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Impact of Oxygen Consumption by Yeast Lees on the Autolysis Phenomenon during Simulation of Wine Aging on Lees

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Potential oxygen consumption by lees, more precisely by nonviable yeasts, during wine aging was recently described. Additionally, yeast autolysis is described as the main mechanism of degradation of lees during wine aging. Thus, to understand the effect of oxygen consumption by yeast lees during wine aging, an accelerated wine aging methodology was tested. Wine aging in the presence of yeast lees was studied both in the presence and in the absence of oxygen. Different markers of yeast autolysis were followed to find a relationship between oxygen consumption by yeast lees and changes in the final wine composition after aging. No differences for compounds tested were found in the wine and in the lees except among sterol compounds in lees: in the presence of oxygen, the concentration of ergosterol in lees was significantly lower than that in the absence of oxygen. It was hypothesized that ergosterol could be oxidized under the influence of oxygen, but none of the known products of ergosterol oxidation were recovered in the corresponding yeast lees. In addition, the decrease of ergosterol content in yeast lees cannot account for the total amount of oxygen consumed by yeast lees during such wine aging.

KEYWORDS: Lees; yeast; autolysis; sterol; oxygen; aging; wine

INTRODUCTION

Wine aging on lees is a traditional wine-making technique used in many countries. This aging technique involves letting wines sit in contact with lees for a few months up to a few years after the alcoholic fermentation. Although the technique is used primarily with white wines, it has more recently been used with red wines. Typical wines aged on lees are Muscadet wines from the Loire valley in France, some famous wines from Burgundy, and sparkling wines from Champagne, as well as some wines from California, Australia, and Japan. Wines aged on lees are generally described as having more body and flavor (for an extensive review, see ref *1*).

The main qualities and properties of yeast lees have been recently described in a review (*1*). Lees are composed mainly of dead yeast and bacteria that have completed both alcoholic and malolactic fermentations. Lees also contain some organic residues (principally plant cell fragments) and tartaric salts (*2*). During wine aging on lees, yeast lees deteriorate themselves after their cellular death. This biological degradation is called "cell autolysis". Autolysis is defined as the hydrolysis of intracellular biopolymers by the endohydrolases activated upon cellular death (*3*). Thus, autolysis releases into the wine many low molecular weight compounds such as nitrogenous compounds, polysaccharides, and lipids, which have been described extensively (*4*−*6*). Most of the studies about aging on lees have focused on compounds released into the wine during yeast autolysis. Moreover, yeast lees are able to adsorb anthocyanins from wines (*7*) and some volatile thiols on their cellular wall (*8*). Also, yeast lees play a role in wine tartaric and proteic stabilization due to the release of specific proteins, which can interact with wine macromolecules (*9*, *10*).

We have recently discovered that yeast lees can consume oxygen during the aging of wine and model solutions (*11*). These oxygen consumptions are clearly attributable to the presence of nonviable yeast in lees. The range of the oxygen consumption rates is between 1 and 4 μmol of O_2 h^{-1} 10^{-10} cells from the second month to the third year of wine aging (*12*, *13*). Oxygen consumption by yeast lees has to be considered during white wine aging for the following reasons: (i) barrel filling (*14*) and simple opening of filled barrels (*15*) are technological steps that strongly favor oxygen dissolution in the wine (up to 0.2 mg of O_2 L^{-1}); (ii) there are many examples of periodic stirring of the lees during wine aging on lees, including repetitive additions of small amounts of oxygen (up to oxygen saturation) to the wines generally associated with a limited homogenization of wine and lees on a traditional and empirical basis (known as "bâtonage" or "stirring" process) (*1*).

Here, we show the impact of oxygen consumption by yeast lees on yeast autolysis capacity and results from the quantification and characterization of the compounds released into the wine. For this study, we simulated wine aging by using wine model solutions and yeast lees to avoid raw material variability and potential oxygen consumption by wine polyphenols (*16*).

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The goal of this study is to better comprehend the autolysis phenomenon during wine aging and to determine the role of oxygen during the process.

MATERIALS AND METHODS

Strains and Culture Conditions. *Yeast Strain.* The *Saccharomyces cerevisiae* strain K1 used was an industrial diploid homothallic wine yeast commercialized as a dried active yeast (K1 ICV-INRA, Montpellier, France).

Culture Media. The synthetic medium MS300 used in this study was a simulated standard grape juice and was strongly buffered to pH 3.3 (*17*). This medium contained, in excess, all of the vitamins essential for yeast strain growth. It contained the following (per liter): glucose, 200 g; citric acid, 6 g; DL-malic acid, 6 g; (mineral salts) KH_2PO_4, 750 mg; KH_2SO_4, 500 mg; $MgSO_4 \cdot 7H_2O$, 250 mg; $CaCl_2 \cdot 2H_2O$, 155 mg; NaCl, 200 mg; $MnSO_4 \cdot H_2O$, 4 mg; $ZnSO_4$, 4 mg; $CuSO_4 \cdot 5H_2O$, 1 mg; KI, 1 mg; $CoCl_2 \cdot 6H_2O$, 0.4 mg; H_3BO_3, 1 mg; $NaMoO_4 \cdot 2H_2O$, 1 mg; (vitamins) *myo*-inositol, 20 mg; nicotinic acid, 2mg; calcium panthothenate, 1.5 mg; thiamin hydrochloride, 0.25 mg; pyridoxine hydrochloride, 0.25 mg; biotin, 0.003 mg; (anaerobic growth factors) ergosterol, 15 mg; sodium oleate, 5 mg; Tween 80, 0.5 mL; nitrogen source, 385 mg of N L^{-1} as (% w/w) ammoniacal nitrogen (NH_4Cl) 18.6; and (amino acids) L-proline, 20.5; l-glutamine, 16.9; L-arginine, 1.25; L-tryptophan, 6; L-alanine, 4.9; L-glutamic acid, 4; L-serine, 2.6; L-threonine, 2.6; L-leucine, 1.6; L-aspartic acid, 1.5; L-valine, 1.5; L-phenylalanine, 1.3; L-isoleucine, 1.1; L-histidine, 1.1; L-methionine, 1.1; L-tyrosine, 0.6; L-glycine, 0.6; L-lysine, 0.6; and L-cysteine. 0.4. Because the amino acid proline is not assimilable by the yeast strains used in this study under anaerobic growth conditions, the ammonium salts and α-amino acids (all amino acids except proline) in the medium were considered as assimilable nitrogen. As a consequence, the assimilable nitrogen concentration of MS300 medium is 300 mg of N L^{-1}.

Growth Conditions. The K1 strain, which is available as a commercial dried yeast, was directly inoculated into fermentation medium after the rehydration procedure recommended by the manufacturer. Briefly, 1 g of dry yeast was suspended in 10 mL of warm water (37 °C) containing 0.5 g of glucose. This suspension was kept without stirring for 30 min at 37 °C except for one short stirring after 15 min of incubation. Inoculation of fermentation media was realized by adding 0.5 mL of this suspension per liter of culture medium. Such yeast inoculum corresponded to the enological practice at the industrial scale (5 g hL^{-1}). Fermentation media were normally deaerated by bubbling argon prior to inoculation (initial oxygen concentration < 1 mg L^{-1}). Filling conditions (1.1 L) were controlled, and fermentations were carried out in fermentors (1.2 L) fitted with fermentation locks with permanent stirring under isothermal conditions (28 °C) and anaerobiosis.

Cell Harvesting and Simulation of Wine Aging on Lees. Yeast cells were harvested by centrifugation exactly 100 h after the end of alcoholic fermentation as determined by the absence of residual sugar in the culture medium (concentration < 2 g L^{-1}). The 100-h lag time at 28 °C after the end of fermentation is necessary to get no cell viability (<10^{-3}) in yeast cells (*12*). The yeast cells were then considered to be yeast lees. The yeast lees were washed twice in a synthetic wine that simulates a standard wine (12%, v/v) and resuspended to the same cell concentration as the MS300 culture medium concentration at the end of alcoholic fermentation. The synthetic wine was buffered to pH 3.3 and contained the following (per liter): citric acid, 6 g; DL-malic acid, 6 g; (mineral salts) KH_2PO_4, 750 mg; KH_2SO_4, 500 mg; $MgSO_4 \cdot 7H_2O$, 250 mg; $CaCl_2 \cdot 2H_2O$, 155 mg; NaCl, 200 mg; and ethanol, 120 mL.

Yeast lees were then divided into three parts: one part was centrifuged and lyophilized and represents the reference yeast [reference lees (T)]. The second and third parts, suspended in pure argon-saturated wine medium, were poured in sterile closed bottles under argon headspace in order to avoid further biological contaminations. Two modalities of autolysis were then tested: Lees were either left alone under constant stirring (500 rpm) at 28 °C for 21 days [argon lees (A)] or regularly received sterile air, thus oxygen, during 21 days under constant stirring (500 rpm) at 28 °C, as previously described [oxygen lees (O)] (*16*). The oxygen transfer coefficient (K_La) was calculated to be 2 h^{-1} when air was introduced. After the simulated wine aging, each sample of lees was harvested by centrifugation, rinsed with saline buffer, and lyophilized. Simulated aged wines on lees were conserved [oxygen medium (OM); argon medium (AM)] for further analysis.

Analytical Methods. *Cell Growth.* Cell growth was monitored as cell number by using an electronic particle counter (Beckman-Coulter, Z2 model).

Residual Glucose. Glucose consumption during growth was measured with dinitrosalicylic acid reagent (*18*).

Nitrogen Determination. Total nitrogen in the wine and the yeast lees was determined by the Kjeldahl method: 5 mL of wine or 20 mg of dry yeast was combined in a tube with pure sulfuric acid (15 mL), catalysis minerals, and two glass beads (5-mm diameter). After mineralization and alkanilization on a Büchi 421 apparatus, boric acid (5 mL) and 10 mL of pure water were added to the distillate. The resulting solution was titrated with hydrochloric acid (0.02 M) to reach a pH value of 4.6.

Total protein was extracted from yeast lees as described by Jayamaran et al. (*19*). Total protein in wine and extracts was determined with the BCA protein assay reagent (Pierce Chemicals, Rockford, IL), according to the method of Smith et al. (*20*).

Total Yeast Polysaccharide Determination. Five milligrams of lyophilized yeast was poured into pure sulfuric acid and allowed to stand for 3 h with intermittent stirring. Five milliliters of water was then added. The yeast suspension was then allowed to stand at 100 °C for 4 h. After cooling on ice, 0.25 mL of sample was added to 2.5 mL of an anthrone solution (50 mL of water, 250 mL of pure sulfuric acid, and 570 mg of anthrone) and allowed to stand for exactly 12 min at 100 °C. Optical density of the chilled samples was read at 625 nm using various glucose solutions as a reference (*21*). Repeatability of this method was >95%.

Polysaccharide Composition as Neutral Monosaccharides. Neutral monosaccharides were released from wine (2 mL brought to dryness at 40 °C) by hydrolysis with 2 M trifluoroacetic acid (0.5 mL, 1.25 h, 120 °C) (*22*), derivatized as their alditol acetates, and then analyzed by GLC at 210 °C on a DB-225 capillary column (30 m × 0.32 mm i.d., 0.25 μm film, J&W Scientific) with H_2 as the carrier gas (*23*). Identifications were based on retention times and confirmed by GLC-MS using the same column, coupled to a Finnigan Mat ITD 400 mass spectrometer.

Lipid Determination. Lipids were measured according to two methods (*24*, *25*). Butylated hydroxytoluene [BHT; 0.01% (w/v)] was added to all solvents to prevent further oxidation.

(1) Quantitative Method. One gram of lyophilized yeast was added to hydrochloric acid (5 mL) and 1.5 mL of water in a sealed tube. After the mixture had boiled for 10 min, 5 mL of chloroform was added. The mixture was boiled again for 10 min. Then 37.5 mL of diethyl ether/ethyl petroleum (1:1, v/v) was added. After vigorous shaking, the two obtained phases were separated. The upper phase (25 mL) was recovered, dried on Na_2SO_4, and then evaporated. The obtained lipids were weighed, and lipid concentration in 100 g of yeast dry weight was determined according to the formula

$$\text{lipid concn} = [(a/b)(37.5 + 5)]/25 \times 100$$

where a is the isolated lipids weight and b is the initial dry yeast weight.

(2) Qualitative Method. One gram of lyophilized yeast was dissolved in 100 mL of methanol/chloroform/water (2:1:0.8, v/v/v) with the addition of an internal standard [100 μL of C13:0 (13.33 g L^{-1})]. The first lipid extraction was performed by intermittent shaking of the suspension for one night at room temperature. The suspension was centrifuged (11000*g*, 4 °C, 10 min), and the yeast was resuspended in 33 mL of methanol/chloroform/water (2:1:0.8, v/v/v). This suspension was shaken for 1.5 h. After centrifugation, both of the obtained supernatants were mixed and completed with 50 mL of chloroform and 50 mL of water. After drying on Na_2SO_4, the organic phase was evaporated under vacuum and weighed. After saponification of samples with 4 N KOH (1 h at 80 °C in sealed tubes), the unsaponifiable fraction was recovered after three extractions with 1.5 mL of hexane and three washing cycles with 1.5 mL of ethanol (50%, v/v). This fraction was evaporated and weighed before analysis. The aqueous fraction, including the fatty acids in potassium salt form, was acidified with 0.5 mL of 4 N HCl. Free fatty acids were then extracted by three extractions with

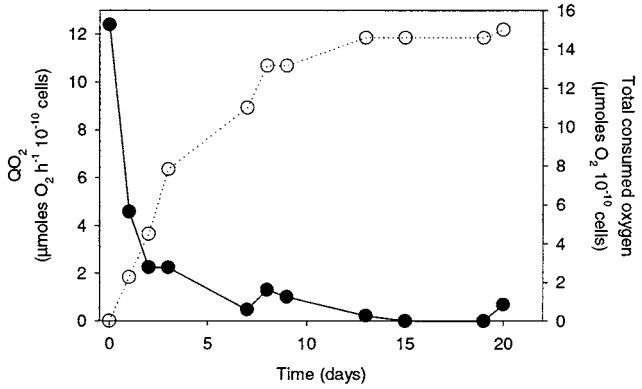

Figure 1. Specific oxygen consumption rates (solid symbols) and total oxygen consumed by lees (open symbols) during storage at 28 °C of *S. cerevisiae* lees in a synthetic model wine (lees concentration = 2×10^8 cells mL^{-1}) in the presence of oxygen ($K_La = 2\ h^{-1}$).

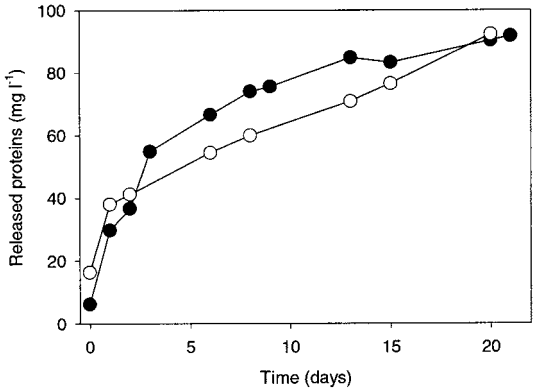

Figure 2. Protein release by lees in the medium during storage at 28 °C of *S. cerevisiae* lees in a synthetic model wine (lees concentration = 2×10^8 cells mL^{-1}): (solid symbols) stirred lees + oxygen ($K_La = 2\ h^{-1}$); (open symbols) stirred lees + argon.

1.5 mL of hexane and three washing cycles with 1.5 mL of ethanol (50%, v/v). After drying by evaporation and weighing, each sample was resuspended in 1 mL of hexane.

(3) GPC-MS Lipid Analysis. All samples were silylated before injection on GPC-MS: 0.4 mL of sample, 50 μL of methylsilyltrifluoroacetamide (MSTFA, Pierce Chemicals), and standards [50 μL of C11:0 (20 mg mL^{-1} in pyridine for fatty acid analysis) or 50 μL of cholesterol (0.5 mg mL^{-1} in pyridine for sterols analysis)] were incubated at 50 °C for 20 min. Silylated compounds were then separated using a Hewlett-Packard 5989-II gas chromatograph fitted with a DB5 apolar column (J&W Scientific, 60 m × 0.32 mm × 1 μm) and coupled to a Hewlett-Packard 5890A mass spectrometer.

Dry Weight. Ten milliliters of yeast suspension sample was filtered through a preweighed filter (0.45 μm). After two washes with water, cell cakes were dried at 80 °C until constant weight.

Ashes. Ashes were calculated after incineration (525 °C, 20 min) of 0.25 g of dried yeast. Ashes are expressed in percent of dry yeast weight.

Cellular Viability. Cellular viability was obtained by plating ~1000 cells, as determined with the electronic particle counter, on YPD agar medium on Petri dishes [agar, 20 g L^{-1}; yeast extract (Difco, Detroit MI), 10 g L^{-1}; bactopeptone (Difco), 20 g L^{-1}; and glucose, 20 g L^{-1}]. Petri dishes were then incubated for 48 h at 28 °C and examined for the presence of colonies.

Statistical Methods. For each aging modality, five or six different aging experiments were performed to determine statistical differences. Statistica software (Statsoft, Tulsa, OK) was used for variance analysis and comparison tests by least significant difference (LSD) test. Test feasibility was confirmed by the Levene test, which showed that the variances and averages are similar and comparable to one another (*26*).

RESULTS AND DISCUSSION

The first aim of this study was to precisely identify the influence of oxygen consumption by yeast lees during wine aging on lees. We tested several accelerated wine aging methods to compare oxygen (O) and no-oxygen (A) modalities with lees harvested at the end of fermentation as references (T). During experimental accelerated wine aging in the presence of oxygen, yeast lees consumed large amounts of oxygen (**Figure 1**), as previously described (*11*). Changes in compounds, which are usually described as autolysis markers, were followed during simulations of wine aging on yeast lees. Thus, we analyzed both the synthetic wine media and the yeast lees composition at the end of the accelerated wine aging.

Synthetic Wine Analysis. The amount of released nitrogen was followed for 2 weeks and the total nitrogen released was measured after 3 weeks of aging. Released nitrogen was previously described as the main measurable factor of yeast autolysis in wine (*27*). Indeed, we noticed that nitrogenous compounds (**Figure 2**) were released during simulation of wine aging: protein release ranged from 67.5 to 85.5 mg L^{-1} (**Figure 2**), and total nitrogen release was estimated between 17 and 32.2 g of N g [dry weight (dw)]$^{-1}$ (set of six repeatable experiments). Nevertheless, as previously described (*11*), no differences were detected between aging modalities, either in nitrogen release profile or in final nitrogen concentration in the aging media (**Table 1**).

Polysaccharides released during aging were analyzed after hydrolysis and derivatization of the corresponding sugar components into alditol acetates. A release of polysaccharides by the yeast was noticed after aging (between 45 and 72 mg L^{-1}, according to our experiments) as noted by others (*28*, *29*). However, no significant differences were observed between agings performed in the presence (O) and absence (A) of oxygen (**Table 1**). Further qualitative analysis of released polysaccharides revealed the major release of two monosaccharides—glucose and mannose—and, for only one sample, trace amounts of fucose and galactose. The obtained mannose/glucose ratios were in accordance with the corresponding literature (*29*).

Yeast Lees Analysis. The yeast lees samples from all modalities tested were nonviable after only 2 days of aging (data not shown). Yeast dry weight was followed every 2 days during the 3 weeks of aging (**Figure 3**).

Compared to the initial yeast lees sample (T), the dry weight of aged yeast lees decreased similarly between 16.6% in the presence of argon (A) and 18.7% in the presence of oxygen (O) (**Table 1**). Such a yeast dry weight decrease has been described by many authors and was considered to be one of the manifestations of yeast autolysis (*5*, *6*, *30*). However, dry weight decrease did not differ significantly between agings performed in the presence (O) and absence of oxygen (A). Thus, oxygen addition did not influence the yeast lees dry weight during aging. With the aging conditions tested, we did not notice an initial increase followed by a decrease of ~40% in dry weight as observed by others (*6*). The different physiological states of the yeasts used could account for this observation. Indeed, to simulate the autolysis phenomenon, most of the authors used dried active yeasts directly inoculated into the wine or model wine (*31*, *32*), or fresh precultures (*5*, *30*). Therefore, such yeast cells had not performed the whole fermentation process. This was not the case in the present experiments, when the yeast used was harvested after a complete alcoholic fermentation without any residual cell viability. Therefore, despite their null viability, yeast lees may activate enzymes, especially proteases and glucanases, which can attack cell wall and membranes and allow the liberation of autolysis products. The observed dry

Table 1. Evolution of Several Parameters after 21 Days of Conservation at 30 °C of *S. cerevisiae* Lees in a Synthetic Model Wine (Lees Concentration = 10^8 Cells mL^{-1})

	aging modality[a]			no. (*n*) of experiments
	T	A	O	
protein release in the aging medium (mg L^{-1})		80.5 ± 5.5	75.6 ± 8.0	5
polysaccharide release in the aging medium (mg of glucose equiv L^{-1})		62.4 ± 8.1	64.7 ± 13.6	4
mannose/glucose ratio of released polysaccharides		2.86 ± 0.38	2.17 ± 0.23	4
yeast lees dry weight (g L^{-1})	4.28 ± 0.15	3.57 ± 0.10	3.48 ± 0.15	5
yeast lees protein [g g $(dw)^{-1}$]	0.41 ± 0.03	0.35 ± 0.02	0.35 ± 0.02	5

[a] T, yeast lees at the end of alcoholic fermentation; O, stirred lees + oxygen (K_La = 2 h^{-1}); A, stirred lees + argon. Mean and standard error of *n* different aging experiments.

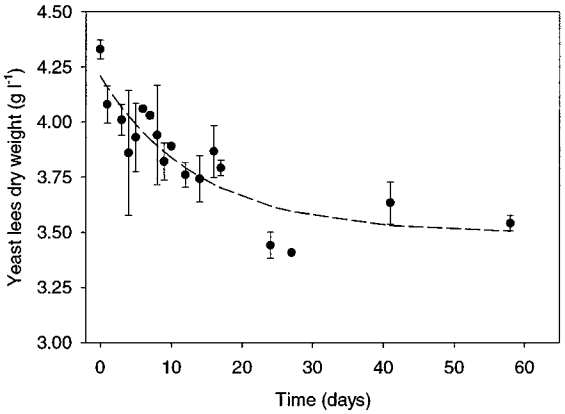

Figure 3. Yeast lees dry weight during storage at 28 °C of stirred *S. cerevisiae* lees in a synthetic model wine under argon gas (lees concentration = 2×10^8 cells mL^{-1}). Mean and range of two different aging experiments.

weight decrease may be attributed to this phenomenon. A complementary study of the total ashes of recovered yeast lees did not reveal any differences between the aging modalities (data not shown).

A significant difference between protein contents of aged (A and O lees) and reference (T lees) lees was also noticed. However, the presence or absence of oxygen did not have any effect (**Table 1**). By using nitrogen concentration in standard lees as a reference, a total recovery of nitrogen between final aging medium and yeast lees was achieved with <5% error (data not shown). After 3 weeks of aging, a relative enrichment of yeast lees in polysaccharides was observed. This observation has been noted by Charpentier and Freyssinet (*33*) during studies on yeast autolysis. Indeed, during autolysis, cell wall loosening allows a greater release of nitrogenous compounds than of polysaccharides, which explains the relative sugar increase within the biomass. The increase in polysaccharides in yeast lees was clearly unaffected by the aging modality (**Table 1**).

Therefore, the effectiveness of yeast lees autolysis in our experimental model was verified by the observation of a decrease in yeast dry weight and a significant release of nitrogenous compounds and polysaccharides in the aging medium after 3 weeks. However, all of these classical autolysis markers were not affected by the presence or absence of oxygen during the aging process.

Evolution of Lipids in Yeast Lees: Quantification and Identification of Fatty Acids. In any living organism, oxidation of lipids and lipoproteins may arise, either by the action of free radicals or by the action of enzymatic activities (*34*, *35*). Therefore, an exhaustive analysis was performed on yeast lipids, because they may easily interact with oxygen by simple oxidation. Lipids are a minor component of yeast (*36*) and therefore required large amounts of cell dry weight to be correctly quantified and analyzed. Two methods of cell lipid quantification were used: the Lamacka method (*24*), described as the best among several methods, and the Kates method (*25*). Repeatability of the second method was estimated to be 21%. Lipids extracted with the second method were verified by GPC-MS as sterol and free-acid forms.

Table 2. Total Lipid Contents of Yeast Lees after 21 Days of Storage at 28 °C of *S. cerevisiae* Lees in a Synthetic Model Wine (Lees Concentration = 2×10^8 Cells mL^{-1})

	total lipid contents [mg g $(dw)^{-1}$] by aging modality[a]					
	method 1 (*24*)			method 2 (*25*)		
expt	T	A	O	T	A	O
1	7.5	1.8	1.1	4.8	5.3	4.8
2	2.7	2.8	2.4	5.2	5.3	5.9
3	1.9	2.0	1.4	4.9	6.5	7.4
4	nd[b]	nd	nd	6.1	8.5	9.5
5	nd	nd	nd	5.6	4.5	5.0
6	nd	nd	nd	5.6	6.0	6.1

[a] T, yeast lees at the end of alcoholic fermentation; O, stirred lees + oxygen (K_La = 2 h^{-1}); A, stirred lees + argon. [b] nd, not determined.

Table 3. Total Lipid Contents of Yeast Lees after 21 Days of Storage at 28 °C of *S. cerevisiae* Lees in a Synthetic Model Wine (Lees Concentration = 2×10^8 Cells mL^{-1})

	total lipid contents [mg g $(dw)^{-1}$] by aging modality[a]					
	gravimetric method			sum of GPC-MS peaks		
expt	T	A	O	T	A	O
1	14.8	17.0	14.8	11.8	13.3	13.3
2	18.9	25.7	23.4	11.6	13.2	15.4
3	16.9	21.9	18.1	10.0	14.9	13.8
4	10.5	143	13.7	10.8	11.5	9.2
5	16.7	30.1	18.1	15.8	17.6	15.0

[a] T, yeast lees at the end of alcoholic fermentation; O, stirred lees + oxygen (K_La = 2 h^{-1}); A, stirred lees + argon.

As shown in **Table 2**, the two lipid quantification methods produced differing results: the lipidic fraction represents 2−3% of cell dry weight with the first method, whereas it represents 4−10% with the second. Lipid accessibility to the solvents could explain these differences previously observed by Lamacka (*24*). However, with both methods, no significant differences concerning the quantities of extracted lipids were noted between the different wine aging modalities.

After saponification of lipids, free fatty acids were more precisely quantified and identified. Their respective amounts were estimated by gravimetry or by simple addition of the different free fatty acids detected by GPC-MS (**Table 3**). This second method (sum of GPC-MS peaks) appeared to be more precise as shown by the small standard deviation values. Indeed, trace amounts of the solvents could easily influence the

Table 4. Analysis of Unsaturation in the Lipids of Yeast Lees after 21 Days of Conservation at 30 °C of *S. cerevisiae* Lees in a Synthetic Model Wine (Lees Concentration = 10^8 Cells mL^{-1})

total lipid contents [mg g (dw)$^{-1}$] by aging modality[a]					
unsaturation degree[b]			unsaturation index[c]		
T	A	O	T	A	O
1.044 ± 0.011	1.066 ± 0.0195	1.058 ± 0.018	0.408 ± 0.024	0.454 ± 0.022	0.452 ± 0.038

[a] T, yeast lees at the end of alcoholic fermentation; O, stirred lees + oxygen ($K_La = 2\ h^{-1}$); A, stirred lees + argon. Mean and standard error of five different experiments. [b] Ratio Σ(unsaturated fatty acids × no. of unsaturation)/Σ(unsaturated fatty acids) in moles. [c] Ratio Σ(unsaturated fatty acids)/Σ(total fatty acids) in moles.

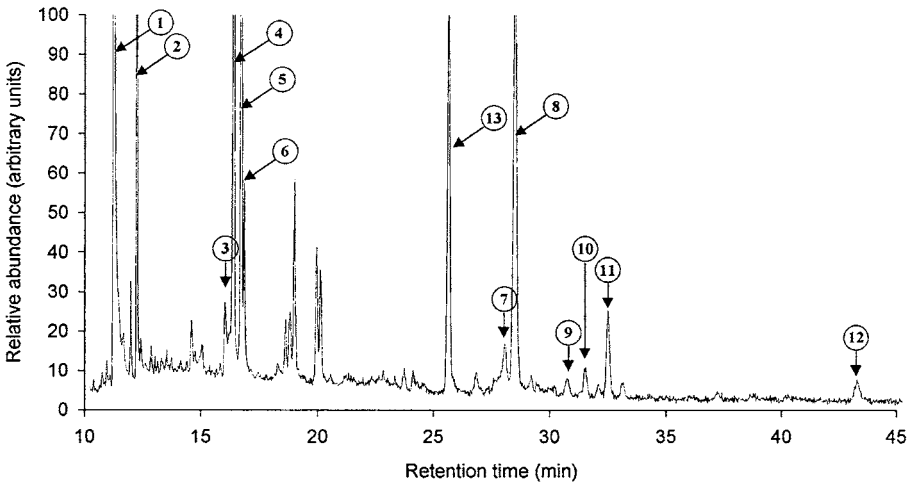

Figure 4. Typical sterol chromatogram of yeast lees after 21 days of storage at 28 °C in a synthetic model wine (lees concentration = 2×10^8 cells mL^{-1}). Peaks: (1) unknown compound; (2) unknown compound; (3) unknown compound; (4) unknown compound; (5) squalene; (6) unknown compound; (7) neoergosterol; (8) ergosterol; (9) unknown compound; (10) ergost-7-en-3β-ol; (11) lanosterol; (12) unknown compound; (13) cholesterol (internal standard).

gravimetric determination of extracted free fatty acids. An analysis of variance performed on the quantities of free fatty acids determined by GPC-MS revealed that there were no differences between the two aging modalities (data not shown).

The yeast lees exhibited a range of fatty acids containing from 8 (caprylic acid C8:0) to 26 carbon (C26:0) atoms. The major free fatty acids were, by weight, oleic acid (C18:1), palmitic acid (C16:0), and stearic acid (C18:0), which combined represented between 68 and 84% of the total identified free fatty acids. Individual free fatty acid release did not statistically reveal any differences according to the aging modality (test by pairs, data not shown).

Ferrari et al. (*37*) previously described an increase of free fatty acids in autolysis medium between 5.3 and 12 μmol L^{-1} during yeast autolysis (40 °C, 7 days). For four sets of experiments, we also observed an initial fall and then a release of free fatty acids into the autolysis medium estimated between 15 and 52 μmol L^{-1}, but we did not see any release for two sets of experiments (data not shown). Unsaturation index and degree were also studied to follow the proportion of saturated and unsaturated free fatty acids in the final yeast lees after 3 weeks of aging (**Table 4**). Again, no differences were observed for the two different aging modalities tested.

Evolution of Lipids in Yeast Lees: Quantification and Identification of Sterols. A similar analysis was performed on yeast sterols to study the eventual influence of oxygen during yeast autolysis on sterols. The weight of sterol compounds was estimated by the sum of all identified sterols by mass spectrometry. This methodology suffers from some key limitations: (i) some peaks could not be identified (and therefore not included in the final calculation); (ii) a coefficient of response of 1 was applied to all identified sterols; (iii) squalene, which is only a precursor of sterols, was included in this calculation.

The total sterol content was estimated to represent only 0.13 and 0.64 mg g^{-1} of dry yeast, which correlates to 0.2−1% of the extracted lipids. The values appeared to be very low compared to the literature values and the initial unsaponifiable material estimated by weighing. Le Fur et al. (*38*) detected a sterol content between 4 and 9 mg g^{-1} in dry yeast after 14 days of autolysis in similar conditions. The sterol extraction conditions, and more precisely the derivatization step, could explain the observed differences. However, the analysis of variance on the identified sterol contents revealed a significant effect at a level of 0.05 dependent on the aging modality (data not shown).

Five sterol-related compounds of yeast lees were clearly identified by mass spectrometry (**Figure 4**): squalene (not silylated), ergosterol, ergost-7-en-β-ol, neoergosterol, and lanosterol. Other sterol peaks were observed but not identified.

Previously, Le Fur et al. (*38*) detected in yeast cells up to 10 different sterols (and 5 unidentified) and some sterols in the autolysis medium, too. The differences between the numbers of identified sterols in both studies were probably due to the different physiological statuses of the yeast used. Le Fur et al. (*38*), in their autolysis model, used directly dried active yeasts, which are known to be particularly rich in lipids due to their production method (*39*, *40*). In contrast, the yeast used in the present study underwent a classical alcoholic fermentation before their use for wine aging. Ergosterol, squalene, lanosterol, and ergost-7-en(3β)-ol were the main sterols detected in yeast lees. Ergosterol is described as the main sterol found in *Saccharomyces* species. Most *Saccharomyces* strains produce only ~0.1% of ergosterol (by dry weight), whereas some strains can produce up to 7−10% (by dry weight) (*41*). In the present study, ergosterol was provided exogenously to the yeast cells during fermentation via the synthetic MS300 culture medium, because

Table 5. Analysis of the Sterol Contents of Yeast Lees after 21 Days of Storage at 28 °C of *S. cerevisiae* Lees in a Synthetic Model Wine (Lees Concentration = 2×10^8 Cells mL^{-1})

	yeast lees contents [mg g $(dw)^{-1}$] by aging modality[a]		
compound[b]	T	A	O
unknown **1**	0.300 ± 0.097	0.351 ± 0.087	0.247 ± 0.090
unknown **2**	0.022 ± 0.006	0.036 ± 0.010	0.042 ± 0.009
unknown **3**	0.027 ± 0.004	0.039 ± 0.008	0.027 ± 0.007
unknown **4**	0.120 ± 0.012	0.131 ± 0.018	0.126 ± 0.027
squalene **5**	0.147 ± 0.022	0.198 ± 0.034	0.171 ± 0.010
unknown **6**	0.192 ± 0.075	0.050 ± 0.020	0.054 ± 0.024
neoergosterol **7**	0.014 ± 0.002	0.016 ± 0.002	0.014 ± 0.002
ergosterol **8**	0.130 ± 0.038	0.176 ± 0.047	0.037 ± 0.008
unknown **9**	0.006 ± 0.001	0.007 ± 0.001	0.006 ± 0.002
ergosten-7-en-3β-ol **10**	0.007 ± 0.007	0.012 ± 0.002	0.012 ± 0.001
lanosterol **11**	0.017 ± 0.006	0.024 ± 0.014	0.025 ± 0.016
unknown **12**	0.008 ± 0.001	0.007 ± 0.001	0.009 ± 0.001

[a] T, yeast lees at the end of alcoholic fermentation; O, stirred lees + oxygen ($K_La = 2\ h^{-1}$); A, stirred lees + argon. Mean and standard error of five different experiments. [b] Numbers correspond to identified compounds in **Figure 4**.

Table 6. Effect of Aging Modality on the Sterol Contents [mg g $(dw)^{-1}$] of Yeast Lees after 21 Days of Storage at 28 °C of *S. cerevisiae* Lees in a Synthetic Model Wine (Lees Concentration = 2×10^8 Cells mL^{-1}): Analysis of Variance on Five Sets of Experiments

	modality	comparison of modalities by pair[a]		
compound[b]	effect	T/A	T/O	A/O
ergosterol **8**	*	NS	*	*
ergosten-7-en-3β-ol **10**	*	NS	*	NS

[a] T, yeast lees at the end of alcoholic fermentation; O, stirred lees + oxygen ($K_La = 2\ h^{-1}$); A, stirred lees + argon; *, significant effect at $p < 0.05$; NS, not significant. [b] Numbers correspond to identified compounds in **Figure 4**.

the yeast could not synthesize sterols under strict anaerobiosis (*42*). A preliminary GPC-MS analysis of the composition of the pure ergosterol (Sigma Chemicals) used for the preparation of anaerobic growth factors revealed that several sterol contaminants were present in trace amounts (*43*): neoergosterol and ergost-7-ene(3β)-ol were clearly identified as contaminants of ergosterol by mass spectrometry. This may explain the detection of residual neoergosterol in aged yeast lees.

Analysis of variance on all identified unsaponifiable compounds indicates that only the evolution of ergosterol concentration in yeast lees during aging in the presence of oxygen (O) is significantly different from those observed in initial lees (T) and in lees conserved under argon gas (A) (**Table 6**). Moreover, the ergosterol concentration in yeast lees decreased during aging in the presence of oxygen (O) from 34 to 74%, depending on the experiments.

The following hypotheses may explain the observed decrease:

(a) Ergosterol could be oxidized during aging and transformed into other oxidized compounds. Several authors have described ergosterol oxidation by singlet oxygen (*44*, *45*), causing yeast membranes to produce 9(11)-dehydroergosterol, 5α,6α-epoxy-(22*E*)-ergosta-8,22-diene-3β,7α-diol or ergosterol epidioxide depending on cell viability (*45*). However, we were not able to detect any of these oxidized sterol compounds by mass spectrometry in our experiments.

(b) Ergosterol could be oxidized until complete vanishing of the sterol core, creating small fragments not detectable by GPC-MS. This phenomenon was recently described by our laboratory upon oxygen addition at moderate and excessive levels during fermentation by viable yeast cells (*43*). In the latter case, the overall decrease in cell sterols upon oxygen addition is due to the complete oxidation of yeast ergosterol, consequently leading to the disappearance of the sterol nucleus and the release of polyenic fragments.

(c) Ergosterol could be released into the aging medium during autolysis in the presence of oxygen. The release of sterols has already been demonstrated during an accelerated autolysis, but in rather small amounts (*6*, *38*). More particularly, in the latter study, the release of ergosterol into the medium was estimated to represent only 0.2 $\mu g\ g^{-1}$ of dry weight of biomass, whereas the decrease of ergosterol in yeast biomass was 290 $\mu g\ g^{-1}$ of dry weight (*38*). In the present study, the average loss of ergosterol in the presence of oxygen was estimated to be 120 $\mu g\ g^{-1}$ of yeast dry weight by averaging all experimental sets. However, the direct detection of sterol compounds in the corresponding aged wines was not performed due to the very low amounts of expected sterols.

It is therefore difficult to reach a conclusion about the exact mechanism for the decrease of ergosterol content in yeast lees upon oxygen addition. Complementary analysis is necessary to confirm the actual participation of the previous postulated assumptions in the final hypothesis. However, previous works performed on the same experimental model showed that the generation of reactive oxygen species (ROS) was detectable upon contact with oxygen, even at very low oxygen concentration in yeast lees characterized by a null viability (*11*). This ROS generation parallels the ability of yeast lees to consume oxygen and accompanies the generation of lipid peroxides in biomass, leading to a strong modification of plasma membrane integrity (*11*). In the present study, oxygen consumption by yeast lees exerted no changes in the overall autolysis phenomenon except for ergosterol levels. This may indicate that ergosterol was the main substrate of the observed lipid oxidation. Another observation showed the potential oxidation of ergosterol as the main mechanism for ergosterol disappearance from yeast lees upon aging in the presence of oxygen: the total amount of oxygen consumed by yeast lees during such wine aging [~50 μmol of $O_2\ g^{-1}$ of dry weight (*11*)] greatly exceeded the observed decrease of ergosterol contents in yeast lees (~0.3 μmol of ergosterol g^{-1} of dry weight). Such a discrepancy may indicate a multiple attack of oxygen-derived ROS on the ergosterol molecule, which will likely lead to the release of small polyenic fragments. Some of these final ergosterol oxidation products may play a role in the organoleptic equilibrium of wines aged on lees. Additional studies need to be conducted to test such a hypothesis.

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