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Conventional and enzyme-assisted autolysis during ageing over lees in red wines: Influence on the release of polysaccharides from yeast cell walls and on wine monomeric anthocyanin content

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Abstract

HPLC with refractive index detection (HPLC/RI) was used to study the autolytic release of polysaccharides from the cell walls of six strains of *Saccharomyces cerevisiae* and the commercial yeast species *Sacch. uvarum* in a model medium over a nine month period of ageing over lees. The effect of adding β-glucanase was also studied. In the presence of this enzyme autolysis was complete within 2–3 weeks; conventional autolysis needed to proceed for at least five months before large quantities of released polysaccharides could be detected. The final polysaccharide profile of the model medium obtained by enzyme-assisted autolysis was, however, different to that obtained by conventional autolysis, with more fragments of smaller molecular weight and a greater grouping of molecular sizes in the HPLC/RI peaks. The influence on the monomeric anthocyanin content of commercial red wines was examined by HPLC with photodiode array detection (HPLC/PDAD). Ageing over lees without enzymes appeared to have a protective effect on the total monomeric anthocyanin content, while the use of β-glucanase led to a large reduction in their concentration, probably via the undesirable activity of β-glucosidase impurities.
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1. Introduction

Ageing over lees is relatively new to red wine production. The wine ages over the cellular remains of the yeasts that fermented it; it therefore becomes enriched in volatile aromatic compounds, and its density is increased through the release of high molecular weight polysaccharides from the cell walls of the dead yeasts. Historically, ageing over lees has been employed in the manufacture of white wines fermented in barrels (Bourgogne wines), natural sparkling wines (Champagne, Cava), and aged biological wines produced with *flor* yeasts (Sherry). Currently, the technique is being used by many wineries to make red wines since it affords good quality products of better structure, aromatic profile and colour stability.

Ageing over lees begins with the autolysis of the yeasts. This involves the hydrolysis of their cell walls and the release of their biopolymers and other molecules into the medium (Babayan et al., 1981; Babayan & Bezrukov, 1985). The cell wall of *Saccharomyces cerevisiae* is made of mannoproteins crossed by fibres of glucan and chitin (Pretorius, 2000). During autolysis, the cell wall is gradually degraded through the breakage of these glucan and chitin fibres; a task performed by the enzymes belonging to the dead yeasts themselves. These include glucanases (which are present in the cell walls for up to four months after the death) and mannosidases (Charpentier & Freyssinet, 1989). As a consequence of this degradation, the cell

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walls become less rigid and polysaccharides are released (Feuillat, 1998). Hydrolysis of the proteins increases the content of nitrogenous compounds in wine (Lurton, Segain, & Feuillat, 1989; Sato et al., 1997). In addition, the lipids released from the lees enrich the aromatic fraction of the wine via the formation of esters and aldehydes (Pueyo, Martínez-Rodríguez, Polo, Santa-María, & Bartolomé, 2000).

Some authors define a yeast's capacity for autolysis as the quantity of nitrogen released into the medium per unit time and gram of yeast dry weight (Charpentier, Nguyen Van Long, Bonaly, & Feuillat, 1986). Suzzi (1990) studied the aptitude for autolysis in different strains of *Saccharomyces* in terms of the maximum quantities of aminoacids released. The literature indicates that protease activity varies considerably depending on the yeast strain (Arizumi et al., 1994). The polysaccharides released act as stabilizers against tartaric (Lubbers, Charpentier, Feuillat, & Voilley, 1994) and proteic (Moine-Ledoux, Perrin, Paladau, & Debourdieu, 1997) precipitations. They also interact with the phenolic fraction, leading to a greater stability of the monomeric anthocyanin content (Saucier, Little, & Glories, 1997; Morata, Calderón, González, Colomo, & Suárez, 2005a). The affinity of the lees for oxygen, and its potential consumption of oxygen, are much higher than those of the polyphenol fraction of the wine (Fornairon-Bonnefond & Salmon, 2003; Salmon, 2005). This largely explains the greater stability of the pigment material and the lesser degradation of the anthocyanins in wines thus aged.

Many years ago, the winemaking industry developed commercial enzyme preparations with the aim of accelerating autolysis in wines aged over lees. These products are mixes of several enzymes such as β-glucanase and pectinase that considerably increase the polysaccharide concentration of both white and red wines (Pellerin & Tessarolo, 2001; Trione & Martínez, 2001). The β-glucanase enzymes authorised for use in winemaking are synthesised by species of *Trichoderma* (growing under conditions that optimise their production) (Humbert-Goffard et al., 2004).

Appropriate yeast selection may help optimise ageing over lees. By selecting strains that undergo rapid autolysis and which absorb anthocyanins poorly, Morata et al. (2005a) showed that shorter ageing times could be achieved, along with better wine structure and stability.

The aims of the present work were to study the release of polysaccharides from the cell walls of selected yeasts strains during autolysis, the accelerating effect of adding β-glucanase, and the effect of growth over lees on the stability of the monomeric anthocyanin content in red wine made from *Vitis vinifera* L. cv. Tempranillo grapes.

2. Methods and materials

2.1. Yeasts used in experimental aging over lees

The *Sacch. cerevisiae* yeast strains used were 9CV, 5CV, 4CV, 7VA, 3VA and 2EV, all of which were isolated and selected for red winemaking by the *Laboratorio de Enología*, *Dept. Tecnología de Alimentos*, *Universidad Politécnica de Madrid* (Madrid, Spain). The commercial *Sacch. uvarum* strain S6U was also used (Lallemand, Danstar Ferment, Montreal, Canada).

The yeast biomass for the over-lees ageing assays was obtained by fermentation in YEPD medium (Kurtzman & Fell, 1998) enriched with glucose to 100 g/l. This biomass was washed with sterile distilled water (ratio 10:1 v/v) and centrifuged (3000 rpm, 2 min). The supernatant was then removed. This operation was performed twice in order to obtain a yeast biomass with no nutrient impurities. These yeasts were then lyophilised and stored at 4 °C.

2.2. Autolysis in model media

To quantify the release of polysaccharides, ageing over lees in wine was simulated in a model medium composed of a water–alcohol solution (water: ethanol 9/1, v/v), acidulated to pH 3.5 with tartaric acid. Volumes of 50 ml of this medium were then placed in 100 ml Erlenmeyer flasks and 0.8 g l^{-1} of lyophilised yeast added. The polysaccharides released by each strain over time were then recorded. A control assay was established which contained no yeast.

In three further assays (with the yeasts 2EV, 7VA and 4CV) β-glucanase (LALLZYME MMX; Lallemad, Danstar Ferment, Montreal, Canada) was added at a dose of 50 mg/l, as recommended by the manufacturer.

All eleven assays were performed in triplicate. The Erlenmeyer flasks were maintained at 30 °C and subjected to weekly orbital agitation for 1 h to reproduce normal winemaking conditions (*bâtonnage*).

2.3. Recovery of polysaccharides from the model medium

Polysaccharides were recovered from 1 ml of autolysate (centrifuged at 5000 rpm for 15 min to remove the lees) by precipitation in an apolar acidic medium (ethanol:HCl, 5 ml 96% ethanol and 50 μl HCl 1 N v/v). Precipitation was facilitated by refrigerating the samples at 4 °C for 24 h. The precipitate was then centrifuged at 8000 rpm for 15 min and the supernatant discarded. The polysaccharides thus separated, were then washed three times with 1 ml of ethanol and dried in an oven at 40 °C. They were then resuspended in 1 ml of 0.1 M $NaNO_3$, filtered through a membrane filter (pore size 0.45 μm) (Teknokroma, Barcelona, Spain), and maintained refrigerated until analysis by HPLC/RI.

2.4. Analysis of polysaccharides by HPLC/RI

The polysaccharides released were analysed by HPLC (Doco, Brillouet, & Moutounet, 1996) (using a Waters chromatograph (Waters, MA) equipped with a 600E pump, a 717p injector and an RI 2412 detector), separating them in an ultrahydrogel 250 (Waters, MA) molecular

exclusion column and employing 0.1 M $NaNO_3$ in MilliQ water as an eluent. Detection was performed by measuring the refractive index.

The sizes of the polysaccharides released during autolysis were compared against those of known pullulan (polymaltotriose) standards (Shodex, Japan): P-100 (112 KDa), P-50 (47.3 KDa), P-20 (22.8 KDa), P-10 (11.8 KDa) and P-5 (5.9 KDa). The polysaccharide content was determined using a calibration curve constructed using these same standards. In the conventional autolysis assays in the model medium, polysaccharides were determined at 1, 3, 5, 7 and 9 months. In the assays with added enzymes, these determinations were made at 1, 2 and 3 weeks.

2.5. Wines

Vitis vinifera L. cv. Tempranillo grapes (a red variety) from the *Appellation Contrôllé* 'Ribera del Duero' (Spain) area were used to produce over-lees aged and enzyme-assisted over-lees aged wines in which the monomeric anthocyanin content was measured. The pH of the must was 3.7 and the sugar content 210 g/l. Lyophilised yeast (0.3 g/l) was then added and the change in monomeric anthocyanin content measured over 420 days.

2.6. Analysis of anthocyanins by HPLC with photodiode array detection (HPLC/PDAD)

The anthocyanins in the wines were analysed using a Waters (Milford, MA) HPLC chromatograph equipped with a 600-MS controller, a 717 plus autosampler, and a 996 photodiode-array detector. Gradients of solvent A (water/formic acid, 90:10, v/v) and B (methanol/formic acid, 90:10, v/v) were used in a reverse-phase Nova-pack C_{18} column (300 × 3.9 mm) as follows: 0–20 % B linear (0.8 ml/min) from 0 to 5 min, 20–50% B linear (0.8 ml/min) from 5 to 70 min, and re-equilibration of the column from 70 to 95 min. Detection was performed by scanning in the 500–600 nm range. Quantification was performed by comparison against an external standard at 525 nm and expressed as a function of the concentration of malvidin-3-*O*-glucoside (Extrasynthèse, Genay, France). 100 µl samples of previously filtered fermentations were injected into the HPLC apparatus.

The different anthocyanins were identified by their retention times with respect to the majority anthocyanin malvidin-3-*O*-glucoside, and by taking into account their UV–Vis absorption spectra (Monagas, Núñez, Bartolomé, & Gómez-Cordovés, 2003).

2.7. Colour measurements

The absorbance by the wine at 420, 520 and 620 nm was determined using a JASCO V-530 (Japan) spectrophotometer with a 1 mm path length quartz cell, following the Glories procedure (Glories, 1984a; Glories, 1984b). The colour intensity and hue were recorded.

2.8. Statistical analysis

Means, standard deviations, ANOVA and least significant differences (LSD) were calculated using PC Statgraphics v.5 software (Graphics Software Systems, Rockville, MD, USA). Significance was set at <0.05 (95.0% confidence level) for the ANOVA matrix F value. The multiple range test was used to separate the means.

3. Results and discussion

3.1. Release of polysaccharides during ageing over the lees of the selected yeasts

Table 1 shows the polysaccharide content of the model media after 1, 3, 5, 7 and 9 months of ageing over lees. In all assays a slow but gradual increase was seen. Significant differences ($p < 0.05$) were found to exist each month between some strains, but these results were not maintained over the ageing process. Therefore, it cannot be affirmed that some of the selected yeasts undergo autolysis more quickly than others.

The elution of polysaccharides in HPLC/RI occurred between minutes 7 and 10.5; however, these peaks were not resolved at the baseline since the methodology does not afford such high resolution. A clear peak was seen at approximately 7.3 min, and other less marked peaks at 8.4 and 9.5 min (continuous lines in Fig. 1). Comparisons with the molecular weight markers show these bands to correspond approximately to 120 000, 23 000 and 7000 Da (Morata, Calderón, Colomo, González, & Suárez-Lepe, 2005b). This is consistent with the random breakage of the cell wall, which produces fragments of different size. The greater quantity of polysaccharides seen in the ninth month corresponded mostly to an increase in the number of fragments of larger size (100.000–120.000 Da).

The study of polysaccharide release by HPLC/RI is complicated since this technique does not have the resolution of reverse phase chromatography. Separation is based on size, with longer polymers eluting before shorter ones. Separation of polymers of the same size, however, does not occur; rather, the peaks produced encompass polymers of similar size.

These difficulties render the repeatability between autolysis assays involving the same biomass of the same strain of yeast lower than those afforded by other chromatographic methods. This is logical given the random breakage of the cell walls, which is influenced by the resistance shown to the mechanical damage caused by agitation, temperature, and the release of lytic enzymes.

3.2. Effect of adding β-glucanase

The addition of β-glucanase to the model medium notably accelerated the process of autolysis. In just two weeks, amounts of polysaccharides similar to or even larger than those produced in nine months by the conventional method

Table 1
Polysaccharides released by conventional autolysis in the studied yeasts at 1, 3, 5, 7 and 9 months

Yeast strain	Expressed as mg/l of pullulans				
	1	3	5	7	9
9CV	0.13 ± 0.23^{a}	11.03 ± 1.60^{b}	9.47 ± 0.91^{a}	16.43 ± 1.61^{a}	36.83 ± 4.40^{c}
5CV	0.10 ± 0.17^{a}	9.53 ± 0.38^{b}	12.43 ± 0.95^{b}	17.07 ± 1.60^{a}	27.83 ± 2.97^{b}
4CV	0.23 ± 0.11^{a}	8.77 ± 0.40^{b}	12.33 ± 2.56^{b}	17.87 ± 1.87^{a}	23.67 ± 0.73^{ab}
7VA	0.37 ± 0.06^{a}	9.47 ± 0.70^{b}	12.57 ± 0.38^{b}	17.60 ± 2.22^{a}	21.77 ± 1.58^{a}
3VA	1.13 ± 0.58^{b}	9.33 ± 1.70^{b}	12.30 ± 0.75^{b}	24.63 ± 4.69^{b}	26.93 ± 3.78^{b}
2EV	0.07 ± 0.11^{a}	9.53 ± 0.60^{b}	12.57 ± 0.40^{b}	22.90 ± 0.95^{b}	26.17 ± 1.05^{ab}
S6U	1.13 ± 0.11^{b}	4.57 ± 2.34^{a}	11.30 ± 1.13^{ab}	18.20 ± 2.01^{a}	21.67 ± 1.33^{a}

Values are means ± standard deviations ($n = 3$). Means in the same column with the same letter are not significantly different ($p < 0.05$).

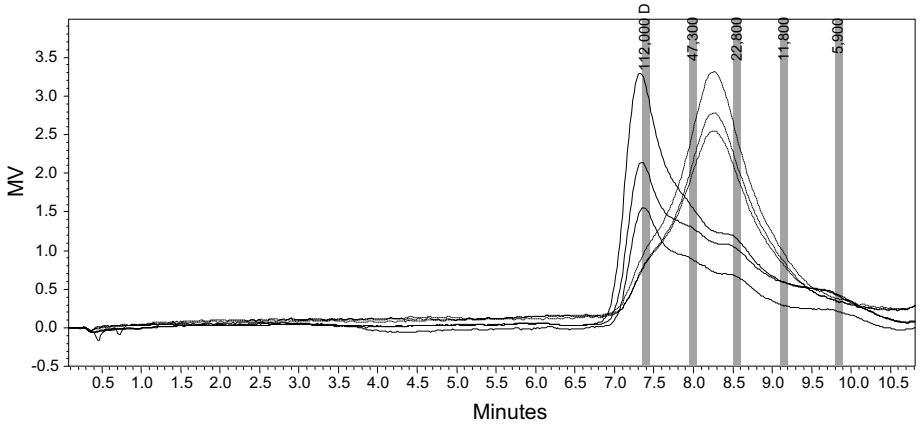

Fig. 1. HPLC/RI chromatograms of the polysaccharides produced by strain 9CV in conventional ageing over lees (AOL; continuous line) and after the addition of β-glucanase (β; dashed line); both assays were performed in triplicate. Also shown are the retention times (bands) for the molecular weight markers (pullulans).

were recorded (Table 2). When these enzymes were used, the repeatability between replicates was better. This is probably because the breakdown process is less random. In the first week practically no autolysis was seen (1.3–3.9 mg/l); the enzyme had not worked for sufficient time for the polysaccharides released to be detectable. From the second week, however, autolysis was well underway, leading to a notable release of polysaccharides in all the yeast strains studied.

In general, the process was the same for all the yeasts, except for strain 2EV. This released a greater quantity of polysaccharides; the final concentration was 111.6 mg/l compared to 53.1–78.8 mg/l for the other strains (differences which were significant at weeks 2 and 3 Table 2). This suggests that yeasts can be selected for cell wall compositions that allow more rapid autolysis, and which might favour the use of commercial β-glucanase. Studies of different strains of *Sacch. cerevisiae* that could be used in winemaking should be undertaken to determine their autolysis rates, whether they release more polysaccharides, and whether they allow the production of wines with better sensorial characteristics.

Table 2
Polysaccharides released by autolysis in the studied yeasts in the presence of β-glucanase at weeks 1, 2 and 3

Yeast strain	Expressed as mg/l of pullulans		
	1	2	3
9CV	2.70 ± 0.96^{b}	34.87 ± 4.69^{abc}	57.20 ± 10.44^{a}
5CV	2.50 ± 0.90^{b}	37.43 ± 0.96^{bc}	61.30 ± 10.29^{ab}
4CV	3.87 ± 0.71^{c}	44.63 ± 3.68^{c}	71.87 ± 16.24^{ab}
7VA	1.83 ± 0.06^{ab}	27.90 ± 3.83^{ab}	53.10 ± 5.44^{a}
3VA	1.37 ± 0.49^{a}	25.63 ± 4.96^{a}	53.80 ± 6.50^{a}
2EV	1.90 ± 0.26^{ab}	68.80 ± 11.38^{d}	111.60 ± 14.06^{c}
S6U	1.30 ± 0.17^{a}	35.80 ± 6.94^{abc}	78.77 ± 13.70^{b}

Values are means ± standard deviations ($n = 3$). Means in the same column with the same letter are not significantly different ($p < 0.05$).

Although the addition of β-glucanase led to a much more rapid breakdown of the yeast cell walls, the polysaccharide profile was not the same as that obtained with conventional ageing over lees. The fragments produced were usually smaller and more uniform in size.

Fig. 1 shows the existence of a band at a retention time of 8.2 min that corresponds to a molecular weight of approximately 35000 Da. The quantities of larger (120000 Da) and smaller (7000 Da) polysaccharides were much less than in conventional autolysis. This is to be expected since β-glucanase hydrolyses the glucan fibres in a more regular fashion, releasing fragments of more similar size. The effect of this at the sensorial level needs to be further studied since the influence of smaller polysaccharides on the properties of the wine might be less perceptible in the mouth than that of larger polymers released by conventional

autolysis. The colloidal stability of the wine could, however, be increased by these smaller polysaccharides, which would be less likely to precipitate.

3.3. Identification of anthocyanins and pyranoanthocyanins by HPLC/PDAD

Attention was paid to the following anthocyanins owing to their importance in terms of quantity and stability: malvidin-3-*O*-glucoside (M3G), malvidin-3-*O*-glucoside-pyruvate or vitisin A (VITA), malvidin-3-*O*-(6-acetyl)-glucoside (M3G6Ac), malvidin-3-*O*-(6-*p*-coumaroyl)-glucoside (M3G6Cm), and 4-vinylphenols (VPH1, VPH2, VPH3). The vinylphenolic derivatives are derived from pyranoanthocyanins of very stable structure and low polarity; they therefore elute after malvidin-3-*O*-(6-*p*-coumaroyl)-glucoside.

Normally, the aging of red wines involves the loss of monomeric anthocyanins since these degrade, turn into non-coloured forms, or become polymerised into more stable forms. However, these process are less likely to occur during ageing over lees since the polysaccharides and mannoproteins released during autolysis exert a protective effect on monomeric anthocyanins. The red–blue colour for which they are responsible therefore lasts longer.

The highest concentration of total anthocyanins was recorded at the first measurement (60 days) (Fig. 2). At this point, the samples to which β-glucanase was added showed an anthocyanin concentration 30% lower than that of the controls and than that of the wine in which conventional autolysis was allowed to proceed (see peak for M3G in Fig. 3). This is probably because commercial enzymes contain small quantities of β-glucosidase; this enzyme hydrolyses the glucose that esterifies the anthocyanins, leading to the production of less stable molecules (Wrolstad, Wightman, & Durst, 1994; Wightman & Wrolstad, 1996).

The tendency of all three enzyme-assisted assays in terms of total anthocyanin content was the same from the outset. After an initial period of activity (in this case two months), the influence of the enzymes on wine anthocyanin content decreased – probably due to their denaturation.

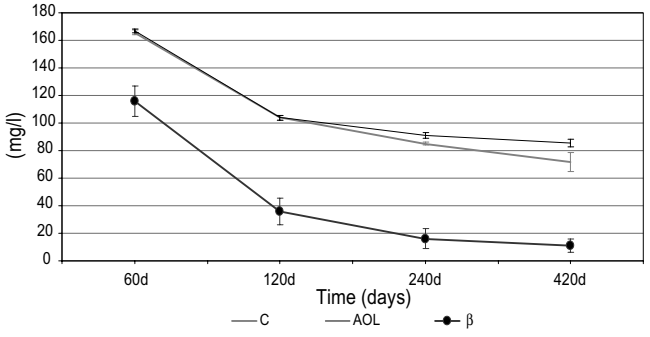

Fig. 2. Change in the concentration of total anthocyanins in the control assay (C), and in the means (SD shown as error bars; $n = 3$) for the samples aged conventionally over lees (AOL) for all strains, and those that underwent enzyme-assisted autolysis (β).

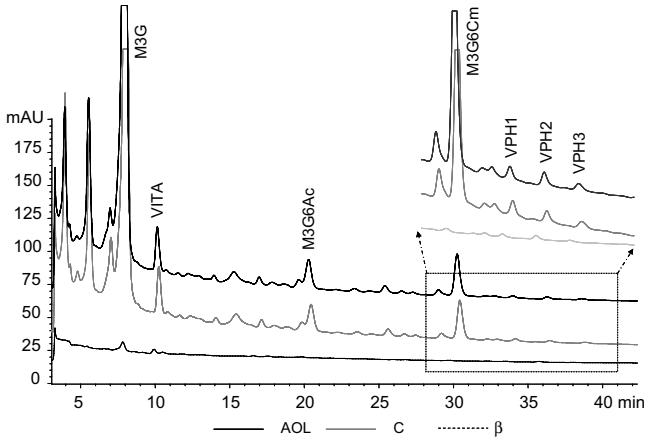

Fig. 3. HPLC/DAD chromatograms at 520 nm (day-420 of ageing) for the control sample (C) and the samples aged conventionally over lees (AOL) and with enzyme-assistance (β) with yeast 7VA.

Although the development of the controls and the conventional samples aged over lees were very similar for some time, differences began to appear at 120 days. The conventional samples aged over lees had significantly higher concentrations of total anthocyanins at 420 days (Table 3, Fig. 3), possibly due to the reducing nature of the lees.

Rosenfeld, Beauvoit, Blondin, and Salmon (2003) and Fornairon-Bonnefond and Salmon (2003) indicate that the indices of potential oxygen consumption for viable yeast cells and lees are much higher than that of the polyphenol fraction of wine. Vivas and Saint Cricq de Gaulejac (2000) obtained less oxidised wines when these were aged over lees, and attributed this to the release of cell wall components during autolysis. It is also reported that lees impede the oxidation of phenolic compounds during ageing (Salmon, 2005). The release of polysaccharides during autolysis and the competition of the lees for oxygen are responsible for the greater stability of the monomeric anthocyanin content. However, an initial period lasting until the fourth month was seen in all conventional lees ageing assays, during which the degradation of the total anthocyanins is greater. In this period the total anthocyanin content fell 76.26%, 66.24% and 76.34% in the media aged over lees, the controls and the media to which β-glucanase was added, respectively.

3.4. Effect of ageing over lees on the content of highly stable pyranoanthocyanin pigments

Vitisins are highly stable pigments formed by the condensation of anthocyanins and metabolites released during fermentation. The vitisins are more resistant than other anthocyanins to discoloration by SO_2 (Bakker & Timberlake, 1997), and express more intense colours than other pigments at pH 4 (Atanasova, Fulcrand, Chenyer, & Moutounet, 2002). A strong correlation has also been recorded between the quantities of pyruvic acid and acetaldehyde released into the medium and the formation of vitisins A and B, respectively (Morata et al., 2003). The addition of

Table 3
Malvidin-3-*O*-glucoside (M3G), vitisin A or malvidin-3-*O*-glucoside pyruvic acid adduct (VITA), malvidin-3-*O*-(6-*O*-acetyl)-glucoside (M3G6Ac), malvidin-3-*O*-(6-*O*-coumaroyl)-glucoside (M3G6Cm), 4-vinylphenols (VPH1, VPH2, VPH3) and total anthocyanin contents (mg/l)

T (days)	Assay	M3G	VITA	M3G6Ac	M3G6Cm	VPH1	VPH2	VPH3	Total
60	2EV-β	75.67 ± 1.73^{a}	2.16 ± 0.09^{a}	3.54 ± 0.07^{a}	4.92 ± 0.15^{a}	0.14 ± 0.01^{a}	–	–	111.41 ± 2.80^{a}
	7VA-β	76.73 ± 11.65^{a}	2.17 ± 0.18^{a}	3.67 ± 0.58^{ab}	5.13 ± 0.98^{ab}	0.09 ± 0.08^{a}	–	–	113.62 ± 18.93^{ab}
	4CV-β	81.65 ± 2.52^{b}	2.33 ± 0.11^{b}	3.88 ± 0.11^{b}	5.53 ± 0.24^{b}	0.09 ± 0.08^{a}	–	–	112.07 ± 4.54^{b}
	T	105.13 ± 0.73^{b}	2.97 ± 0.09^{b}	5.07 ± 0.05^{c}	7.49 ± 0.16^{c}	0.13 ± 0.01^{a}	–	–	165.80 ± 1.88^{c}
	9CV	107.34 ± 0.65^{b}	2.88 ± 0.05^{b}	5.15 ± 0.04^{c}	7.84 ± 0.07^{c}	0.13 ± 0.01^{a}	–	–	165.92 ± 1.25^{c}
	5CV	108.22 ± 0.45^{b}	2.86 ± 0.03^{b}	5.21 ± 0.09^{c}	7.89 ± 0.02^{c}	0.14 ± 0.07^{a}	–	–	166.76 ± 0.66^{c}
	S6U	108.36 ± 0.37^{b}	2.91 ± 0.07^{b}	5.20 ± 0.02^{c}	7.69 ± 0.06^{c}	0.12 ± 0.00^{a}	–	–	165.88 ± 0.70^{c}
	2EV	108.41 ± 0.89^{b}	2.96 ± 0.04^{b}	5.24 ± 0.01^{c}	7.78 ± 0.11^{c}	0.14 ± 0.01^{a}	–	–	165.61 ± 1.49^{c}
	7VA	108.46 ± 0.86^{b}	2.91 ± 0.02^{b}	5.22 ± 0.13^{c}	7.88 ± 0.08^{c}	0.13 ± 0.01^{a}	–	–	167.15 ± 0.96^{c}
	4CV	108.15 ± 0.66^{b}	3.22 ± 0.21^{b}	5.25 ± 0.08^{c}	7.92 ± 0.09^{c}	0.14 ± 0.01^{a}	–	–	168.60 ± 1.15^{c}
	3VA	109.24 ± 0.70^{b}	3.23 ± 0.12^{b}	5.35 ± 0.07^{c}	7.89 ± 0.08^{c}	0.14 ± 0.01^{a}	–	–	168.17 ± 1.24^{c}
120	2EV-β	19.80 ± 6.24^{a}	1.15 ± 0.19^{a}	0.85 ± 0.26^{a}	0.74 ± 0.64^{a}	0.05 ± 0.00^{bc}	0.00 ± 0.00^{a}	–	26.93 ± 8.70^{a}
	7VA-β	20.05 ± 7.68^{b}	1.31 ± 0.24^{ab}	1.23 ± 0.3^{b}	1.53 ± 0.48^{b}	0.06 ± 0.05^{bc}	0.00 ± 0.00^{a}	–	38.34 ± 10.93^{a}
	4CV-β	30.25 ± 0.97^{b}	1.39 ± 0.04^{b}	1.30 ± 0.05^{b}	1.63 ± 0.06^{b}	0.08 ± 0.01^{c}	0.00 ± 0.00^{a}	–	41.71 ± 1.20^{a}
	T	70.06 ± 0.98^{c}	2.20 ± 0.19^{c}	2.93 ± 0.21^{c}	4.78 ± 0.06^{c}	0.01 ± 0.02^{a}	0.07 ± 0.01^{bc}	–	103.87 ± 1.68^{bc}
	9CV	72.66 ± 0.09^{c}	2.09 ± 0.05^{c}	2.95 ± 0.02^{c}	4.88 ± 0.02^{c}	0.06 ± 0.01^{bc}	0.03 ± 0.05^{ab}	–	102.30 ± 0.43^{ab}
	5CV	73.01 ± 0.54^{c}	2.17 ± 0.02^{c}	3.00 ± 0.02^{c}	4.91 ± 0.05^{c}	0.06 ± 0.01^{bc}	0.08 ± 0.01^{c}	–	103.34 ± 0.75^{c}
	S6U	73.74 ± 0.77^{c}	2.24 ± 0.06^{c}	3.11 ± 0.2^{c}	4.96 ± 0.07^{c}	0.04 ± 0.00^{ab}	0.08 ± 0.01^{c}	–	105.11 ± 1.46^{c}
	2EV	73.89 ± 0.94^{c}	2.17 ± 0.08^{c}	3.02 ± 0.03^{c}	4.96 ± 0.08^{c}	0.05 ± 0.00^{ab}	0.07 ± 0.00^{bc}	–	104.26 ± 1.43^{bc}
	7VA	72.50 ± 0.49^{c}	2.13 ± 0.06^{c}	2.92 ± 0.02^{c}	4.85 ± 0.04^{c}	0.06 ± 0.01^{bc}	0.05 ± 0.05^{bc}	–	102.28 ± 0.93^{bc}
	4CV	73.40 ± 0.54^{c}	2.17 ± 0.05^{c}	2.99 ± 0.02^{c}	4.91 ± 0.07^{c}	0.06 ± 0.00^{bc}	0.03 ± 0.00^{ab}	–	104.29 ± 1.30^{ab}
	3VA	74.27 ± 0.34^{c}	2.21 ± 0.08^{c}	3.02 ± 0.01^{c}	4.99 ± 0.03^{c}	0.04 ± 0.01^{ab}	0.08 ± 0.01^{c}	–	105.55 ± 0.70^{c}
240	2EV-β	9.73 ± 5.42^{a}	1.14 ± 0.30^{a}	0.35 ± 0.25^{a}	0.28 ± 0.22^{a}	0.01 ± 0.02^{a}	0.01 ± 0.02^{ab}	–	13.43 ± 7.43^{a}
	7VA-β	10.59 ± 6.87^{ab}	1.16 ± 0.53^{a}	0.39 ± 0.34^{a}	0.33 ± 0.29^{a}	0.03 ± 0.03^{ab}	0.05 ± 0.04^{abc}	–	14.66 ± 9.70^{ab}
	4CV-β	14.74 ± 1.69^{b}	1.40 ± 0.03^{a}	0.59 ± 0.08^{a}	0.52 ± 0.09^{a}	0.00 ± 0.00^{b}	0.06 ± 0.01^{c}	–	20.49 ± 2.23^{b}
	T	55.31 ± 0.52^{c}	2.64 ± 0.09^{b}	2.74 ± 0.03^{b}	3.02 ± 0.18^{b}	0.08 ± 0.02^{c}	0.01 ± 0.02^{a}	–	85.03 ± 1.17^{c}
	9CV	62.61 ± 0.65^{d}	2.83 ± 0.04^{b}	3.14 ± 0.04^{c}	3.63 ± 0.06^{c}	0.08 ± 0.00^{c}	0.05 ± 0.01^{bc}	–	91.19 ± 2.41^{cd}
	5CV	63.32 ± 1.17^{d}	2.84 ± 0.03^{b}	3.22 ± 0.07^{c}	3.67 ± 0.13^{c}	0.08 ± 0.00^{c}	0.04 ± 0.04^{abc}	–	92.98 ± 1.98^{cd}
	S6U	61.47 ± 1.01^{d}	2.70 ± 0.01^{b}	3.12 ± 0.04^{c}	3.56 ± 0.05^{c}	0.08 ± 0.00^{c}	0.04 ± 0.03^{abc}	–	89.31 ± 1.14^{cd}
	2EV	62.51 ± 0.71^{d}	2.71 ± 0.03^{b}	3.15 ± 0.03^{c}	3.60 ± 0.04^{c}	0.07 ± 0.01^{c}	0.05 ± 0.01^{abc}	–	89.99 ± 0.98^{cd}
	7VA	62.56 ± 1.07^{d}	2.81 ± 0.04^{b}	3.14 ± 0.06^{c}	3.62 ± 0.07^{c}	0.08 ± 0.01^{c}	0.07 ± 0.00^{c}	–	91.67 ± 1.76^{cd}
	4CV	61.90 ± 2.13^{d}	2.65 ± 0.03^{b}	3.10 ± 0.08^{c}	3.60 ± 0.17^{c}	0.08 ± 0.00^{c}	0.07 ± 0.01^{c}	–	90.71 ± 3.53^{d}
	3VA	63.19 ± 0.71^{d}	2.77 ± 0.06^{b}	3.17 ± 0.05^{c}	3.64 ± 0.06^{c}	0.08 ± 0.01^{c}	0.05 ± 0.00^{abc}	–	92.29 ± 1.16^{d}
420	2EV-β	6.29 ± 4.10^{a}	1.04 ± 0.35^{a}	0.00 ± 0.00^{a}	0.41 ± 0.16^{a}	0.23 ± 0.03^{a}	0.26 ± 0.03^{a}	0.28 ± 0.03^{a}	8.88 ± 4.77^{a}
	7VA-β	7.61 ± 6.01^{a}	1.11 ± 0.59^{a}	0.00 ± 0.00^{a}	0.51 ± 0.26^{a}	0.25 ± 0.05^{a}	0.26 ± 0.05^{a}	0.30 ± 0.07^{ab}	10.43 ± 7.16^{a}
	4CV-β	10.01 ± 1.87^{a}	1.33 ± 0.10^{a}	0.00 ± 0.00^{a}	0.59 ± 0.10^{a}	0.27 ± 0.01^{a}	0.29 ± 0.01^{ab}	0.33 ± 0.01^{b}	13.25 ± 2.11^{a}
	T	44.69 ± 3.87^{b}	2.71 ± 0.14^{b}	2.43 ± 0.023^{b}	3.20 ± 0.35^{b}	0.38 ± 0.03^{b}	0.31 ± 0.02^{bc}	0.26 ± 0.01^{a}	71.51 ± 6.83^{b}
	9CV	54.82 ± 1.05^{c}	2.84 ± 0.05^{b}	3.00 ± 0.06^{c}	3.87 ± 0.13^{c}	0.35 ± 0.01^{b}	0.34 ± 0.00^{cde}	0.29 ± 0.01^{ab}	83.60 ± 1.36^{c}
	5CV	56.66 ± 1.40^{c}	2.94 ± 0.08^{b}	3.00 ± 0.05^{c}	4.00 ± 0.09^{c}	0.38 ± 0.01^{b}	0.37 ± 0.02^{e}	0.30 ± 0.00^{ab}	87.32 ± 2.39^{c}
	S6U	57.02 ± 0.23^{c}	2.98 ± 0.01^{b}	3.01 ± 0.01^{c}	4.04 ± 0.03^{c}	0.36 ± 0.01^{b}	0.33 ± 0.01^{cd}	0.30 ± 0.01^{ab}	87.22 ± 0.27^{c}
	2EV	54.50 ± 2.78^{c}	2.87 ± 0.05^{b}	2.87 ± 0.18^{c}	3.81 ± 0.27^{c}	0.36 ± 0.01^{b}	0.35 ± 0.00^{de}	0.30 ± 0.02^{ab}	82.29 ± 4.41^{c}
	7VA	55.93 ± 0.72^{c}	2.90 ± 0.05^{b}	2.97 ± 0.01^{c}	3.94 ± 0.08^{c}	0.35 ± 0.01^{b}	0.37 ± 0.00^{e}	0.30 ± 0.01^{ab}	85.24 ± 1.84^{c}
	4CV	55.86 ± 2.21^{c}	2.93 ± 0.03^{b}	2.97 ± 0.11^{c}	3.93 ± 0.14^{c}	0.38 ± 0.01^{b}	0.37 ± 0.02^{e}	0.30 ± 0.01^{ab}	86.10 ± 3.30^{c}
	3VA	56.28 ± 0.45^{c}	2.94 ± 0.01^{b}	2.96 ± 0.05^{c}	3.97 ± 0.05^{c}	0.36 ± 0.01^{b}	0.37 ± 0.01^{e}	0.29 ± 0.01^{ab}	86.12 ± 0.53^{c}

Means in the same column with the same letter are not significantly different ($p < 0.05$).

pyruvic acid and acetaldehyde encourages the formation of vitisins A and B (Morata, Calderón, González, Gómez-Cordovés, & Suárez, 2007; Hayasaka & Asenstorfer, 2002; Romero & Bakker, 2000).

Fig. 4 shows the trends in the development of malvidin-3-*O*-glucoside and vitisin A in all the assays. The graph reflects the reduced degradation and permanence of pyranoanthocyanin pigments such as vitisin A compared to glucoside anthocyanins such as malvidin-3-*O*-glucoside. While the changes in the content of malvidin-3-*O*-glucoside follows the same trend as that described for total anthocyanins, the concentration of vitisin A is maintained during ageing over lees. Especially noticeable is the behaviour of both pigments in the samples treated with β-glucanase. In these, vitisin A was much more persistent than malvidin-3-*O*-glucoside, indicating less degradation of the colour when these enzymes are used to accelerate autolysis. This justifies the selection of yeast strains that produce large amounts of pyruvic acid and acetaldehyde – strains that induce the synthesis of these pigments - especially during ageing over lees (such wines usually are treated with large amounts of SO_2 and have a high pH).

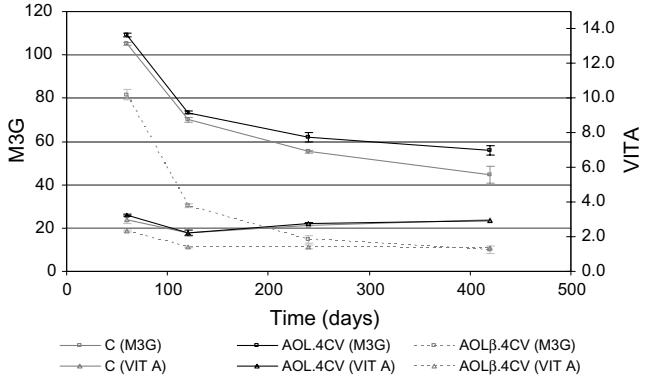

Fig. 4. Change in the concentrations of malvidin-3-*O*-glucoside (M3G) and vitisin A (VIT A) in the control sample (C) and samples aged (conventionally (AOL) and enzyme-assisted [β]) over the lees of yeast strain 4CV. Values are means plus SD (shows as error bars; $n = 3$).

Other long-lasting pigments exist whose changes in concentration are also closely linked to the processes underway in ageing over lees, e.g., the vinylphenolic pyranoanthocyanin (VPH) derivatives. Monomeric anthocyanins naturally condense with vinylphenols (formed in wine by the decarboxylation of hydroxycinnamic acids, especially when yeasts with high hydroxycinnamic decarboxylase activities are used) (Morata, Gómez-Cordovés, Calderón, & Suárez, 2006). The great stability of these pigments is due to their fourth heteroaromatic ring, which allows the delocalisation of the positive charge of the anthocyanin across two molecules of oxygen. These pigments are therefore more resistant to discoloration by SO_2, and to the loss of colour intensity caused by high pHs; the condensation reaction involved in their production means position 4 of the anthocyanin is no longer accessible.

Table 3 shows the content of some of the major anthocyanins and the vinylphenols in the wines produced. The progressive formation of vinylphenols during ageing overlaps with the above-mentioned process of condensation.

The addition of β-glucanase leads to large losses of total anthocyanins, while the degradation of more stable compounds such as the vitisins or vinylphenolic adducts is much reduced (Fig. 4, Table 3). The variable effect of the commercial enzymes on the anthocyanin-3-glucoside content was much greater than on the remaining pigments (Table 3). In contrast, no great differences were seen between the different assays in terms of the formation of vinylphenols. Hydroxycinnamic acids are found in wine before ageing since they accumulate in the grapes before *veraison*. The concentration of vinylphenol precursors was therefore similar in all the assays, explaining the absence of great differences between their vinylphenol concentrations.

3.5. Effect of ageing over lees on colour intensity and hue

Fig. 5a shows the trends in the colour intensity of the control samples, of wine aged over lees, and of the same

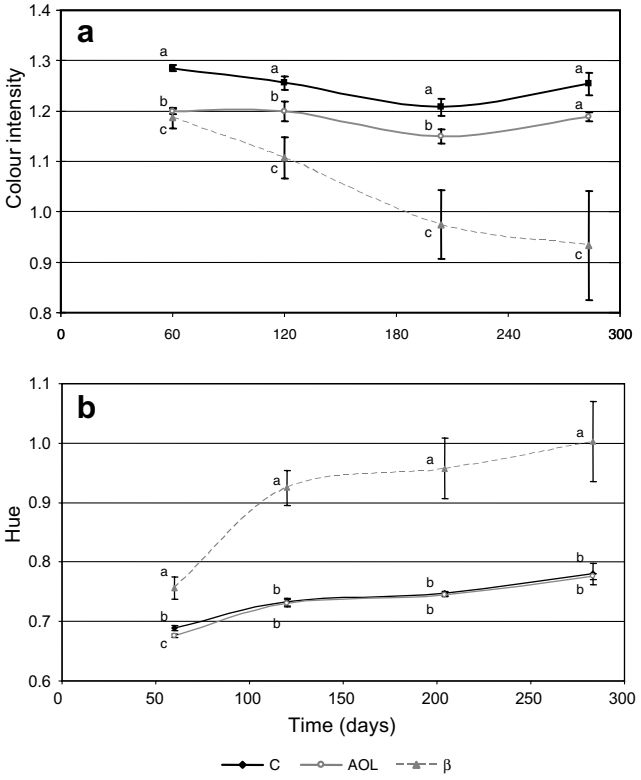

Fig. 5. (a) Colour intensity ($A_{420\ nm} + A_{520\ nm} + A_{620\ nm}$) kinetics in the control (C) and aged samples (conventionally (AOL) and by enzyme-assistance [β]) for all strains studied. Mean values, standard deviations and least significant differences (LSD) are provided. (b) Colour hue ($A_{420\ nm}/A_{520\ nm}$) kinetics for the control sample (C) and aged samples (both conventionally [AOL] and enzyme-assisted [β]) for all the strains studied. Mean values, standard deviations and least significant differences (LSD) are provided.

but with added β-glucanase. Under the first two conditions, the colour intensity changed in the same way; no significant differences were seen at 280 days. The use of β-glucanase, however, led to a great loss of colour intensity.

Fig. 5b shows the change in hue under each of the above conditions. As with colour intensity, the changes in hue seen with the control and conventional ageing over lees treatments were very similar; no significant differences were seen over the experimental period. The addition of β-glucanase, however, led to a marked degradation of the wine's colour, and a corresponding increase in yellow hue. The great loss of monomeric and derived anthocyanins observed, along with the results for the colour obtained when these enzymes were used, should be borne in mind if they are to be employed for ageing red wines.

All the commercial preparations of β-glucanase authorised for use in winemaking are isolated from species of *Trichoderma* cultivated under conditions that favour this (Humbert-Goffard et al., 2004). Products with β-1,3-glucanase and β-1,6-glucanase are produced, although non-specific β-glycosidase activity is also obtained which has negative effects in red winemaking (Debourdieu, Desplanques, Villetaz, & Ribéreau-Gayon, 1985). In wines aged over

lees, this is largely that responsible for the great loss of colour intensity and the increase in yellow hue.

The small differences in colour intensity between the control wines and those aged over lees in the conventional fashion can be explained by the fixing of the colouring material by cell membranes, walls and fragments (Morata et al., 2003; Mazauric and Salmon, 2005). The parallel changes recorded over the study period support this hypothesis.

4. Conclusions

Ageing red wines over lees allows a better wine structure and density – and therefore sensorial characteristics – to be obtained, a consequence of the release of polysaccharides from the walls of the dead yeast cells. It also allows the monomeric anthocyanin content to be stabilised, favouring the stability of the wine's colour. The addition of β-glucanase greatly reduces the time required for the release of such polysaccharides, although these are of smaller molecular weight. Commercial preparations of these enzymes may also be contaminated with β-glucosidase, which negatively affects the anthocyanin content. The use of yeasts that show rapid autolysis may allow faster ageing over lees, free of the problems of commercial enzyme contamination and organoleptic deviation.

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