4.8 ± 0.47	3.9 ± 0.72	5.4 ± 0.53	1.4 ± 0.06
	S. capensis	1.3 ± 0.06	0.68 ± 0.14	1.8 ± 0.47	0.39 ± 0.10	1.3 ± 0.28	0.71 ± 0.16
(*E*)-nerolidol	*S. cerevisiae*	ND	ND	0.19 ± 0.01	ND	0.42 ± 0.03	0.19 ± 0.04
	S. bayanus	ND	0.28 ± 0.06	0.26 ± 0.04	0.88 ± 0.12	0.42 ± 0.07	0.57 ± 0.03
	S. capensis	0.78 ± 0.18	0.41 ± 0.11	0.39 ± 0.03	0.37 ± 0.04	0.08 ± 0.02	0.33 ± 0.06
(*Z*)-nerolidol	*S. cerevisiae*	ND	ND	0.12 ± 0.02	0.13 ± 0.01	0.32 ± 0.03	0.60 ± 0.14
	S. bayanus	ND	ND	ND	0.37 ± 0.03	0.40 ± 0.07	3.9 ± 0.18
	S. capensis	ND	0.15 ± 0.01	0.22 ± 0.01	0.34 ± 0.04	0.27 ± 0.06	0.44 ± 0.04
total terpenes	*S. cerevisiae*	7.5 ± 0.80	8.7 ± 1.62	7.8 ± 1.10	8.2 ± 0.81	29.4 ± 1.08	11.1 ± 1.90
	S. bayanus	3.7 ± 0.44	4.5 ± 1.05	5.9 ± 0.64	5.9 ± 0.78	6.4 ± 0.60	7.9 ± 0.33
	S. capensis	2.2 ± 0.17	1.4 ± 0.26	2.6 ± 0.45	1.6 ± 0.00	2.1 ± 0.38	1.8 ± 0.21

[a] Concentrations are expressed in milligrams per liter of cellular volume. [b] ND, not detected.

Table 3. Mean and Standard Deviation of the Must Contents (Micrograms per Liter) in Terpenic Compounds during the Vinification

		days						
compound	yeast	0	1	2	3	10	31	134
geraniol	*S. cerevisiae*		13.6 ± 2.46	17.4 ± 4.10	13.4 ± 1.57	15.6 ± 3.82	11.6 ± 1.16	6.0 ± 0.60
	S. bayanus	10.3 ± 0.52	3.2 ± 0.95	7.0 ± 1.10	9.4 ± 3.04	15.7 ± 1.51	26.5 ± 4.35	11.8 ± 3.15
	S. capensis		ND[a]	2.2 ± 0.17	3.1 ± 0.59	13.2 ± 1.81	10.1 ± 0.81	25.5 ± 4.50
linalool	*S. cerevisiae*		1.4 ± 0.15	3.4 ± 0.89	4.1 ± 0.55	7.1 ± 1.05	5.1 ± 0.26	6.8 ± 0.95
	S. bayanus	28.9 ± 2.35	1.0 ± 0.10	2.1 ± 0.42	2.8 ± 0.50	8.1 ± 1.41	35.9 ± 8.35	53.6 ± 3.30
	S. capensis		6.2 ± 0.56	8.9 ± 1.50	10.8 ± 1.36	9.7 ± 1.56	9.5 ± 2.00	16.1 ± 1.42
α-terpineol	*S. cerevisiae*		24.5 ± 2.45	29.1 ± 7.52	40.3 ± 2.14	77.0 ± 12.0	50.1 ± 10.8	77.0 ± 12.6
	S. bayanus	5.2 ± 1.20	11.2 ± 2.26	1.4 ± 0.06	3.4 ± 0.10	6.5 ± 0.40	17.3 ± 3.99	29.9 ± 7.96
	S. capensis		5.1 ± 0.20	6.1 ± 1.57	5.6 ± 0.64	5.7 ± 1.25	7.0 ± 0.66	15.7 ± 2.89
farnesol	*S. cerevisiae*		156 ± 22.4	132 ± 5.71	118 ± 17.8	39.2 ± 7.49	34.6 ± 6.44	10.1 ± 2.15
	S. bayanus	24.2 ± 2.75	12.2 ± 0.85	17.9 ± 0.31	17.4 ± 0.36	14.3 ± 2.00	26.7 ± 2.12	87.7 ± 4.75
	S. capensis		6.0 ± 1.01	6.1 ± 0.12	1.9 ± 0.46	25.4 ± 4.25	6.4 ± 0.55	14.8 ± 3.93
(*E*)-nerolidol	*S. cerevisiae*		10.6 ± 1.22	15.2 ± 3.01	115 ± 11.8	324 ± 20.0	320 ± 32.3	216 ± 24.3
	S. bayanus	32.3 ± 3.51	43.0 ± 7.26	81.0 ± 0.00	107 ± 6.1	312 ± 35.4	439 ± 77.7	391 ± 53.8
	S. capensis		62.8 ± 4.05	85.9 ± 14.1	84.7 ± 8.93	204 ± 10.9	263 ± 27.6	320 ± 28.2
(*Z*)-nerolidol	*S. cerevisiae*		ND	ND	ND	15.2 ± 0.68	3.6 ± 0.55	10.2 ± 0.40
	S. bayanus	ND	2.0 ± 0.35	5.0 ± 0.40	6.0 ± 0.51	13.3 ± 3.06	13.9 ± 3.53	18.7 ± 6.64
	S. capensis		0.9 ± 0.23	ND	ND	10.1 ± 2.30	1.8 ± 0.33	20.6 ± 2.24
total terpenes	*S. cerevisiae*		206 ± 22.3	197 ± 19.7	290 ± 11.7	478 ± 35.6	425 ± 36.6	326 ± 34.5
	S. bayanus	101 ± 10.4	72.5 ± 9.24	114 ± 0.84	146 ± 4.40	370 ± 35.4	560 ± 88.9	593 ± 42.2
	S. capensis		81.0 ± 5.52	109 ± 14.1	106 ± 11.1	268 ± 8.39	298 ± 27.9	412 ± 39.6

[a] ND, not detected.

no veil formation was observed in the *cerevisiae* race. Ethanol contents in the wines obtained by the three races were 12.5−13.6% v/v after 134 days.

Changes in Terpenic Contents in Yeast Cells during Fermentation. The yeast cells studied were analyzed for the monoterpenic alcohols geraniol, linalool, α-terpineol, and (*E*)- and (*Z*)-nerolidol. The nerolidols are sesquiterpenic alcohols. The contents of alcohols are given in milligrams per liter of cell volume in Table 2.

α-Terpineol was found to occur at the highest concentrations of all the terpenic alcohols studied in the three yeast races, with a maximum at the end of the postfermentation period (31 days), and was particularly high in the *cerevisiae* race. The contents of this alcohol decreased during the veil formation period. On the other hand, (*Z*)-nerolidol was found to accumulate in cells after alcoholic fermentation (10 days) and to peak during veil formation (134 days) by the three yeast races.

Linalool was the major alcohol in *cerevisiae* race cells during the first 2 days of fermentation, after which time its concentration dropped, possibly as a result of its cyclization to α-terpineol. On the other hand, geraniol occurred at undetectable concentrations in this race. Geraniol content in the cell of *bayanus* race increased

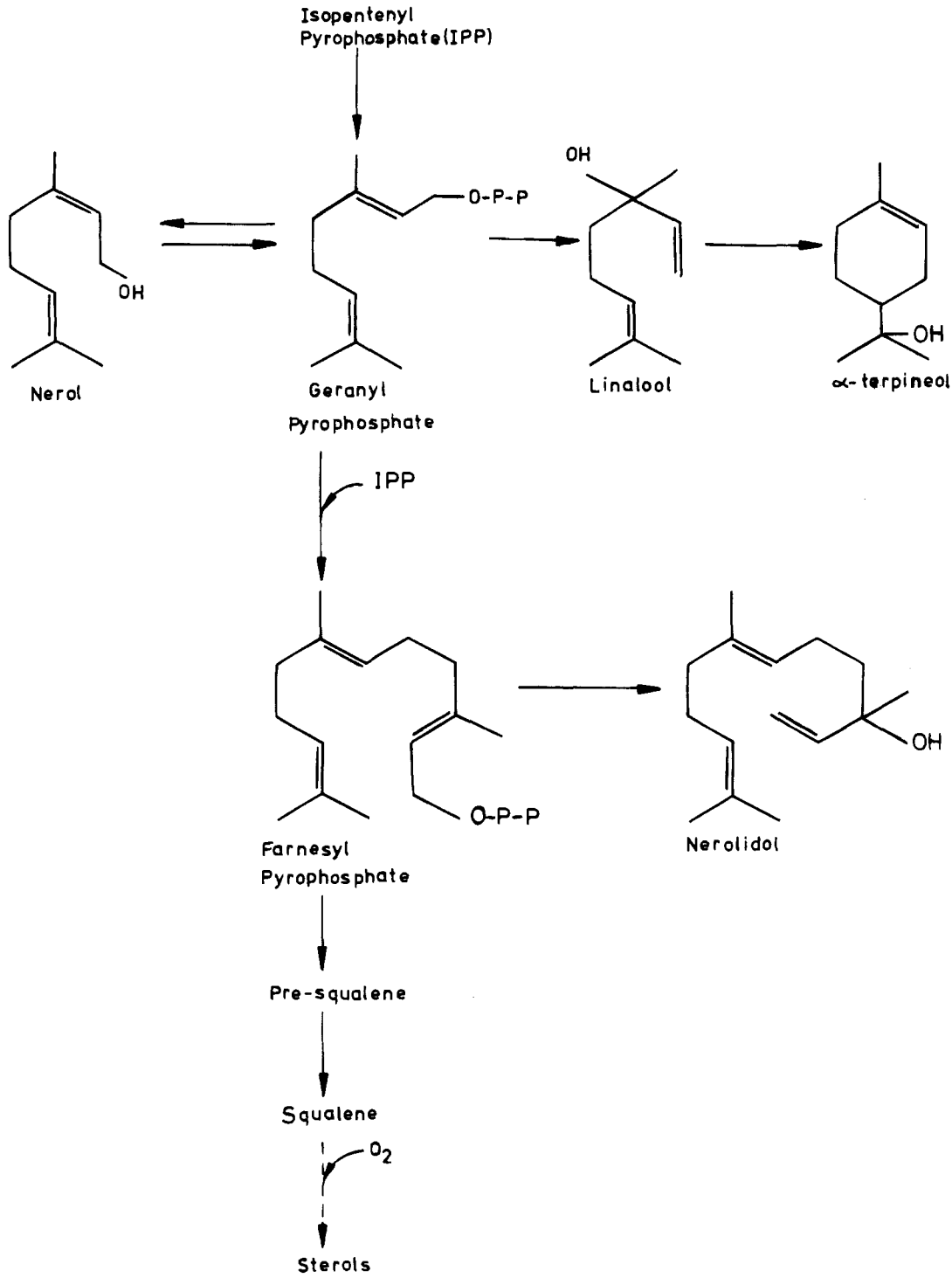

Figure 1. Sterol biosynthesis pathway and terpene formation in yeasts.

during the first 3 days of fermentation and peaked at 134 days. At this point, linalool also peaked, though both decreased between 10 and 31 days. Linalool in the *capensis* race cell was detected at the end of alcoholic fermentation (10 days) and then remained constant. Finally, the geraniol contents in this race were constant throughout the study.

Changes in Terpenic Compounds in Musts during Fermentation. Table 3 gives the geraniol, linalool, α-terpineol, (*E*)- and (*Z*)-nerolidol, and farnesol contents in musts and wines. Farnesol was not detected in yeast cells.

(*E*)-Nerolidol was found to be the terpene accumulated in highest concentration during fermentation. Its concentration peaked at the end of fermentation carried out by the *cerevisiae* race or during veil formation for the *capensis* and *bayanus* races. Its *Z* isomer was found to accumulate after alcoholic fermentation, though at much lower concentrations.

As a rule, linalool and α-terpineol accumulated during the period studied (134 days) in the three wines. The decrease in the linalool content within the first 24 h was concomitant with an increase in the α-terpineol content, except in the must fermented by the *capensis* race.

Geraniol accumulated in the musts during fermentations carried out by the three yeast races. This alcohol was partly depleted during the veil formation period in the wines produced by the *cerevisiae* and *bayanus* races

but continued to accumulate in the wine produced by the *capensis* race.

The overall terpenes (Table 3) reveal that the highest increases of concentrations were detected between 3 and 10 days, when the alcoholic fermentation was virtually concluded. After this point, their contents in the wines containing dead cells of the *cerevisiae* race fell, while those in the wines produced by veil-forming yeasts increased markedly.

DISCUSSION

Experiments performed by Cañete et al. (1993) using cv. Pedro Ximenez grapes and Pectinol V.R. (BASF Röhm) as a source of β-glycosidase activity revealed the overall content of linalool, nerol, geraniol, and α-terpineol in this grape variety to be 56 μg/L, of which only 15 μg/L was found to be glycosidically bound. Results obtained following the Dimitriadis and Williams (1984) method, together with the data reported by Moreno et al. (1988) and Toledano (1990) and those data obtained prior to the beginning of fermentation in this work, show that the Pedro Ximenez variety contains scanty free and bound terpenes, varying with the vintages.

According to Darriet (1992) the changes in terpene contents during alcoholic fermentation are the result of the joint action of several phenomena including mutual conversions, formation of terpene oxides, sweeping by CO_2 released during alcoholic fermentation, enzymatic and chemical hydrolysis of glycosidic bonds, and even absorption in the walls of yeast cells. Nevertheless, the potential effects of all of these factors, the differences in terpene changes during the fermentations performed with the three *S. cerevisiae* yeast races tested and the high α-terpineol concentrations found in the wines produced by race *cerevisiae*, cannot be fully accounted for unless a direct effect of the physiological activity of the yeast is considered. This is particularly true when the low contents of bound terpenes in this grape variety are taken into account.

During the growth phase, when some oxygen is still present in the fermentation medium, yeasts synthesize terpenic compounds via isopentenyl pyrophosphate (Coolbear and Threlfall, 1989). They use those compounds as a biosynthetic intermediate for sterol synthesis (Figure 1). After dissolved oxygen in the must is depleted, squalene cannot be epoxidized, so further sterol synthesis is inhibited (Mauricio et al., 1990, 1991), and squalene and its biosynthetic intermediates (geranyl P-P, farnesyl P-P, and pre-squalene P-P) may accumulate within yeast cells. This accumulation could facilitate their excretion to the must, with a different extent depending on the particular yeast race. Thus, the *cerevisiae* race may excrete more geraniol and α-terpineol than do the two veil-forming races. In fact, in this race these compounds were not detected in cells, the former during the entire fermentation and the latter in the first few days. However, both alcohols were detected at higher concentrations in the resulting wines obtained by the *cerevisiae* race than in those produced by the other two races, particularly in the first few days. Also, the *capensis* race excreted more linalool, which was undetectable in cells during the first 3 days but occurred at higher concentrations in the wines produced by this yeast race than in those yielded by the other two.

The terpenes may undergo intracellular isomerizations, mutual enzymatic conversions, and cyclizations. Transformations of this type have been observed with in vitro cultured grape cells (Ambid et al., 1983). Besides, prenyl pyrophosphate carbocyclases, carboligase, and intramolecular pyrophosphate eliminating enzymes have been found in fungi (Coolbear and Threlfall, 1989), so probably the yeasts may also develop reactions of this type (Figure 1). As a result, geraniol may isomerize to linalool in *cerevisiae* cells from the first day of fermentation, and linalool may in turn cyclize to α-terpineol from the third day. Similar conversions may also take place, to a lesser extent, in the veil-forming races. Such reactions may account for the fact that α-terpineol was the terpene that reached the highest concentration within cells of the three yeast races during the postfermentation phase. A rapid excretion of farnesol to the wine and its intracellular isomerization to nerolidol may account for its absence from cells and the accumulation of both terpenols in the wines.

The higher linalool, (*E*)-nerolidol, and farnesol contents in the wines obtained by the *bayanus* and *capensis* races after 134 days are consistent with the results reported by Fagan et al. (1981), suggesting a biosynthetic activity of veil-forming yeasts during their formation.

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