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"EFFECT OF BIOLOGICAL "SURLIE" AGEING ON SPARKLING WINES QUALITY AND ELABORATION"

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INTRODUCTION

The production quality sparkling wine like Cava and Champagne represents a very important contribution for the European economy. Then, their quality must be controlled and guaranteed and even enhanced by means of objectives parameters.

Surlie ageing after second fermentation is a fundamental operation in the production of these wines by the traditional method. It takes place in anaerobic conditions and in contact with lees, during at least 9-12 months, resulting in an increase in product structure, richness and roundness (Fornairon-Bonnefond et al., 2002). Nevertheless, the conditions influencing the organoleptic evolution of sparkling wines during the surlie ageing period are largely unknown. The base wine, the autolysis of lees and the ageing period seem to be the factors that mainly influence the sensory quality of this sparkling wine. Some author suggested that the special ageing of sparkling wines only depends on the physical and chemical processes that occur in a medium saturated with CO_2 (Usseglio-Tomasset et al., 1983). In contrast, several studies pointed out the process of enzymatic auto-destruction of yeast cells during the ageing of sparkling wines elaborated by the *champenoise* method *(*Colagrande, 1986; Silva et al., 1988; Sato et al., 1997).

During this practice, a narrow interaction is established between wine and lees, which undergo important modifications of their structure, due to the self-degradation process known as autolysis (Leroy et al., 1990). Recently, the importance of the interaction between wine and lees cell surface has been reported (Pérez-Serradilla et al., 2008; Gallardo-Chacón et al., 2008; Mazauric et al., 2005; Lubbers et al., 1994; Chassagne et al., 2005; Jiménez-Moreno et al., 2007). Cell surface properties are considered to be major determinants in microbial interactions, having important repercussions in several technological aspects. Phenomena such as adhesion, flocculation and sorption capacity could have important implication in the ageing on lees practice, and they are reportedly governed by distinct cell surface properties (Smit et al., 1992; Jin and Speers, 1998; Mercier-Bonin et al., 2004; Lubbers et al., 1994; Pradelles et al., 2008).

Among other lees technological aptitudes, sorption and flocculation capacity are especially important in the production of sparkling wine, and are governed by distinct cell surface properties (Smit et al., 1992; Jin and Speers, 1998; Mercier-Bonin et al., 2004; Lubbers et al., 1994; Pradelles et al., 2008). During sparkling wine *surlie* aging, the interaction between aroma compounds and lees cell walls could largely affect the aroma of sparkling wines due to sorption phenomena (Caridi, 2006; Pozo-Bayón et al., 2009). In fact, the parietal capacity of wine yeast to adsorb wine volatile compounds (Lubbers, 1994; Gallardo-Chacón et al., 2008) can reduce the concentration of some volatile compounds once lees are removed, as was already reported in white wines after lees contact (Medina-Carnicer, 2002). Several studies report and characterise the sorption of various wine molecules by yeast surfaces, including phenolic compounds (Vasserot *et al.*, 1997), sulfur products (Palacios *et al.*, 1997) and aroma compounds (Lubbers *et al.*, 1994). Previous studies have demonstrated the sorption capacity of *Saccharomyces cerevisiae* cells for 4-ethylphenol (Guilloux-Benatier *et al.*, 2001, Chassagne *et al.*, 2005). More recently, Pradelles *et al.* (2008) showed that 4-ethylphenol sorption by yeast is greatly influenced by the nature of the yeast strain, the medium and mode of culture and the yeast wall nature and composition. These factors affect the physico-chemical surface properties of cells and that there is a correlation between cell surface characteristics and their sorption capacities. It was suggested that physico-chemical changes in the yeast surface have consequences on the sorption capacity (Pradelles et al., 2008; 2009).

Moreover, by the *méthode champenoise* the yeast cells can be removed from the bottle only by their being settled. Yeast flocculation is thought to be due to cell wall interactions mediated by specific lectins (Miki et al., 1982), but nonspecific interactions governed by cell surface properties such as hydrophobicity (Wilkocks et al., 1995; Amory et al., 1988; Kamada et al., 1984; Smit et al., 1992; Straver et al., 1996) and charge (Amory et al., 1988; Kamada et al., 1984) are supposed to be major determinants in cell flocculation.

Surface properties of yeast cells depend on the wall biochemical composition. Lees are composed mainly by yeast cells (Fornairon-Bonnefond et al., 2002) and it is the outer part of yeasts, which is in constant contact with wine. The cell wall makes up between

25-50 % of cell volume (Lipke et al., 1998) and is composed of a three-dimensional internal skeletal layer of 1,3-_ glucan (30-45% of wall mass) stabilized by hydrogen bonds. Mannoproteins (30-40% of wall mass), or cell-wall proteins, are linked to the nonreducing ends of 1,3- _-glucan (Pir-CWPs) or 1,6-_-glucan molecules (5-10% of wall mass) (GPI-CWPs). These compounds confer to cells physicochemical properties that enable yeasts and lees to interact with other compounds. Mannoproteins are the most highly exposed cell-wall molecules and may therefore form sorption sites.

The deep modifications in cell wall structure by the autolytic process occurring during the ageing on lees, involve cell wall components thought to confer to cells physicochemical properties (Piton et al., 1988; Guilloux-Benatier and Chassagne, 2003; Alexandre and Guilloux-Benatier, 2006), and could induce relevant modifications in the cell surface physicochemical properties and thus in the sorption and flocculation capacity. Autolysis has been defined as the hydrolysis of cellular components by hydrolytic yeasts enzymes. Breakdown of cell membranes, release of hydrolytic enzymes liberation of intracellular constituents, and hydrolysis of intracellular biopolymers into products with a low molecular weight are the main events that occur during this process (Fornairon-Bonnefond et al., 2002; Martínez-Rodriguez et al., 2001; Guilloux-Benatier et al., 2003). In wine production, yeast autolysis has been observed in the case of sparkling wines stored in contact with lees during ageing (Moreno-Arribas et al., 1996). This process entails the release of nitrogen compounds, volatile substances, lipids, carbohydrates and mannoproteins (Fornairon-Bonnefond et al., 2002). During the ageing on lees, mannoproteins are released by yeast by enzymatic action (Caridi, 2006; Leroy et al., 1990) The initial effects of autolysis begin with the hydrolysis of bonds connecting β-1-3 glucans associated with mannoproteins on the outer layer of the cell. Following this step, β-glucanases further hydrolyze the β-1-3 glucans that make up the cell wall (Fornairon-Bonnefond et al., 2002). Doco et al. (2003) reported that mannoproteins release during wine ageing on lees is a progressive and linear phenomenon.

The changes in lees wall structure caused by the autolytic process during sparkling wine *surlie* ageing could induce relevant modifications in the cell surface physicochemical properties and thus in the sorption and flocculation capacity. Although surface properties of live *Saccharomyces cerevisiae* cells have been largely studied, to our knowledge no data on the modifications in cell surface properties during wine ageing and their impact on lees technological attributes are reported.

OBJECTIVES

The aim of the present study was to know the possible implication of lees surface modifications occurring during *surlie* ageing on sparkling wine quality and elaboration. Sorption of volatile compounds and flocculation were taken into account for their role in these aspects and for involving cell surface interactions.

First, to better understand the relationship between physico-chemical properties such as hydrophobicity, charge and electron-donor characteristics, and the yeast surface sorption capacities, we determined these factors in a model system after modifying the cell surface properties of an enological yeast strain. It was subjected to three drying processes which produced significant modifications to cell surface properties and/or composition (Hromádková *et al.*, 2003), by temperature shock to wall macromolecules (Prestrelski *et al.*, 1993), or by the induction of specific transciptionnal mechanisms for cell wall adaptation (Singh *et al.*, 2005).

Then, to confirm the suitability of the results obtained in model system, real industrial lees samples were investigated. The surface properties of sparkling wine lees from the same strain of *Saccharomyces cerevisiae* were characterized according to the time of *surlie* ageing, and their possible influence on lees sorption and flocculation capacity was then evaluated.

METHODOLOGY

Chemicals

The following chemicals were used: glucose, fructose, peptone, tartaric acid, yeast nitrogen base without amino acids, malic acid, ammonium chloride, 99% potassium hydroxide, 99% potassium sulphate, 99% sodium chloride, Sodium acetate trihydrate, acetic acid glacial, hydrochloric acid, calcium chloride$_2$, 99% 4-ethylphenol, 99% 3,4-dimethylphenol, 98% acetic acid, 98% magnesium sulphate, 99% malic acid, 99% decan, 99% hexadecan, 99.8% absolute ethanol, 99% chloroform, and 99.5% ethyl acetate. These chemicals were obtained from Sigma (St Louis, MO, USA). Solutions were made up with ultra pure water, obtained from a Milli-Q system (Millipore, Bedford, MA).

Yeasts and culture medium

The enological *Saccharomyces cerevisiae* strain BM45 Lallemand™ was used. The strain was cultivated in YPD medium (Yeast extract 5 g.L^{-1}, Peptone 10 g.L^{-1} and D-Glucose 20 g.L^{-1}) at 30°C in dynamic mode (80 rpm) under aerobic conditions to obtain a sufficient quantity of biomass.

Yeast cells were harvested in the early stationary phase of growth. Cells were washed three times with sterilized distilled water.

Drying conditions

Lyophilization

The yeast biomass was resuspended in the same mass of distilled water, and was then frozen at -20 ºC for 12 h and lyophilized at 60 mBar and -40 ºC for 24 h using a Christ Loc 1M Alpha 2-4 apparatus.

Spray drying

The yeast biomass was resuspended in distilled water and spray-dried by means of a Mini Spray Dryer B-290, Büchi (Essen, Germany). Initial and outlet temperatures were 180 and 80 ºC, respectively, and the flow was regulated at 70 % of the maximum

allowed by the apparatus. A volume of 50 mL of yeast suspension (10 mg.L^{-1} of fresh biomass) was dried approximatively in one minute. For this temperature, at the cell or at the droplet scale, the evaporation occurs instantaneously (Gharsallaoui *et al.*, 2007).

Evaporative drying at low water activity

The yeast biomass was resuspended in distilled water. Six ml aliquots of yeast suspension were placed on opened Petri plates to form a thin layer. The Petri plates were then introduced into a controlled humidity chamber at an a_w of around 0.09 (saturated potassium hydroxyde solution) and maintained at 20 ºC (Greenspan, 1977) for a period of 48h.

After drying, all the samples (dried at low water activity, lyophilized and spray dried) were placed into an evaporative drying at low water activity apparatus for three days to reach a water activity of 0.09.

Rehydrating dried cells

Two kinds of rehydration methods were used. Treatment 1 corresponds to the direct hydration in the model wine matrix (ethanol 12.5% V/V), simulating a wine with added 4-ethylphenol. In treatment 2, the dried biomass was rehydrated in water before contact with 4-ethylphenol diluted in model wine. The rehydration was carried out in sterile distilled water (ten-fold the mass of dried yeast) for 30 min at 30°C. Then the biomass was harvested by filtration using cellulose acetate membrane (0.45 µm pore size, 47mm, Whatman) and cells were resuspended in model wine enriched with 4-ethylphenol at 10 mg. L^{-1}.

Industrial lees samples

Lees of the same strain of Saccharomyces cerevisiae ssp bayanus belonging to the collection of the winery were from industrial sparkling wines obtained from two distinct coupages of base wines produced in distinct crop years. Lees were collected from wines at 2, 10, 18 and 40 months of “surlie” ageing. Two different bottles were analyzed for each lees sample (total 8x2 bottles), for a total of 16 independent samples analyzed in triplicate.

Lees were prepared as follows: the content of 1 bottle was centrifuged 15 min at 1410 g and 4ºC (Rotina 48CR); the pellet was washed three times with 10 ml of acetate buffer (pH 3.6, 0.3 M) and resuspended in 5 ml of the same buffer. The lees were maintained refrigerated and under nitrogen atmosphere.

Model wine

The model wine buffer contained 12.5 %, v/v ethanol, 3 $g.L^{-1}$ D-L malic acid, 0.1 $g.L^{-1}$ acetic acid, 0.1 $g.L^{-1}$ potassium sulphate, and 0.025 $g.L^{-1}$ magnesium sulphate. The pH was adjusted to 3.5 with 2N sodium hydroxide.

Physico-chemical properties of yeast cell surface

Electron-donor/ -acceptor properties of cells surface

The microbial adhesion to solvent (MATS) technique, based on cell surface affinities for a monopolar and nonpolar solvent (Bellon-Fontaine *et al.*, 1996), was used to determine the electron donor or acceptor properties of dried yeasts and lees suspended in acetate buffer (pH 3.5, 0.3 M). The electron donor/Lewis base character (EDC) was calculated as the difference between the % adherence of lees to chloroform and hexadecane, and the electron acceptor/Lewis acid character (EAC) was calculated as the difference between the % adherence of lees to ethylacetate and decane.

Cell surface hydrophobicity

Yeast cell surface hydrophobicity was determined by adhesion of yeast cells to paramagnetic, polystyrene-coated latex beads (Straver and Kijne, 1995).

Zeta potential.

Lees and dried yeast surface charge was quantified by measurement of the electrophoretic mobility of cells on laser Zetacompact equipment (CAD Instrumentation, Limours, France). This allowed calculating the zeta potential (mV) using the Smoluckowsky's equation. After equilibration of suspensions by magnetic stirring for 15 min, an electric field of 8 $V.cm^{-1}$ was applied to a lees suspension of 10^6 cells mL^{-1}. Results were calculated as mean values of six repetitions.

Cell number, diameter and surface measurements.

The number of cells of each sample and the correspondent cell diameter were measured by using a Coulter counter (Multisizer II, with a 15 µm aperture tube), at which 1.0 mL of cell suspension was added to 49.0 mL of NaCl 0.9% solution (Panreac, Barcelona, Spain). In order to avoid the formation of floc, the stock solution was properly agitated during measurements. A narrow and sharp Gaussian type distribution curve of cell size was obtained, indicating a good cell separation in the stock solution. The number of cells/ml was established by dividing the number of events by the volume of the loop (500 µl). For each suspension the measure was made in triplicate.

The cells surface in each sample was determined considering the mean surface equivalent diameter of the yeast population.

Sorption measurements in model wine

The sorption capacity was determined in model wine with the yeast biomass (14 g of dried biomass per liter) stored in 37 mL capacity flasks, with Teflon caps to prevent the loss of volatile compounds. The initial concentration of standard 4-ethylphenol in the model wine was 10 ppm. This concentration was used to allow direct gas chromatography analysis of 4-ethylphenol and to minimize variability in measurements.

Samples were stirred (550 rpm) at 10°C until equilibrium was reached (4 h of contact). The pH (3.5), the ethanol content (12.5 %) of the synthetic wine and the temperature of the studied system (10°C) were selected to optimize 4-ethylphenol sorption by yeast (Chassagne *et al.*, 2005). The amount of volatile phenols sorbed was calculated from the difference of concentrations between control samples (samples without biomass) and the experimental samples. Experiments of 4-ethylphenol sorption in yeast cells were carried out in triplicate.

Given that the key-step of 4-ethylphenol sorption by yeast is thought to be the binding of the volatile phenol to the cell surface (Chassagne *et al.*, 2005, Pradelles *et al.*, 2008), the sorption capacity of yeast was expressed as the quantity of 4-ethylphenol sorbed

(mg) per cell surface area (m^2), allowing sorption to be normalized against the yeast surface.

Volatile analysis in model wine

Following the protocol outlined by Pradelles *et al.* (2008), samples were centrifuged to remove yeast cells at the end of contact time and 5 $mg.L^{-1}$ of 3,4-dimethylphenol (internal standard) was added to supernatants. The analysis was performed by gas chromatography (Focus GC apparatus, Thermo-Finnigan). The apparatus included a capillary column CP Wax 57 CB (25 m x 0.25 mm i.d., 0.2 µm bonded phase) (Varian) and an FID detector. Data acquisition and treatment were carried out using Chrom-card workstation version (Thermo Electron Corporation).

Sorption capacity of real lees samples towards mayor wine volatiles*.*

Headspace solid phase microextraction (SPME) coupled to Gas chromatography/mass spectrometry (GC/MS).

The analysis of volatiles sorbed on lees surface during the *surlie* ageing was carried out as previously reported (Gallardo-Chacón et al., 2008). Briefly, 15 mg of lees were suspended in 1 ml of 10 mM phosphate buffer in 0.9 % NaCl (pH 7) into a 10 mL vial and maintained at 50 ºC under magnetic stirring (700 rpm). A divinylbenzene/Carboxen/polydimethylsiloxane SPME fibre (DVB/CAR/PDMS) 50/30 µm, 2 cm long, from Supelco (Bellefonte, PA, USA) was exposed to the sample headspace during 40 min, then immediately desorbed in the GC injection port. 4 mL of the corresponding sparkling wines were also analyzed at the same extraction conditions.

Identification of compounds was performed by gas chromatography coupled to quadrupolar mass selective spectrometry using an Agilent 5973 Network detector (Agilent Technologies, Palo Alto, CA, USA). Analytes were separated on a Supelcowax-10, 30m×0.25mm I.D., 0.25 µm film thickness. Column temperature was held at 60 ºC for 3 min, increased to 75 ºC at 4 ºC/min, then to 260 ºC at 8 ºC/min, holding 5 min. The injector temperature was 260 ºC and the time of desorption of the fibre into the injection port was fixed at 5 min. Helium was the carrier gas, at a linear velocity of 38

cm/s. The temperature of the ion source was 175 ºC and the transfer line, 280 ºC. GC–MS analysis was performed in the complete scanning mode (SCAN), in the 40–300 u mass range.

Semi-quantitative determination of major volatile compounds.

Major volatiles recovered from lees headspace were ethyl octanoate, ethyl decanoate and ethyl-9-decenoate, as previously reported (Gallardo-Chacón et al., 2009), and their sum accounted up to 80 % of the total chromatographic area. As the amount of volatiles on lees surface is suppose to depend on the their concentration in wine, lees sorption capacity was estimated as the ratio between sum of major volatiles on lees surface (sum of chromatographic areas/millions of cells) and sum of major volatiles in the corresponding wines (sum of chromatographic areas).

***Lees flocculation assay*.**

The flocculence of yeast cells was determined on the basis of the Helm's method. Briefly, $CaCl_2$ was added to the lees suspension in acetate buffer (pH 3.5, 0.3 M) to a final concentration of 0.1%. The degree of flocculation of the different strains was determined in terms of the ratio between the initial absorbance at 620 nm of the lees suspension (Abs_0) and that obtained after 20 min at rest (Abs_{20}). The Abs_0 of lees suspensions was adjusted around 1.5. The flocculation capacity of lees was expressed as $(1-Abs_{20}/Abs_0)x100$.

Statistical analysis

The Statgraphics Plus (1999) packages were used for the statistical analysis of data, which were subjected to factorial analysis of variance and stepwise multiple regression analysis. The percent of variance explained by each factor and interaction was calculated from partial eta-square values. Fisher's LSD (least significant differences) method was applied to assess significant differences for each surface characteristic between lees samples with distinct ageing periods. For all the statistical analyses performed, differences were considered significant at $p \leq 0.05$. The degree and significance of the correlations were expressed by regression coefficient (r) and p values, respectively.

RESULTS

Experimental conditions

The concentration of fresh biomass used during the various drying processes, the yields after dehydration and cell viability in relation to each process were gathered in **Table 1**. The three processes had different rates of drying, with no large differences in yields after drying (**Table 2**). All tested samples were used after equilibration at the same water activity (0.09) before their direct hydration in the hydroalcoholic solution (rehydration treatment 1). Therefore, we decided to test an additional method for biomass rehydration. We added a rehydration step in water alone (rehydration treatment 2) before biomass contact with the model wine solution, containing 4-ethylphenol, to possibly modify the 4-ethylphenol sorption capacity of the biomass. This step of rehydration was based upon the common protocol used in industrial DAY (Dry Active Yeasts) activation.

Table 1. Fresh yeast biomass concentration before drying, dehydration yields, and viability of yeast cells after the three dehydration processes.

	Biomass concentration ($mg.L^{-1}$ of sterilized water)	Yield (%, w/w)	Viability (%)
Lyophilization	600	28.7	0
Spray-drying	200	29.4	0
Evaporative drying at low water activity	800	26.7	0

Influence of drying processes on sorption by yeasts

Seven samples were tested, with the sorption capacities expressed in mg of ethylphenol sorbed per square meter of yeast wall (**Figure 1**): fresh biomass (one sample), considered to be the control test as it was not dried; biomass dried by the three processes and rehydrated directly in the contaminated synthetic wine (three

samples) (rehydration treatment 1); and the same dried biomass rehydrated in water before contact with the contaminated wine (three samples) (rehydration treatment 2).

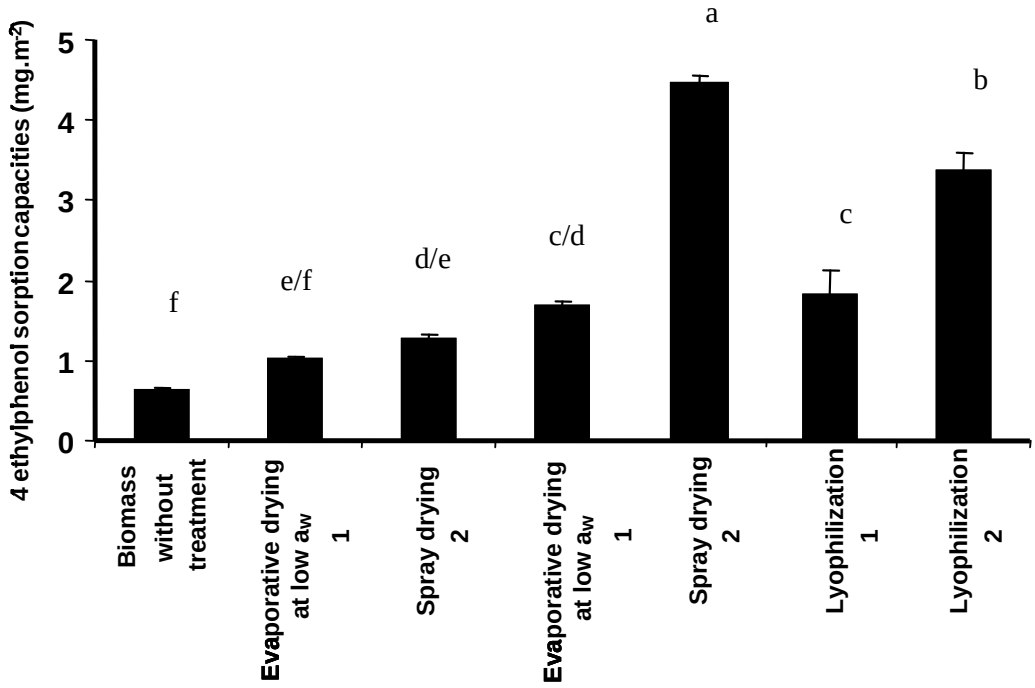

Figure 1. Sorption capacities of the enological strain as a function of the biomass drying and rehydration processes (50 $g.L^{-1}$ of fresh biomass). Samples were stored under stirring (550 rpm) at 10°C. (1: rehydration treatment 1 ; 2: rehydration treatment 2). Different letters indicate significant difference ($p < 0.05$) indicated by one-way ANOVA followed by the Tukey comparison test.

Actually, for rehydration treatment 1, the sorption capacity measurements were significantly different for each drying process (**Figure 1**). Biomass dried using the three drying processes (spray-drying, lyophilization and evaporative drying at low water activity) followed by rehydration treatment 1 had significantly higher 4-ethylphenol sorption capacities values than biomass without drying treatment. Evaporative drying at low water activity, spray-drying and lyophilization induced 4-ethylphenol sorption capacity increases of 61.5%, 169% and 192%, respectively. By contrast, no significant differences were reported between sorption values for spray-drying and lyophilization. This positive effect on 4-ethylphenol sorption was larger for the two most rapid drying processes (spray-drying and lyophilization). Significant structural changes of the yeast surface caused by these drying processes (Hromádková *et al.*, 2003, Türker *et al.*, 2006)

may explain the sorption capacity differences in comparison with fresh biomass. Furthermore, dessication stress induces a specific transcriptional response in the yeast cell to preserve its integrity: being the first barrier against environmental stresses, the yeast cell wall is subjected to composition modifications, such as the down regulation of some mannoprotein genes (Singh *et al.*, 2005). In our study, this mechanism could only have occurred during evaporative drying at low water activity, as cell death occurs immediately during spray-drying. Singh *et al*. (2005) reported that *GAS1* is down-regulated during yeast dessication, but that the Hsp12p mannoprotein is synthesized in response to this stress. Yeast strain mutants deleted for these genes had 74.4% lower and 51.5% higher 4-ethylphenol sorption capacities, respectively, than the wild type strain under similar conditions (Pradelles *et al.*, 2008). Thus, the lower 4-ethylphenol sorption capacity observed in the evaporative drying at low water activity sample may be explained by alternate modes of yeast wall modification: physico-chemical action from dessication in the spray-dried sample and lyophilized samples, and phenotypic changes to the yeast wall in the evaporative drying at low water activity case. These data are consistent with the nature of the yeast wall and mannoprotein composition having a significant effect on its 4-ethylphenol sorption capacity (Pradelles *et al.*, 2008). Drying treatments appeared to have a positive effect on 4-ethylphenol sorption. Moreover, yeasts that underwent the two most rapid drying processes (spray-drying and lyophilization) displayed the strongest 4-ethylphenol sorption.

The samples rehydrated in water (treatment 2) had significantly higher 4-ethylphenol sorption capacity values than yeasts that were directly rehydrated in model wine (treatment 1): the hydration step in water led to sorption capacities that were 27%, 85.5% and 160.5% greater for evaporative drying at low water activity, lyophilization and spray-drying, respectively, than yeasts undergoing the various drying treatments with treatment 1 (**Figure 1**). Interestingly, sorption increases with treatment 2 were strongly dependent on the drying process (**Figure 1**). Differences between sample behavior stemming from the three drying processes may be linked to differences in the rates of drying: the faster the process, the greater the sorption capacity increases in yeasts undergoing treatment 2 than those undergoing treatment 1. The nature of the

drying process may also explain these differences: drying by heat treatment (spray-drying) or by vacuum after freezing (lyophilization) probably modifies yeast surface structures, leading to different behavior after rehydration in water. Indeed, a previous study reported on the specific effects of lyophilization on *S. cerevisiae* wall: *S. cerevisiae* were characterized by cell wall collapse, imperviousness and an amorphous form (Sherrer *et al.*, 1977), which may explain the differing influences that rehydration exerts on the spray-dried sample.

These differences in the 4-ethylphenol sorption capacities of yeasts that underwent rehydration treatment 1 and 2 may be explained by alternate yeast surface organization and refolding in water than that observed in the hydroalcoholic solution. Therefore, bearing in mind that the rehydration step increases the yeast cell wall permeability (Crowe *et al.*, 1989), increases in 4-ethylphenol sorption may be favored by a higher contact interface associated with increased permeability. The rehydration step could also modify the yeast surface, through for instance the onset of a better refolding of the yeast surface macromolecules involved in 4-ethylphenol sorption. Dry membranes and lipid bi-layers force transient permeability changes during rehydration, due to gel to liquid-crystalline phase transitions (Crowe *et al.*, 1989). Furthermore, the processes of drying and rehydration certainly increases the leakage of ions and other soluble cell components into the surrounding medium (Beker *et al.*, 1984, Rapoport *et al.*, 1995). In our case, a loss of 19% of dry weight was recorded. Dry yeasts are impermeable to molecules, whereas hydrated yeast samples allow molecule transfer through the cell wall structure (Normand *et al.*, 2005). Also, during rehydration, the external mannoprotein layer is first hydrated and then the glucan layer is hydrated by diffusion of the water through the mannoprotein layer (Dardelle *et al.*, 2007). Thus, model wine that contains ethanol reduces the rehydration of the cell wall structure by its preferential localization on the mannoprotein layer according to the presence of several apolar interactions.

Therefore, the water prehydration step clearly enhanced the sorption capacity of yeasts when put in contact with the 4-ethylphenol enriched model wine. The presence of ethanol during hydration of the samples appears to have a negative effect on the

refolding of the yeast wall structure: the number of the 4-ethylphenol sorption sites on yeast cell wall decrease.

Cell surface physico-chemical properties

Table 2 presents the results of the measurements of the physico-chemical properties of cell surface in relation to the drying processes: surface hydrophobicity, electron donor characteristics and zeta potential. We studied physico-chemical property changes caused by the drying processes, as the techniques for cell surface characterization were unable to reveal differences in the physico-chemical properties between dried samples (treatment 1) and their rehydrated forms (treatment 2).

All three drying processes induced significant changes in terms of the physico-chemical characteristics compared with fresh biomass. The surface hydrophobicity of cells increased after drying, and all samples were characterized as hydrophobic. This increase was higher in the case of the two fastest drying processes (lyophilization and spray-drying). Conversely, these two processes led to a significantly lower zeta potential than evaporative drying at low water activity. The electron-donor characteristic was also increased by these drying steps, with the largest increase observed after spray-drying. Freezing and drying by heat greatly modify *S. cerevisiae* cell walls and membranes (Deere *et al.*, 1998): this leads to a release of amino acids and proteins, disruption of the phosphodiester bridges, and degradation of the phospholipid membrane in the case of heat treatment (Marcellino *et al.*, 2008). Therefore, these treatments may induce different physico-chemical properties among dried and non-dried yeast cell surfaces, including the different properties resulting from each separate drying treatment. Spray-drying induces the largest changes to cell surface properties. Spray-drying causes significant structural modifications on the yeast surface, such as the enhancement of surface hydrophobicity due to an increase of H bond donor availability (Hromádková *et al.*, 2003). This possibly increases the number of potential sorption sites for 4-ethylphenol on the yeast cell wall, as cell surface hydrophobicity was greater positively affecting 4-ethylphenol sorption (Pradelles *et al.*, 2008). The drying processes in this study had similarly significant effects on the physico-chemical properties of cell surface.

Table 2. Yeast cell surface characteristics after three different dehydration processes and after equilibration at a_w 0.09.

	Hydrophobicity (%)	Electron donor character	Zeta Potential (mV)	Cell diameter (µm)
Fresh biomass	91.58 ^{c} ±4.20	-4.48^{d} ±2.29	-12.83^{a}±0.84	5.02 ^{c}±0.02
Lyophilization	98.90^{a}±0.95	12.70 ^{c}±0.70	-20.20 ^{c} ±0.80	4.44 ^{b} ±0.12
Spray-drying	98.50^{a}±0.37	34.50 ^{a}±1.5	-17.00 ^{c} ±0.48	3.87 ^{c} ±0.02
Evaporative drying at low water activity	96.50^{b}±0.25	21.50 $^{a/b}$±4.71	-15.80 ^{b} ±0.30	5.78 ^{a} ±0.06

Different letters indicate significant difference ($p < 0.05$) indicated by one-way ANOVA followed by the Tukey comparison test.

Modeling of 4-ethylphenol sorption according to the physico-chemical surface properties of cells

The model of prediction obtained through multiple linear regressions treatment is,

4-ethylphenol sorption capacity ($mg.m^{-2}$) = $6{,}11 - 3{,}16.10^{-2} . X_{EDC} + 4{,}6 .10^{-2} .X_{ZP}$

with, $X_{EDC,}$ the electron-donnor character in percentage and X_{ZP}, the zeta potential in percentage.

The *p*-values of the regressor contributions for the electron-donnor character and for the zeta potential variables were respectively 0.000 and 0.046, so these variables had significant effects (*p*-values smaller than 0.05). However, the surface hydrophobicity has a non-significant effect because the *p*-value of the regressor contribution was higher than 0.05 (0.37).

According to the equation of the predictive model, the coefficient of the electron-donor character variable ($-3{,}16.10^{-2}$) suggests that this variable has a negative effect on 4-ethylphenol sorption. By contrast, a high zeta potential has a positive effect. Thus, the variation in the 4-ethylphenol sorption capacities of yeasts as a function of the drying treatment may be correlated with changes to the physico-chemical surface properties. **Figure 2** presents the corelation between the model of prediction and the

4-ethylphenol sorption calculated with the model obtained from analysis, and 4-ethylphenol sorption capacities measured was quite high (R^2 = 0.897).

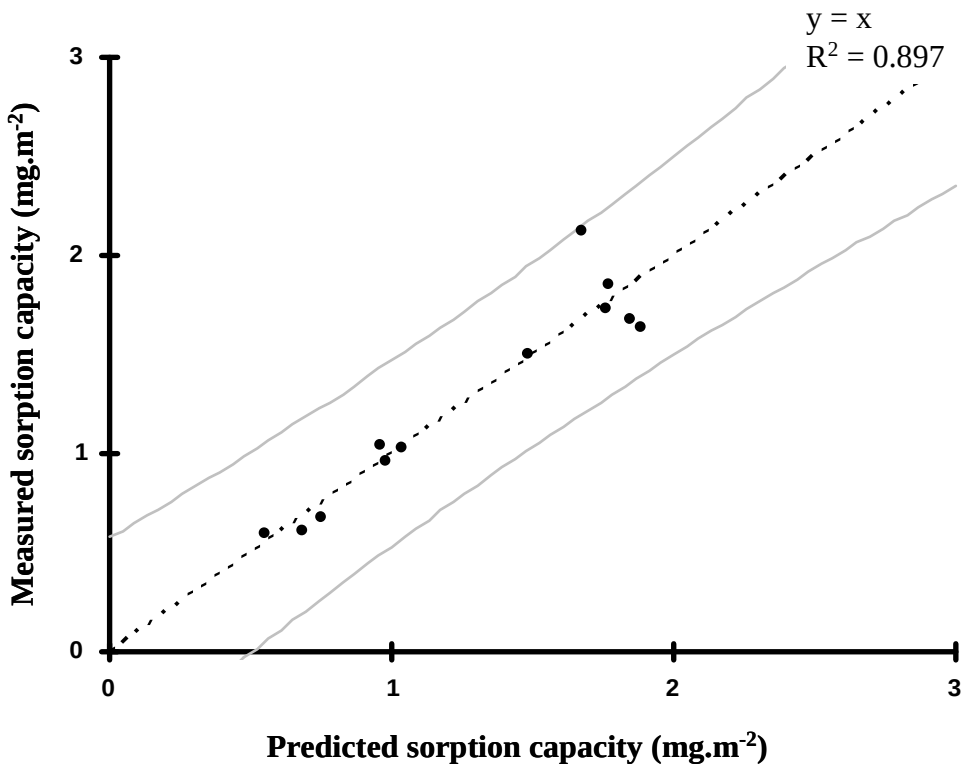

Figure 2. Correlation between measured (y) and predicted (x) 4-ethylpenol sorption capacities obtained through the multiple linear regression model (4-ethylphenol sorption capacity ($mg.m^{-2}$) = 6,11 –3,16.10^{-2} . X_{EDC} + 4,6 .10^{-2} .X_{ZP}, with, $X_{EDC,}$ the electron-donnor character (%) and X_{ZP}, the zeta potential (%)).

In a previous study, modeling of 4-ethylphenol sorption according to the physico-chemical surface properties of cells in *S. cerevisiae* mutant strains deleted for genes encoding mannoproteins of the yeast wall revealed various correlations: surface hydrophobicity has a positive effect (major factor), electron-donor nature has a negative effect and zeta potential has no influence on 4-ethylphenol sorption in the case of hydrophilic strains (Pradelles *et al.*, 2008). Samples in this study displayed greater hydrophobicity than those previous reported, clearly suggesting that the zeta potential is strongly correlated with the hydrophobic character of yeast cells. Thus, the samples studied here allowed us to observe the effects of the zeta potential, as the surface hydrophobicity factor can be considered to be a constant. This assumption was

based on the fact that all the samples have similarly high surface hydrophobicity and that is why the statiscal analysis expresses the no significant effect of the surface hydrophobicity. Using model media, some studies conclude that the rate of sorption by yeasts is directly correlated with the hydrophobicity of the aroma compounds (Lubbers *et al.*, 1994). However, Jiménez Moreno and Ancín Azpilicueta (2007) showed that lees can have a considerable sorption capacity for volatile phenols with low hydrophobicities, such as eugenol, 4-propylguaiacol or 4-methylguaiacol, in real wine. However, although the nature of these compounds was more or less hydrophobic, the yeast cell surface characteristics used in these studies were not unambiguously characterized. Moreover, the results obtained seem to confirm that hydrophobic interactions are not the sole interactions involved in 4-ethylphenol sorption, as previously demonstrated for 4-ethylphenol sorption in lees or by yeast cells (Chassagne *et al.*, 2005, Pradelles *et al.*, 2008). More precisely, it seems that sorption 4-ethylphenol via the yeast surface is a balance between hydrophobic, electron acceptor and electrostatic interactions.

Influence of ageing time and wine coupage on cell size and physicochemical properties of lees surface.

The MATS and Z potential results obtained for *Saccharomyces cerevisiae* lees at different periods of sparkling wine *surlie* ageing are reported in **Table 3**. The cell surface hydrophobicity (CSH) of yeast lees, calculated by the solvent partition assay and expressed as the percent of adherence to hexadecane, was observed to be inversely related to the time of sparkling wine ageing. Independently from the wine *coupage*, at short ageing periods yeast lees possessed hydrophobic character, showing an adherence to the non-polar solvent hexadecane of around 40-50%. In correspondence of 10 and 20 months of ageing lees presented CSH values around 20-30%, which then dropped to approximately 10% after 40 months, when lees acquired a hydrophilic character, according to Li and McLandsborough (1999).

Moreover, during the entire period of ageing tested the yeast lees showed larger tendency to donate electrons from their surface than to accept electrons, (**Table 3**), indicating a Lewis' basic character of lees cell wall. This was in accordance with

previously reported observations on viable cells of *Saccharomyces cerevisiae* (Mercier-Bonin et al., 2004). The electron donating capacity (EDC) of cells surface did not show a marked tendency during the biological ageing. At 10 and 40 months of ageing, lees samples presented the highest EDC (**Table 3**). Conversely, the weak electron acceptor character (EAC) progressively rose in lees collected at increasing ageing periods. The Z potential of lees became more positive in the course of the biological ageing, going from slightly negative values in lees of 2 and 10 months of ageing, to clearly positive values after 40 months of biological ageing (**Table 3**). At the same analytical conditions viable cells of different strains of *S. cerevisiae* grown in synthetic must were reported to show Z potential values between approx. -6 and -15 mV, and EDC between approx. 8 and 48 %, (Pradelles et al., 2008).

Table 3. Cell surface properties and cell size of lees from different sparkling wine coupages at different periods of ageing, and results of factorial analysis of variance. The percent of variance explained by each factor is also reported. Different numbers indicate significant differences (p<0.05).

Month	EDC[a]	EAC[b]	CSH[c]	Z[d]	Cell diameter
2	47.9 ± 7.4 a	-27.5 ± 8.2 a	44.0 ± 6.8 a	-4.1 ± 0.5 a	4.7± 0.1 a
10	69.0 ± 6.1 b	-17.8 ± 9.9 b	19.9 ± 2.4 b	-3.2 ± 0.7 a	4.6 ± 0.2 a
18	58.3 ± 7.1 a	-15.1 ± 8.4 b	24.7 ± 10.5 b	-0.3 ± 1. b	4.5 ± 0.1 a
40	70.1 ± 5.7 b	10.1 ± 3.4 c	11.2 ± 3.7 c	6.2 ± 0.4 c	3.8 ± 0.0 b
MANOVA results					
month	<0.05 (41%)[e]	<0.05 (73%)	<0.05 (75%)	<0.05 (97%)	<0.05 (82%)
wine *coupage*	ns	<0.05 (18%)	ns	ns	ns
interaction	ns	ns	ns	ns	ns

[a]: EDC, electron donor character calculated as % adherence to chloroform - % adherence to hexadecane; [b]: EAC, electron acceptor character calculated as % adherence to ethyl acetate - % adherence to decane; [c]: CSH, cell surface hydrophobicity calculated as % of adherence to hexadecane; [d]: Z potential (mV); ns: not significant; [e]: percent of variance explained by each factor.

A clear influence of the time of ageing on cell lees surface properties could be evidenced in all the cases. Although compositional characteristics of yeasts surface depends on the medium where they are grown (Caridi 2006; Klis et al., 2002), the effect of the type of wine on cell surface physicochemical characteristics was only

noticed for lees EAC. In any case the interaction between these factors was proved to be significant (**Table 3**).

Moreover, some of the surface properties resulted inter-correlated (**Table 4**). CSH was correlated to EAC and in particular to EDC, according to previous findings describing a hydrophilic behavior for *S. bayanus* cells with Lewis basic character and strong hydrophobic character for cells with more pronounced acidic nature and high affinity for apolar solvents (Mercier-Bonin, 2004). This was also in agreement with results reported for other yeast genera (Amaral et al., 2006). Z potential was in accordance with lees EAC, and thus inversely correlated to CSH (**Table 4**).

Table 4. Matrix of correlation between cell surface properties assesses in lees from different sparkling wine *coupages* at different periods of ageing. For significant correlations ($p<0.05$), the coefficient of regression (r) is reported.

	EDC[a]	EAC[b]	CSH[c]	Z[d]
EDC	-	0.59	-0.78	ns
EAC		-	-0.71	0.87
CSH			-	-0.67
Z				-

[a]: EDC, electron donor character calculated as % adherence to chloroform - % adherence to hexadecane; [b]: EAC, electron acceptor character calculated as % adherence to ethyl acetate - % adherence to decane; [c]: CSH, cell surface hydrophobicity calculated as % of adherence to hexadecane; [d]: Z potential (mV); ns: not significant

The differences in cell surface properties determined in lees along the ageing could be reasonably ascribed to the cell wall degradation due to the yeast autolysis. In fact, hydrophobicity of the yeast cell surface has been positively correlated with the presence and concentration of proteins in the cell wall (Amory et al., 1988, Kamada et al., 1984; Smit et al., 1992; Suzzi et al. 1994). Likewise, S. cerevisiae cells tend to exhibit negatively charged surfaces due to the presence of phosphate groups in the outer cell wall mannoprotein layers (Amory et al., 1988; Mestdagh et al., 1990; Caridi, 2006), whose surface concentration demonstrated a linear relationship with Z potential (Amory et al., 1988). It is well known that a release of yeast wall mannoproteins occurs during fermentation and during cell wall degradation due to the yeast autolysis (Fornairon-Bonnefond et al., 2002; Leroy et al., 1990). This progressive

loss of proteins during the *surlie* ageing could then determine the observed decrease of both CSH and negative charge of lees surface. Electron acceptor character of *S. cerevisiae* cells has been related to the cell wall content of β-glucan (Mercier-Bonin, 2004). In the case of cells undergoing autolysis, the release of mannoproteins from cell surface could possibly favor the exposition of the glucan layer and then influence the Lewis basic/acid character of the cell surface.

Apart from cell surface properties, cell size is expected to control the sedimentation of particles with diameter >0.2 μm (Mortensen et al., 2005). Possible modification of cell lees size during the ageing period could then influence their flocculation capacity due to the implication of gravity forces. The cell diameter resulted significantly shorter for lees collected at 40 months of ageing, independently from the wine *coupage* (Table 3).

Effect of surface characteristics on the flocculation and sorption capacities.

As cell wall properties are thought to be a major determinant in flocculation and sorption capacity (Smit et al., 1992; Jin and Speers, 1998; Mercier-Bonin et al., 2004; Lubbers et al., 1994; Pradelles et al., 2008), the changes in lees wall structure caused by the autolytic process during sparkling wine *surlie* ageing are able to induce relevant modifications in these technological aptitudes.

Both flocculation and sorption capacities of lees showed a decreasing trend according to the ageing time of lees samples (**Table 5**).

Sorption capacity toward the major wine volatiles was expressed as the ratio between the same volatiles measured on lees and in the corresponding wine. It seemed to be influenced to comparable extents by wine *coupage* and ageing time, showing significant differences between lees at 18 and 40 months of ageing. Flocculation resulted significantly dependant on both the ageing period and the wine *coupage*. The percent of variance explained by each factor reported in Table 3 indicates as lees flocculation capacity was principally influenced by the ageing period, while wine characteristics determined significant but minor differences on flocculation capacity.
As most of the lees surface properties resulted inter-related (**Table 4**), the influence of cell surface properties and cell diameter on lees flocculation and sorption capacity was

evaluated by stepwise multiple regression analysis, taking into account the relationship among the factors.

Table 5. Flocculation and sorption capacities of lees from different sparkling wine *coupages* at different periods of ageing, and results of factorial analysis of variance. The percent of variance explained by each factor is also reported. Different numbers indicate significant differences (p<0.05).

Month	Flocculation [a] Acetate buffer (pH 3.5, 0.3 M) 0.1 % $CaCl_2$	Sorption[b]
2	88.1 ± 4.1 a	1.13 ± 0.28 a
10	62.5 ± 9.3 b	0.85 ± 0.17 a
18	71.9 ± 2.4 c	0.86 ± 0.13 a
40	40.6 ± 10.4 d	0.47 ± 0.03 b
MANOVA results		
month	<0.05 (82%)[c]	<0.05 (54%)
wine *coupage*	<0.05 (17%)	<0.05 (33%)
interaction	ns	ns

[a]: flocculation capacity calculated as $(1-OD_{20}/OD_0)x100$, where OD_{20}=optical density of lees suspension after 20 min, OD_0= initial optical density of lees suspension; b: sorption capacity estimated as the ratio between sum of major volatiles on lees surface (sum of chromatographic areas/millions of cells) and sum of major volatiles in the corresponding wines (sum of chromatographic areas). Different letters in columns indicate significant differences (p<0.05) between lees of different ageing; [c]: percent of variance explained by each factor.

Although *S. cerevisiae* flocculation is reportedly mediated by different mechanisms (Suzzi et al., 1994), flocculation capacity of sparkling wine lees at different ageing periods was significantly correlated only to hydrophobicity (r=0.70, p<0.05). These results are in agreement with earlier studies pointing out the major role played by cell wall hydrophobicity in yeast cells flocculation (Wilkocks et al., 1995; Smit et al., 1992; Straver et al., 1996). Cell to cell contact should become progressively easier as surface charge is lowered, resulting in an increase of the extent and rate of flocculation (Stratford and Keenan, 1988). Nevertheless, although lees Z potential was higher at longer periods of ageing, its influence on the flocculation capacity did not result statistically significant by stepwise multiple regression. Anyway, the influence of surface charge on *S. cerevisiae* flocculation was reported to be strain-dependent (Wilcocks et al., 1995). Sorption of volatiles resulted to be dependent on both CSH and EAC, according to the linear multiple regression model obtained: sorption (volatiles sorbed by lees/volatiles in wine) = $0.840 - 0.015.\ X_{EAC} + 0.0128.X_{CSH}$ (r=0.48, p<0.05),

where the contribution of Lewis acid-base properties of cells was higher (p=0.014) than that of hydrophobicity (p=0.044). This was in accordance with previous findings reporting that Lewis acid-base characteristics of yeast cell surface and not hydrophobicity were the main determinant of sorption capacity, and that the these factors do not fully account for the variation observed in volatiles sorption by yeast surface, as indicated by the low regression coefficient (Pradelles et al., 2008).

CONCLUSIONS

In conclusion, drying the yeast biomass greatly modified the physico-chemical surface properties of the cells. These modifications were correlated with significant differences in sorption capacities, particularly in the case of the fast drying methods (spray-drying and lyophilization): these changes ensured the most positive effects on sorption, by increasing cell surface hydrophobicity caused by damages to the cell wall structure. A pre-rehydration step of the yeast biomass in water before contact with 4-ethylphenol also appears to enhance the sorption capacity. Thus, our findings demonstrate that these factors appear to be more important than the nature of the yeast strain, or the culture method, as they significantly affect the nature of the yeast cell wall and its structure.

Regarding real lees samples during sparkling wine rime, yeast lees surface properties were proved to be significantly dependant on the time of sparkling wine *surlie* ageing, while a weaker influence of wine *coupage* was only noticed on lees EAC. The release of mannoproteins from the cell wall external layer occurring during the autolytic process could explain the decrease of surface hydrophobicity and the increase of Z potential and EAC observed according to the time of ageing. Most of the lees surface properties resulted inter-correlated, but stepwise multiple regression analysis indicated that only lees surface hydrophobicity and lewis acid/base characteristics significantly influenced lees flocculation and sorption capacity, which showed a decreasing trend along the period of *surlie* ageing.

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