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New genera of yeasts for over-lees aging of red wine

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ABSTRACT

The osmophilic yeast genera *Schizosaccharomyces* and *Saccharomycodes*, and the primary fermentation-phase genera *Pichia*, were studied for possible use in the over-lees aging of red wines. The molecular architecture and chemical composition shown by the cell walls of these yeasts is quite unusual within the family *Saccharomycetaceae*. Their kinetics of cell wall polysaccharide release were examined by HPLC-RI molecular exclusion chromatography; two columns were coupled in series to increase the capacity of resolution since these yeasts all show an early elution peak corresponding to the largest polysaccharides. The anthocyanin content was determined by HPLC with photodiode array and mass spectrometry detection (HPLC-PDAD/ESI-MS). During aging over the lees of these yeasts, colour was monitored by UV–VIS spectrophotometry. In order to quantify any anomalies of potential sensorial importance, the change in the profile of the majority volatile compounds was monitored using gas chromatography with flame ionisation detection (GC-FID).

1. Introduction

The technique of aging over-lees is gaining importance in the production of red wines since it affords an efficient means of obtaining high quality products with their own identity; something of importance in a saturated market showing a great deal of homogeneity. This technique, however, demands appreciable investment in resources (vats, barrels, labour, sensorial analyses and *batonnâges*) and is not free of problems. Many research groups are now working on how to minimise these difficulties, and on how to obtain balanced products more quickly and simply.

The polysaccharide fraction released from the yeast cell wall due to the action of the cell's own enzymes, e.g., β-glucanase and cell wall mannosidase (Charpentier & Freyssinet, 1989), is, among all the products of autolysis, that which exerts one of the major influences on the sensorial and physico-chemical properties of wines aged over-lees. The qualitative composition of yeast cell walls, as well as the formation of their wall polysaccharides, can vary significantly from one species to another. In fact, knowledge of the general composition of the cell wall has allowed great advances to be made in the classification of yeasts (Bartnicki-García, 1968; Prillinger et al., 1993; Weijman & Golubev, 1987). However, the cell wall carbohydrate composition of only a few species has been studied in depth, and in even fewer has the distribution of carbohydrate components been investigated (Phaff, 1998).

Weijman and Golubev (1987) distinguish between three types of cell walls within the ascomycetous yeasts, including the *Saccharomyces*-type, which contain glucose and mannose and the *Schizosaccharomyces*-type, which contain galactose, glucose and mannose. The main structural component of the cell wall of *Saccharomyces cerevisiae* is β-(1→3) glucan, which has β-(1→6) glucan lateral ramifications (Fleet & Phaff, 1981). These fibres intertwine with small quantities of chitin (Molano, Bowers, & Cabib, 1980) to form a three dimensional structure that support the glucomannan–protein complex, which is the major component of the cell wall (Ballou, 1976).

Schizosaccharomyces pombe is an yeast that reproduces asexually by binary fission. Its cell wall has a particular structure and composition owed to the presence of polysaccharides and sugar derivatives that are unusual within the family *Saccharomycetaceae*. In 1995, Kopecká et al. studied the cell wall formation of this yeast using electron microscopy and enzyme techniques, and found the main differences between *Sacch. cerevisiae* and *Schiz. pombe* to be the possession of α-galactomannose rather than mannose, along with the presence of α-(1→3) glucan. Fig. 1 compares the composition and distribution of carbohydrates in the cell walls of *Schizosaccharomyces* spp. and *Saccharomyces* spp.

The kinetics of polysaccharide release varies, depending on the yeast strain (Morata, Calderón, Colomo, González, & Suárez-Lepe, 2005; Palomero, Morata, Benito, González, & Suárez-Lepe, 2007).

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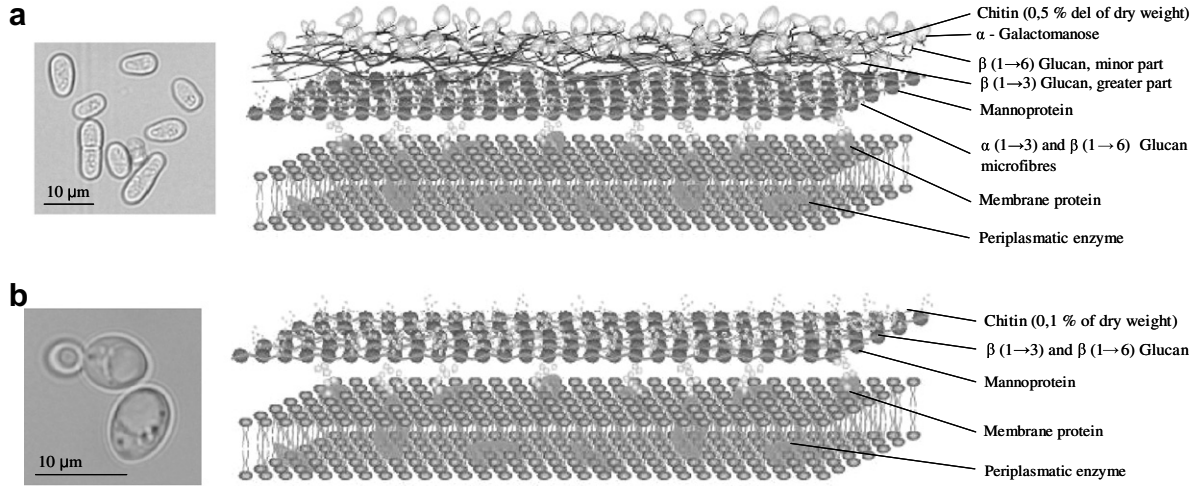

Fig. 1. a. Illustration of the cell wall and optical microscopy image (1000×) of *Schizosaccharomyces pombe*. b. Illustration of the cell wall and optical microscopy image (1000×) of *Saccharomyces cerevisiae*.

The combination of cell wall structure and autolytic behaviour suggests different yeasts may possess different polysaccharide release kinetics, which should be investigated in order to improve aging over-lees; this would be of particular interest with respect to *Schizosaccharomyces*-type walls (Weijman & Golubev, 1987).

In agreement with the new over-lees aging technique developed by Suárez-Lepe and Morata, (2006), the use of non-*Saccharomyces* yeasts requires the exogenous generation of a biomass for later addition to the wine. This method has the advantages that it allows the use of yeasts selected for their autolytic properties while minimising the supply of nutrients and contaminants associated with traditional over-lees aging.

In addition to the release of polysaccharides, which is a fundamental criterion in yeast selection for aging over-lees, the reducing nature of the lees provides protection from the oxidation of polyphenols (Fornairon-Bonnefond & Salmon, 2003; Salmon, 2005); the selection of yeasts that exhibit this trait strongly is of great interest.

The aim of the present work was to study the release of polysaccharides during the autolysis of different genera of non-*Saccharomyces* wine yeasts, the possible use of these yeasts in the acceleration of aging over-lees, and their effect on the stability of the anthocyanin monomer content in red wine made from *Vitis vinifera* L. cv. Garnacha grapes.

2. Materials and methods

2.1. Yeasts used in experimental over-lees aging

Five different strains of yeast were examined: *S. cerevisiae* G3(7) (selected for its high autolytic capacity at the Department of Food Technology, UPM, Madrid, Spain), which was used as a control, *Schiz. pombe* 936, *Saccharomycodes ludwigii* 980, *Pichia anomala* 930, and *Pichia membranifaciens* 956 (all from the collection of the *Instituto de Fermentaciones Industriales*, CSIC, Madrid, Spain).

The yeast biomass used in the over-lees aging assays was obtained by growing the different strains in YEPD medium (Yarrow, 1998) enriched with glucose (up to 100 g/l). The yeast biomass was washed with 10:1 volumes of sterile distilled water, centrifuged at 3000 rpm for 2 min, and the supernatant discarded. This procedure was repeated twice to provide yeast biomass with no remains of nutrients. These yeasts were then lyophilised and conserved at 4 °C.

2.2. Autolysis in a model medium

To quantify the release of polysaccharides during autolysis, aging over-lees was simulated in a model medium composed of water/ethanol (90:10 v/v) acidulated to pH 3.5 with tartaric acid. This medium was divided into 200 ml aliquots and placed in 250 ml translucent plastic flasks.

Polysaccharide release was studied in all four non-*Saccharomyces* strains and the control strain *Sacch. cerevisiae* G3(7). Lyophilised yeast (0.8 g/l) were added to the prepared flasks, which were then isothermically maintained at 28 °C for up to 140 days, with orbital agitation for 1 h at 180 rpm once a week to simulate *bâtonnage* aging conditions. All assays were performed in triplicate.

2.3. Recovery of polysaccharides from the model media

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 1 N HCl). Precipitation was facilitated by refrigerating the samples at 4 °C for 24 h. The precipitate was centrifuged at 8000 rpm for 15 min and the supernatant discarded. The separated polysaccharides were washed three times with 1 ml ethanol and dried in an oven at 40 °C. They were resuspended in 1 ml of 0.1 M $NaNO_3$ solution, filtered through an acetate methyl ester membrane with 0.45 < mu > m pore size (Teknokroma, Barcelona, Spain), and kept at 4 °C until analysis by HPLC-RI.

2.4. Analysis of polysaccharides by high performance liquid chromatography with refractive index detection (HPLC-RI)

The polysaccharides released were analysed by HPLC (Doco, Brillouet, & Moutounet, 1996), using a Waters chromatograph (Waters, Milford, MA) equipped with a 600E pump, a 717p injector and a RI 2412 detector. Separation was achieved using both Ultrahydrogel 250 and Ultrahydrogel 500 molecular exclusion columns (Waters, Milford, MA), employing 0.1 M $NaNO_3$ in de-ionised water (MilliQ) as an eluent.

The sizes of the polysaccharides released during autolysis were compared to those of known pullulan (polymaltotriose) standards (Shodex, Showa Denko K.K, Japan): P-800 (788 kDa), P-400 (404 kDa), P-200 (212 kDa), 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 of the model medium was determined using a calibration curve constructed from the above standards. In the autolysis assays in the model medium, polysaccharides were determined at days 28, 58, 85, 112 and 142.

2.5. Wines

Red wine obtained from *V. vinifera* L. cv. Garnacha grapes from the 'Navarra' (Spain) *Denominación de Origen* area were used to produce over-lees aged wines in which the monomeric anthocyanin content, colour variables and volatile compounds were measured. After adding the lyophilised yeast (0.8 g/l) the change in monomeric anthocyanin content was measured over 100 days.

The red Garnacha grapes of the 2004 vintage were processed into wines by conventional procedures at the Irache winery in Navarra, Spain. Once fermented, the wine was racked for clarification and was cold stabilised to eliminate potassium bitartrate. It was bottled as a commercial wine before four months of aging in oak barrels. The ethanol content was 12.5% v/v with pH 3.6.

2.6. Analysis of anthocyanins by HPLC with photodiode array detection and mass spectrometry (HPLC-PDAD/ESI-MS)

Attention was paid to the following anthocyanins owing to their importance in terms of wine quality and stability: Malvidin-3-*O*-glucoside (M3 G), malvidin-3-*O*-glucoside-pyruvate adduct or vitisin A (VITA), malvidin-3-*O*-(6″-*p*-coumaroylglucoside)-pyruvate adduct (PcVITA), malvidin-3-*O*-(6″-*p*-acetylglucoside) (M3G6Ac), malvidin-3-*O*-glucoside-4-vinylcatechol (VPH1), malvidin-3-*O*-glucoside-vinyl-epicatechin (M3GVcat) and malvidin-3-*O*-glucoside-4-vinylphenol (VPH2).

The anthocyanins in the wines were analysed using an Agilent HPLC (Agilent Technologies Palo Alto, CA), equipped with a PDAD and a quadrupole mass spectrometer with an electrospray

Table 1
Polysaccharides (expressed as mg/l of pullulans) released by autolysis in the studied yeasts at 28, 58, 85, 112 and 142 days

Yeast strain	28	58	85	112	142
Saccharomyces cerevisiae [G3(7)]	4.07 ± 0.22[a]	7.92 ± 0.12[a]	22.47 ± 6.52[b]	33.09 ± 0.42[a]	36.65 ± 5.65[a]
Saccharomycodes ludwigii (980)	52.26 ± 9.14[b]	69.00 ± 4.46[b]	71.76 ± 3.28[c]	107.49 ± 15.40[c]	110.51 ± 10.02[b]
Schizosaccharomyces pombe (936)	62.02 ± 13.71[b]	68.34 ± 7.29[b]	72.01 ± 9.74[c]	75.11 ± 10.88[b]	103.61 ± 24.88[b]
Pichia anomala (930)	2.40 ± 0.90[a]	8.09 ± 0.34[a]	12.04 ± 2.89[ab]	17.8 ± 1.47[a]	30.04 ± 4.90[a]
Pichia membranifaciens (956)	1.66 ± 0.38[a]	4.98 ± 1.46[a]	11.63 ± 3.54[a]	19.09 ± 2.45[a]	21.15 ± 4.78[a]

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

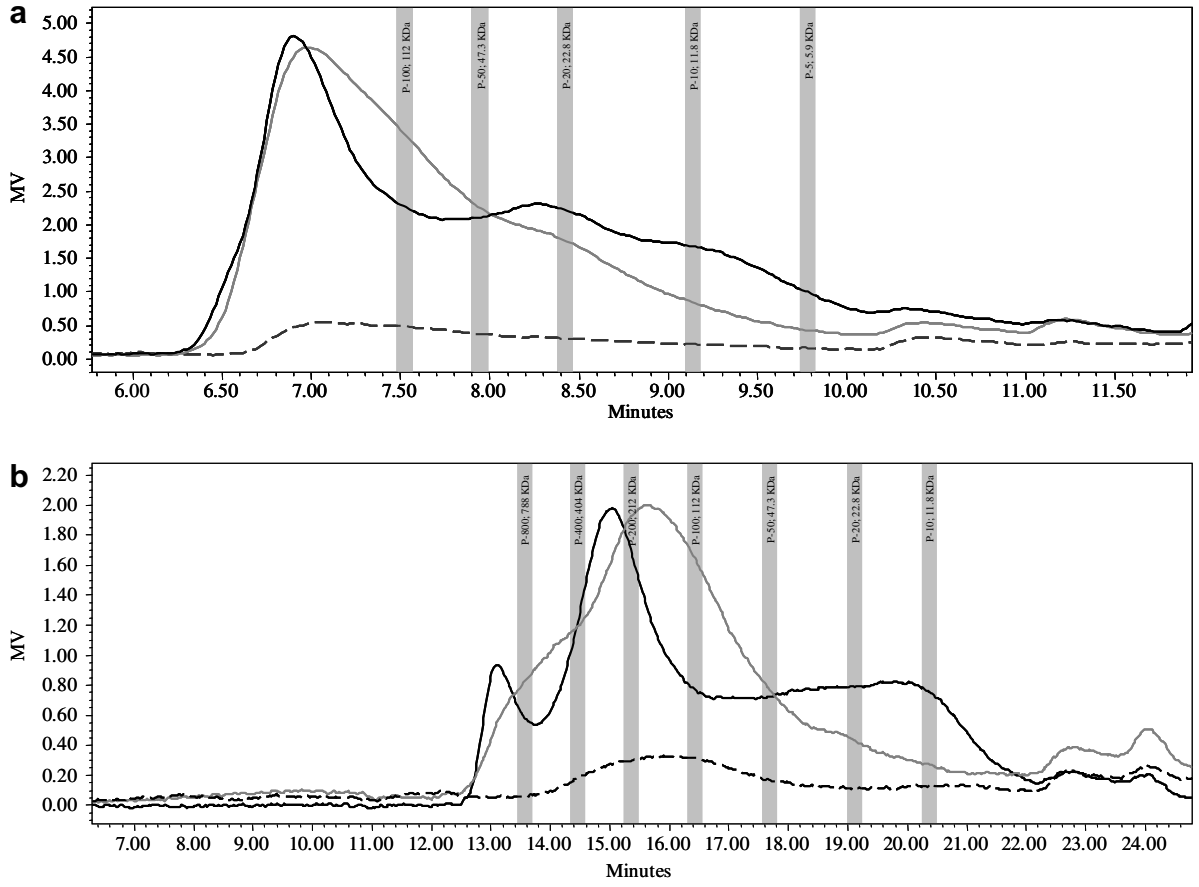

Fig. 2. HPLC-RI chromatograms of the polysaccharides produced by *Saccharomyces cerevisiae* G3(7) (dashed black line), *Schizosaccharomyces pombe* (continuous black line) and *Saccharomycodes ludwigii* (continuous grey line) at 58 days, both assays were performed in triplicate. Also shown are the retention times (bands) for the molecular weight markers (pullulans). a. Chromatogram obtained using a Waters Ultrahydrogel 250 molecular exclusion column. b. Chromatogram obtained using two molecular exclusion columns (Waters Ultrahydrogel 250 and 500).

interface. Solvent A (water/formic acid, 90:10 v/v) and solvent B (methanol/formic acid, 90:10 v/v) were used as eluents.

Chromatographic separation was achieved, using a reverse-phase Nova-Pak C_{18} analytical column (300 mm × 3.9 mm i.d.) working at room temperature (25 °C approximately). The gradient was linear at a flow rate of 0.8 ml/min from 20 to 50% solvent B for 0–50 min and from 50 to 20% solvent B for 50–52 min. The column was re-equilibrated for 5 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). Samples (100 μl) of previously filtered fermentations were injected onto the HPLC column.

The different anthocyanins were identified by their retention times with respect to the majority anthocyanin malvidin-3-*O*-glucoside, and by comparing the UV–Visible and mass spectra with data in the literature (Heier, Blaas, Drob, & Wittowski, 2002; Morata, González, & Suárez-Lepe, 2007). The structures of the anthocyanin-derived pigments formed were determined by liquid chromatographic/electrospray mass spectrometry (HPLC/ESI-MS) using the same liquid chromatography conditions. Electrospray interface parameters were: Drying gas (N_2); flow rate, 10 ml/min; temperature, 350 °C; nebulizer pressure, 380 Pa (55 psi); and capillary voltage, 4000 V. Electrospray interface was performed in positive mode scanning from *m/z* 100 to *m/z* 1500 using 150 V as the fragmenter voltage from 0 to 57 min.

2.7. Colour measurements

Wine absorbance at 420, 520 and 620 nm was determined using a JASCO V-530 (Tokio, 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.

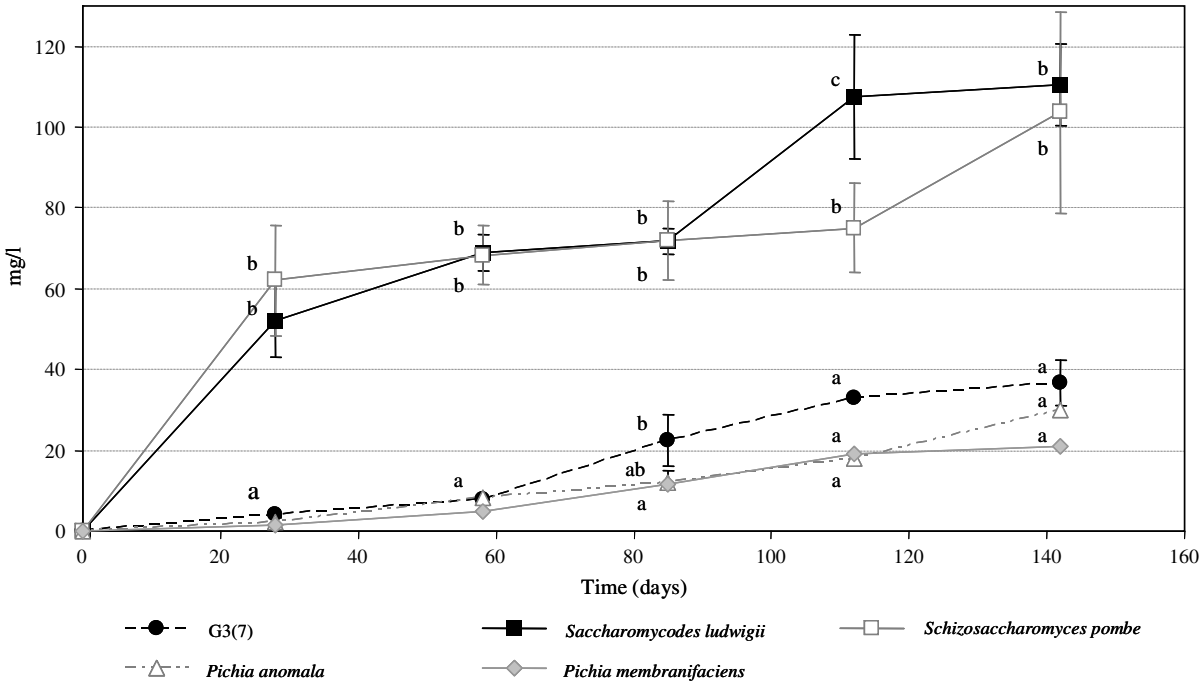

Fig. 3. Polysaccharides released in over-lees aging by all the strains studied. Mean values, standard deviations ($n = 3$) and least significant differences (LSD) are provided.

Table 2
Change in content of malvidin-3-*O*-glucoside (M3G), malvidin-3-*O*-glucoside-pyruvate adduct or vitisin A (VITA), malvidin-3-*O*-(6″-*p*-coumaroylglucoside)-pyruvate adduct (PcVITA), malvidin-3-*O*-(6"-acetylglucoside) (M3G6Ac), malvidin-3-*O*-glucoside-4-vinylcatechol (VPH1), malvidin-3-*O*-glucoside-vinyl-epicatechin (M3GVcat) and malvidin-3-*O*-glucoside-4-vinylphenol (VPH2)

T (days)	Assay	M3G	VITA	PcVIT A	M3G6Ac	VPH1	M3GVcat	VPH3	Total
0	Sample wine	7.73 ± 0.32	2.76 ± 0.01	1.77 ± 0.01	0.98 ± 0.00	0.78 ± 0.05	0.36 ± 0.00	0.47 ± 0.00	21.44 ± 0.36
8	Control	7.67 ± 0.02^{a}	2.65 ± 0.00^{ab}	1.76 ± 0.00^{a}	0.83 ± 0.00^{a}	0.59 ± 0.00^{a}	0.32 ± 0.00^{a}	0.40 ± 0.00^{a}	21.2 ± 0.07^{c}
	Sacch. cerevisiae [G3(7)]	8.11 ± 0.01^{ab}	2.71 ± 0.00^{bc}	1.81 ± 0.00^{a}	0.91 ± 0.00^{b}	0.62 ± 0.00^{a}	0.34 ± 0.00^{a}	0.43 ± 0.00^{b}	20.04 ± 0.12^{a}
	S'codes. ludwigii (980)	7.67 ± 0.01^{a}	2.64 ± 0.00^{a}	1.76 ± 0.00^{a}	0.88 ± 0.00^{ab}	0.63 ± 0.00^{a}	0.34 ± 0.00^{a}	0.43 ± 0.00^{b}	20.58 ± 0.03^{b}
	Schiz. pombe (936)	7.67 ± 0.01^{a}	2.64 ± 0.00^{a}	1.76 ± 0.00^{a}	0.88 ± 0.00^{ab}	0.63 ± 0.00^{a}	0.34 ± 0.00^{a}	0.43 ± 0.00^{b}	20.16 ± 0.07^{ab}
77	Control	2.18 ± 0.00^{a}	2.24 ± 0.00^{a}	0.65 ± 0.00^{a}	0.37 ± 0.00^{a}	0.62 ± 0.00^{c}	0.33 ± 0.00^{b}	0.44 ± 0.00^{c}	10.94 ± 0.03^{a}
	Sacch. cerevisiae [G3(7)]	2.30 ± 0.00^{b}	2.22 ± 0.00^{a}	0.70 ± 0.00^{ab}	0.39 ± 0.00^{b}	0.55 ± 0.00^{a}	0.31 ± 0.00^{a}	0.40 ± 0.00^{a}	10.87 ± 0.03^{a}
	S'codes. ludwigii (980)	2.30 ± 0.00^{b}	2.24 ± 0.00^{a}	0.67 ± 0.00^{a}	0.38 ± 0.00^{a}	0.59 ± 0.00^{b}	0.32 ± 0.00^{b}	0.43 ± 0.00^{bc}	10.85 ± 0.01^{a}
	Schiz. pombe (936)	2.31 ± 0.00^{b}	2.26 ± 0.00^{a}	0.66 ± 0.00^{a}	0.37 ± 0.00^{a}	0.59 ± 0.00^{b}	0.33 ± 0.00^{ab}	0.43 ± 0.00^{b}	11.05 ± 0.01^{a}
100	Control	1.95 ± 0.14^{b}	1.99 ± 0.00^{b}	0.40±0.01^{a}	0.25 ± 0.00^{a}	0.46 ± 0.00^{a}	0.25 ± 0.00^{a}	0.38 ± 0.00^{a}	9.63 ± 0.11^{b}
	Sacch. cerevisiae [G3(7)]	1.79 ± 0.12^{b}	1.95 ± 0.00^{a}	0.42 ± 0.00^{b}	0.31 ± 0.00^{b}	0.44 ± 0.00^{a}	0.23 ± 0.00^{a}	0.38±0.00^{a}	9.14 ± 0.28^{a}
	S'codes. ludwigii (980)	1.34 ± 0.03^{a}	1.97 ± 0.00^{ab}	0.43 ± 0.00^{bc}	0.30 ± 0.00^{b}	0.47±0.00^{a}	0.24 ± 0.00^{a}	0.39 ± 0.00^{a}	9.96 ± 0.27^{b}
	Schiz. pombe (936)	1.18 ± 0.05^{a}	1.98 ± 0.00^{ab}	0.44 ± 0.00^{c}	0.30 ± 0.00^{b}	0.44 ± 0.00^{a}	0.26 ± 0.00^{a}	0.38 ± 0.00^{a}	9.74 ± 0.21^{b}

Values are means ± standard deviations ($n = 3$), expressed as mg/l, over time with each yeast.
Means in the same column with the same letter are not significantly different ($p < 0.05$).

2.8. Analysis of volatile compounds by gas chromatography with flame ionisation detection (GC-FID)

The changes in the concentrations of 10 characteristic wine volatile compounds (acetaldehyde, ethyl lactate, diacetyl, *n*-propanol, hexanol, isobutanol, isoamylic alcohol, acetoin, ethyl lactate, and 2,3-butane diol) were monitored by gas chromatography using an HP 5890 series II gas chromatograph equipped with a flame ionization detector (Hewlett Packard, Palo Alto, CA). The instrument was equipped with a Sugelabor SGL-20 (60 m × 0.25 mm × 0.25 μm df) column (Madrid, Spain) and calibrated with 4-methyl-2-pentanol. Gas chromatography reference samples (Fluka, Sigma–Aldrich Corp., Buchs SG, Switzerland) were used to provide standard peaks. Injector temperature and detector temperature were both 250 °C. Column temperature was held at 40 °C for 4 min and linearly programmed at a rate of 2 °C/min up to 60 °C, increased by 5 °C/min up to 140 °C, held for 10 min, and finally increased from 40 °C/min to 220 °C held for 7 min. Carrier gas was helium with a flow rate of 1.5 ml/min. Injection mode was split; with a split ratio of 1:20.

2.9. Sensorial analysis

Wines were evaluated by a panel of ten judges, comprising members of the staff of the Food Technology Department, experienced in wine tasting. The assessment took place in standard sensory analysis chambers with separate booths.

Prior to the generation of consistent terminology by consensus, five aroma and four gustatory attributes were chosen to describe the wines. The formal evaluation consisted of two sessions that were held on different days. The panellists used a 10 cm unstructured scale, from 0 to 100, to rate the intensity of each attribute, previously selected. Low values were "attribute not perceptible" and on the contrast high values were "attribute strongly perceptible". The overall impression was also evaluated by each panellist, bearing in mind olfactory and gustatory aspects, as well as the lack of defects.

2.10. Statistical analysis

Means, standard deviations, analysis of variance (ANOVA) and least significant differences (LSD) were calculated using PC Statgraphics v.5 software (Graphics Software Systems, Rockville, MD, USA). Significance was set at $P < 0.05$ (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 aging over the lees of the selected yeasts

Table 1 shows the polysaccharides released. From the very first measurement great differences were seen between the behaviour of the osmophilic and remaining yeasts. At 28 days the concentrations of polysaccharides produced by *Schiz. pombe* and *S'codes. ludwigii* were more than ten times greater than those produced by the *Saccharomyces* and *Pichia* strains. This rapid enrichment in polysaccharides of different molecular size should be studied in depth to determine whether its potentially favourable effects on wine protein (Lubbers, Charpentier, Feuillat, & Voilley, 1994) and tartaric acid (Moine-Ledoux, Perrin, Paladau, & Dubourdieu, 1997) stabilisation actually occur.

Three models of autolytic kinetics were discernable. The primary fermentation-phase yeasts *P. anomala* (930) and *Pichia membranifaciens* (956) released polysaccharides slowly and progressively, *Schiz. pombe* (936) and *S'codes. ludwigii* (980) released polysaccharides in much greater quantities, and *Sacch. cerevisiae* G3(7) released polysaccharides at an intermediate rate. Two months into the overlees aging process, the polysaccharide concentrations of the osmophilic yeast autolysates were those that would be reached at six or seven months by *Saccharomyces*. This rapid release may be explained by differences in the chemical composition and structure

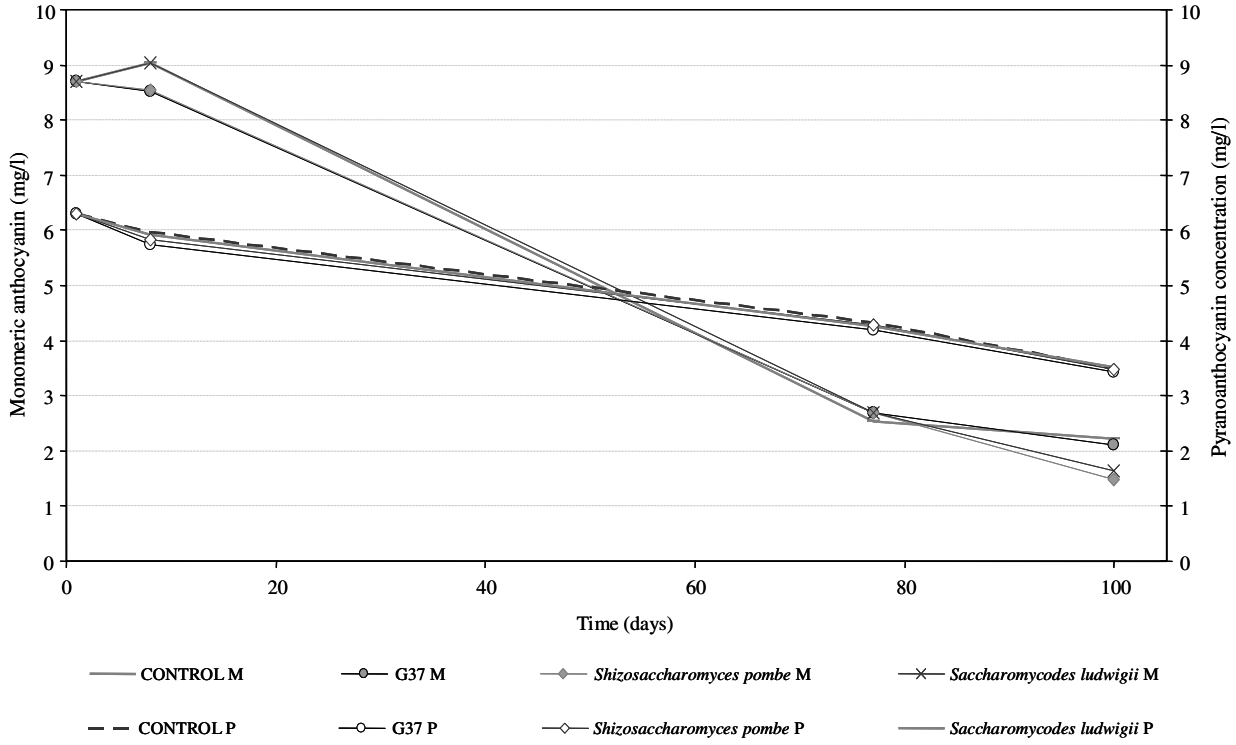

Fig. 4. Changes in the concentrations of total monomeric anthocyanins (M) compared to those shown by highly stable pigments (P) in all assays.

of their cell walls (Weijman & Golubev, 1987), but also by the large quantities of resistance elements in the walls of these yeasts that help them support high osmotic pressures (Kopeckà, Fleet, & Phaff, 1995; Fig. 1).

Fig. 3 shows the change in the release of polysaccharides. The osmophilic yeasts showed biphasic autolytic kinetics, a consequence of the composition of their cell walls into layers (Kopeckà et al., 1995; Fig. 1a). A rapid increase in the polysaccharide concentration of the medium was observed over the first 25 days of over-lees aging; the polysaccharides released may correspond to the constituents of the outer cell wall layer of amorphous glucan and α-galactomannose residues (Fig. 1a). The concentration of polysaccharides remained constant for 50 days – the same period required by *Sacch. cerevisiae* G3(7) to release the first polysaccharides into the medium (see Fig. 1a). The new rise in concentration after this period might mark the time when the internal layer of fibrous glucan comes into contact with the medium.

The elution of polysaccharides under the chromatographic conditions described took place between 6.5 and 10.5 min. With *Saccharomyces* spp., the yeasts normally used at our laboratory for the study of autolysis, a clear peak is seen at approximately 7.3 min, and others (less well defined) at 8.5 and 9.5 min (Palomero

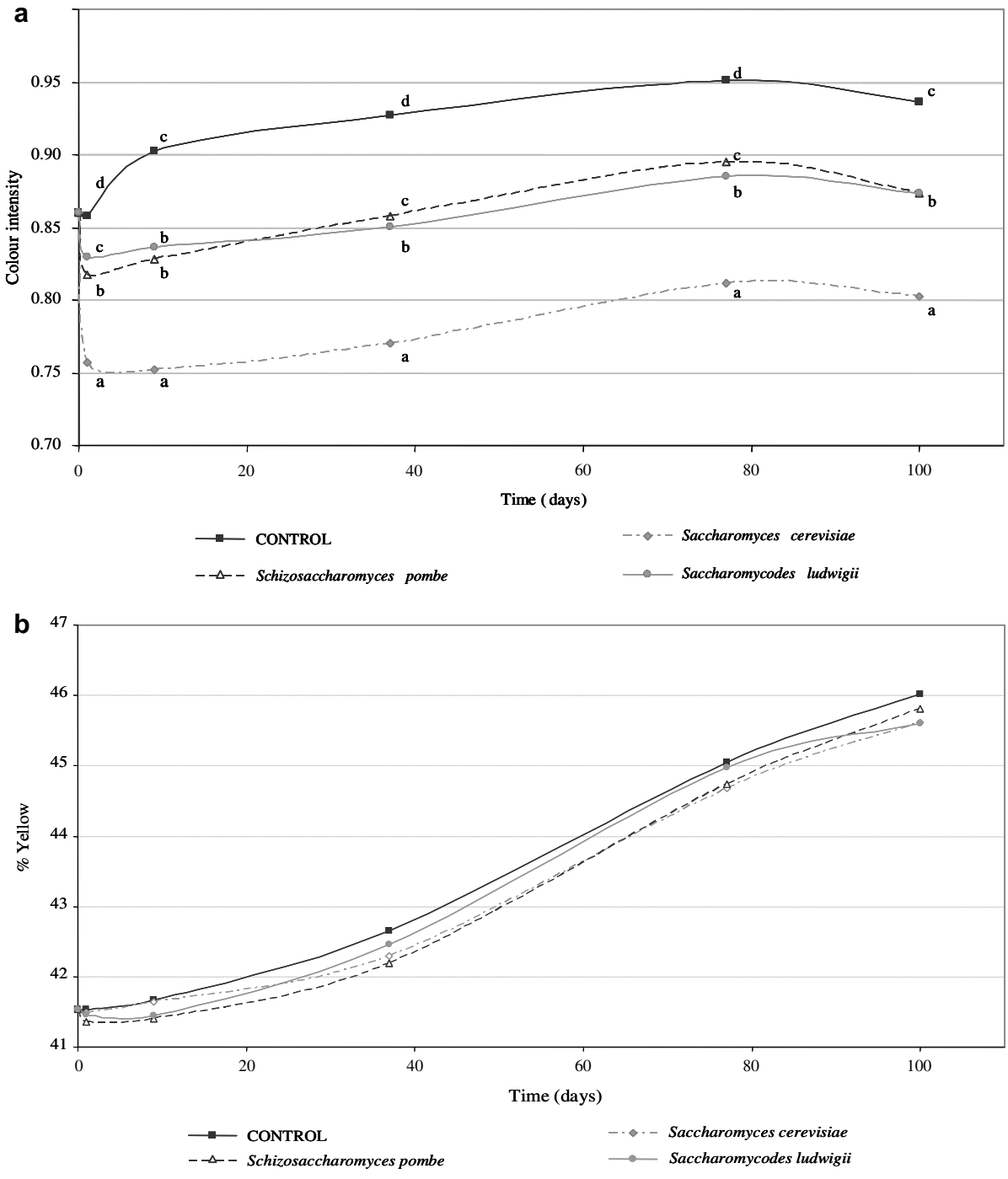

Fig. 5. a. Colour intensity ($A_{420} + A_{520} + A_{620}$) kinetics in the control and over-lees aged samples (all strains). Mean values, standard deviations and least significant differences (LSD) are provided. b. % Yellow ($A_{420} \cdot 100$)/IC kinetics for the control and over-lees aged samples (all strains).

et al., 2007). Fig. 2a shows the molecular exclusion chromatograms of the autolysates of *Sacch. cerevisiae* G3(7), *Schiz. pombe* (936) and *S'codes. ludwigii* (980) at 58 days of autolysis. Coupling two molecular exclusion columns (Ultrahydrogel 500 and Ultrahydrogel 250, Waters, Milford, MA) in series, provided improved resolutions. The chromatograms in Fig. 2b show the osmophilic yeasts to produce an early elution peak. Comparison to the pullulan molecular weight markers show that these peaks correspond to biopolymers of over 788 kDa (Morata et al., 2005); they are therefore larger than most of the fragments released by *Sacch. cerevisiae* and could be of oenological interest owing to their sensorial properties, palatability, and colour preserving potential.

3.2. Identification of anthocyanins and pyranoanthocyanins by HPLC-PDAD

Table 2 shows the concentrations of the majority anthocyanin monomers and the colour-stable forms. The aging of red wines is associated with either a degradation of the pigments present or their development into other compounds, which in both cases can lead to lower colour indices and more orange hues. In aging over-lees, the release of biopolymers such as mannoproteins and cell wall polysaccharides exerts a protective effect on anthocyanin monomers. These anthocyanin monomers are responsible for the lasting blue–red colours. Indeed, aging over-lees is now considered to be a technique that helps preserve wine colour (Escot, Feuillat, Dulau, & Charpentier, 2001; Vivas & Saint Cricq de Gaulejac, 2000) (Fig. 4).

In studies of the influence of this technique on the anthocyanin-type phenolic content of wines, grapes with good chromatic characteristics have been used. In the present work, however, the wine used was made from *V. Vinífera* cv. Garnacha grapes and had a low initial colour intensity and a low total anthocyanin content. Table 2 shows the wine anthocyanin concentration achieved by the different yeasts during aging over-lees to be significantly different, however these differences did not last until the end of the experiment. Aging over-lees did not appear to protect the anthocyanins from oxidation and deterioration. Normally, the reducing power of lees (Palomero et al., 2007) and their greater affinity for oxygen (Salmon, 2005) affords protection to anthocyanins from oxidation. However, wine also contains chemical species such as quinones – which are found in large quantities in Garnacha musts – that promote the oxidation of the colorant material whether it comes into contact with oxygen or not. The tartaric hydroxycinnamic esters are the first compounds to be oxidised by polyphenoloxidases and quinones, producing compounds that undergo reactions with anthocyanins leading to discoloration. In fact, the anthocyanin concentration (especially that of monomeric anthocyanins) was very low in the present wine. Fig. 4 shows the changes in the concentration of these pigments and of the studied pyranoanthocyanins. Greater stability of the pyranoanthocyanin adducts was observed in all assays, as a consequence of the fourth heteroaromatic ring in the anthocyanin

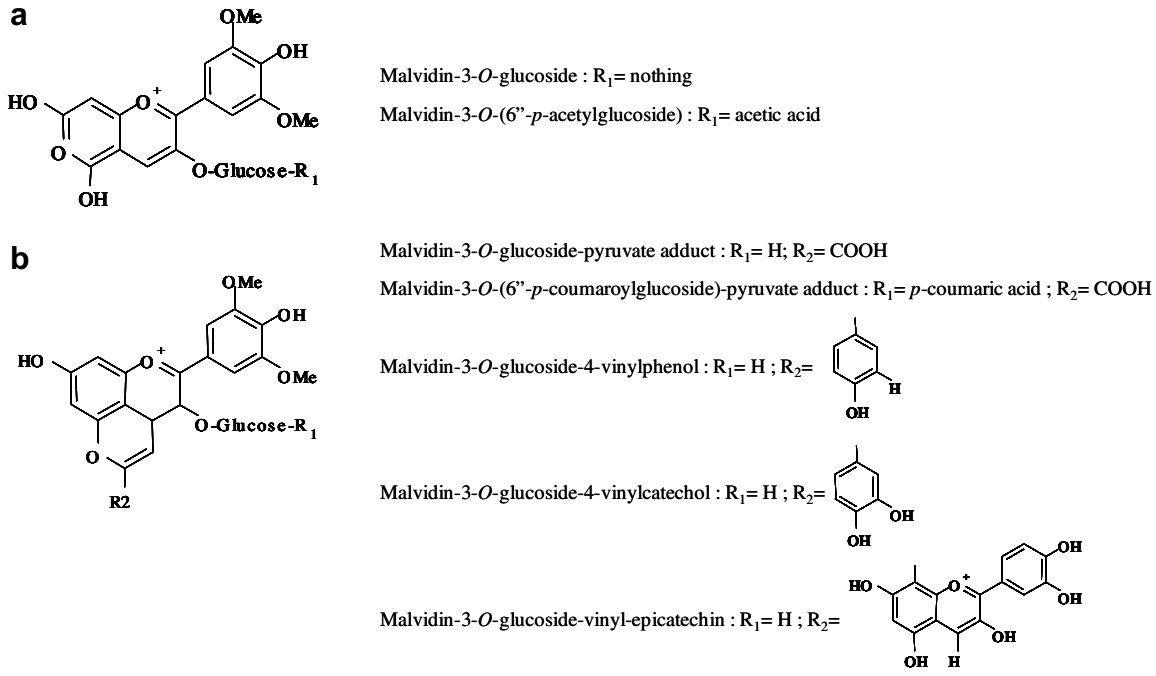

Fig. 6. Structures of the wine pigments studied: a. Monomeric anthocyanins, b. Pyranoanthocyanins.

Table 3
Anthocyanins and anthocyanin-derived pigments identified by HPLC-PDAD/ESI-MS in wines aged over lees

R_t (min)	Pigment	Compound	$[M]^+$ (*m/z*)	Fragments (*m/z*)	λ_{max}
4.95	M3G	Malvidin-3-*O*-glucoside	493	331	520
6.11	VIT A	Malvidin-3-*O*-glucoside-pyruvate adduct	561	369	512
9.78	PcVIT A	Malvidin-3-*O*-(6″-*p*-coumaroylglucoside)-pyruvate adduct	707	399	518
12.87	M3G6Ac	Malvidin-3-*O*-(6″-*p*-acetylglucoside)	535	331	530
23.04	VPH1	Malvidin-3-*O*-glucoside-4-vinylcatechol	625	463	510
25.39	M3G Vcat	Malvidin-3-*O*-glucoside-vinyl-epicatechin	805	643	508
25.73	VPH2	Malvidin-3-*O*-glucoside-4-vinylphenol	609	447	504

which promotes the delocalisation of the positive charge between two oxygen molecules (Morata, Gómez-Cordovés, Calderón, & Suárez, 2006). Thus, high stability pigments such as vitisin A (a malvidin-3-*O*-glucoside-pyruvate adduct) and the 4-vinylphenols acquire greater importance in the colour of wines with low anthocyanin contents (Fig. 6; Table 3).

The metabolic characteristics of living osmophilic yeasts render them spoilage microorganisms; they are capable of causing re-fermentation, the excessive formation of carbonyl compounds (acetoin and diacetyl), and in the case of *Schizosaccharomyces* spp., excessive de-acidification of the must. The maloalcoholic fermentation that members of this genus can undergo leads to a reduction in acidity and an increase in pH. This leaves wines more susceptible to anomalous microbial growth and leads to a displacement of the equilibrium of the wine anthocyanins (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2000), causing them to turn into less coloured or even colourless forms. However, adding exogenous, lyophilised yeast biomass to a medium without nutrients, such as a fermented, stabilised wine, should cause no colour degradation.

The presence of different enzyme activities in the cell wall of yeasts is an ecophysiological feature that must be borne in mind in over-lees aging. Some authors (Manzanares, Rojas, Genovés, & Vallés, 2000; Rosi, Vinella, & Domizio, 1994; Strauss, Jolly, Lambrechts, & van Rensburg, 2001) have studied the β-glucosidase (anthocyanin-β-glucosidase or anthocyanase) activity of several genera of non-*Saccharomyces* yeasts, including *Saccharomycodes* and *Schizosaccharomyces*. Strauss et al. (2001) indicate, however, that β-glucosidase activity can be confused with that of exoglucanase given that the substrate employed by the above authors can be used to detect either activity. β-glucosidase activity in red wines provokes colour loss due to the breaking of the bonds between glucose and anthocyanidin moieties (Wightman & Wrolstad, 1996; Wrolstad, Wightman, & Durst, 1994).

In the present assays with *Schiz. pombe* and *S'codes. ludwigii*, no important reduction was seen in the total anthocyanin content of the wine, nor was any significant differences seen in percentage yellow compared to the control.

3.3. Effect of aging over-lees on colour intensity and hue

Fig. 5a shows the colour intensity values for all the samples over the entire experimental period. The trends seen in all assays were analogous, but significant differences were seen between the values for the control and the osmophilic yeasts. The adsorption of the coloured compounds by the cell walls, membranes and fragments produced during the lytic process (Morata et al, 2003) might explain the small differences in colour intensity between the control and the assay samples. This process of fixation is partially reversible when wines are aged over-lees, depending on the conditions of the medium (Mazauric & Salmon, 2005; Mazauric & Salmon, 2006). The outer amorphous glucan layer of the osmophilic yeasts, which is responsible for the rapid release of polysaccharides by these strains, influences the reversibility of pigment fixation on the cell walls; the rapid destruction of the wall may release any fixed pigments.

Fig. 5b shows the percentage yellow of the samples over the experimental period. No significant differences were detected, which suggests the oxidation conditions in the different assays were similar.

3.4. Analysis of volatile compounds

Over the prolonged contact of the wine with the lees, the mannoproteins and polysaccharides released by the lees can interact with a number of aromatic compounds (Jiménez & Ancín, 2007).

Table 4
Volatile compounds found in all the assays studied: Means, standard deviations and least significant differences (LSD) are provided ($n = 3$), expressed as mg/l

T (days)	Assay	Acetaldehyde	Ethyl acetate	Diacetyl	*n*-Propanol	Hexanol	Isobutanol	Isoamylic alcohol	Acetoin	Ethyl lactate	2,3-Butane diol
8	Control	10.68 ± 1.04^{a}	53.12 ± 10.44^{a}	0.31 ± 0.44^{a}	12.92 ± 1.84^{a}	32.61 ± 2.44^{a}	25.80 ± 5.02^{a}	111.92 ± 20.54^{a}	3.85 ± 0.54^{a}	2.39 ± 0.26^{a}	74.62 ± 4.05^{a}
	Sacch. cerevisiae [G3(7)]	10.52 ± 0.46^{a}	53.49 ± 2.13^{a}	0.25 ± 0.05^{a}	11.60 ± 0.36^{a}	30.18 ± 2.58^{a}	25.35 ± 1.34^{a}	109.46 ± 5.87^{a}	6.16 ± 0.82^{b}	3.00 ± 0.14^{b}	89.51 ± 7.78^{a}
	S'codes. ludwigii (980)	10.59 ± 0.67^{a}	52.23 ± 5.92^{a}	0.30 ± 0.06^{a}	12.44 ± 1.47^{a}	29.30 ± 4.41^{a}	26.40 ± 2.16^{a}	115.13 ± 9.99^{a}	6.09 ± 0.23^{b}	3.02 ± 0.40^{b}	87.51 ± 11.60^{a}
	Schiz. pombe (936)	10.29 ± 0.90^{a}	48.64 ± 5.91^{a}	0.27 ± 0.02 ^{a}	11.03 ± 1.31^{a}	29.13 ± 1.10^{a}	24.58 ± 0.29^{a}	105.20 ± 9.28^{a}	5.69 ± 0.17^{ab}	2.77 ± 0.34^{ab}	84.72 ± 8.03^{a}
77	Control	10.36 ± 1.02^{a}	55.74 ± 9.07^{a}	0.27 ± 0.01^{a}	10.14 ± 1.10^{a}	25.56 ± 2.79^{a}	22.17 ± 2.03^{a}	95.90 ± 9.74^{a}	8.25 ± 0.56^{a}	2.78 ± 0.00^{a}	79.90 ± 3.79^{ab}
	Sacch. cerevisiae [G3(7)]	10.36 ± 1.60^{a}	70.22 ± 29.47^{a}	0.32 ± 0.04^{a}	10.54 ± 1.29^{a}	26.59 ± 4.09^{ab}	24.05 ± 3.78^{a}	104.10 ± 17.17^{a}	8.78 ± 1.21^{ab}	2.95 ± 0.00^{a}	78.02 ± 5.08^{a}
	S'codes. ludwigii (980)	9.58 ± 0.49^{a}	66.10 ± 22.23^{a}	0.19 ± 0.17^{a}	10.23 ± 0.19^{a}	26.02±1.57^{a}	21.90 ± 0.91^{a}	94.63 ± 3.73^{a}	9.95±0.49^{bc}	2.65 ± 0.08^{a}	76.21 ± 4.04^{a}
	Schiz. pombe (936)	10.40 ± 0.03^{a}	70.99 ± 14.74^{a}	0.31 ± 0.01^{a}	11.48 ± 0.31^{a}	32.05 ± 1.12^{b}	23.20 ± 0.68^{a}	100.31±3.34^{a}	11.36 ± 0.87^{c}	3.02 ± 0.15^{a}	87.69 ± 1.38^{b}
100	Control	10.91 ± 0.36^{a}	50.45 ± 2.88^{a}	0.29±0.03^{a}	10.53 ± 0.10^{a}	29.26 ± 2.11^{a}	22.06 ± 0.61^{b}	80.26 ± 8.89^{a}	8.57 ± 1.84^{a}	2.78 ± 0.08^{b}	86.31±4.91^{ab}
	Sacch. cerevisiae [G3(7)]	10.33 ± 1.41^{a}	48.35 ± 5.96^{a}	0.33 ± 0.09^{a}	9.08 ± 1.39^{a}	30.62 ± 1.67^{a}	18.95±3.24^{a}	95.14±0.61^{b}	9.34 ± 0.30^{a}	2.38 ± 0.23^{a}	92.66 ± 0.25^{b}
	S'codes. ludwigii (980)	10.46 ± 0.74^{a}	48.70 ± 6.99^{a}	0.28±0.02^{a}	10.00 ± 0.97^{a}	27.40±3.14^{a}	20.86 ± 1.58^{ab}	90.37 ± 7.44^{ab}	8.44 ± 0.77^{a}	2.70 ± 0.34^{ab}	82.73 ± 6.91^{a}
	Schiz. pombe (936)	10.67 ± 0.45^{a}	50.04 ± 3.53^{a}	0.29 ± 0.03^{a}	9.99±0.63^{a}	29.87 ± 0.96^{a}	20.94 ± 1.28^{ab}	90.87±5.58^{ab}	9.68 ± 0.14^{a}	2.80 ± 0.12^{b}	94.17 ± 2.97^{b}

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

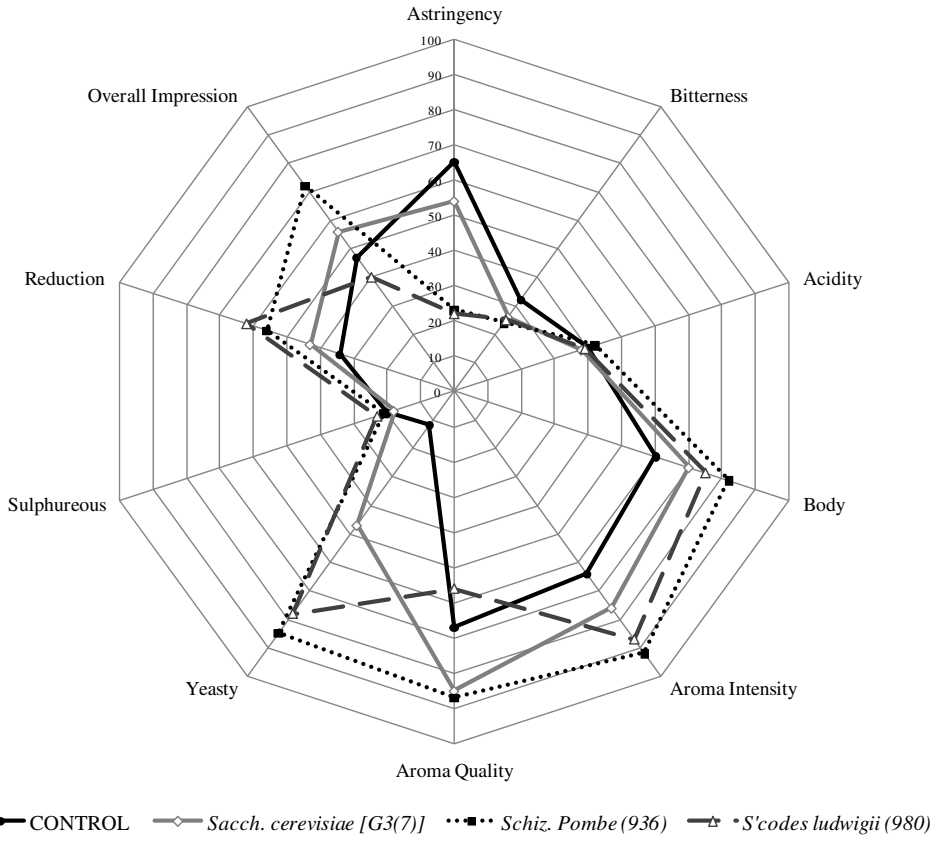

Fig. 7. Gustatory and Olfactory attribute scores of the control wine and the wines obtained through aging over-lees (all strains).

This can be advantageous, for example, in the prevention of wood flavours that often appears over long aging periods (Chatonet, Dubourdieu, & Boidron, 1992). The interactions that occur depend primarily on the nature and concentration of the aromatic molecules involved (Lubbers et al., 1994), but also on the physico-chemical properties of the medium (pH, temperature, ionic strength), which can influence the aroma-retaining capacity of the lees by causing conformational changes in the tertiary structure of their proteins (Kinsella, 1990). Chalier, Angot, Delteil, Doco, and Gunata (2007) established the aroma-retaining capacity of the polysaccharide size fractions of the lees of different yeast species.

Table 4 shows the change in concentration of the different aromatic compounds studied. No significant differences were seen between the yeasts used. Since the yeasts added were lyophilised and added to a nutrient-free medium, no aromatic compounds such as diacetyl or acetoin were produced.

3.5. Sensory analysis

Fig. 7 shows "spider web" diagrams for the average scores of some olfactory and gustatory attributes – of importance in relation to the aging over-lees methodology, of the control wine versus those wines aged over-lees for all the strains assayed. The wines aged with lyophilised yeast biomass from *Schiz. pombe* presented the maximum aroma intensity as well as aroma quality, and they were preferred by the tasters.

The resultant wines from the aging over-lees with *S'codes. ludwigii* showed similar aroma intensity; however their yeast lees biomass produced a low pronounced olfactory off-flavour not easily discernable (only two tasters were able to detected it) related to aroma quality, and they obtain the less average score for overall impression. This new attribute described as over-ripe green apple odour, was not detectable in the control samples, so it supposed to derive entirely from the yeast; however it must be further studied in order to verify its origin.

4. Conclusions

The yeast genera studied in this work showed very positive polysaccharide release kinetics, and are of potential interest in the new over-lees aging method of Suárez-Lepe and Morata (2006) (patent P-200602423). Their use would allow aging times to be reduced since the wine content of yeast cell wall polysaccharides and mannoproteins would increase rapidly. This would invest wines with more complex molecular size profiles with beneficial effects on their physico-chemical (tartaric and protein stability), sensorial and palatability properties. It would also improve the density of these wines in the mouth. Importantly, these benefits seem not to be accompanied by any loss of colour.

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