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Immobilisation of yeasts on oak chips or cellulose powder for use in bottle-fermented sparkling wine

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ABSTRACT

Sparkling wine production comprises two successive fermentations performed by *Sacharomyces cerevisiae* strains. This research aimed to: develop yeast immobilisation processes on two wine-compatible supports; study the effects of yeast type (IOC 18–2007 and 55A) and the immobilisation support type (oak chips and cellulose powder) on the fermentation kinetics, the deposition rate of lees and the volatile composition of the finished sparkling wine; compare the fermentation parameters of the wines inoculated with immobilised or non-immobilised cells. Proper immobilisation of yeast on oak chips and cellulose powder was demonstrated by electron microscopy. Total sugar consumption occurred in under 60 days in all bottles, regardless of the strain used and the way they were inoculated in wine. Deposition of lees was 3-fold faster in the bottles containing immobilised cells than in those with free cells; no addition of adjuvants was necessary. The analysis of the volatile compounds of the finished sparkling wines showed significant differences in the formation of esters, acids, alcohols, aldehydes and lactones according to the yeast and the immobilisation support used. Oak chips were the more appropriate support for yeast immobilisation. No significant differences in the sensorial analysis of the sparkling wines produced by the different strategies were found.

1. Introduction

Sparkling wine production requires two successive alcoholic fermentations (AF). Firstof all, fermentation is regular white winemaking that results in a dried wine to be bottled. A mixture of sugars and yeasts (*tirage liqueur*) and riddling agents is added to each bottle to perform a second fermentation inside capped bottles. Given sugar fermentation by yeast, the resulting wines are higher in ethanol and contain dissolved CO_2. The second fermentation is followed by an ageing period in which wine comes into contact with dead yeast cells (Kemp et al., 2015). High quality sparkling wines, such as Champagne (France), Cava (Spain) or Talento (Italy), are fermented in closed bottles following the traditional or "champenoise" method, and remain in contact with yeast lees in a bottle for several months and even for years (Buxaderas and López-Tamames, 2012). Ageing is followed by the riddling process to move proteins and yeast sediments to the neck of the bottle (Torresi et al., 2011). The gradual and controlled turning of slanted and inverted bottles brings yeasts and adjuvants together towards the neck of the bottle (Jeandet et al., 2000). In recent years, the riddling time has been cut to 2–4 days with automated riddling machines called gyropalettes that hold 504 bottles per cage (Jeandet et al., 2000). Disgorging is the process followed to remove the yeast sediment and adjuvants from bottles (Kemp et al., 2015). It is currently performed by inserting the neck of the bottle into a glycol or calcium chloride solution, which freezes ($-25\,^{\circ}C$) the yeast sediment. Bottles are then picked up and quickly placed neck up; the crown cap is removed, and pressure ejects the iced sediment (Garofalo et al., 2016; Torresi et al., 2011).

The second fermentation and ageing lees completely change the organoleptic properties of the base wine, and confer the sparkling wine its characteristic aroma, flavour, foamability and roundness. During the ageing process, dead yeasts undergo autolysis and release their cell components to wine, with mannoproteins and proteins among them (Kemp et al., 2015; Martínez-Lapuente et al., 2015b; Pozo-Bayón et al., 2010; Velázquez et al., 2016). Winemakers generally use active commercial dried yeast to perform the second fermentation. Sometimes the yeast used for this secondary fermentation is the same as that employed

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for the first fermentation but, invariably, this yeast must be chosen for its ability to ferment high-acidity and low-pH wines, and it must be ethanol-tolerant (Garofalo et al., 2016; Pozo-Bayón et al., 2009). Since yeasts must be removed once the second fermentation has been completed, the use of flocculation yeasts can improve their sedimentation in bottles. Apart from this technological advantage, the ability to floccule confers yeast greater resistance to the stressful conditions inside bottles (Nedović et al., 2015). Yeast cells capable of flocculating appear more resistant to ethanol, peroxide, high temperature or antibiotic exposure (Smukalla et al., 2008; Zhao et al., 2012). Flocculation apparently represents a community behaviour in which aggregated cells are physically protected from stress by an outer layer of sacrificial cells (Nedović et al., 2015). Some companies have developed "agglomerated yeast" which, apart from being more resistant to the second fermentation conditions, avoids using riddling agents (Pozo-Bayón et al., 2003). Bentonite is the most widespread riddling agent. This clay efficiently removes proteins from wine thanks to its negatively charged surface that attracts and binds the positively charged proteins of the grape must (Lambri et al., 2012; Vanrell et al., 2007). Some other riddling agents can contain potassium alginate. This polysaccharide forms an anionic gel under acidic conditions that becomes a very stable gel in the presence of calcium cation, which enables the rapid formation of film type sediments that allow quicker riddling (Kemp et al., 2015). An alternative is to use riddling agents, and some authors have developed techniques to encapsulate or immobilise yeasts. The first applications of immobilised yeasts were performed in κ-carrageenan by Wada et al. (1979), and in sodium alginate by Veliky and Williams (1981). Over the years, immobilised yeasts have been frequently studied in sparkling wine production, and the first commercial application was reported by Fumi et al. (1987). Despite many immobilisation supports having been suggested for winemaking applications, the industrial use of this technology remains uncertain (Kourkoutas et al., 2004). Other supports used to immobilise yeasts employed for wine, beer or bioethanol production are alginate beads, DEAE-cellulose, delignified-cellulose, wood, sawdust, gluten pellets, apple pieces, grape skins, polygorskite, montmorilonite, hydromica, porous porcelain, porous glass, lightly cross-linked poly-acrylic acid, and even the hyphae of the fungus *Penicillium chrysogenum* (Kourkoutas et al., 2004; Peinado et al., 2006; Sroka et al., 2017). According to Mantaluta et al. (2011), the immobilisation of yeasts by including gellan gum beads results in the production of transparent wines and allows riddling stages to be done away with. In addition, immobilisation techniques provide protection to yeast, and in a similar way to flocculation (Kemp et al., 2015). As regards the effect of immobilisation on the final characteristics of beverages, Puig-Pujol et al. (2013) have revealed that no relevant oenological and sensorial differences exist among the sparkling wines produced by *S. cerevisiae* yeast immobilised on biocapsules or calcium alginate beads, and those fermented by free cells.

Our research objectives were to initially develop yeast immobilisation processes on two wine-compatible supports: oak chips and cellulose powder; secondly, to study the effects of type of yeast and type of immobilisation support on the fermentation kinetics, the deposition rate of lees and the volatile composition of finished sparkling wine; finally, to compare the fermentation parameters of the wines inoculated with immobilised or non-immobilised cells.

2. Material and methods

2.1. Microorganisms and immobilisation supports

Two different *S. cerevisiae* yeast strains were used to inoculate base wines: the *S. cerevisiae* strain Enolab55A (55A) isolated from an organic Spanish wine from Utiel-Requena D.O.P. in Spain; the commercial yeast *prise de mousse* IOC18-2007 (Institute OEnologique de Champagne). The used immobilisation supports were oak chips Spirit NATURE, (Agrovin S.A.) and cellulose powder Radicel 200 (Agrovin S.A).

2.2. Yeast immobilisation

Yeast strains 55A and IOC 18–2007 were cultured in liquid GPY culture (4% glucose [Panreac, Madrid], 0.5% peptone [Oxoid, Valladolid], and 0.5% yeast extract [Pronadisa, Madrid]) to reach 2×10^8 colony-forming units (CFU)/mL. The culture was centrifuged at $6842 \times g$ for 15 min (Heraeus Multifuge 1 S-R) and the supernatant was removed. A mixture composed of 7% (w/w) cell biomass, 15% (w/w) oak chips or cellulose powder and 78% (w/w) cryoprotectant (15% w/v glucose, Panreac) was incubated with stirring (70 rpm) for 30 min to encourage yeasts to adhere to the support. Wheat starch (Fluka, Germany), 8% in water, was prepared and heated to 90 °C to obtain gel consistency, cooled to 45 °C and was added to the cells and oak chips/cellulose powder mixture. Then preparations were frozen at −20 °C for 6 h and lyophilised for 27 h under vacuum conditions (15.9 millitorrs) in a Virtis Sentry equipe. The lyophilised preparations were stored at 4 °C in the dark and were sheltered from air. The efficacy of the yeast immobilisation on supports after lyophilisation was checked under an electron microscopy. The chips with starch-coated immobilised yeast were gold-covered for 2 min by a Balzers SCD 004 sputter coater and were then examined under a JSM-6300 scanning electron microscope.

2.3. Estimating the immobilised viable cell concentration

S. cerevisiae viable cell counts per gram of the concentrate obtained after centrifugation (CFU/g) were obtained by serial dilutions on GPYA (Belloch et al., 1998). The number of yeast cells per gram of immobilised preparation was calculated by taking into account the total wet weight of the set (yeast, oak chips/cellulose powder and starch gel). After lyophilisation, the percentage of viable and dead cells was calculated by the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen). To calculate these percentages, 0.1 g of immobilised yeasts was resuspended in 100 μL of distilled water and 0.3 μL of the mix (1v solution A: 1v solution B of the LIVE/DEAD BacLight Bacterial Viability Kit) was added to the preparation. The mixture was incubated for 20 min in the dark and was then observed at 1000X with immersion oil under a fluorescence microscope (Leica). Viable cells emitted green fluorescence, whereas dead cells emitted red fluorescence. By considering the percentages of the viable/death cells of the analysed suspension, the number of viable/death cells per gram of immobilised yeasts was calculated.

2.4. Sparkling wine production and sampling times

All the vinification trials were run in a base wine that consisted in a coupage of 80% Macabeo and 20% Chardonnay wines at the Bodega Dominio de la Vega winery S.L. (D.O.P. Utiel-Requena, Spain). The second fermentation was performed by the traditional *Champenoise* method (inside capped bottles), according to EU and Spanish government specifications (BOE-189278, 1991; EEC-358/79, 1979). Base wine (1 g/L reducing sugars; 11% ethanol v/v; pH: 3.15, total acidity 8.50 g/L; expressed as tartaric acid; volatile acidity 0.16 g/L, expressed as acetic acid) was distributed (135 bottles) in transparent glass bottles (45 bottles/experiment). The *tirage* liqueur (12 g/L glucose and 12 g/L of fructose) and the immobilised oak chips/cellulose powder yeasts (1 g/L), or a free cell yeast culture (2×10^6 CFU/mL) grown in GPY (Belloch et al., 1998), were added. Besides, 1 g/L of the oak chips/cellulose powder without yeast cells was added to some bottles that were taken as the non-inoculated controls. No riddling agents were added. Bottles were kept at 11-13 °C and at a relative humidity of 75–85% for 9 months. Each week during the first month and at the end of the second month of ageing, three bottles per experiment were opened to determine the residual sugars and ethanol concentrations in order to know the evolution of the second alcoholic fermentation. After 9 months, yeast sediment deposition efficiency was measured by considering the total time that the gyropalette required to make wines transparent. The volatile composition of the resulting sparkling wines was determined at the end of the ageing period (9 months). Before the analysis, bottles were riddled and disgorged. Brut nature sparkling wines were obtained and no expedition liqueur was added. The experiments were performed in triplicate.

2.5. Analytical methods

Glucose, fructose and ethanol contents were quantified by High Pressure Liquid Chromatography (HPLC) (Agilent series 1200), equipped with an isocratic pump (Agilent G1310A), following the procedure described by Frayne (1986) with minor modifications. The mobile phase consisted of a solution of 0.75 mL of 85% H_3PO_4 per litre of deionised water at a flow rate of 0.7 mL/min. An Agilent G1322A degasser was employed. Samples (5 μL) were automatically injected (Agilent G1367B). Components were separated in an Aminex HPX-87H precolumn (Bio-Rad) coupled with two ion exclusion columns of 300 mm by 7.8 mm, Aminex HPX-87H (Bio-Rad), which were thermostatically controlled at 65 °C (Agilent G1316A). Compounds were detected by a G1314B variable-wavelength detector (Agilent) set at 210 nm and a refractive index detector (Agilent G1362A) set in series. The elution time was 45 min. External calibration was performed with reference standards of glucose, fructose and ethanol. All the samples were centrifuged at 6000 g for 10 min. Then the supernatant was filtered through a membrane filter with a mean pore size of 0.22 μm before injection. Quantification was performed by measuring the peak height compared to those of the external standards.

The analytical methods recommended by the OIV were used to determine titratable acidity and volatile acidity (OIV, 2009a). pH was determined by a HANNA Instruments HI 8424 pH meter. Foaming, proteins and polysaccharides measurements were taken as Esteruelas et al. (2014) described.

2.6. Volatile aroma compound analysis

Volatile compounds were analysed by the procedure proposed by Ortega et al. (2001) with slight modifications. A volume of 2.7 mL of the samples was transferred to a 10-mL screw-capped centrifuge tube that contained 4.05 g of ammonium sulphate (Panreac, Barcelona) to which the following compounds were added: 6.3 mL of milliQ water (Panreac), 20 μL of a standard internal solution (2-butanol, 4-methyl-2-pentanol and 2-octanol from Aldrich, at 140 μg/mL each, in absolute ethanol from LiChrosolv-Merck), and 0.25 mL of dichloromethane (LiChrosolv-Merck) The tube was shaken mechanically for 120 min and was later centrifuged at 2900 g for 15 min. The dichloromethane phase was recovered with a 0.5-mL syringe, transferred to the autosampler phial and analysed. The chromatographic analysis was carried out in a HP-6890, equipped with a ZB-Wax plus column (60 m × 0.25 mm x 0.25 μm) from Phenomenex. The column temperature, initially set at 40 °C and maintained a this temperature for 5 min, was then raised to 102 °C at a rate of 4 °C/min to 112 °C at a rate of 2 °C/min, to 125 °C at a rate of 3 °C/min and this temperature was maintained for 5 min and then raised to 160 °C at a rate of 3 °C/min; to 200 °C at a rate of 6 °C/min and was then kept at this temperature for 30 min. The carrier gas was helium, which was fluxed at rate of 3 mL/min. The injection was done in the split mode 1:20 (injection volume 2 μL) with a flame-ionisation-detector (FID detector).

In addition, Kovats retention indices (KI) were calculated for the GC peaks corresponding to identify substance by the interpolation of the retention time of normal alkane (C8 −C20) by Fluka Buchs, Schwiez, Switzerland), analysed under the same chromatographic condition. The calculated KI were compared with those reported in the literature for the same stationary phase.

2.7. Sensory analysis

A sensory analysis of the resulting sparkling wines was done by a panel of 16 experts. The visual, aroma, and flavour characteristics were analysed according to the score sheet for sparkling and pearl wines, as published by the OIV (2009b). The intensity of each attribute was rated on a scale from 0 to 10 with indented anchor points of 'low' and 'high', respectively.

2.8. Statistical analysis

Final residual sugar, ethanol, total and volatile acidities, pH, volatile aroma, the total polysaccharide and protein contents and foam properties of cava wines were statistically analysed with the Statgraphic Plus 5.1. software. ANOVA and discriminant analyses were employed. The statistical significance of each considered factor was calculated at $\alpha = 0.05$ by the Student's *t*-test.

3. Results and discussion

3.1. Cell immobilisation and cell viability

Adherence of both 55A and IOC 18–2007 cells to oak chips and cellulose powder after lyophilisation was confirmed by electron microscopy. As shown in Fig. 1, *S. cerevisiae* cells adhered to the surfaces of oak chips/cellulose powder and appeared to be coated with the polysaccharide layer of starch. The cells immobilised on the supports retained their shape, and did not shrink after the process. The number of viable yeasts per gram of immobilised culture was $1.80 \times 10^9 \pm 1.01 \times 10^9$ CFU/mL and $1.96 \times 10^9 \pm 1.13 \times 10^9$ CFU/mL, respectively, for strains IOC 18–2007 and 55A on oak chips, and $1.60 \times 10^9 \pm 6.85 \times 10^8$ CFU/mL and $2.10 \times 10^9 \pm 1.30 \times 10^9$ CFU/mL, respectively, for strains IOC 18–2007 and 55A on cellulose powder. In all cases, cells retained a viability over 86% after the lyophilisation process. No significant differences ($p = 0.988$) in viability after lyophilisation between strains or immobilisation supports were found. Lyophilisation was used here for a twofold purpose: to promote cell adhesion to the solid support and to preserve yeast over time. Lyophilisation is a method to preserve food, and mainly microorganisms (Berny and Hennebert, 1991; Gehrke et al., 1992). In accordance with

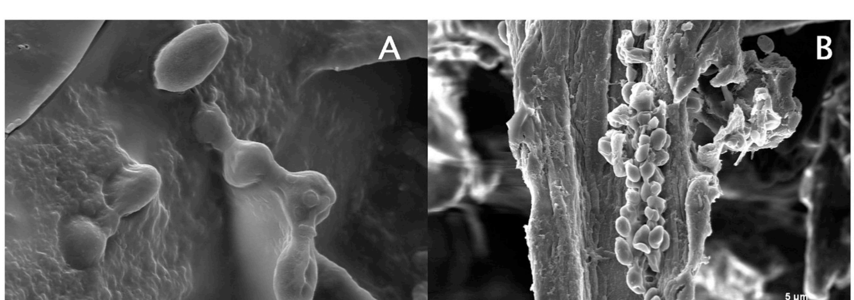

Fig. 1. Electron microscopy photograph showing *S. cerevisiae* cells immobilised on oak chips (A) and cellulose powder (B) by lyophilisation.

Bekatorou et al. (2001) and with Kandylis et al. (2010) the results showed that the lyophilisation technique was suitable for cell immobilisation and preservation. The supports provided a dry mass by protecting living cells biochemically against damage during freeze-drying (Kourkoutas et al., 2004). In addition to the protective effect, lyophilisation protects cell preparation from contamination or infestation during storage, ensures long viability and makes biocatalyst distribution easy (Kandylis et al., 2010).

The advantage of using starch-coated oak chips/cellulose powder is that these materials are allowed in winemaking, unlike other used previously materials (alginate, silica, polyacrylamide, apple, etc.). (Callone et al., 2008; Fumi et al., 1987; Kourkoutas et al., 2006; Rossi and Clementi, 1984). The unique flavour profile of fermented wines can be attributed to the biochemical activities that take place in the yeast cell during fermentation (Lodolo et al., 2008). High volumetric productivities of aroma and other metabolites with high volumetric cell densities can be achieved by packing cells into a small defined volume by either entrapment within a carrier matrix or adsorption onto the surface of a porous material. This approach is known as immobilised cell technology and has been widely investigated since halfway through the 20th century and designed for different stages in the fermentations of alcoholic beverages (Nedović et al., 2015). A main criterion for the successful application of cell immobilisation for bioflavour production is to choose a suitable carrier material since a number of factors should be taken into account, namely safety, legality, stability, product quality and operating costs (Nedović et al., 2015).

3.2. Kinetics of sugar consumption and ethanol production in capped bottles

The kinetics of glucose and fructose consumption and the kinetics of ethanol production during the second fermentation were recorded to know if differences between immobilised/free state of the cells, between both yeasts and between types of supports, existed. The final sugar and ethanol concentrations were similar in the sparkling wines fermented with the free cells or those immobilised on oak chips/cellulose powder cells, regardless of the yeast strain used; glucose and fructose were consumed in under 60 days and ethanol increased by 1.5% (Fig. 2). However, sugar fermentation began 1 week later in the wines inoculated with immobilised cells, regardless of the strain used. The differences in the fermentation kinetics exhibited by the immobilised and free cells were bigger for strain IOC 18–2007, which exhibited a higher sugar consumption and ethanol production rates in the free form, although the maximum ethanol produced was similar (Fig. 2A, C and E). Strain 55A in the immobilised form started glucose consumption 1 week later than the free one (Fig. 2B). Fructose consumption also showed a delay, but only when fermentation was performed with the yeast immobilised on cellulose powder (Fig. 2D). Nevertheless, the final residual sugars (Fig. 2A, B, C and 2D) and ethanol contents (Fig. 2E and F) showed no significant differences between the fermentations performed with immobilised and free cells ($p = 0.8847$).

The second fermentation occurs under very particular conditions for yeasts; the base wine presents high alcohol content, and not all strains can grow and ferment under these conditions (Torresi et al., 2011). Low fermentation temperatures (12-18 °C), typical in sparkling wine making, slow down fermentation activity, but this is useful for sparkling wines' quality improvement. Yeasts must tolerate more than 4-atm pressure, low pH and high alcohol content conditions (Ribéreau-Gayon et al., 2003).

3.3. Efficiency of yeast sediment (lees) removal

In traditional sparkling wine production, lees removal is a very labour-intensive and time-consuming process, and using immobilised yeasts could reduce and simplify the riddling and disgorging procedures. During the riddling performed automatically (for 48 h) with a gyropalette, the immobilised yeasts on oak chips/cellulose powder settled in the neck of the bottles 3-fold faster than in the bottles that contained free cells. No appreciable differences were found between the time needed for yeasts to settle, regardless of whether they were immobilised on oak chips or cellulose powder. Completely transparent wines were obtained without having to resort to riddling agents, such as bentonite, which results in less manipulated and more natural products. These results are in accordance with those reported by Mantaluta et al. (2011), although those authors used another immobilisation support.

3.4. Chemical characteristics of sparkling wines

The total and volatile acidities and the pH values of sparkling wines 9 months after the second fermentation began can be seen in Table 1. The less affected parameter was wine pH, which was very similar in all wines, no matter what the condition used to perform the second fermentation. However, a significant difference in the total acidity of free cells-inoculated wines was found, and it was related to the type of yeast (Table S1). The yeast strain is a key element that affects the quality of the product, which also applies to sparkling wines, as already reported by some authors (Martí-Raga et al., 2016; Martínez-Rodríguez et al., 2002). In our case, the IOC18-2007 strain provided higher total acidity than 55A when inoculated in the free form. The IOC18-2007 strain provided the highest total acidity when inoculated as free cells, and the lowest was obtained when immobilised on oak chips. In contrast, the 55A strain provided the highest total acidity when immobilised on cellulose powder, and the lowest when inoculated as free cells (Table 1). The volatile acidity values (Table 1) of the different sparkling wines were not significantly different, and neither the yeast nor the inoculation strategies seemed to influence this parameter (Table 1 and Table S1). These results agree with those obtained by Silva et al. (2002), who observed that the immobilisation of *S. cerevisiae* in Ca-alginate did not increase wine volatile acidity compared to that of the free cells-inoculated wines.

3.5. Foaming properties and parameters related to yeast autolysis

It can be deduced from Fig. 3 that no significant differences existed between sparkling wine foamability (HM) and foam persistence (HS), and between their protein concentrations, nor for *Saccharomyces cerevisiae* IOC18-2007 or *Saccharomyces cerevisiae* 55A, regardless of the inoculation strategy used. Although no significant differences were found in the foam parameters between the free yeast- and the immobilised yeast-inoculated sparkling wines, those wines produced by oak chips-immobilised IOC-2007 cells had a slightly higher HM and HS, and total protein values (Fig. 3). Strains IOC18-2007 and 55A gave sparkling wines whose HM, HS values and protein content did not significantly differ (Table S2). Many papers have corroborated the existence of a relationship between the concentration of proteins and the quality of sparkling wines (Alexandre and Guilloux-Benatier, 2006; Brissonnet and Maujean, 1993; Esteruelas et al., 2015; Pueyo et al., 1995). Most studies indicate a positive correlation between protein concentration and maximum height or foamability (HM) and foam stability (HS) (Malvy et al., 1994). Esteruelas et al. (2015) found that intermediate and a low-molecular-weight protein fraction correlates positively with HM.

Polysaccharides come from the glucanes and mannoproteins present in the yeast cell wall and are released from it during yeast autolysis. Polysaccharides contribute to the mouth-feel properties of wine by providing ‘mellowness’ and body sensations, but can also influence sparkling wine foam characteristics (Culbert et al., 2017; Gawel et al., 2016; Vidal et al., 2004).

The total polysaccharides content of the sparkling wines fermented with strain 55A was similar no matter what the inoculation strategy (Fig. 3). In contrast, the sparkling wines fermented with the oak chips-immobilised IOC18-2007 cells showed the lowest total polysaccharides

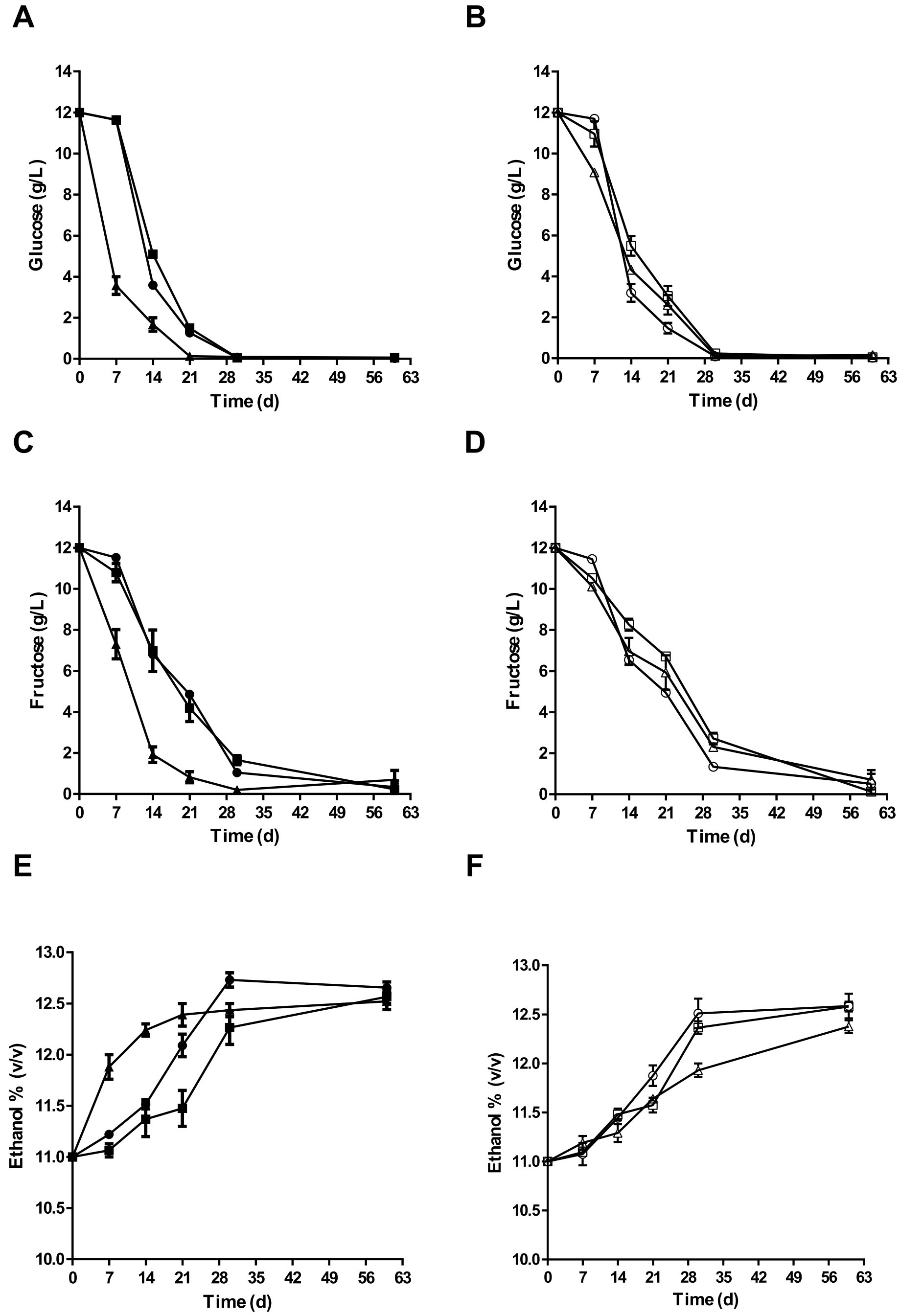

Fig. 2. Fermentation kinetics during the base wine second fermentation by *S. cerevisiae* immobilised cells and free cells; A and B glucose consumption (g/L), C and D fructose consumption (g/L), E and F ethanol production (% v/v). Symbols: IOC 18–2007 free cells (▲), IOC 18–2007 immobilised cells on oak chips (●), IOC 18–2007 immobilised cells on cellulose powder (■), 55A free cells (Δ), 55A immobilised cells on oak chips (○), 55A immobilised cells on cellulose powder (□).

Table 1
Chemical parameters of the resulting sparkling wines fermented with *Saccharomyces cerevisiae* strains IOC 18–2007 and 55A inoculated in the free form, immobilised on oak chips and immobilised on cellulose powder.

	IOC18-2007			55 A		
	Free cells	Oak chips cells	Cellulose powder cells	Free cells	Oak chips cells	Cellulose powder cells
Total acidity[1]	7,65 ± 0,21a	6,55 ± 0,49a	7,10 ± 0,07a	6,00 ± 0,21a	6,98 ± 0,11a	7,35 ± 1,06a
pH	3,23 ± 0,49a	3,20 ± 0,02a	3,24 ± 0,06a	3,17 ± 0,02a	3,18 ± 0,01a	3256 ± 0,22a
Volatile acidity[2]	0,25 ± 0,09a	0,19 ± 0,01a	0,23 ± 0,04a	0,35 ± 0,09a	0,21 ± 0,07a	0,28 ± 0,14a

The ANOVA analysis have been performed for each yeast separately. Significance levels between different inoculation strategies are shown as letters on the data: different letters on the same line indicate significant differences of 95%. [1]: expressed as g/L as g/L of tartaric acid. [2]: expressed as g/L of acetic acid.

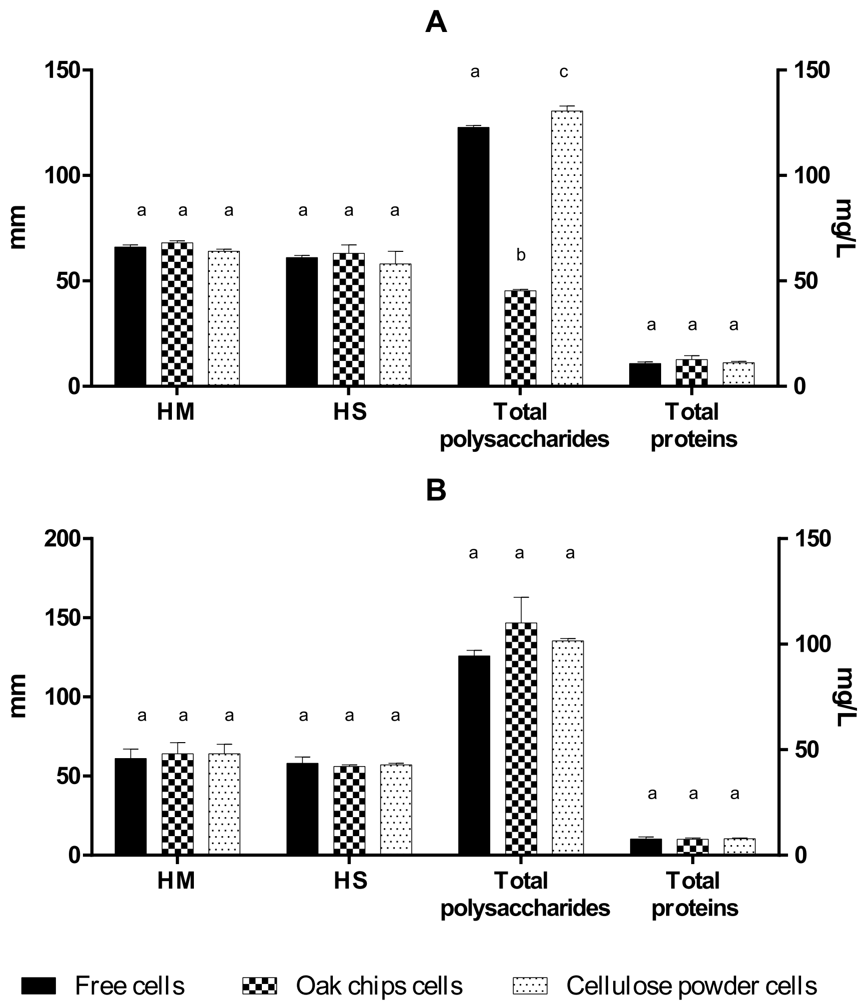

Fig. 3. Effect of the different ways in which yeasts were added to the base wine on foaming properties: foamability (HM) and foam persistence (HS) (expressed in mm), and on total polysaccharides and proteins (expressed in mg/L). A: *Saccharomyces cerevisiae* IOC18-2007; B: *Saccharomyces cerevisiae* 55A. Equal letters indicate the absence of statistically significant diferences ($P > 0.05$).

content. In this case, significant differences of this parameter were found among the wines fermented by the different strategies. Genisheva et al. (2013) found that *O. oeni* cells were more protected when immobilised on corncobs, grape skins and grape stems than when free. Perhaps IOC18-2007 cells are more protected in an oak chips support and result in lower autolysis and less polysaccharides release. When comparing the influence of yeast type on the polysaccharides concentration of sparkling wines, larger amounts were found in the wines fermented with strain 55A regardless of the inoculation strategy used (Fig. 3). Differences in autolysis abilities have been one of the criteria used to select an appropriate second fermentation yeast (Martínez-Rodríguez et al., 2001). Taking into account the higher polysaccharide releasing by strain 55A and the fact that it exhibited similar fermentation dynamics to commercial strain IOC18-2007, it would seem that yeast 55A is the best choice to perform the second fermentation in such wines.

Although differences in polysaccharides content existed among wines, the influence of these compounds on foam parameters HM and HS was null (Fig. 3). Contradictory results about the relationships between polysaccharides and foam have been published; Moreno-Arribas et al. (2000) found that they correlated positively, whereas (Martínez-Lapuente et al., 2015a) did not find any correlation. The higher polysaccharide content of some wines does not result in differences in sensory characteristics, as we corroborate later.

3.6. Volatile aroma analysis

Thirty-two volatile compounds were identified in sparkling wine. The concentrations of these compounds differed in the wines fermented with immobilised or free cells, with cells immobilised on oak chips or on cellulose powder, and with different yeast strains (Tables 2 and 3).

From the detected volatile compounds, only those with compound concentrations/odour threshold value ratios (AOVs) above 1 contributed to the sparkling wine aroma. Eight compounds (the free cells-fermented wines) and seven (in both the oak chips- and cellulose powder-fermented wines) had AOVs above 1 with strain IOC 18–2007, whereas the AOVs of nine (the free cells- and oak chips-fermented wines) and eight compounds (the cellulose powder-fermented wines) went above 1 with strain 55A.

Good volumetric productivities of aroma and other metabolites can be achieved with high volumetric cell densities by packing cells into a small defined volume, either by entrapment inside a carrier matrix or adsorption onto the surface of a porous material. This approach is known as immobilised cell technology, has been widely investigated since halfway through the past century and has been designed for different stages in the fermentations of alcoholic beverages (Nedović et al., 2015).

Alterations to cell growth, physiology and metabolic activity may be induced by cell immobilisation, which influences flavour formation during fermentation processes. Many studies have discussed these

Table 2
Odour descriptor, odour threshold value, concentration for each component of the aromas found in the sparkling wines fermented with *S. cerevisiae* IOC 18–2007 and 55A (free cells, and immobilised on oak chips or cellulose powder).

Group	Aromatic compound	Odour descriptor	Odour threshold values (μg/L)	Compound concentration ± SD (μg/L)					
				IOC 18-2007			55A		
				Free cells	Oak chips cells	Cellulose powder cells	Free cells	Oak chips cells	Cellulose powder cells
Aldehydes	Acetaldehyde	Apple[4]	500[5]	184.7 ± 78a	189.4 ± 84a	235.7 ± 92a	299.6 ± 44c	207.4 ± 51b	133.5 ± 61a
	Benzaldehyde	Almonds[1]	350[8]	35.7 ± 5.6b	nd	nd	4.1 ± 0.2a	4.0 ± 0.2a	14.8 ± 0.2b
	Diacetyl	Butter[3]	100[2]	97.8 ± 58b	13.0 ± 2a	68.5 ± 90ab	151.4 ± 46b	58.0 ± 28a	70.540a
	5-Methylfurfural	Spicy[3]	20000[6]	184.8 ± 87a	210.6 ± 44a	225.0 ± 36a	149.5 ± 6b	161.8 ± 21b	94.3 ± 19a
Total aldehydes				503.0a	413.0 ± b	529.2c	604.6a	431.2b	313.3c
Esters	Diethyl glutarate	Fruity[1]	–	30.7 ± 17a	28.4 ± 4a	58.9 ± 25b	58.8 ± 5b	30.8 ± 7a	96.3 ± 29c
	Diethyl succinate	Fruity[1]	1200000[7]	953.5 ± 181a	1238.0 ± 173a	2203.0 ± 1124b	556.8 ± 99a	551.0 ± 45a	834.9 ± 244b
	Ethyl acetate	Fruity, sweet[1]	12300[3]	134.8 ± 45b	100.6 ± 15ab	83.8 ± 32a	109.0 ± 33a	95.3 ± 12a	112.5 ± 55a
	Ethyl butyrate	Apple[2]	20[5]	73.6 ± 16a	66.3 ± 22a	82.0 ± 18a	49.1 ± 6a	66.4 ± 19ab	78.7 ± 21b
	Ethyl hexanoate	Fruity, anise[1]	14[3]	100.2 ± 6.3b	nd	nd	nd	309.2 ± 21b	nd
	Ethyl octanoate	Pineapple, pear, floral[1]	2[5]	335.7 ± 14a	436.5 ± 24.2a	422.0 ± 45a	632.4 ± 36c	473.5 ± 61a	546.8 ± 33b
	Ethyl decanoate	Fruity[1]	200[3]	496.5 ± 63a	547.8 ± 22a	612.8 ± 225a	569.9 ± 100a	540.5 ± 111a	526.2 ± 67a
	Ethyl isovalerate	Fruity[2]	3[2]	56.6 ± 0.3a	58.6 ± 91a	nd	nd	nd	nd
	Ethyl lactate	Sour[1]	155000[3]	34224.2 ± 6.7a	38079.7 ± 2661.1a	35233.6 ± 5766a	29587.5 ± 6435a	37641.2 ± 9706ab	42942.4 ± 1737b
	Hexyl acetate	Fruity, pear[1]	670[3]	258.8 ± 65.3a	266.5 ± 27.6a	224.7 ± 30a	301.2 ± 38b	262.2 ± 38a	298.6 ± 10ab
	Isobutyl acetate	Solvent[1]	1600[6]	28.7 ± 7a	32.2 ± 9a	22.6 ± 15a	34.4 ± 5b	nd	17.7 ± 27ab
	Methyl acetate	Fruity[2]	470000[7]	51.0 ± 12a	42.1 ± 7a	61.5 ± 28a	98.9 ± 92a	47.6 ± 13a	143.9 ± 250a
	2- Phenethyl acetate	Pleasant, floral[1]	250[3]	208.9 ± 89b	106.1 ± 23a	155.3 ± 51ab	1091.0 ± 228b	1133.5 ± 158b	477.0 ± 55a
Total esters				36953.2a	41002.8b	39160.2a	33089.0a	41151.2ab	46075.0b
Acids	Butyric acid	Stale, cheese[2]	10000[5]	155.4 ± 27a	168.7 ± 52a	155.1 ± 26a	149.7 ± 15a	177.4 ± 16b	177.2 ± 17b
	2-Ethylhexanoic acid	Herbaceous[1]	–	97.2 ± 10c	76.8 ± 4b	7.3 ± 1a	40.0 ± 14b	12.9 ± 2a	9 ± 1a
	Hexanoic acid	Cheese, fatty, stale[1]	420[3]	2179.4 ± 493a	2195.3 ± 169a	3060.2 ± 1030b	1801.6 ± 132a	2384.9 ± 571b	2240.9 ± 438ab
	Octanoic acid	Cheese, rough, sour[1]	500[3]	3940.8 ± 944a	3905.7 ± 316a	6534.8 ± 3444b	3196.4 ± 373a	4662.8 ± 1106b	4155.3 ± 835ab
	Decanoic acid	Fatty[1]	1000[3]	613.1 ± 130a	603.7 ± 61a	1095.3 ± 603b	537.1 ± 62a	618.7 ± 33ab	714.7 ± 187b
	Isobutyric Acid	Fatty[1]	200000[5]	nd	121.9 ± 9c	14.4 ± 1b	15.6 ± 3b	44.2 ± 19c	nd
	Isopentanoic acid	Stale[1]	–	184.7 ± 33b	156.5 ± 11a	137.2 ± 15a	122.8 ± 11a	141.7 ± 15b	134.9 ± 12ab
Total acids				7170.6a	7228.6a	11004.3b	5863.2a	8042.6b	7432.0c
Alcohols	Benzyl alcohol	Citric, fruity[1]	200000[5]	18.0 ± 10a	34.8 ± 16ab	62.8 ± 39b	46.1 ± 10b	23.0 ± 4a	90.2 ± 8c
	2,3-Butanediol	Butter[1]	150 000[4]	44.2 ± 14a	33.5 ± 9a	69.0 ± 8b	347.1 ± 105b	nd	50.2 ± 24ab
	1-Propanol	Fresh, alcohol[1]	830[4]	nd	nd	nd	8739.1 ± 526a	11390.9 ± 2550b	22336.4 ± 2070c
	1-Butanol	Medicine, alcohol[1]	150000[3]	47.5 ± 4.3a	206.7 ± 243a	49.0 ± 3a	593.0 ± 125b	549.0 ± 67b	59.1 ± 4a
	Isoamyl alcohol	Cheese[1]	30000[2]	40208.9 ± 6545a	42022.9 ± 3255ab	52223.1 ± 14061b	40343.0 ± 2195a	42275.7 ± 2818a	42777.4 ± 2644a
	Cis-3-hexenol	herbaceous[1]	400[5]	nd	4.5 ± 0.4b	84.6 ± 4c	5.8 ± 3b	nd	nd
	2-Phenylethanol	Floral, pollen[1]	14000[3]	7508.1 ± 1625a	7521.7 ± 552a	11005.9 ± 4289b	5796.5 ± 533a	6879.8 ± 352b	7111.7 ± 1263b
Total alcohols				4782.6a	49824.1a	63494.4 ± b	55870.6a	64118.4ab	7242.5b
Lactones	γ- Butyrolactone	Sweet, toasted, caramel[4]	50000[6]	814.2 ± 78b	940.5 ± 92c	699.2 ± 91a	1462.1 ± 199b	1236.2 ± 349b	421.4 ± 328a

The ANOVA analysis have been performed for each yeast separately. Significance levels between different inoculation strategies are shown as letters on the data that correspond to the concentration of aromatic compounds: different letters on the same line indicate significant differences of 95%. nd: not detected. Odour descriptor references: [1]Jiang and Zhang (2010) [2]Francis (2013) [3]Gambetta et al. (2014) [4]Sánchez-Palomo et al. (2012). Odour threshold value references [5]Guth (1997) [6]Aznar et al. (2003) [7]Zea et al. (2001) [8]Belitz et al. (2009).

issues (Kregiel et al., 2013; Melzoch et al., 1994; Norton and D'Amore, 1994; Walsh and Malone, 1995; Willaert and Nedovic, 2006). The results in the present work agree with the above research works, and show that the aromatic composition of the final wine depends on both the *S. cerevisae* strain and the inoculation strategy followed (in the free cells form, immobilised on oak chips or on cellulose powder). With both immobilisation supports, the immobilisation with oak chips was the best option as they generated more esters with a value of OAV > 1 with both commercial strain IOC 18–2007 and *S. cerevisiae* 55A.

3.6.1. Influence of the way in which cells were added to base wines: free or immobilised

As deduced from Table 2, the way in which cells performed the second fermentation (free or immobilised) affected the aromatic profile of sparkling wines. Thus immobilisation, regardless of the substrate used, significantly affected the concentration of 20 (in the wines fermented with IOC 18–2007) and 27 (in the wines fermented with 55A) of the 32 volatile compounds. No common pattern was deduced from the two yeasts, and only diacetyl and 2-ethyl hexanoic acid were significantly higher in the wines fermented with free cells than in those fermented with immobilised cells, irrespectively of the strain used. The concentration of isopentanoic acid was higher in the wines fermented with the IOC 18–2007 free cells than in those fermented with immobilised ones, whereas the opposite occurred with yeast 55A. Benzyl alcohol and cis-3-hexenol were found at lower concentrations in the wines fermented with the IOC 18–2007 free cells than in those fermented with immobilised ones, whereas the opposite was observed with yeast 55A. When the total concentrations of the families of volatile compounds were taken into account, more esters, fatty acids and alcohols were found in the wines fermented with immobilised yeasts than in those fermented with free cells, regardless of the strain used. Unlike we the authors believing that each volatile compound having OIV > 1 contributed to aroma, Cacho (2006) stated that the aromatic profile of a wine is caused by families of odorants, and not by individual compounds, because the effect of each component of an aromatic family can be additive or synergic. As a general rule, a large amount of ester confers wine a fruitier aroma, whereas high concentrations of fatty acids and higher alcohols (or fusel alcohols) contribute negatively to aroma (Mingorance-Cazorla et al., 2003). In spite of the different concentrations of volatile compounds found in the different wines, only those with OAVs above 1 contribute to wine aroma (Ferreira et al., 2002; Moyano et al., 2002). By taking this into account, we detected that the compounds which contributed to aroma were diacetyl, ethyl butyrate, ethyl hexanoate, ethyl octanoacte, ethyl decanoate, ethyl isovalerate, hexanoic, octanoic and decanoic acids, isoamyl alcohol and 1-propanol. Diacetyl confers wines a buttery smell, which is more noticeable in free cells fermented wines, ethyl esters of hexanoic, octanoic, decanoic and isovaleric acids provide fruity and floral aromas, and some such as ethyl octanoate (pineapple, pear, floral smell) contribute greatly to wine aroma as it scores the highest OAVs (from 167 to 316). However, precursor acids hexanoic, octanoic and decanoic confer fatty and rough notes to wines, but only the first two have a sensorial impact on wines (with OAVs ranging between 4 and 9). Isoamyl alcohol confers a cheese aroma, whereas 1-propanol provides fresh or alcoholic notes. The way in which the yeast was added to the base wine (free or immobilised) affected the aroma of sparkling wines because it influenced the concentrations of the above-described compound, although the nature of the differences was strain-dependent. The sparkling wines fermented with the free cells of yeast IOC 18–2007 contained diacetyl and ethyl hexanoate, which were not detected in the wines fermented with immobilised cells. These compounds confer butter, fruity and anise aromas. On the contrary, the ethyl octanoate, ethyl decanoate, hexanoic acid, decanoic acid and isoamyl alcohol OAVs were higher in the immobilised cells-than in the free-cells fermented wines. For yeast 55A, diacetyl, ethyl octanoacte, ethyl decanoate contributed more to aroma in the free cells-fermented wines than in the immobilised cells-fermented ones. The presence of diacetyl in sparkling wine provides butter aromas (Nielsen and Richelieu, 1999), as already said, but could be detrimental if in excess (Clarke and Bakker, 2004). Conversely, ethyl butyrate, hexanoic and octanoic acids, isoamyl alcohol and 1-propanol were more noticeable in the wines fermented with immobilised cells. The presence of 1-propanol in moderate amounts can confer pleasant aromas by contributing to the complexity of wine aroma (Ribéreau-Gayon et al., 2003).

3.6.2. Influence of the substrate on which cells were immobilised

The type of substrate used to immobilise yeast cells affected the volatile compounds. Thus diethyl succinate, diethyl glutarate, 2-phenethyl acetate, hexanoic, octanoic isobutyric acid, and decanoic acids 2,3-butanodiol, benzyl alcohol, and γ-butyrolactone concentrations differed significantly between the wines fermented with oak chips and cellulose powder-immobilised cells, regardless of the yeast strain used. The concentrations of diacetyl, ethyl isovalerate, ethyl acetate, γ-butirolactone and total esters were higher in the wines fermented with oak chips-immobilised IOC 18–2007 yeast than in those fermented with cellulose powder-immobilised IOC 18–2007 cells, whereas 2-phenylethanol, cis-3-hexenol, total aldehydes, total acids, and total alcohols were lower in the former than in the latter. When considering that esters and lactones are desired compounds in wine (Ferreira et al., 2004; Jarauta, 2004), yeast IOC108-2007 immobilisation on oak chips provided better results than when immobilised on cellulose powder. With yeast 55A, acetaldehyde, 5-methylfurfural, ethyl hexanoate, pentanoic acid, γ-butyrolactone, total aldehydes and the total fatty acids concentrations were higher in the oak chips-immobilised cells than in the cellulose powder-immobilised cells, whereas benzaldehyde, ethyl butyrate, ethyl octanoacte, isobutyl acetate, hexyl acetate, ethyl lactate, 1-propanol, total esters, total alcohols were lower in the former than in the latter. The formation of fatty acids by themselves is undesirable (Ferreira et al., 2004; Jarauta, 2004), but they can be esterified with different alcohols to form esters that contribute positively to the final aromatic profile. Alcohol production is also essential, especially 2-phenylethanol formation. This is the only compound from the alcohol group that contributes a pleasant aroma to wines (Hua and Xu, 2011). Although no significant differences in esters content were found between the wines fermented with cellulose powder or oak chips immobilised cells, the formation of alcohols and acids was lower when strain 55A was used on oak chips. Moreover, the lactone content was higher with oak chips than with cellulose powder, and provided desirable toasted and caramel aromas (Robinson, 2011).

The volatile compounds with different concentrations in the wines fermented with oak chips-immobilised or cellulose powder-immobilised cells and with AOVs higher than 1 were ethyl butyrate, ethyl hexanoate, ethyl decanoate, ethyl isovalererate, 2-phenyl acetate, hexanoic and octanoic acids, isoamyl alcohol and 1-propanol. The variations in these compounds were strain-dependent. Ethyl isovalerate had a very strong sensory impact, but only when strain IOC 18–2007 was immobilised on oak chips, and not when it was on cellulose powder. This compound provided a fruitier character to the wine fermented with oak chips. In contrast, this compound did not contribute to aroma in any of the wines fermented with strain 55A. Overall, the wines fermented with IOC 18–2007 immobilised on oak chips showed more esters with OAV > 1 than those produced with cell immobilised on cellulose powder, which provided a fruitier aroma to sparkling wines. Furthermore, acids (hexanoic and octanoic acids) and alcohols (isoamylic alcohol) had an OAV > 1. These compounds are responsible for unpleasant odours; therefore oak chips would be a better choice as the immobilisation support. With yeast 55A, the number of compounds with OAV > 1 was 9 in the wines fermented with oak chips-immobilised cells and 7 in those fermented with cellulose powder-immobilised cells. The contribution of esters (ethyl hexanoate, ethyl decanoate, 2-phenyl acetate) to aroma was greater in the wines fermented with oak chips-immobilised cells, so using this immobilisation system of obtain a better

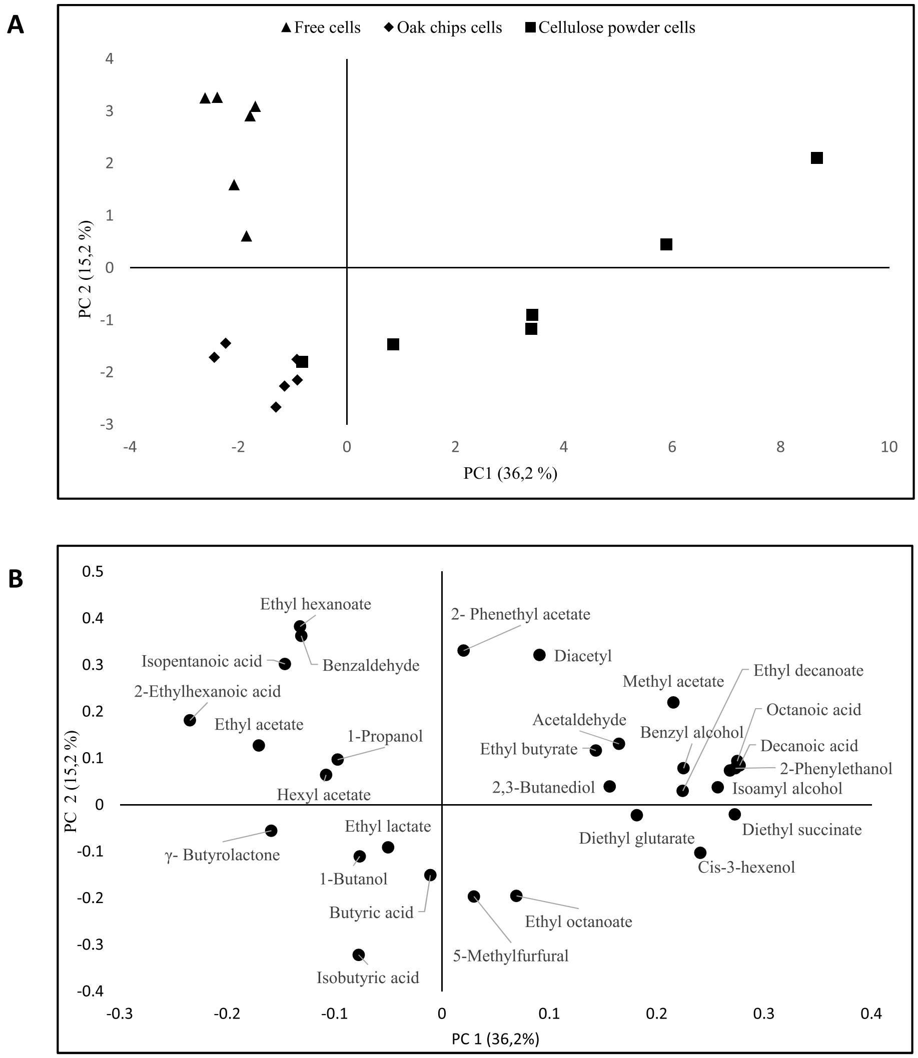

Fig. 4. Principal component analysis (PCA) of volatile compounds in sparkling wines inoculated fermented with *Saccharomyces cerevisiae* IOC18-2007 inoculated as free cells, oak chips-immobilised cells or cellulose powder immobilised cells. (A) Plot of the two principal component scores. (B) Plot of the two principal component loadings.

aroma is recommended, as in the case of yeast IOC-2007. A PCA analysis of the volatile data was made for each yeast in order to know if the strategy of inoculation discriminates the sparkling wines s. In addition to the wines fermented with immobilised yeasts, those fermented with free yeasts have been included in the analysis. Figs. 4 and 5 show the plots of the two principal component loadings and the plots of the two principal component scores. The sparkling wines separated in the plane, which indicates that the volatile composition of the wines differs significantly depending on the inoculation system that has taken place. This behaviour is common for the two yeasts, although the separation of the three groups is less evident for *S. cerevisiae* IOC-20017 than for *S. cerevisiae* 55A. In Fig. 4A, wines fermented with free and oak chips-immobilised *S. cerevisiae* IOC-20017 cells placed in the left part of the PC1 component, while the wines fermented with cellulose powder-immobilised cells placed in the right part of the PC1 component. The PC2 component separated clearly the wines fermented with free cells from those fermented with oak chips-immobilised cells; this explained that differences between them were mainly related to this component. Wines fermented with cellulose powder-immobilised cells partially merged with those fermented with oak chips-immobilised cells in the left bottom quadrant, and showed a much more scattered location than the other two types of wine. Fig. 4B shows the arrangement of the different aromatic compounds in the plane. This arrangement provided an idea of which were the main differences in wine volatile composition relating to the inoculation system used. In the case of wines fermented with the yeast *S. cerevisiae* 55A, the PC1 component explains the differences between free cells and immobilised cell fermented wines, whereas the component PC2 clearly separates the wines fermented with oak chips-immobilised cells from the other fermented with cellulose powder-immobilised cells and free cells (Fig. 5A). Fig. 5B shows the layout of the different aromatic compounds of the wines fermented with *S. cerevisiae* 55A in the plane. López de Lerma et al. (2018) found that the sparkling wines fermented with two different yeast strains that were inoculated as free cells, alginate bed-immobilised cells and biocapsules-immobilised cells, were also grouped by a PCA analysis into three clusters according to the inoculation strategy. They suggested that, although some volatile compounds rely more on the yeast strain than on the inoculation format, some specific aroma compounds were associated with the immobilisation format.

Although controls were carried out to see the influence of immobilisation substrates on the aroma of sparkling wines, this influence could not be deduced because those wines fermented spontaneously due to the growth of some unknown yeast from the wine base.

3.6.3. Influence of yeast strain

Given the interaction detected between the way in which yeasts

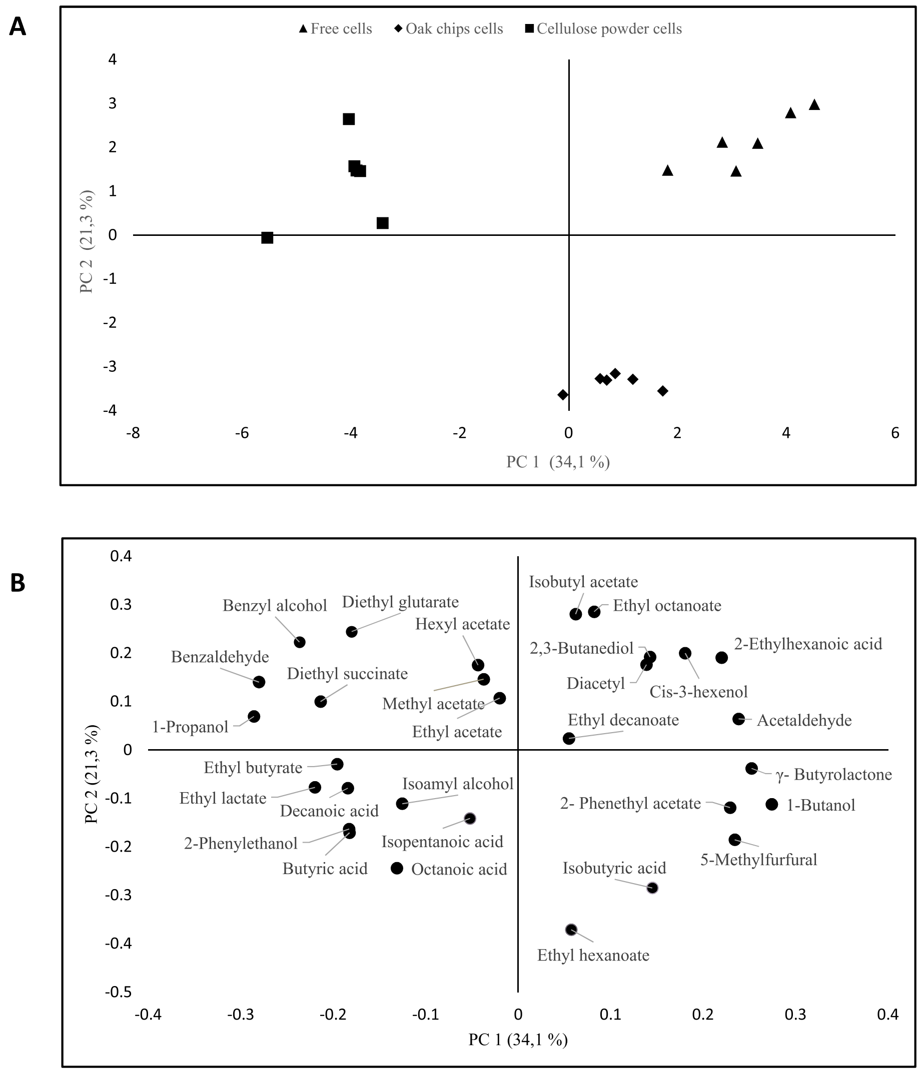

Fig. 5. Principal component analysis (PCA) of volatile compounds in sparkling wines inoculated fermented with *Saccharomyces cerevisiae* 55A inoculated as free cells, oak chips-immobilised cells or cellulose powder immobilised cells. (A) Plot of the two principal component scores. (B) Plot of the two principal component loading.

were added to the base wine and the yeast strains, a comparison of volatile compound concentrations and a statistical analysis to know the influence of the yeast strain were performed separately for the free form, the oak chips immobilised form and for the cellulose powder immobilised form. Regardless of the way in which cells were inoculated, the concentrations of 9 of 32 coumpounds significantly differed between strains, whereas 6 others did not (methyl acetate, ethyl acetate, ethyl decanoate, and hexanoic, octanoic and decanoic acids). When comparing the columns entitled "Free cells" in Table 2 it appears that yeast IOC 18–2007 produced higher concentrations of methylfurfural, ethyl esters (except for ethyl octanoate and ethyl decanoate), and diethyl succinate than 55A (Table 2). This yeast also produced higher concentrations of fatty acids (except for isobutyric hexanoic, octanoic and decanoic acids). A high concentration of medium-chain fatty acids conferred rough notes to wines. Conversely, IOC 18–2007 synthesised fewer alcohols (excepting 2-phenylethanol) and less γ-butyrolactone. Strain 55A produced more diacetyl (buttery aroma), benzaldehyde, fewer esters, (except for diethyl glutarate, ethyl octanoate, ethyl decanoate and esters hexyl, methyl and isobutyl of acetate), more alcohols (save 2-phenylethanol), and more γ-butyrolactone than IOC 18–2007. The yeast strain factor significantly affected the concentration of 17 of the 32 volatile compounds (Table 3). The majority of the significant differences between yeasts are in the group of alcohols. In fact, total alcohol concentrations were significantly different between the two yeasts (Table 3). Taking into account that the compounds which OAVs higher than 1, different contributions to aroma were found for diacetyl, ethyl butyrate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl isovalerate, 2-phenylethyl acetate, hexanoic and octanoic acids, and 1-propanol andisoamyl alcohols when comparing both yeast strains. Thus strain 55A provided more diacetyl, ethyl octanoate and 2-phenetyl acetate than IOC 18–2007, which confer butter, pineapple, pear, fruity, fresh and floral notes to wines, whereas IOC 18–2007 provided more ethyl butyrate and ethyl hexanoate and conferred apple and anise notes. Yeast 55A provided less cheese, fatty and sour notes than IOC-18-2017.

When comparing the columns "Oak chips cells" in Table 2, the wines fermented with IOC 18–2007 showed lower aldehydes concentrations (excluding 5-methylfurfural), smaller amounts of esters (except for diethyl succinate, ethyl isovalerate, ethyl lactate and isobutyl acetate), fewer fatty acids (save 2-ethyl hexanoic, isobutyric and isopentanoic acids), and fewer alcohols and less γ-butyrolactone than 55A. Only the total concentrations of the alcohol group were significantly different between the two yeasts (Table 3). Significant differences in the concentrations of 14 of the 32 volatile compounds were found between the yeast strains (Table 3), but only seven of the 32 compounds contributed differently to wine aroma: ethyl hexanoate, ethyl octanoate, ethyl isovalerate, 2-phenethyl acetate, hexanoic and octanoic acids and 1-propanol. All of them, except for ethyl isovalerate, presented lower AOVs in the wines fermented with IOC 18–2007 than

Table 3
ANOVA analysis results from the volatile compounds found in the sparkling wines fermented with *Saccharomyces cerevisiae* strains IOC 18–2007 and 55A inoculated in the free form, immobilised on oak chips and immobilised on cellulose powder.

Group	Aromatic compound	Free cells		Oak chips cells		Cellulose powder cells	
		F-ratio	P	F-ratio	P	F-ratio	P
Aldehydes	Acetaldehyde	9.90	0.0104[a]	0.20	0.6623	5.11	0.0474[a]
	Benzaldehyde	190.61	0.0000[a]	1567.20	0.0000[a]	1876.49	0.0000[a]
	Diacetyl	1.37	0.2682	15.47	0.0028[a]	0.00	0.9580
	5-Methylfurfural	0.98	0.3450	0.68	0.0333[a]	62.2	0.0000[a]
Total aldehydes		2.00	0.1872	0.36	0.5599	10.37	0,0092[a]
Esters	Diethyl glutarate	14.78	0.0032[a]	0.56	0.4698	5.64	0.0390[a]
	Diethyl succinate	10.65	0.0085[a]	88.97	0.0000[a]	8.50	0.0154[a]
	Ethyl acetate	1.29	0.2820	2.49	0.1457	1.21	0.2970
	Ethyl butyrate	12.21	0.0058[a]	0.00	0.9891	0.09	0.7594
	Ethyl hexanoate	1498.76	0.0000[a]	1275.11	0.0000[a]	nd	nd
	Ethyl octanoate	25.46	0.0005[a]	1.92	0.6100	29.82	0.0030[a]
	Ethyl decanoate	2.28	0.1617	0.03	0.8774	0.82	0.3867
	Ethyl isovalerate	2.50	0.1449	2.50	0.1451	nd	nd
	Ethyl lactate	1.22	0.2951	0.01	0.9171	9.83	0.0106[a]
	Hexyl acetate	1.90	0.1986	0.05	0.8263	33.84	0.0002[a]
	Isobutyl acetate	2.84	0.1226	72.05	0.0000[a]	0.13	0.7218
	Methyl acetate	1.61	0.2332	0.77	0.4004	0.64	0.4424
	2- Phenethyl acetate	24.48	0.0006[a]	247.06	0.0000[a]	110.12	0.0000[a]
Total esters		0.86	0.3764	0.00	0.9667	8.1	0.0173[a]
Acids	Butyric acid	0.21	0.6538	0.15	0.7038	2.90	0.1192
	2-Ethylhexanoic acid	66.57	0.0000[a]	1011.65	0.0000[a]	5.43	0.0420[a]
	Hexanoic acid	3.29	0.0998	0.61	0.4528	3.21	0.1033
	Octanoic acid	3.23	0.1025	2.60	0.1380	2.71	0.1310
	Decanoic acid	1.67	0.2254	0.28	0.6097	2.18	0.1708
	Isobutyric acid	173.67	0.0000[a]	78.90	0.0000[a]	1849.00	0.0000[a]
	Isopentanoic acid	18.77	0.0015[a]	3.95	0.0751	0.09	0.7746
Total acids		3.91	0.0762	1.41	0.2628	2.78	0.1265
Alcohols	Benzyl alcohol	25.57	0.0005[a]	3.09	0.1090	2.87	0.1209
	2,3-Butanediol	2.77	0.1269	81.42	0.0000[a]	3.20	0.1041
	1-Propanol	1657.45	0.0000[a]	119.70	0.0000[a]	698.44	0.0000[a]
	1-Butanol	114.4	0.0000[a]	11.06	0.0077[a]	21.41	0.0009[a]
	Isoamyl alcohol	0.00	0.9630	0.02	0.8885	2.61	0.1369
	Cis-3-hexenol	22.77	0.0008[a]	490.00	0.0000[a]	3173.44	0.0000[a]
	2-Phenylethanol	6.01	0.0342[a]	1011.65	0.0000[a]	4.55	0.0587
Total alcohols		5.48	0.0413[a]	20.06	0.0012[a]	1.35	0.2723
Lactones	γ- Butyrolactone	55.01	0.0000[a]	4.02	0.0728	4.00	0.0735

[a] Significant differences between yeasts.

in those fermented with 55A. Thus strain 55A provided sparkling wines more anise, pineapple, pear, fruity, floral and fresh notes than IOC 18–2007, whereas IOC 18-2007conferred wine a more intense fruity character due to the higher sensorial impact of ethyl isovalerate. Yeast 55A conferred a cheesier rough aroma as a result of its higher octanoic acid production and a fresher/alcoholic note due higher 1-propanol production.

Finally, the comparison of the data in the columns entitled “Cellulose powder cells” in Table 2 shows that yeast IOC 18–2007 produced a significantly larger quantity of aldehydes (except for benzaldehyde and diacetyl) significantly lower esters (save diethyl succinate ethyl isovalerate, ethyl lactate and isobutyl acetate), lower fatty acids (except 2-ethyl hexanoic, isobutyric, isopentanoic acids), lower alcohols (mainly 1-propanol, 1-butanol and isoamyl alcohol), and lower γ-butyrolactone than 55A (Tables 2 and 3). Significant differences in the concentrations of 14 of the 32 compound concentrations were found between yeast strains (Table 3), but only nine conferred distinct sensorial impacts: ethyl butyrate, ethyl octanoate, ethyl decanoate, 2-phenyl acetate, hexanoic, octanoic and decanoics acids, n-propanol and isoamyl alcohol. Taking into account only those compounds with OAVs above 1, yeast 55A conferred wine more pineapple, pear, floral and fresh notes and less detrimental notes due to lower fatty acid production than IOC 18–2007. This latter yeast provided a slightly more apple and fruity aromas to sparkling wine.

Differences in the volatile aroma profiles found in wines between those fermented with two different *Saccharomyces cerevisiae* strains has been previously recorded by Ubeda Iranzo et al. (2000), Regodón Mateos et al. (2006), and Berthels et al. (2008). Torrens et al. (2008) have demonstrated that chemical and volatile compositions, as well as the sensorial profile of sparkling wines, depend on the yeast strain used for fermentation. From our results, more significant differences in individual compounds were recorded between strains when in the free form than in the immobilised form, but when groups of compounds were considered, only total alcohols and lactones allowed us to significantly discriminate both yeasts. Only the alcohol group significantly differentiated both yeasts when they were immobilised on oak chips cells, whereas both aldehyde and ester groups did so when cells were immobilised on cellulose powder.

During the sparkling wine-making process, the second fermentation occurs under very particular conditions for yeasts; the base wine presents a high alcohol content, and not all strains can grow and ferment under these conditions (Garofalo et al., 2016; Torresi et al., 2011). Low fermentation temperatures (12-18 °C), typical in sparkling wine making, slow down fermentation activity, but this is useful for sparkling wines' quality improvement. Yeasts must tolerate more than 4-atm pressure, low pH and high alcohol content conditions (Ribéreau-Gayon et al., 2003). Therefore, careful yeast strain selection and temperature control of the second fermentation are sufficient requirements to guarantee a proper fermentation process inside bottles. In this case, strain 55A endured the second fermentation in-bottle conditions in both the immobilised and free cells forms by fermenting sugars and the same ethanol content arising as with the commercial strain.

3.7. Sensorial analysis results

Despite the differences observed in the profile of volatile wines, at the sensory level no significant differences appeared between the fermented wines with both yeast types and those with the free or immobilised yeast with the different immobilisation substrates (Table S3). These results suggest that the new technology can be used with yeast 55A as an alternative to commercial starter IOC18-2007, and that the use of immobilisation supports reports the advantages of rapid yeast elimination and not adding bentonite, and does not have a negative impact on the wine sensory profile.

4. Conclusions

A new procedure to immobilise *S. cerevisiae* strains on natural supports (oak chips and cellulose powder, both accepted for use in oenology) has been developed and the usefulness of the new products has been demonstrated at the industrial level. Sugar consumption with immobilised cells was delayed 1 week compared to that done with free cell fermentations, but the riddling process was 3-fold quicker. In addition, it was not necessary to add riddling agents. Significant differences were obtained in the formation of esters, acids, alcohols, aldehydes and lactones depending on the yeast used and the immobilisation support. Oak chips were the more appropriate support for yeast immobilisation. The yeast released higher polysaccharide quantities, which produced a larger number of esters when immobilised on oak chips was 55A. No significant differences in wine sensorial characteristics were found no matter what way the second fermentation occurred.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.09.016.

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