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Use of *Trichoderma* enzymatic extracts on vinification of *Palomino fino* grapes in the sherry region

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Abstract

The influence of *Trichoderma* hydrolytic enzymes, with pectinase, cellulase, chitinase and/or glucanase activities, the supraextraction and the health stage of *Palomino fino* grape on juice clarification, fermentation process and the final characteristic of young wine were studied. The effect of enzymatic preparation on juice clarification depended of the grape sanitary stage (healthy or infected) and on the system applied to obtain the juice (frozen or fresh juices of grapes). The highest effectivity was shown with the use of enzymes in infected juice. The fermentation kinetics were not affected by the enzymatic preparations although a decrease in turbidity (below 20 NTU) was noted. Also, the highest differences in the wine characteristic were detected when comparing wines from juices subjected to different conditions (healthy of infected, frozen or fresh) independently of enzymes use. Supraextracted juices gave rise to wines with increased acidity and higher alcohols such as methanol, propanol and isobutanol.

Keywords: *Trichoderma* enzymes; *Palomino fino* grapes; Sherry young wine; Juice decantation; Fermentation kinetic; Supraextraction

1. Introduction

The Jerez area has traditionally been involved in high quality sherry wine production. Together with the specific edaphic conditions and climate, what stands out in the production process is the biological (fino sherry), physico-chemical (oloroso sherry) or a mixture of both processes (amontillado sherry) aging by the solera system (Martínez de la Ossa, Caro, Bonat, Pérez, & Domecq, 1987; Martínez de la Ossa, Pérez, & Caro, 1987). This aging has motivated for vineyards to be formed that are almost exclusively *Palomino fino* grape variety for the idoneous kind of grape for sherry elaboration (Puertas García, 1989).

Different procedures applied during the harvest and storage of the grapes, different fungal enzymatic preparations obtained from commercial sources or from *Trichoderma* strains have been added to enhance the decantation process. This rationale is based on the fact that the cell wall components slow down decantation and may retain aromatic compounds (Pretorius, 2000). These components, mainly pectin content depend on the grape variety, maturity and the technology used in the wine production (Pretorius, 2000; Visser & Voragen, 1996). Pectin makes the settling of particles and the clarification very slow. Pectinases are the enzymes with the most important technological effects. They enable the increase of the free run juice volume by decreasing its viscosity, and improve the clarification of the juice and the wine filtration. In association with cellulases, glucanases and hemicellulases, pectinases speed up the natural process of winemaking and improve the quality of the wine (Rhem & Reed, 1996; Visser & Voragen, 1996).

Enzymes being the grape and the yeast release the aromatic compounds of juice during fermentation

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(González, Ramón, & Pérez-González, 1992). This can also be achieved by exogenous commercial enzymes (Visser & Voragen, 1996) principally from the fungus *Aspergillus niger* which contain pectinases and glycosidases that release the major part of terpenols and lead to more fruity and aromatic wines.

However, commercial enzymes used in winemaking are mixtures containing different amounts of pectin esterases, polygalacturonases, pectin lyases and minor of hemicellulases, cellulases and other activities. Their exact nature and ratios have not yet been established which may be crucial in obtaining optimal performance in winemaking (Harman & Kubicek, 1998). Preparations higher in pectin lyase than in pectin methyl esterase are preferred so that methanol liberation from methylated polygalacturonic acid is minimized (Harman & Kubicek, 1998), but these preparations have a limited effect in the improvement of problematic juices such as obtained from *Botrytis cinerea* infected grapes. In this case, *Trichoderma* B-glucanases could be successfully used in the processing of wine (Dubourdieu, Desplanques, Villettaz, & Ribereau-Gayon, 1985; Harman & Kubicek, 1998). However, significant and reproducible improvement is therefore obtained only with a correct balance of exogenous activities added to complement the poor endogenous enzyme activities of the grape. This balance is found by experimenting with various enzyme mixtures. Some positive results have been obtained using a macerating enzyme preparation having a blend of activities (pectinases, cellulases, hemicellulases) from *Trichoderma* and *Aspergillus* over four successive vintages (Harman & Kubicek, 1998). Alternatively other authors have suggested that to speed juice extraction producing aromatic compounds, yeast can increase the liberation of varietal aroma by producing enzymes that hydrolyze both grape cell walls and the aromatic precursors in the grape juice (Dequin, 2001; Nevoigt et al., 2002; Ostergaards, Olsson, & Nielse, 2000).

In this work, the influence of *Trichoderma* hydrolytic enzymes on the supraextraction and the health stage of *P. fino* grape on juice clarification and fermentation process and the final characteristic of young wine were studied.

2. Materials and methods

2.1. Strains

Trichoderma harzianum Rifai strain CECT 2413 and *Trichoderma reesei* strain CECT 2414 were obtained from Colección Española de Cultivos Tipo, Burjassot, Valencia, Spain (Dawson, Belloch, García-López, & Uruburu, 1990). They were maintained on PPG agar medium (Limón, Pintor-Toro, & Benítez, 1998). A *Saccharomyces cerevisiae* wine strain isolated in Bodegas Domecq, S.A., Jerez de la Frontera, Spain (PDC strain) was used to carry out *P. fino* juice fermentations.

2.2. Enzymatic preparations and determination of enzymatic activities

A two-step culture was used to produce enzymatic extracts from minimal media supplemented with different carbon sources. *T. harzianum* and *T. reesei* were grown in Czapeck salt minimal medium supplemented with 2% glucose and then filtered mycelia were resuspended in 10 L containers with 5 L of the same minimal medium with either 0.5% crabshell chitin for *T. harzianum* cultures or 0.5% cellulose or 0.5 citrus pectin for *T. reesei* cultures, instead of glucose (Limón et al., 1998). The cultures were incubated for 3–5 days with magnetic stirring, following procedures described (Limón et al., 1998). The cultures were then filtered and the supernatants were brought to 80% saturation with ammonium sulfate. The resultant precipitates were collected and dialyzed and the extracts lyophilized and kept for further protein and enzyme analysis, according to procedures described (Limón et al., 1998).

Determination of dry weight and protein content present in the extracts, as well as protease, chitinase, cellulase, B-1,3-glucanase, B-1,6-glucanase, polygalacturonase, pectate-lyase and pectin methyl esterase activities were measured following procedures already described (De la Cruz et al., 1993; García-Maceira, 2000).

2.3. Juice preparation procedures

Samples of *P. fino* grapes were taken in the Jerez–Xérèz–Sherry D.O. wine production area. They were used in all the assays carried out, although their characteristics varied according to their sanitary state and the way the grapes were kept after being harvested and/or pressed: Healthy juice (HJ) corresponded to juice obtained from healthy grapes once pressed; infected juice (IJ) referred to juice obtained from *B. cinerea* infected grapes; frozen juice (FZJ) was the juice obtained from healthy grapes and kept frozen until its utilization; juice from frozen grapes (JFG) was the juice obtained from healthy grapes that were frozen after the harvest and kept frozen until their use for juice extraction once thawed (supraextraction). The samples collected, either fresh or frozen, were pressed mechanically to extract the juice. The sugar content oscillated between 11° and 12° Be and the total acidity oscillated between 5 and 8 g/L tartaric acid. Before decantation to eliminate solids in suspension, the juices were corrected for acidity to pH 3.2–3.3 and 80 mg/L SO_2 were added.

After the grape pressed juices were obtained, six samples of 3 L each were taken for each assay carried out. The samples were placed into 5 L glass containers and the enzymatic preparations obtained from *T. reesei*

grown in pectin—(E1) (10 mg), or cellulose—(E2) (40 mg), or *T. harzianum* grown in chitin-supplemented minimal media (E3) (25 mg), as well as two fungal commercial extracts, Ultrazym 100 G (E4) (10 mg), and Novoclairzym FCE G (E5) (2.5 mg) a pectolytic enzyme preparation used for juice decantation were added. Different amounts of each extract were added to get the same or near the same final enzyme units as the ones recommended in the commercial preparations. Controls with no enzymes added were also carried out (TG).

Once the enzymatic preparations were homogeneously distributed, the juice of each glass container was newly divided into two 2 L containers to carry out the experiments in duplicate. The containers were maintained at room temperature (20–22 °C) for 24 h to allow decantation of the juices to occur. The cleared juices were transferred to new glass containers and subjected to a direct fermentation by inoculating (in duplicate). During fermentation the alcohol content (by the decrease in density of the juice) and increase in cell biomass (by re-count) were determined every day to follow fermentation kinetics. After the fermentation process was completed, wines were clarified by storage at 4 °C for one week, the solids were removed, and the wines were maintained at the same conditions until the analytical and sensorial analyses were carried out.

2.4. Physico-chemical analyses

The turbidity (NTU) was determined with a 2100AN turbidimeter (Hach). The density was determined with a densitometer (Atom–Paar). The pH was determined with a pH-meter Crison 2001. The titratable acidity was determined by titration according to the method of the American Society of Enologists (Office International de la Vigne et du Vin, 1978). The alcoholic content was determined by distillation and later density meter. The volatile acidity was determined by the García Tena method (Métodos Oficiales de Análisis, 1976). Absorbances at 420, 340 and 280 nm were determined with a UV–visible spectrophotometer (Perkin–Elmer 200, Perkin–Elmer Corp., Norwalk, CT). And Na^+ and K^+ cations were measured by atomic absorption with a Pye Unicam SP9 atomic absorption spectrophotometer.

2.5. Glycerol and organic acid determination

The organic acids (citric, tartaric, malic, succinic and lactic acids) and glycerol were determined, in samples previously filtered, by HPLC in a Waters chromatograph. The chromatographic conditions were: a ION 300 column prewarmed at 50 °C, a mobile phase of 0.005 M H_2SO_4, a 0.3 mL/min flux and 60 min chromatogram time. A PDA detector at 210 nm to determine organic acids and a refraction index to determine glycerol respectively, were used.

2.6. Volatile compound determinations

Volatile compounds (acetaldehyde, di-acetyle, ethyl-acetate, methanol, *n*-propanol, isobutanol, isoamyl-acetate, 2-methyl-butanol and 3-methyl-butanol) were determined by gas chromatography, through direct injection of distilled samples using 4-methyl-2-pentanol as an internal standard. The chromatographic conditions used were a Carbowax 20 column (50 m, 0.25 mm ID, 0.25 μm), 250 °C injector, 250 °C detector and H_2 as carrier gas; the temperatures programmed were 35 °C for 10 min, 4 °C/min (200 °C) and 200 °C 5 min.

2.7. Terpenes determination

To determine terpenes (terpineol, linalool, citronellol, nerol and geraniol) an extractive procedure in solid phase as a preconcentration technique was first applied, previous to terpene analysis by gas chromatography. To do so 600 mg Sep-Pack C18 were pretreated in the following way: 5 mL methanol, 5 mL bi-distilled water and 25 mL sample were mixed and the remaining water was eliminated by bubbling N_2. The terpenes were then extracted with 5 mL of di-ethyl-ether. The chromatographic conditions were an MFE-1000 column (50 m, 0.25 mm ID, 0.25 μm), 250 °C injector, 275 °C detector, H_2 as carrying gas and the programmed temperatures were 80 °C for 1 min, 4 °C/min to 200 °C, and 200 °C for 20 min. 2-Octanol was used as internal standard.

2.8. Sensorial analyses

The final products (young wines) obtained from each of the fermentations carried out were assessed by five professional testers with regards to their organoleptic properties, mostly color and aromatic features. Precisely, they were asked to indicate the most significant features shown by each wine and those features which may differentiate wines from each other. The main parameters to be considered were wine color, intensity, acidity, intensity of aromas, and aroma differences between floral, fruity and herbaceous.

2.9. Statistical analyses

Results of the physico-chemical analyses were processed with the statistical program STATWIN 4.0. A cluster analysis was carried out in order to establish whether or not the different wines could be gathered according to the treatment to which they had been subjected (different procedures for juice formation, addition or not of enzymatic preparations etc.).

3. Results and discussion

3.1. Extracts enzymatic activities

Supernatants from *Trichoderma* strains cultures, once precipitated, dyalyzed and lyophilized (Ait-Lahsen et al., 2001) were weighed, and enzyme and protein determinations were further carried out. Data shown are averages of three experiments with standard deviations of less than 5%. Dry mass obtained were 850 mg from the pectin supplemented medium, with a protein content of 9.4%; the cellulose-supplemented media produced 150 mg of dry mass, and its protein content was 8.1%; finally, the chitin-supplemented media gave rise to 250 mg dry mass and a protein content of 5.1%. Table 1 shows the enzymatic activities detected in the supernants of *Trichoderma* strains grown in chitin (CHS) (E3), pectin (PCS) (E1) and cellulose (CLS) (E2) supplemented media. Whereas PCS and CLS enzyme preparations posses pectate-lyase, cellulase and B-1,3-glucanase activities, CHS lacks pectinases. Instead, CHS had B-1,3 and B-1,6 glucanase, protease and chitinase activities (Table 1). PCS and CLS seemed to be better enzyme preparations than CHS to be added to juices. In addition, no pectin-methyl-esterase activity was detected in any enzymatic preparation.

3.2. Effects on juice decantation

As can be observed in Table 2, the initial turbidity varied enormously in the different juices. The juices infected with *B. cinerea* (IJ) displaying the highest values. This result already indicates that the solid content varied according to the sanitary stage of the grapes and the storage and extraction procedures that had been applied to the juices. Due to this initial variability, the effectiveness of the static decantation was also highly variable, as indicated by comparing decantation of the juices with no enzymes added (TG). In all juices except that infected with *B. cinerea* (IFJ), turbidity decreases rapidly, reaching values of 200 NTU or lower. Therefore, static decantation is effective enough in all cases, with acceptable values (50–200 NTU final turbidity values) (Dubordieu & Ollivier, 1989; Visser & Voragen, 1996), except IFJ, where a high concentration of solids, and of B-glucans (Dubourdieu et al., 1985) have been liberated to the media, impeding clarification.

The role that enzymatic preparations play on juices also depends on the procedure applied to each juice specifically. No differences were detected when comparing the addition of E1, E2, E4, E5 and the control with no enzymes added (TG) to fresh (HJ) or frozen juices (FZJ) obtained from healthy grapes. A significant positive effect on juice clarification was observed with enzymatic commercial preparations (E4 and E5) added to juice obtained from frozen grapes (supraextraction) (JFG), and with *Trichoderma* (E1 and E2) and commercial (E4 and E5) enzymatic preparations when they were added to juices infected with *B. cinerea* (IFJ). This mold attacks nearly-ripe grapes producing a high molecular weight colloidal polysaccharide, a B-1,3-glucan with short side chains linked through B-1,6-bonds, that causes great difficulties in wine filtration (Dubourdieu et al., 1985; Harman & Kubicek, 1998). The presence of glucanase activities in E1 and E2 may be partly responsible for the positive effect detected in the IFJ juice decantation. Glucanases of E1 and E2 enzyme preparations may also help to hydrolyze other glucans such as those from lysed yeasts that can also cause serious clarification and filtration problems (Harman & Kubicek, 1998). Both in JFG and IFJ, E5 commercial enzyme preparation showed the highest effectivity, followed by the second commercial enzyme preparation used, E4. As indicated in Section 2, E5 is a mixture of pectinases and cellulases used to clarify juices and E4

Table 1
Enzymatic activities[a] detected in the lyophilized supernatants of *Trichoderma* strains, grown in minimal medium supplemented with either pectin (E1), cellulose (E2) or chitin (E3)[b,c]

Activity	E1	E2	E3
Pectate-lyase	0.045	0.004	0.000
Polygalacturonase	0.000	0.000	0.000
Pectin methyl esterase	0.000	0.000	0.000
Cellulase	0.112	0.479	0.000
β-1,3-Glucanase	0.055	0.214	0.413
β-1,6-Glucanase	0.000	0.039	0.483
Protease	0.000	0.000	0.115
Chitinase	0.000	0.000	0.102
Xylanase	0.000	0.000	0.000

[a] Activity is expressed as nmol/min/μg dry mass. Results are the average of three experiments in triplicate, with standard deviations of less than 5%.
[b] The protein content was 9.4% (E1), 8.1% (E2) and 5.1% (E3) dry mass.
[c] The commercial enzyme preparations Novoclairzym (E4) and Ultrazym (E5) have polygalacturonase (0.302 and 0.029 respectively) and cellulase (0.080 and 0.082 respectively) activities. E5 also has xylanase activity (0.752), defined as in (a).

Table 2
Effects of enzyme preparation on turbidity (NTU) of the different juices after decantation

Enzyme preparations	HJ[a] (1900)[e]	FZJ[b] (1795)[e]	JFG[c] (311)[e]	IJ[d] (2500)[e]
E1	73	32	215	178
E2	–	35	218	–
E4	87	36	25	229
E5	71	34	19	168
Tg	76	34	218	494

[a] HJ, juice from healthy grapes.
[b] FZJ, frozen juice from healthy grapes.
[c] JFG, juice from frozen healthy grapes (supraextraction).
[d] IJ, juice from fresh grapes infected with *B. cinerea*.
[e] Data in parenthesis indicated initial turbidity.

is a mixture of the former enzymes plus xylanases used to liberate aromatic compounds.

3.3. Effects on fermentation kinetics

Preliminary results obtained after adding the E3 enzymatic preparation to juice gave rise to a slight delay in fermentation, and above all, a decrease in the final yeast cell number as compared to the control with no enzymes added (TG) (data not shown).

Enzyme preparations may have a direct negative effect on yeasts due to the presence of glucans as components of the yeast cell walls (Dequin, 2001; Pretorius, 2000), and an indirect effect due to the removal of essential nutrients during solid precipitation (Martínez, Valcárcel, Pérez, & Benítez, 1998). To detect any of these possible effects of fungal hydrolytic preparations on yeasts during fermentation, frozen juice (FZJ) and juice from frozen grapes (JFG) were chosen. FZJ was selected because apparently there does not seem to exist major differences in this juice prior and after decantation; JFG (supraextraction) was selected because after adding the commercial enzyme preparations E4 and E5, the final turbidity reached was far too low (Table 2) and therefore the juice may be low nutrient and not suitable in order to get appropriate fermentation kinetics (Visser & Voragen, 1996). Low-nutrient juices give rise to a delay in the start of fermentation, the fermentation may take a long time or it ever may stop (Pretorius, 2000), so that turbidity not lower than 100–150 NTU is desirable to allow a good alcoholic fermentation (Visser & Voragen, 1996). However, when JFG (supraextraction) was fermented, the lag period was shorter and the yeast growth rate slightly higher than those parameters in JFG (Table 3). It may happen that during freeze and thaw of JFG, grape cell walls get very seriously damaged, liberating higher amounts of cell components (González et al., 1992) that become nutrients for the yeast cells. Furthermore, the addition of the commercial enzyme preparations E4 and E5 to JFG reduces considerably the NTU value, below advisable values (Table 2). However, neither delay in the start of fermentation nor decrease in the rate of fermentation as compared to the juice with no enzymes added (TG) was observed (Table 3), indicating that soluble nutrients that have not been removed during decantation are present in concentrations appropriated to allow optimal fermentation kinetics.

Table 3
Effects of enzyme preparation on fermentation kinetics

Enzyme preparations	Lag phase (days)		Growth rate (h^{-1})	
	FZJ	JFG	FZJ	JFG
E1	2.3	1.8	0.264	0.329
E2	2.3	1.8	0.274	0.334
E4	1.4	1.8	0.259	0.326
E5	2.3	1.8	0.261	0.326
Tg	2.3	1.7	0.264	0.341

FZJ: frozen juice from healthy grapes; JFG: fresh juice from frozen healthy grapes (supraextraction).

The presence of hydrolytic enzymes such as pectinases and cellulases may also liberate and solubilize pectic, cellulosic, and other monomers and oligomers (Visser & Voragen, 1996) which could be assimilated by the wine yeasts as carbon, nitrogen and/or energy sources, to carry our the fermentation under optimal conditions. In support of this suggestion, Varela, Calderón, Suárez, and Garcia (1997) already reported that addition of commercial enzymes (E4 and E5) to juices gave rise to a decrease in the total nitrogen content present in the juices, but almost no variation was observed in either the concentration of aminic nitrogen or in that of easily assimilable nitrogen sources.

3.4. Effects on wine physico-chemical characteristics

The effects of adding fungal hydrolytic enzymes to juices on the properties of the wines finally obtained, after juice fermentation and clarification, were studied in the frozen juices (FZJ) and juices obtained from frozen grapes (JFG) (supraextraction) and the results obtained were subjected to statistical analyses. Applying a cluster analysis (Fig. 1), in which it was taken into account all the parameters that were analyzed in the wines, it was observed that wines obtained from juices with different characteristics (healthy versus infected, fresh versus frozen, etc.) can be gathered into two blocks, independently of whether or not fungal enzymes were added or the origin (commercial, obtained in the laboratory) of these enzyme preparations.

Also, when the average values (obtained from the different parameters analyzed among the enzymes of each group, reflected in the cluster analysis) were compared, significant differences were detected between the two blocks (Table 4). Juices obtained from frozen grapes (JFG) (supraextraction) possess a substantial increase in total acidity with regards to the remaining juices. This increase in acidity is the result of the high content of organic acids, mainly citric and malic acids present in these juices. In addition, higher Na^+ and K^+ concentrations and glycerol, lower ethanol, and significantly higher concentrations of methanol, *n*-propanol and isobutanol, and lower concentrations of acetaldehyde, ethyl-acetate and isoamyl-acetate were also detected. Supraextraction, resulting from grapes which have been first frozen and then thawed, produces very seriously damaged skin tissues. Compounds very relevant for wine color and aroma such as polyphenols, organic acids or cations are present mostly in the skin (Harman & Kubicek, 1998; Visser & Voragen, 1996), and the supraextraction procedure allows all these compounds to be liberated from the skin, contributing to wine color and aroma. On the

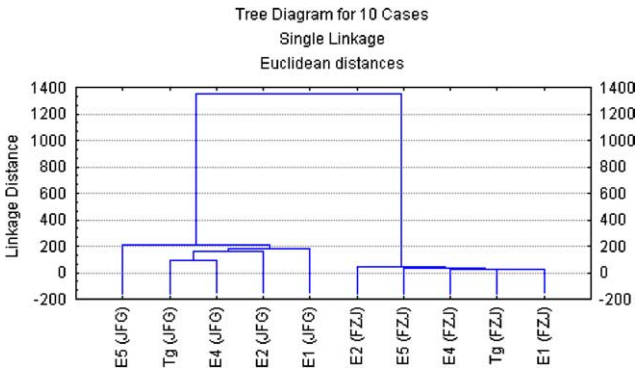

Fig. 1. Diagram of cluster.

Table 4
Parameters measured in FZJ and JFG (average values ± standard deviations) ($n = 10$)

	FZJ	JFG
Density	0.9893 ± 0.0001	0.9922 ± 0.0001
pH	3.05 ± 0.07	2.94 ± 0.01
Ethanol (% v/v)	12.0 ± 0.1	10.9 ± 0.2
AT (g/L TH_2)	5.39 ± 0.08	7.90 ± 0.01
AV (g/L AcH)	0.42 ± 0.08	0.19 ± 0.03
Absorbance A420	0.095 ± 0.004	0.115 ± 0.006
Absorbance A320	2.620 ± 0.035	1.275 ± 0.042
Absorbance A280	2.605 ± 0.007	2.880 ± 0.007
Na	13.12 ± 1.53	82.95 ± 10.05
K	639.20 ± 10.05	2225.00 ± 220.92
Glycerol (g/L)	5.64 ± 0.37	6.76 ± 0.21
Citric acid (mg/L)	113.56 ± 0.22	443.00 ± 0.32
Tartaric acid (g/L)	3.32 ± 0.22	4.53 ± 0.25
Malic acid (mg/L)	211.22 ± 0.12	547.20 ± 25.57
Succinic acid (g/L)	1.03 ± 0.09	2.25 ± 0.06
Lactic acid (g/L)	1.15 ± 0.28	nd
Acetaldehyde (mg/L)	105.47 ± 4.49	58.16 ± 14.84
Ethyl-acetate (mg/L)	23.78 ± 1.64	20.73 ± 4.48
Di-acetyle (mg/L)	3.93 ± 0.78	5.32 ± 1.63
Methanol (mg/L)	31.73 ± 2.30	40.69 ± 2.20
n-Propanol (mg/L)	15.55 ± 0.61	31.37 ± 1.56
Isobutanol (mg/L)	19.43 ± 0.21	36.06 ± 2.86
2-Methyl-butanol (mg/L)	27.17 ± 1.27	18.79 ± 3.77
3-Methyl-butanol (mg/L)	136.64 ± 3.45	108.27 ± 19.01
Terpenes (mg/L)	2.14 ± 0.92	2.54 ± 0.52

nd: Non-detected; FZJ, frozen juice from healthy grapes; JFG, fresh juice from frozen healthy grapes (supraextraction).

other hand, more pectins and pectic oligomers are extracted from the cell tissues formed mostly by cellulose and pectins (Visser & Voragen, 1996). Pectin and pectic oligomers are induced and substrate of the pectolytic system, among them pectin methyl esterases, which would act on pectic residues liberating methanol. Similarly, nitrogenous residues are liberated from damaged skins in great amounts. These compounds are precursors of higher alcohols such as isobutanol and *n*-propanol, decreasing the concentration of other alcohols such as isoamyl-alcohol (González et al., 1992; Pretorius, 2000).

The results obtained indicate that the different procedures used for juice extraction (frozen or fresh grapes, frozen or fresh juices) seem to be more relevant with regards to the final characteristics of the wine than the addition of hydrolytic enzymes. However, in the supraextraction process there were significant differences according to whether or not enzyme preparations were added and their origin, so that commercial enzyme preparations appear closely gathered (Fig. 1). Wines obtained after fermentation of juices obtained by supraextraction (FGJ), and further addition of commercial enzyme preparations (E4 and E5) possess characteristics and composition very similar and totally different from the characteristics shown by wines obtained from FGJ with no enzymes added (TG) or with the addition of non-commercial enzyme preparations (E1 and E2) (Fig. 1).

Table 5 shows the average data obtained after analyzing these wines resulting from supraextracted juices (FGJ) with no enzyme addition (TG) or after adding commercial (E4 and E5) or non-commercial enzymes (E1 and E2). Wine from juices (FGJ) treated with commercial enzymes (E4 and E5) had lower concentrations of acetaldehyde, ethyl-acetate and di-acetyle and higher concentrations of 2- and 3-methyl-butanol and phenethyl-alcohol than wines from juices (FGJ) with no enzymes added (TG) or treated with *Trichoderma* enzyme preparations (E1 and E2); on the other hand, wines from juices (FGJ) treated with *Trichoderma* enzyme preparations (E1 and E2) possessed higher levels of malic acid, acetaldehyde, ethyl-acetate and isoamyl-acetate, this latter compound being detectable only in this case. The remaining parameters that were measured (Table 5) showed similar values in wines from juices (FGJ) with no enzymes added or treated with any enzyme preparation, commercial or no, what indicates that their concentration does not depend on enzymatic treatments. According to Revilla, Pérez-Magariño, and Gonzalez-Sanjosé (1997), juices treated with pectolytic enzymes have higher concentrations of methanol. However, this is not the case in the wines analyzed in this study. A possible reason accounting for this result is that the methylation percentage of the grape's pectin is very low, so that polygalacturonases but no methyl esterases are responsible for the decrease in the juice viscosity (Harman & Kubicek, 1998) with almost no formation of methanol. However, both juices with or without enzymes added had a similar methanol content, whereas the characteristics of the juices or the procedures of extraction do establish significant differences in methanol contents and the concentration of many other components (Table 5).

According to the enzyme preparations used in this study, both commercial (E4 and E5) and obtained from *Trichoderma* strains (E1 and E2) had cellulolytic, glucanolytic and/or pectolytic activities (E1, E2, E4 and E5) (Table 1). Also, it was expected aromatic compounds in general and terpenes in particular would

Table 5
Composition of wines obtained from JFG treated with different enzyme preparations, or with no enzymes added (TG) (average values ± standard deviations)

	Commercial ($n = 4$)	*Trichoderma* ($n = 4$)	TG ($n = 2$)
Density	0.9923 ± 0.0001	0.9922 ± 0.0001	0.9923 ± 0.0001
pH	2.95 ± 0.003	2.94 ± 0.003	2.95 ± 0.001
Ethanol (% v/v)	10.8 ± 0.3	11.1 ± 0.1	10.8 ± 0.0
AT (g/L TH_2)	7.90 ± 0.00	7.90 ± 0.00	7.87 ± 0.00
AV (g/L AcH)	0.187 ± 0.001	0.217 ± 0.001	0.155 ± 0.035
Absorbance A420	0.113 ± 0.001	0.110 ± 0.011	0.112 ± 0.004
Absorbance A320	1.315 ± 0.010	1.268 ± 0.020	1.220 ± 0.008
Absorbance A280	2.885 ± 0.001	2.877 ± 0.010	2.874 ± 0.019
Na	80.6 ± 0.78	76.87 ± 5.90	99.80 ± 17.68
K	2414.7 ± 148.8	2019.0 ± 123.04	2257.5 ± 77.07
Glycerol (g/L)	6.81 ± 0.24	6.60 ± 0.11	7.0 ± 0.03
Citric acid (mg/L)	443.00 ± 0.03	417.00 ± 0.02	490 ± 0.03
Tartaric acid (g/L)	4.78 ± 0.04	4.37 ± 0.04	4.56 ± 0.13
Malic acid (mg/L)	533.00 ± 0.01	570.00 ± 0.03	530.00 ± 0.00
Succinic acid (g/L)	2.28 ± 0.06	2.19 ± 0.01	2.31 ± 0.09
Acetaldehyde (mg/L)	43.58 ± 19.26	68.51 ± 6.60	66.60 ± 0.41
Ethyl-acetate (mg/L)	16.87 ± 1.22	25.51 ± 0.19	18.90 ± 3.91
Di-acetyle (mg/L)	3.70 ± 0.73	5.95 ± 0.39	7.30 ± 0.06
Methanol (mg/L)	39.84 ± 2.50	41.92 ± 2.85	39.94 ± 1.29
n-Propanol (mg/L)	32.27 ± 0.95	31.77 ± 0.39	28.78 ± 1.05
Isobutanol (mg/L)	38.57 ± 2.28	34.27 ± 2.50	34.59 ± 1.61
Isoamyl-acetate (mg/L)	nd	4.32 ± 2.18	nd
2-Methyl-butanol (mg/L)	22.92 ± 0.45	16.24 ± 0.33	15.65 ± 4.26
3-Methyl-butanol (mg/L)	128.80 ± 2.60	96.96 ± 0.92	89.83 ± 27.39
Ethyl-lactate (mg/L)	2.79 ± 1.98	2.77 ± 1.96	2.49 ± 1.76
Phenethyl-alcohol (mg/L)	14.14 ± 0.04	9.78 ± 3.39	10.57 ± 2.21
Terpenes (mg/L)	2.02 ± 0.04	3.04 ± 0.13	2.58 ± 2.70

nd: Non-detected; JFG: juice from frozen healthy grapes (supraextraction).

increase considerably in wines from juices treated with these enzyme preparations, as compared to those from juices with no enzymes added (TG). Precisely, the commercial enzymatic preparation E5 is recommended after fermentation in order to increase the level of glycosilated aromatic compounds.

The differences in the wines terpene content were not significatives. These results can indicate that, during fermentation, terpenic compounds are abundantly liberated, being probably steadily maintained in the wine in a soluble form. If this terpene liberation takes place too early, prior to fermentation, there seems to occur a loss of these terpenes which are probably removed by CO_2 emission or converted into cyclic compounds by biotransformation reactions (King & Dickinson, 2000). Supporting this suggestion, it has been reported that, due to biotransformation reactions during fermentation, free terpenoids are not direct products of the corresponding bound terpenoids (King & Dickinson, 2000; Pretorius, 2000).

3.5. Effects of addition of fungal hydrolytic enzyme preparations on the organoleptic characteristics of the wines

Tests were carried out by five professionals to determine the organoleptic properties of wines from juices treated or not with the different enzyme preparations, mostly color, intensity of aroma and characteristics of aroma such as fruity, floral and herbaceous. Results indicated that there were no significant differences or peculiarities among the different wines with regards to either these sensorial features.

As indicated above, terpenoids are compounds with strong sensory qualities, and important indicators of varietal differences in grapes. The *Muscat* variety in particular has a high level of linalool which is thought to be responsible for the grape-like aroma of wines produced from this grape (King & Dickinson, 2000). On the contrary, young wines obtained from the *P. fino* grape do not possess fruity flavour. For either reason, biotransformation or lack of abundant precursors in this variety and the addition of fungal enzymes does not seem to dramatically increase terpene content and fruity aroma of wine.

4. Conclusions

The role that enzymatic preparations play on juice decantation depends on the sanitary stage of grape and the procedure applied to extract each juice. However, commercial enzyme preparations showed the greatest effect.

Even though the addition of the enzyme preparations considerably reduces the turbidity (<100 NTU), the decantation process is favoured. Therefore, the soluble nutrients are present in concentrations to allow optimal fermentation kinetics.

The characteristics of the final wine depend of the different process used for juice extraction (frozen or fresh grape, frozen or fresh juice), but independently of the hydrolytic enzymes. The supraextraction process lead to a increase in acidity and to a higher concentrations of Na^+, K^+, methanol, *n*-propanol and isobutanol. The concentrations of these components are related to the compounds extracted from skin grape when damage skin tissues are produced.

According to the sensorial analysis there were no significant differences among the wine with no enzyme addition and treated with enzyme extracts, therefore the addition of fungal enzymes does not increase terpene content and the fruity aroma of wine.

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