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Evaluation of the Combined Effects of Enzymatic Treatment and Aging on Lees on the Aroma of Wine from *Bombino bianco* Grapes

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In this study, two different doses of commercial β-glucanase enzyme preparation were tested to verify their effect on wines aged on lees. These wines were compared with two samples with no enzymatic treatment. The former was aged on lees (control), and the latter was readily filtered off from the yeast cell biomass (standard). Analysis of variance (one-way ANOVA), the Tukey test, and principal component analysis (PCA) were applied to all of the samples, which were analyzed for aroma composition, along with galacturonic acid, total acidity, pH, and color. Results showed a large number of statistically significant differences among samples. In general, wines treated with β-glucanase were characterized by higher concentration of many volatile compounds. The presence of lees and even more the exogenous enzymatic action enhanced almost all volatile compounds. Besides the high presence of ethyl esters, it is worth mentioning the behavior of hexanol and *trans*-3-hexenol, which are strongly enhanced by the presence of lees and by enzymatic treatments.

KEYWORDS: "*Bombino bianco*"; yeast lees; wine aroma; white wine; enzyme in winemaking

INTRODUCTION

In the past few years, consumer preferences for white wines are addressed to full-bodied, aromatic, and natural products. *Vitis vinifera* L. *Bombino bianco* is a cultivar of uncertain origin, widespread since ancient times in the Apulia region and present in other regions of the South of Italy, where it has been sometimes indicated with synonyms (*Bonvino*, *Ottenese*, *Trebbiano d'oro*, *Trebbiano d'Abruzzo*, *Uva d'Oro*, or *Gold Trauben*, etc.).

Bombino bianco grapes are characterized by high yield, good resistance to bad weather conditions as well as to grape diseases, such as *Plasmopara* or *Botrytis* (*1*). In the past, *Bombino bianco* was also utilized as a table grape and largely exported to Germany due its late-harvest and for its good resistance to transport (*1*). However, this cultivar is very poor in varietal aroma. For this reason, *Bombino bianco* is an ideal substrate to study the performance of wine aging on lees (or *élevage sur lies*), and at the same time, it might have a great advantage from this treatment. This technology is already employed in the manufacture of Bourgogne wines, Champagne, and aged wines produced with flor yeasts (Sherry) (*2*). In a few words, wines are improved on aging over the yeast cells after fermentation, for a variable period (*2*). During this time, yeasts undergo natural autolysis of the cell (*3*), enriching the wine of volatile substances, as well as high molecular weight substances from the cell walls (*2*).

In particular, cell walls of *Saccharomyces cerevisiae* contain mannoproteins crossed by fibers of glucan and chitin (*4*). During autolysis, the cell wall is gradually degraded by breaking its glucan and chitin fibers. This degradation is promoted by the naturally occurring enzymes of yeasts. These include glucanases and mannosidases. Because of this degradation, the cell walls become less rigid, and polysaccharides and other externally adhered substances are more easily released in the medium (*5*). In addition, lipids released from the lees enrich the aromatic fraction of the wine, enhancing the formation of esters and aldehydes (*6*).

Moreover, polysaccharides stabilize wines against tartaric and proteic precipitations (*7, 8*). They stabilize the polyphenolic fraction as well, mainly the monomeric anthocyanin content (*9*).

Finally, yeast autolysis promotes the releasing of mannoproteins (*10*), high molecular weight polysaccharides conjugated with proteins, as already well documented (*11*). Mannoproteins increase density, contributing to fullness and full-body of wine. However, the knowledge of the potential contribution to fermentative aromas, some of which are absorbed onto the cell wall of the yeasts and eliminated by filtration, is still poor (*12*).

In the past few years, the winemaking industry has developed commercial enzyme preparations able to accelerate the autolysis

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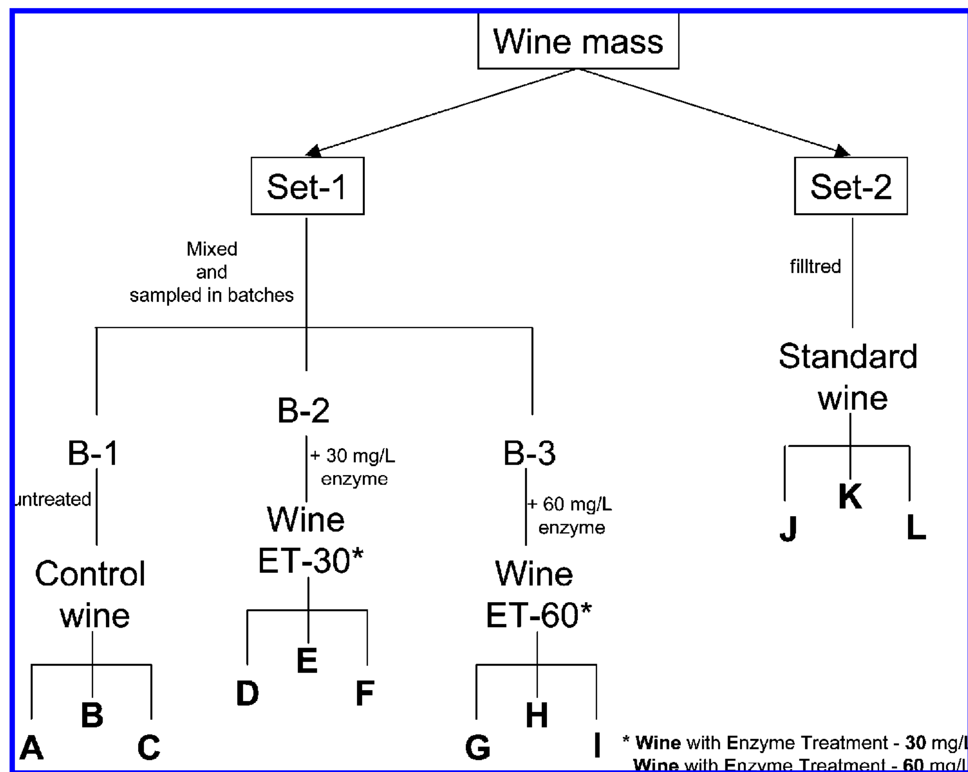

Figure 1. Schematization of the sample set.

process in wines aged over lees (*2*). On the market, several enzymatic preparations are now available, such as β-glucanase and pectinase, that considerably increase the polysaccharide concentration of both white and red wines (*13*).

In this research, wine aging on lees combined with enzymes with β-1,3-glucanase activity was tested in order to verify their influence on volatile content and on some wine parameters.

MATERIALS AND METHODS

Enzyme. A β-glucanase commercial preparation (Lallzyme MMX; Lallemand Inc.; Castel D'Azzano, VR, Italy) was used. Its standard activity is (a) 1840 poly galacturonase units; (b) 25 pectin lyase units; and (c) 545 pectin esterase units.

Must and Fermentation. Grapes (*Vitis vinifera* L., *Bombino bianco* cultivar) were harvested in late September from a vine, around San Severo and Torremaggiore, in "Tavoliere delle Puglie", a plain in the South-East of Italy. The whole grapes were pressed with a horizontal pneumatic press at the Società Cooperativa Agricola Fortore of Torremaggiore (Foggia, Italy).

The must was cooled down to about 4 °C, added with charcoal Enoblack super (100 mg/L; Esseco srl, S. Martino Trecate, NO, Italy) and pectolytic enzymes Lallzyme C.MAX (5 mg/L; Lallemand Inc.; Castel D'Azzano, VR, Italy), and set aside for 24 h. Then, static defecation was carried out, decanting the clear must into a clean tank.

Alcoholic fermentation was carried out at 20 °C, by adding the selected yeast strain (300 mg/L; Lalvin ICV-D47; Lallemand Inc.; Castel D'Azzano, VR, Italy). This yeast is a high polysaccharide producer, particularly specific for wine aging on lees. Moreover, 60 mg/L of sulfur dioxide (SO_2) and ammonium sulfate−ammonium phosphate−thiamine fermentation activator (300 mg/L) were used.

At the end of alcoholic fermentation, SO_2 was increased up to 80 mg/L, and an aliquot (225 L) of the whole wine mass was divided into two sets. The former set with yeast lees was mixed and sampled into three batches stored in 25-L stainless steel kegs. Two batches were added at 30 and 60 mg/L of Lallzyme MMX, and the third one was used as reference wine (control) without further manipulations. The latter set of wine was filtered with diatomaceous earth filter (2 g/L; Randal 2, Randal 7, and Randal USA, Dal Cin Gildo SpA, Sesto S. Giovanni, MI, Italy), and it was considered as the standard, with neither lees nor enzyme. Each batch was sampled three times for a total number of 12 samples (**Figure 1**).

To prevent oxidation, batches were stored under N_2, as inert gas between 18 and 20 °C. Each batch was daily mixed (*bâtonnage*) to homogenize the product, improving enzyme distribution and enhancing the dissolution of the macromolecules released by the yeast cell walls. The process was carried out by keg overturning.

After 70 days, the kegs were opened, and the wines were monitored for alcoholic strength and SO_2 content, which was reintegrated at 110 mg/L. Finally, the wines were filtered and introduced into 0.66-L bottles.

Reagents. Pure reference compounds were purchased from Fluka Sigma-Aldrich (Milan, Italy), while high purity solvents were supplied by different companies, and they were purified and redistilled before use.

Deionised water was obtained by a Milli-Q purification system (Millipore, Milan, Italy).

Volatile Analysis. Reference standard solutions were prepared by dissolving each substance in absolute ethanol (10,000 mg/L; stock solutions). In the same way, internal standard solution (dodecan-1-ol) was also prepared.

An aliquot of each stock solution was used to prepare a 100-mg/L standard mixture in ethanol for volatile quantification.

A model wine was prepared with 5 mL of standard mixture solution (100 mg/L), 5 mL of ethanol, up to 100 mL with a solution of tartaric acid (5 g/L), and glycerine (2.5 g/L). The model wine was treated as a common sample, and it was used for correction factor calculation.

Chemical Analysis. Ethanol was determined by densitometry at 20 °C after distillation; reducing sugars were determined according to the Fehling method; total acidity (expressed as g/L of tartaric acid) was determined by titration with 0.1 N sodium hydroxide solution (bromothymol blue as indicator); pH was determined by using the potentiometric method; color absorbance was determined by a spectrophotometric method (420 nm, 1 cm optical path). Details of the used procedures are reported in ref *14* and in the European official methods (*15*).

Galacturonic acid was determined with a method reported by Usseglio Tommaset (*16*), while catechins were determined by HPLC (*17*).

Table 1. Results of the Determinations Performed on the Samples Expressed As the Mean Values of Three Replicates ± Standard Deviation[a]

	control samples mean ± SD	wine ET-30[b] mean ± SD	wine ET-60[b] mean ± SD	standard samples mean ± SD	ANOVA one way F_{values}
total acidity (g/L)	5.08 ± 0.04	5.08 ± 0.04	5.10 ± 0.07	5.18 ± 0.34	0.21 n.s.
pH	3.27 ± 0.01	3.25 ± 0.03	3.21 ± 0.01	3.21 ± 0.04	4.49 n.s.
ethanol (% v/v)	11.25 ± 0.05	11.23 ± 0.07	11.22 ± 0.04	11.26 ± 0.08	0.27 n.s.
sugars (g/L)	1.97 ± 0.42	2.20 ± 0.30	2.37 ± 0.46	2.23 ± 0.11	0.68 n.s.
volatile acidity (g/L)	0.13 ± 0.06	0.10 ± 0.02	0.15 ± 0.03	0.11 ± 0.01	1.20 n.s.
color (λ = 420 nm)	0.17 ± 0.02	0.17 ± 0.00	0.18 ± 0.04	0.18 ± 0.01	0.15 n.s.
galacturonic acid (mg/L)	315.37 ± 30.03	234.87 ± 62.53	357.53 ± 107.34	344.73 ± 54.81	1.88 n.s.
volatile phenols (μg/L)					
4-ethylguaiacol	181.33 ab ± 5.51	227.33 bc ± 21.39	250.33 c ± 17.56	168.67 a ± 24.42	12.70**
4-vinylphenol	11965.33 b ± 794.62	14788.67 b ± 777.51	11949.33 b ± 2902.39	5432.00 a ± 539.34	18.97**
pholyphenol (mg/L)					
catechins	10.67 a ± 0.50	13.43 c ± 0.21	12.57 bc ± 0.21	12.50 b ± 0.36	34.58***
alcohols (μg/L)					
methanol (mg/L)	20.00 ± 2.65	20.00 ± 1.00	18.67 ± 2.08	20.67 ± 1.15	4.64 n.s.
propanol (mg/L)	34.33 ± 0.58	33.00 ± 1.00	32.67 ± 0.58	33.33 ± 1.15	2.07 n.s.
i-butanol (mg/L)	20.33 ± 0.58	18.67 ± 0.58	19.00 ± 1.73	19.00 ± 1.00	1.40 n.s.
i-amyl alcohols (mg/L)	117.00 ± 2.65	121.00 ± 17.35	106.33 ± 12.22	109.00 ± 5.57	1.15 n.s.
n-butanol	1064.00 ab ± 66.91	1322.00 b ± 87.73	1225.33 b ± 306.61	658.33 a ± 78.68	9.15**
3-methylthio-1-propanol	99.67 b ± 3.06	122.33 b ± 10.60	93.00 b ± 19.70	62.00 a ± 4.00	14.15***
3-ethoxy-1-propanol	42.33 a ± 1.53	53.33 b ± 4.73	52.00 b ± 2.65	37.00 a ± 4.58	13.98**
2-phenylethanol	25093.33 b ± 993.91	31256.67 c ± 1360.38	29633.67 bc ± 3239.15	17806.67 a ± 1921.08	68.89***
benzyl alcohol	208.33 b ± 4.51	238.00 b ± 0.00	307.33 c ± 34.50	103.67 a ± 6.11	25.56***
aldehyde (mg/L)					
acetaldehyde	14.00 a ± 1.00	13.67 a ± 0.58	12.67 a ± 1.15	36.33 b ± 2.52	175.0***
C-6 alcohols (μg/L)					
n-hexanol	2005.00 ab ± 42.00	2427.67 b ± 123.86	2521.00 b ± 588.35	1355.33 a ± 123.49	8.94**
trans-3-hexenol	36.33 ab ± 3.51	53.00 c ± 2.00	48.00 bc ± 8.19	26.00 a ± 4.00	17.70***
cis-3-hexenol	51.33 a ± 6.51	62.33 ab ± 8.50	78.33 b ± 13.32	70.67 b ± 2.31	5.41*
fatty acids (μg/L)					
i-butyric acid	93.00 a ± 5.00	149.33 b ± 35.23	87.67 a ± 22.12	74.33 a ± 5.69	7.36**
n-butyric acid	304.67 ± 22.59	327.00 ± 135.53	354.33 ± 121.17	391.67 ± 428.43	0.08 n.s.
2-methylbutyric acid	107.33 ab ± 1.53	144.33 c ± 21.50	125.33 bc ± 15.04	76.33 a ± 5.51	13.92**
hexanoic acid	2226.67 b ± 93.09	2900.33 c ± 107.52	2530.67 bc ± 449.80	1335.33 a ± 130.00	22.37***
octanoic acid	5141.33 b ± 203.50	6778.33 b ± 255.60	5950.67 b ± 1268.94	2666.67 a ± 264.64	21.17***
decanoic acid	1004.33 b ± 53.16	1168.67 b ± 121.49	1104.33 b ± 374.75	300.00 a ± 37.00	12.16**
esters (μg/L)					
ethyl acetate (mg/L)	40.33 ± 1.53	39.33 ± 0.58	41.67 ± 2.52	42.67 ± 9.87	0.24 n.s.
hexyl acetate	3653.67 ± 118.78	3903.00 ± 476.04	3597.00 ± 431.64	2349.67 ± 434.24	0.58 n.s.
i-amyl acetates	595.00 a ± 9.17	846.67 b ± 90.74	830.33 b ± 62.31	473.00 a ± 8.19	32.70***
ethyl 3-hydroxybutyrate	67.33 ab ± 0.58	79.67 b ± 3.06	79.33 b ± 16.56	45.00 a ± 4.58	10.41**
ethyl 4-hydroxybutyrate	127.33 a ± 1.53	160.00 ab ± 16.52	183.67 b ± 30.07	154.33 ab ± 15.50	4,72 n.s.
ethyl hexanoate	352.33 b ± 10.21	427.00 b ± 34.77	381.67 b ± 92.09	212.67 a ± 26.73	9.75**
diethylsuccinate	1140.33 b ± 70.71	1167.33 b ± 93.82	1118.67 b ± 153.45	639.33 a ± 79.54	17.47***
ethyl lactate	22318.00 b ± 699.48	29417.67 c ± 2503.46	29054.00 c ± 1530.42	16281.00 a ± 2338.44	32.13***
lactone (μg/L)					
γ-butyrolactone	2170.33 ab ± 40.77	2698.00 bc ± 164.68	2888.67 c ± 107.22	1932.00 a ± 391.91	12.33**
Total volatiles[c] (μg/L)	80896.00 b ± 2679.00	101840.67 c ± 6465.57	97295.33 bc ± 10268.79	53963.33 a ± 2909.66	34.52***

[a] Results of one-way ANOVA and the Tukey test are reported as F_{value} and superscript letters (for F_{value} are statistically significant), respectively. Different letters identify samples that are significantly different (p = 0.05). ***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$. [b] Wine with enzyme treatment, 30 mg/L and wine with enzyme treatment, 60 mg/L. [c] Only significant substances were considered.

Extraction of Volatile Compounds. Volatile components were determined by gas chromatography with flame ionization detection and gas chromatography coupled with mass spectrometry (GC/MS) after their extraction and concentration by means of a column packed with Extrelut resin (Bracco Merck, Milan, Italy). An aliquot of internal standard (100 μL) was added to 20 mL of filtered wine, and it was applied to an Extrelut column. Volatiles were extracted with 40 mL of dichloromethane (*18*).

Water was removed by filtering the frosted organic phase (−18 °C for 60 min) and then dried over anhydrous sodium sulfate. The solvent excess was removed by distillation using a Snyder column up to 1−2 mL. Finally, it was reduced to 500 μL under a stream of pure N_2.

Identifications were carried out by comparing retention times and the mass spectra of pure standard injected under the same conditions. The quantification was made by using the internal standard method.

Gas Chromatography Conditions. Extracts (1 μL) were analyzed with a GC 9000 Series (Fisons Instruments S.p.A, Rodano, Milan, Italy) equipped with an FFAP homemade column (30 m × 0.25 mm i.d. × 0.25 μm film thickness), a split injector (split ratio 1:50; 250 °C), and an FID (250 °C). The column temperature was kept at 50 °C for 4 min, and then it was raised up to 200 °C (5 °C/min) and held for 10 min. Finally, the temperature was increased to 230 °C (10 °C/min) and held for 5 min. The carrier gas (hydrogen) flowed at 3 mL/min.

Chromatograms were acquired and processed with Chrom-Card 1.15 Software (Fisons Instruments S.p.A, Rodano, Milan, Italy).

GC/MS analyses were carried out with a Hewlett-Packard instrument 6890 series (Hewlett-Packard Waldbronn, Germany), using the same conditions. Mass spectra were recorded from 30 to 400 m/z at 70 eV.

Table 2. Correlation Matrix of the Data Set[a]

	acetaldehyde	methanol	*n*-butanol	3-methylthio-1-propanol	3-ethoxy-1-propanol	2-phenylethanol	benzyl alcohol	*n*-hexanol	*trans*-3-hexenol	*cis*-3-hexenol	4-ethylguaiacol	4-vinylphenol
acetaldehyde	1.00											
methanol		1.00										
n-butanol	−0.81		1.00									
3-methylthio-1-propanol			0.87	1.00								
3-ethoxy-1-propanol			0.88		1.00							
2-phenylethanol	−0.85		0.97	0.84	0.94	1.00						
benzyl alcohol	−0.88		0.85		0.82	0.88	1.00					
n-hexanol			0.82		0.81	0.88	0.85	1.00				
trans-3-hexenol			0.92		0.93	0.97	0.82	0.92	1.00			
cis-3-hexenol										1.00		
4-ethylguaiacol					0.92	0.84	0.84	0.84	0.86		1.00	
4-vinylphenol	−0.87		0.96	0.93	0.83	0.94			0.86			1.00
i-butyric acid												
n-butyric acid												
2-methylbutyric acid			0.94	0.80	0.93	0.96			0.92			0.93
hexanoic acid	−0.85		0.98	0.91	0.90	0.99	0.84	0.85	0.95			0.97
octanoic acid	−0.86		0.99	0.91	0.89	0.99	0.85	0.85	0.94			0.98
decanoic acid	−0.88		0.97	0.86		0.94	0.87	0.83	0.86			0.95
ethyl acetate												
hexyl acetate												
i-amyl acetates			0.88		0.93	0.93	0.87	0.79	0.91		0.86	0.83
ethyl 3-hydroxybutyrate	−0.81		0.91		0.82	0.93	0.87	0.97	0.94		0.81	0.85
ethyl 4-hydroxybutyrate												
ethyl hexanoate	−0.81		0.99	0.87	0.85	0.96	0.81	0.84	0.92			0.96
diethylsuccinate	−0.90		0.93	0.84		0.91	0.82		0.81			0.95
ethyl lactate	−0.81		0.86		0.92	0.93	0.87		0.88		0.82	0.84
γ-butyrolactone					0.94	0.85	0.83		0.83		0.94	

Determination of Higher Alcohols. Acetaldehyde, ethyl acetate, methanol, propanol, 2-methylpropanol, 3- and 2-methylbutanol (these latter two substances were quantified together as *i*-amyl alcohols) were determined by packed gas chromatography after sample distillation (*19*).

Statistical Analysis. Analysis of variance (one-way ANOVA), Tukey test, linear regression analysis, and principal component analysis (PCA) were applied to the data of sample set (**Table 1**). Statistical processing was carried out using Minitab Release 13.13 software (Minitab Inc., Pine Hall, PA, USA).

RESULTS AND DISCUSSION

The results, reported as the means of three replicates ± standard deviation and their statistical analysis are shown in **Table 1**.

Total acidity and pH values in the tested samples were in accordance with those of literature for wines obtained from the *Bombino bianco* cultivar (*1, 20*). As expected, enzymes did not affect these parameters, as yeast cell walls did not release any acidity active compound. The same considerations can be extended to alcoholic strength, residual sugars, volatile acidity, and color.

Volatile phenol concentration depends on the vinification process (*21*). Among them, vinylphenol and ethylguaiacol were quantified in tested samples. In wines, the amount of these compounds is generally low and usually limited by the concentration of their precursors (*22*). The concentration of 4-vinylphenol was particularly remarkable in all wines except for the standard one. In fact, in the other wines the concentration of 4-vinylphenol was more than double, when compared with that of standard wine. For this reason, 4-vinylphenol was influenced by lees occurrence, whereas the presence of enzyme seemed negligible. This suggests an absorption effect on yeast cell walls, and subsequent release because of lees leaching. However, wines obtained from musts treated with pectinase preparations to improve clarification and color extraction (*23*) showed greater levels of volatile phenols (*24*).

The catechin concentration was lower in untreated control samples aged on lees (10.67 mg/L) because these substances are easily absorbed by solid matter. However, statistical analysis revealed significant differences among the samples ($p \leq 0.001$). In fact, its concentration decreased during the treatment on lees, while enzymes had a positive effect. In other words, cell wall tends to absorb these substances that were released by enzyme treatment. In addition, the instability of catechins to oxidation cannot be neglected. The antioxidant activity of lees can further justify the lower figures in control samples with lees, as a consequence of free thiol substance release, able to protect catechins against oxidation.

The total concentration of volatile compounds in each sample (**Table 1**) clearly shows differences among the standard samples not aged on lees (the lowest values), enzyme untreated control samples aged on lees (intermediate values), and the samples with added enzyme (the highest values). However, there was no difference between the two levels of added enzyme.

Acetaldehyde showed a sharp variation among the samples. In particular, its content was about 3 times higher in standard samples not aged on lees (36.3 mg/L) rather than the other treatments. Unlike during wine storage, the antioxidant effect of lees probably prevented the oxidation of ethanol (*25, 26*). On the contrary, methanol was not affected by the experimental conditions. In fact, it was entirely produced by pectinmethylesterase activity during grape crushing.

As expected, higher alcohols (propanol, *i*-butanol, and *i*-amyl alcohols), typical fermentative products, showed no statistical difference. As a consequence of their fair water solubility (around 3 g/L at 30 °C for *i*-amyl alcohols), low molecular weight alcohols were not strongly absorbed on yeast cell walls. On increasing molecular weight, their water solubility decreases, and the effect of lees becomes more and more important. The wide differences between wines with or without lees (control vs standard) for 2-phenylethanol confirm this observation. The enzyme addition further enhanced the 2-phenylethanol concentration because of yeast cell wall lysis. Benzyl alcohol showed a similar behavior, but unlike 2-phenylethanol, its concentration was under the odor threshold (*27*).

Table 2. Continued

i-butyric acid	*n*-butyric acid	2-methylbutyric acid	hexanoic acid	octanoic acid	decanoic acid	ethyl acetate	hexyl acetate	*i*-amyl acetates	ethyl 3-hydroxybutyrate	ethyl 4-hydroxybutyrate	ethyl hexanoate	diethyl succinate	ethyl lactate	γ-butyrolactone
1.00														
	1.00													
		1.00												
		0.96	1.00											
		0.95	1.00	1.00										
		0.89	0.95	0.97	1.00									
						1.00								
						0.92	1.00							
		0.93	0.90	0.90	0.83			1.00						
		0.86	0.92	0.92	0.92			0.84	1.00					
										1.00				
		0.94	0.98	0.98	0.98			0.85	0.93		1.00			
		0.86	0.92	0.93	0.94				0.85		0.93	1.00		
		0.92	0.89	0.89	0.82			0.96	0.84		0.84	0.81	1.00	
		0.80						0.88					0.85	1.00

[a] Correlation coefficient (r) with $p \leq 0.001$ are reported.

A similar consideration can be extended to C6 alcohols as well, with the remarkable exception of *cis*-3-hexenol. This group of alcohols is generally considered as prefermentative substances, whose presence comes from enzymatic degradation of grape lipids (*28*). This fact is certainly true for the *cis*-3 isomer, which is present in all samples at similar concentrations, but *n*-hexanol and even more clearly *trans*-3-hexenol seem involved in yeast metabolism and could also be considered as fermentative products. These latter two alcohols, in fact, behave exactly like 2-phenylethanol and other lees affected substances. It is very likely that yeast lipid oxidation was responsible for these results, acting on fatty acid precursors in a manner similar to prefermentative pathways.

Similar evidence on *trans*-3-hexenol was already observed in flor-aged wines (*29*) and in a previous study on different yeast strains (*30*). In this latter paper, the same must underwent fermentation with different yeast strains. The concentration ranges of two isomers were sharper in the case of *trans*-3-hexenol: minimum−maximum ratio was 1.47 for *trans*-3-hexenol and 1.39 for *cis*-3-hexenol.

Except for *n*-butyric acid, all fatty acids were influenced by the treatments. The very high value of standard deviation reported for standard wine could likely explain the behavior of *n*-butyric acid. As for alcohols, higher congeners are more influenced by yeast cell walls. This characteristic is well known, and it is widely exploited to prevent and resolve fermentation stucks (*12*).

Analogous considerations can be made as for ester compounds. Particularly interesting are the *iso*-amyl acetates, ethyl hexanoate, and ethyl lactate. Since these compounds were present in tested samples in concentration higher than the odor threshold, they play a very important role in sensory quality of the product (*27*). Unfortunately, it was impossible to quantify ethyl octanoate, as it coeluted with acetic acid.

Ethyl lactate was the most abundant ester, ranging from 16.3 mg/L (standard wine) to 29.4 mg/L (30-mg/L enzymed wine), thus exceeding the perception threshold (*27*). On the contrary, there were not significant differences between the samples treated with different amounts of enzyme. A similar consideration can be applied to γ-butyrolactone, as well. The lower quantity of enzyme was likely enough to promote ester enhancement, and a further enzyme increase was ineffective.

The correlation matrix (**Table 2**) shows many linear correlations among the different parameters. To simplify this complex pattern, only very high correlations have been considered ($|r| > 0.808$; $p \leq 0.001$).

A vast majority of substances were positively correlated, while acetaldehyde was the only substance with negative correlations with most of the other compounds.

Only a limited group of substances had no correlation: methanol, *cis*-3-hexenol, *i*-, and *n*-butyric acids. Among them, the absence of correlation for methanol and *cis*-3-hexenol is particularly interesting, as a further confirmation of their prefermentative origin. On the contrary, the *trans*- isomer is widely correlated with typical fermentative substances, such as alcohols, acids, and esters, supporting the evidence already discussed for this substance. Finally, ethyl and hexyl acetate were highly correlated to each other because of the common biochemical pathway, but did not show any other correlation.

These behaviors could likely be due to a yeast−cell wall effect. In other words, all the positively correlated compounds were affected by yeast lees in different ways. In the case of fermentative substances, such as acids, esters, and alcohols, a mechanism of release as a consequence of yeast cell wall maceration and breakdown was likely involved. These compounds, in fact, are produced by yeast metabolism and cross the cell membrane with an intimate interaction with cell wall constituents. In other cases, lees merely adsorb analytes, thus extracting these compounds from the medium, with no actual participation in their synthesis. This is the case of ethyl lactate.

Finally, the tiny molecular dimensions of acetaldehyde and its high water solubility can explain its negative correlation with all those substances that were positively influenced by the presence of lees.

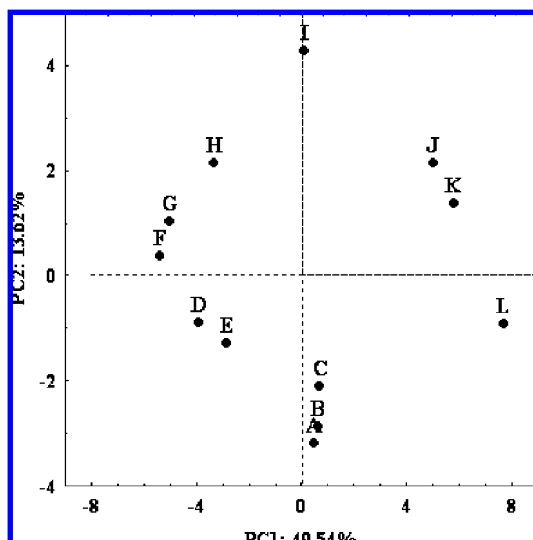

Figure 2. Principal component analysis of samples. Plot of the first two principal components (PC1 and PC2) with the explained variance in parentheses are reported. A, B and C, control samples; D, E, and F, 30 mg/L enzyme treated samples; G, H, and I, 60 mg/L enzyme treated samples; J, K, and L, standard samples.

Table 3. Loading Scores for the First 2 Principal Components

principal component	compound[a]	factor loadings
PC1	acetaldehyde	0.84
	n-butanol	−0.97
	3-methylthio-1-propanol	−0.85
	3-ethoxy-1-propanol	−0.94
	2-phenylethanol	−1.00
	benzyl alcohol	−0.88
	n-hexanol	−0.88
	trans-3-hexenol	−0.96
	4-ethylguaiacol	−0.84
	4-vinylphenol	−0.94
	2-methylbutyric acid	−0.96
	hexanoic acid	−0.99
	octanoic acid	−0.99
	decanoic acid	−0.95
	i-amyl acetates	−0.93
	ethyl 3-hydroxybutyrate	−0.94
	ethyl hexanoate	−0.97
	diethylsuccinate	−0.91
	ethyl lactate	−0.93
	γ-butyrolactone	−0.84
PC2	pH	−0.84
	cis-3-hexenol	0.83
	ethyl 4-hydroxybutyrate	0.88

[a] Only compounds with a loading score (absolute value) higher than 0.80 have been included.

Principal Component Analysis. PCA (**Figure 2**) was applied to the data matrix constituted by three replicated measures of each wine sample obtained by different treatments. The total variability of the sample set (73.06%) was explained by the first 3 PCs.

The loading scores (**Table 3**) suggest that 20 variables (loading value >0.80) were able to explain the variability among the samples.

The selected variables were nearly all loaded on the first component (PC1) showing loading values ranging from −0.83 to −1.00. Only pH, with a negative weight, *cis*-3-hexenol and ethyl-4-hydroxybutyrate, with positive weight, were loaded on the second component (PC2).

Samples distribution in the plane formed by the first 2 PCs is shown in **Figure 2**. Along the first component, the samples were clearly grouped in 3 clusters. Standard samples not aged on lees (J, K, and L) were grouped in a single cluster on the right part of the plot, while the enzyme treated samples (D, E, F, G, and H) are on the other side of the plot grouped into a more scattered cluster. Finally, enzyme untreated control samples (A, B, and C) were tightly grouped in the central part on PC1, aligned with sample I, as a consequence of a very close composition of this sample to those of control ones.

In a few words, the substances of PC1 explain more than half of the whole variability of the samples, while *cis*-3-hexenol, ethyl 4-hydroxybutyrate, and pH (PC2) are less important.

Aging on lees caused a general increase of volatile substances in *Bombino bianco* wine. The use of the enzyme preparation further enhanced this tendency. Almost all substances were influenced by the presence of yeast cell walls as well as by enzymatic action. No additional advantages were verified by doubling enzymatic concentration. Probably the lower concentration was so high that a further enhancement was ineffective.

The main differences occurred for ethyl esters, short chain fatty acids, and in general all the secondary products of fermentation that show poor water solubility. Lees maceration and enzyme action clearly promoted their detaching from the yeast cell wall.

The behavior of *n*-hexanol and *trans*-3-hexenol is particularly interesting because generally they are not considered to be involved in yeast metabolism. However, the great significant differences among the treated samples versus standard wine (neither enzyme nor lees) should indicate a more important involvement of C6 alcohols in side metabolic pathways. Further research on this topic is necessary to confirm this outcome. Methanol and other low molecular weight alcohols were not or very slightly influenced by lees and enzyme treatments.

These results suggest the effectiveness of this kind of enzyme preparation for the elaboration of wines that are richer in flavor, particularly for grapes lacking varietal aroma. In this case, fermentative aroma may compensate and characterize the scent of this kind of products. For these reasons, the use of this mild technology could represent a way to achieve a marketable choice wine instead of a blending wine, which is at the moment one of its main outlets.

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