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Consecutive alcoholic fermentations of white grape musts with yeasts immobilized on grape skins – Effect of biocatalyst storage and SO_2 concentration on wine characteristics

Zlatina Genisheva [a], Mar Vilanova [b], Solange I. Mussatto [a], José A. Teixeira [a], José M. Oliveira [a, *]

[a] CEB – Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal
[b] Misión Biológica de Galicia (CSIC), PO Box 28, 38080 Pontevedra, Spain

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ABSTRACT

Saccharomyces cerevisiae yeasts, immobilized by natural adsorption on grape skins, were used to carry out the alcoholic fermentation step of a winemaking process. The viability of the immobilized cells was evaluated by the implementation of 7 successive fermentations of a white grape must containing 30 mg/L of SO_2. The time to complete alcoholic fermentation, the physicochemical characteristics of the produced wines (ethanol, glycerol, organic acids, volatile compounds, color) and sensory properties were evaluated. A traditional fermentation with free cells was used as control. Three other fermentations were conducted after storage of the immobilized biocatalyst (30 d, 4 °C), the first one in the same conditions of the earlier assays, and the other two with higher amounts of SO_2 (60 mg/L, 90 mg/L).

Wines produced with immobilized cells presented physicochemical and sensory characteristics similar to those traditionally produced with free cells. After three consecutive fermentations, chromatic characteristics became similar to those of traditional wines, but the fermentation time had been reduced from 7 d to 4 d. The fermentative process and the characteristics of the produced wines were not significantly affected by the use of higher amounts of SO_2. Immobilized biocatalysts could be stored at least one month without losing its activity.

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1. Introduction

Cell immobilization systems used for alcoholic fermentations have various technological and economic advantages when compared with free cell systems, including the higher productivities, greater tolerance to inhibitory substances and the possibility of operating the processes in a continuous mode (Diviès & Cachon, 2005; Genisheva, Mussatto, Oliveira, & Teixeira, 2011; Kourkoutas, Bekatorou, Banat, Marchant, & Koutinas, 2004). It is well known that, when compared to free cells, immobilized cells are more resistant against ethanol toxicity, acidity, extreme temperatures and some inhibitors like heavy metals, phenols and sulfur dioxide (Diviès & Cachon, 2005; Yajima & Yokotsuka, 2001).

The immobilization techniques can be divided into four categories: attachment to a support, entrapment in a porous matrix, cell aggregation and containment behind a barrier (Kourkoutas et al., 2004). The supports to be used can be organic or inorganic; however, organic supports from natural origin, such as fruit pieces, can be easily accepted by the consumer (Genisheva, Macedo, Mussatto, Teixeira, & Oliveira, 2012; Kourkoutas et al., 2004). Apple (Kourkoutas, Douma, et al., 2002), quince (Kourkoutas, Koutinas, Kanellaki, Banat, & Marchant, 2002), pear (Mallios et al., 2004), grape skins (Mallouchos et al., 2002) and dried raisin berries (Tsakiris, Sipsas, Bekatorou, Mallouchos, & Koutinas, 2004) have already been studied as support for cells immobilization, and present advantages for application on an industrial scale, as they are of food grade purity and could reduce the cost of the process. Nevertheless, deeper studies on the immobilization practice must be done in order to ease the handling of the process and the use of this tool at the cellar (Kourkoutas et al., 2004; Vila-Crespo, Rodriguez-Nogales, Fernandéz-Fernandéz, & Hernanz-Moral, 2010).

In fermentation processes with immobilized cells, the possibility of storage of the immobilized microorganisms for further use is an important aspect that must be taken into account (Diviès &

* Corresponding author. Tel.: +351 253 604 411; fax: +351 253 604 429.
E-mail address: jmoliveira@deb.uminho.pt (J.M. Oliveira).

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Cachon, 2005; Genisheva, Mussatto, Oliveira, & Teixeira, 2013; Kandylis, Drouza, Bekatorou, & Koutinas, 2010). Additionally, to be used in a winemaking process, the support must satisfy other prerequisites: be abundant and cheap (Bakoyianis, Kanellaki, Kaliafas, & Koutinas, 1992). In this context grape skins are food-grade, abundant and of a low cost, as it is a by-product extensively generated in the wine industry. It is also a natural product from the vine, which supposedly will not interfere negatively on the final quality of the wine.

The aim of the present study was to evaluate the possibility to carry out consecutive alcoholic fermentations using *Saccharomyces cerevisiae* yeasts immobilized on grape skins to produce a white wine. Additionally, the viability of the immobilized biocatalyst after a storage period and the inhibitory effect of SO_2 were also studied. To assess the quality of the final products, physicochemical characteristics, color, volatile compounds and sensory properties were evaluated.

2. Materials and methods

2.1. Inoculum preparation

A commercial *S. cerevisiae* strain (Lalvin QA23®, Lallemand) was used in the experiments. The inoculum was prepared by hydrating 300 mg/L of yeast in sterilized warm water (30 °C) for 30 min, according to the manufacturer's instructions.

2.2. Support material for cell immobilization

Grape skins, a residue obtained after pressing of white grapes, were used as support material for cell immobilization. This support was supplied by a local winemaking company, being washed with distilled water and dried at 60 °C until constant weight before use. Then, the stems and the seeds were removed, and the support was sterilized for 20 min at 121 °C.

2.3. Fermentation assays

Seven alcoholic fermentations were carried out in consecutive batches (from batch 1 to batch 7), using a clarified white grape must with a pH of 2.9 and a density of 1082 g/L. For the first batch, 50 g of dry grape skins were placed in 1 L of grape must and 300 mg of rehydrated yeast cells, prepared as mentioned above, were added. Yeasts were allowed to immobilize spontaneously by natural adsorption. The must/wine density was monitored daily and the fermentation was monitored until density was below 0.997 g/mL. After that, the support was recovered and washed with 500 mL of sterilized water and reused in the next batch. Free cell fermentations, with the same cell concentration, were performed as controls. All the experiments were performed in triplicate, in Erlenmeyer flasks taped with cotton plugs, at 20 °C without agitation, being SO_2 concentration adjusted to 30 mg/L.

After batch 7, the support with immobilized cells was washed with sterilized water (500 mL) and stored at 4 °C for 30 d. Then, after storage of the immobilized biocatalysts, three more successive fermentation batches were performed, using the same grape must. Batch 8 was carried out in the same conditions of batch 7, while batches 9 and 10 were conducted with increased concentration of free SO_2, respectively adjusted to 60 mg/L and 90 mg/L.

Before bottling, all the produced wines were clarified by centrifugation (10 min, $RCF = 6000$), and sulfur dioxide was adjusted to 30 mg/L. Wines were stored at 4 °C before analysis.

The following nomenclature was adopted: FC for fermentations with free cells and B1, B2, B3, B4, B5, B6, B7, B8, B9 and B10 for fermentations using immobilized cells.

2.4. Immobilized cells determination

Immobilized cells concentration was determined at the fermentations' end by dry weight, after washing a small amount of the support (1 g, wet weight ≈ 0.3 g, dry weight) with 30 g/L NaOH solution, for 24 h, at 30 °C and agitation rate of 120 min^{-1}, according to Genisheva et al. (2011). The missing amount of the support was replaced by an equivalent amount of fresh dry material (without cells), so the initial concentration stayed unchanged. Free cells concentration in the fermentation medium was estimated by measuring the absorbance at 600 nm, which was correlated to a calibration curve (dry weight × absorbance). Immobilized death/live yeasts were determined after detachment of the cells by vigorous agitation of 0.5 g of support with 30 g/L solution of NaCl, for 30 min. Then the released cells were further stained with methylene blue and the dead/live cells were counted on a Neubauer chamber.

2.5. General physicochemical analysis

Free SO_2 concentration and total acidy were measured by titration according to the methods OIV-MA-AS323-04A and OIV-MA-AS313-01, respectively (OIV, 2012a).

2.6. HPLC analysis

Glucose, fructose, ethanol, glycerol and organic acids (citric, tartaric, malic, succinic and acetic) concentrations were determined by high performance liquid chromatography (HPLC) according to Genisheva et al. (2013), using a Jasco chromatograph equipped with a refractive index detector (Jasco 830-RI), an ultraviolet detector and a Varian Metacarb 67H column (300 mm × 6.5 mm) operated at 80 °C. A 5 mmol/L H_2SO_4 aqueous solution was used as eluent at a constant flow rate of 0.3 mL/min.

2.7. Gas-Chromatographic analysis

Major volatile compounds were directly analyzed after adding 410 μg of 4-nonanol (internal standard – IS) to 5 mL of wine. A Chrompack CP-9000 gas chromatograph equipped with a split/splitless injector, a flame ionization detector (FID) and a capillary column, coated with CP-Wax 57 CB (50 m × 0.25 mm; 0.2 μm film thickness, Chrompack), was used. The temperatures of the injector and the detector were both set to 250 °C. The oven temperature was initially held at 60 °C, for 5 min, then programmed to rise from 60 °C to 220 °C, at 3 °C/min, and finally maintained at 220 °C for 10 min. The carrier gas was helium 4× (Praxair) at an initial flow rate of 1 mL/min (125 kPa at the head of the column). The analyses were performed by injecting 1 μL of sample in the split mode (15 mL/min). The quantification of major volatile compounds, after the determination of the detector response factor for each analyte, was performed with the software Star-Chromatography Workstation version 6.41 (Varian) by comparing retention times with those of pure standard compounds.

Minor volatile compounds were analyzed by GC–MS after extraction of 8 mL of wine with 400 μL of dichloromethane, spiked with 3.28 μg of 4-nonanol (IS), according to the methodology proposed by Oliveira, Faria, Sá, Barros, and Araújo (2006). A gas chromatograph Varian 3800 with a 1079 injector and an ion-trap mass spectrometer Varian Saturn 2000 was used. A 1 μL injection was made in splitless mode (30 s) in a Varian Factor Four VF-Wax ms column (30 m × 0.15 mm; 0.15 μm film thickness). The carrier gas was helium UltraPlus 5× (Praxair) at a constant flow rate of 1.3 mL/min. The detector was set to electronic impact mode with an ionization energy of 70 eV, a mass acquisition range from 35 m/z to

260 *m*/*z* and an acquisition interval of 610 ms. The oven temperature was initially set to 60 °C for 2 min and then raised from 60 °C to 234 °C at a rate of 3 °C/min, raised from 234 °C to 250 °C at 10 °C/min, and finally maintained at 250 °C for 10 min. The temperature of the injector was maintained at 250 °C during the analysis time and the split flow was maintained at 30 mL/min. The identification of compounds was performed using the software MS Workstation version 6.9 (Varian) by comparing their mass spectra and retention indices with those of pure standard compounds. The minor compounds were quantified in terms of 4-nonanol equivalents only.

2.8. Color analysis

The color of the wines was assayed by the CIELab method as described by Genisheva et al. (2012), by measuring the absorbance between 380 nm and 770 nm (data pitch = 2 nm), using a Jasco UV/Vis V-560 spectrophotometer. The recorded data were processed by an algorithm using the program Matlab version r2010a, developed by the Science of Vision and Colour Laboratory, Centre of Physics, University of Minho, to obtain the CIELab coordinates, L^*, $a^*$and b^*. These coordinates allowed the determination of other three parameters in the produced wines: saturation (C^*), variation in saturation (ΔC^*) and variation in lightness (ΔL^*), according to Almela, Javaloy, Fernández-López, and López-Roca (1995). The following equations were used:

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (1)$$

$$\Delta C^* = C^*_x - \overline{C}^* \quad (2)$$

$$\Delta L^* = L^*_x - \overline{L}^* \quad (3)$$

C^*_x and L^*_x are the saturation and lightness of the wines produced by immobilized cells, and $\overline{C}^*$ and $\overline{L}^*$ are the saturation and lightness, respectively of the reference wines, *i.e.* wines produced with free cells.

2.9. Sensory analysis

Ten tasting panelists (four male and six female), with ages between 40 and 50 years old and all of them having a long experience in sensory analysis, carried out the descriptive sensory analysis of wines in two distinct sessions. In the first session, and to establish the descriptors of wines, the evaluation was performed using QDA methodology (Lawless & Heymann, 1998). Two training periods of 1 h were carried out, where judges generated descriptive terms to define the wines. In the second session, a constant volume of 30 mL of each wine was evaluated in wine-taster glasses at 12 °C as described by the Norm ISO 3591–1977. During the analysis, the wine tasters scored the intensity of each attribute using a ten-point scale. Relative frequency (*F*), relative intensity (*I*) and geometric mean (*GM*) of the different descriptors were calculated for each wine. *GM* was calculated as the square root of the product between relative intensity and relative frequency, *i.e.* $GM/\% = \sqrt{I \times F} \times 100$. The descriptors were classified for each wine by using the *GM*, according to the Norm ISO 11035–1994 which make possible the elimination of relatively low values. Consequently, only descriptors presenting $GM > 15$ % for at least one wine, were considered.

As complementary study, a triangle test was applied for determining whether a perceptible sensory difference exists between samples FC and B1 and between FC and B7 (Norm ISO 4120–2004). For each analysis, two sets of samples were used: FC-B1-B1 and B1-FC-FC; FC-B7-B7 and B7-FC-FC.

2.10. Statistical analysis

The data were analyzed by using the software XLstat-Pro (Addinsoft, Paris 2009). Significant differences among wines intensity were determined by analysis of variance (ANOVA).

As referred previously, three replicate assays were done for the batch fermentations. Accordingly, HPLC, GC, color and general physicochemical analyses were done in triplicate, *i.e.* one per replicate. For sensory analysis, the three replicates were mixed before testing.

3. Results and discussion

3.1. General characterization of fermentation assays

At the end of the fermentation assays the following measurements were carried out: pH, total acidity, free and immobilized cells concentrations, and percentage of immobilized death cells (Table 1). Multiple comparison analysis by Tukey's test ($p < 0.05$) was performed.

In general, the fermentation time diminished with the number of repeated batch fermentations, being initially 7 d, for FC and B1 assays, and stabilizing in 4 d for the later fermentations, B6 and B7. After storage (batch 8) the fermentation time increased to 5 d, but in batches 9 and 10 it diminished again to 4 d. A continuous decrease in the fermentation time (about one half after the 4th assay) for successive batch fermentations of glucose with *S. cerevisiae* immobilized in gluten pellets, have already been referred by Bekatorou, Koutinas, Kaliafas, and Kanellaki (2001); likewise, an acceleration of the alcoholic fermentation by yeasts immobilized in alginate gel beads has been reported (Diviès & Cachon, 2005). On the other hand, the unusual higher concentrations of SO_2 in the fermenting must seem not to affect the yeasts activity. Similar results were stated by Yajima and Yokotsuka (2001) when using yeasts immobilized in double-layer gel beads of Ca-alginate. These authors showed an indubitable reduction of the time needed to complete the alcoholic fermentation, after a previous adaptation of the immobilized yeast cells. Additionally, as reported in other studies (Kandylis et al., 2010; Tsakiris, Bekatorou, et al., 2004), the immobilized biocatalysts did not show any loss of operational stability after the 10 batch fermentations. These features, associated to global end product quality, are very important when an industrial process is planned. The decrease of the fermentation time may be due to various reasons (Genisheva et al., 2011): adaptation of yeasts to the fermentation media, in the early batch fermentations; higher cell concentration of yeasts, achieved by immobilization; improvement of the fermentation rates and efficiency of bioconversion due to the modification of cell immobilization metabolism.

Titratable acidity, determined as tartaric acid, varied between 6.28 g/L and 6.85 g/L, and pH values of the produced wines were found to be between 2.80 and 3.07. These results are in the normal range for white wines (Ribéreau-Gayon, Dubourdieu, Donéche, & Lonvaud, 2006).

The free cell concentration diminished from batch 1 to batch 7, reaching a value around two times lower than those observed in the free cell assays. In contrast, the concentration of immobilized cells increased from batches 1 to 7, demonstrating stronger cell–cell or cell–support interactions. The total cells concentration also increased with the repeated batch fermentations (B1 to B7), being FC assays those with the lowest values. As reported early (Genisheva et al., 2012), both free and immobilized cells may contribute to the fermentation process. After the storage (4 °C, 30 d) of the support with immobilized cells (B8, B9 and B10 assays) free cells concentrations were much lower compared to batch 7

Table 1
General characterization of fermentation assays: fermentation length time (t) and multiple comparison analysis (Tukey's test; $p < 0.05$), including standard deviation (sd), for total acidity as tartaric acid (TA), pH, concentration of immobilized cells (X_{im}), free cells ($X_{f.cel}$), immobilized death cells (D_{im}) and total produced cells (X_t).

	t	pH	sd	TA	sd	$X_{f.cel}$	sd	X_{im}	sd	D_{im}	sd	X_t	sd
	d			g/L		g/L		mg/g		%		g/L	
FC	7	2.88^{c}	0.01	6.28^{c}	0.04	4.90^{a}	0.11	0.00	0.00	0.00	0.0	4.90^{c}	0.11
B1	7	3.07^{a}	0.01	6.58^{abc}	0.04	3.22^{bc}	0.06	24.20^{d}	6.35	9.3^{ab}	4.5	4.43^{c}	0.32
B2	5	2.83^{de}	0.02	5.50^{d}	0.04	3.78^{ab}	1.53	32.70^{d}	4.16	6.2^{ab}	2.0	5.41^{c}	1.34
B3	6	2.80^{e}	0.01	6.28^{c}	0.04	3.02^{bc}	0.13	43.33^{d}	2.24	5.6^{b}	0.9	5.18^{c}	0.11
B4	4	2.92^{b}	0.01	6.35^{bc}	0.04	3.20^{bc}	0.42	55.37^{d}	5.83	4.5^{b}	1.5	5.97^{bc}	0.56
B5	5	2.95^{b}	0.01	6.28^{c}	0.23	2.72^{bcd}	0.17	50.03^{d}	19.38	5.5^{b}	1.1	5.23^{c}	0.81
B6	4	2.86^{cd}	0.01	6.28^{c}	0.09	2.74^{bcd}	0.22	72.23^{cd}	9.76	8.5^{ab}	1.1	6.35^{ab}	0.68
B7	4	2.87^{c}	0.01	6.35^{bc}	0.24	2.47^{bcde}	0.17	87.47^{bcd}	5.13	4.5^{b}	1.5	6.83^{ab}	0.42
B8	5	2.85^{cd}	0.02	6.65^{ab}	0.04	0.95^{cde}	0.11	160.67^{abc}	8.14	8.5^{ab}	1.4	8.99^{ab}	0.52
B9	4	2.86^{cd}	0.00	6.65^{ab}	0.04	1.52^{de}	0.13	183.60^{a}	41.77	11.8^{a}	1.1	10.70^{a}	2.04
B10	4	2.93^{b}	0.03	6.85^{a}	0.09	1.10^{e}	0.16	178.43^{ab}	87.13	11.5^{a}	1.6	10.02^{ab}	4.38

a, b, c, d – for each parameter (*i.e.* each column) values with the same letters mean no significant difference at 95 % confidence level.

(Table 1). Additionally, the concentration of the immobilized cells increased two times, being the highest amount recorded for batch 9 (183.60 mg/g). The highest total concentrations of cells was also recorded for B9 assay (10.70 g/L), followed by B10 assay (10.02 g/L). Therefore, the previous storage of the immobilized yeasts and the increased amount of sulfites in the assays B9 and B10 seem to promote the immobilization of free cells on the support. Batches 9 and 10 had the highest concentrations of immobilized cells but also had the highest percentage of death immobilized cells (11.8 % and 11.5 %, respectively), probably due to the higher concentration of SO_2 used in these experiments.

3.2. Ethanol, glycerol, sugars and organic acids

The obtained concentrations of glucose, fructose, glycerol, ethanol and organic acids (citric, tartaric, malic, succinic and acetic) are shown in Table 2.

Glucose and fructose were present in low concentrations for all produced wines, thus confirming the completion of alcoholic fermentation; furthermore, wines did not show significant differences ($p < 0.05$) in terms of concentration of these compounds. Glycerol is the most important by-product of the alcoholic fermentation. Normally, in wines, glycerol can be found in concentrations from 5 g/L to 15 g/L (Ribéreau-Gayon et al., 2006). In our study, the highest content of glycerol was recorded for wine from batch 1, which was found to be different ($p < 0.05$) of all the other wines. Ethanol content is one of the main characteristics of the wine and is a key factor for its quality, giving body and viscosity (Ribéreau-Gayon et al., 2006). In this study, ethanol concentrations varied from 11.2 % vol. (B8) to 12.1 % (B3), indicating that the produced wines had a good strength. In regard to citric acid concentration, wine produced with free cells was significantly different ($p < 0.05$) from the wines produced in the batch series using immobilized cells, showing the lowest value. Tartaric acid was the acid with the highest concentration in all the produced wines, which could be explained by the fact this acid is usually found in high concentrations in grapes and do not undergo large changes during fermentation. Succinic acid (produced during the alcoholic fermentation) was present in wine B1 at concentrations significantly different with respect to the other wines. The concentration of acetic acid in the wines was lower than 1 g/L, showing that no bacterial contamination occurred during the grape must fermentation. Moreover, all the recorded values were always below the acceptable limit for white wines of 1.2 g/L (OIV, 2012b).

Even though statistical differences were found for the composition of the produced wines, those produced with free cells usually presented similarity with some of the wines produced with immobilized cells in successive batches; the highest differences were found between wines produced from free cells (FC) and the first batch fermentation (B1). This fact demonstrates that the main characteristics of wines produced with free cells and with immobilized cells are not so different. The fermentation time was the main parameter differentiating them (Table 1).

The previous storage of the support with immobilized cells (wine B8) and the use of more elevated concentrations of SO_2 in the medium (wines B9 and B10) yielded higher concentrations of glycerol in the wine when compared to the wine B7. This is in accordance with Ribéreau-Gayon et al. (2006) who observed an increase of glycerol concentration in wines, as high as 20 g/L, when high concentrations of SO_2 were applied. The storage of the immobilized support may negatively influence the ethanol production, while the use of high SO_2 concentrations seems to have no influence over its formation. Wines B8 had higher amounts of citric, tartaric and succinic acids than B7 wines. However, the concentrations of these organic acids diminished in the subsequent fermentations (batches 9 and 10). The concentration of acetic acid was lower in wines produced in batch 8; in contrast, the highest concentration of free SO_2 in the must of batch 10 (90 mg/L) proportioned wines with the highest concentrations of acetic acid. For B10 wines, acetic acid (1.03 g/L) almost reached the acceptable limit for white wines of 1.2 g/L (OIV, 2012b).

3.3. Major volatile compounds

Table 3 shows the 8 major volatile compounds identified in the produced wines. As a whole, statistical significant differences were found between wines, except for 1-propanol.

Acetaldehyde was found in all the samples in concentrations higher than its orthonasal perception threshold of 10 mg/L, and might give “overripe apple” notes to wines (Chaves, Zea, Moyano, & Medina, 2007; Moreno, Zea, Moyano, & Medina, 2005). The highest concentration of this aldehyde was observed in wines produced with free cells (26.5 mg/L). This fact is in agreement with the results published by Tsakiris, Bekatorou, et al. (2004) who also observed higher amounts of acetaldehyde in wines produced with free cells; nevertheless, the obtained values were lower than those detected by Kourkoutas, Douma, et al. (2002) in wines produced with cells immobilized on quince (106 mg/L).

Ethyl acetate was found in all produced wines in concentrations above its perception threshold of 12.3 mg/L (Escudero et al., 2004), contributing to the “pineapple” and “nail polish” character of wines (Chaves et al., 2007). The highest concentration was recorded for wine B7 (45.6 mg/L), which was significantly different from the others.

Table 2
Mean concentrations (*C*) and confidence limits ($p = 0.05$) for sugars, organic acids, ethanol and glycerol at the end of the alcoholic fermentation.

Compound	FC		B1		B2		B3		B4		B5		B6		B7		B8		B9		B10	
	C/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±
Glucose	2.6^{a}	0.1	3.4^{a}	0.2	2.5^{a}	0.7	2.8^{a}	0.3	3.7^{a}	1.22	2.3^{a}	0.4	3.0^{a}	0.3	3.1^{a}_{a}	1.0	2.2_{b}	0.2	2.3_{b}	0.2	2.4_{b}	0.2
Fructose	7.6^{ab}	1.1	3.9^{c}	0.2	8.2^{ab}	3.5	5.3^{bc}	2.1	10.0^{a}	3.94	3.2^{c}	1.1	7.9^{ab}	2.2	7.7^{ab}_{a}	5.6	1.4_{b}	0.2	1.6_{b}	0.2	3.3_{b}	0.3
Glycerol	7.1^{b}	0.2	9.6^{a}	0.6	7.1^{b}	0.5	6.8^{b}	0.4	6.6^{b}	1.47	7.0^{b}	0.3	7.0^{b}	0.2	6.9^{b}_{b}	0.6	7.4_{a}	0.3	7.2_{ab}	0.5	7.4_{a}	0.2
Ethanol	90.2^{ab}	2.9	94.6^{ab}	3.9	89.1^{b}	5.9	95.2^{a}	5.8	90.6^{ab}	8.61	92.4^{ab}	1.6	93.9^{ab}	4.7	92.4^{ab}_{b}	1.8	88.7_{c}	2.5	94.2_{a}	0.7	91.6_{b}	1.6
Citric acid	0.3^{b}	0.0	0.6^{a}	0.1	0.6^{a}	0.0	0.6^{a}	0.0	0.6^{a}	0.10	0.6^{a}	0.0	0.6^{a}	0.0	0.6^{a}_{b}	0.0	0.7_{a}	0.1	0.5_{c}	0.0	0.6_{b}	0.0
Tartaric acid	4.4^{bc}	0.1	5.4^{a}	0.2	4.2^{bc}	0.7	3.8^{cd}	0.2	4.0^{bcd}	0.30	4.6^{b}	0.4	4.5^{b}	1.1	3.6^{d}_{c}	0.7	5.3_{a}	0.5	4.4_{b}	0.9	5.1_{ab}	0.9
Malic acid	2.0^{d}	0.1	3.2^{a}	0.1	2.3^{bc}	0.1	2.3^{bc}	0.2	2.1^{cd}	0.19	2.3^{b}	0.1	2.3^{bc}	0.2	2.3^{bc}_{a}	0.0	2.4_{a}	0.6	2.0_{b}	0.1	2.3_{a}	0.1
Succinic acid	1.4^{bc}	0.1	2.5^{a}	0.3	1.39^{bc}	0.1	2.3^{ab}	1.1	2.3^{abc}	2.16	1.3^{c}	0.0	1.3^{c}	0.1	1.3^{c}_{b}	0.0	1.7_{a}	0.2	1.7_{a}	0.6	1.4_{ab}	0.1
Acetic acid	0.5^{c}	0.0	0.3^{d}	0.0	0.2^{e}	0.0	0.2^{e}	0.0	0.2^{e}	0.09	0.5^{bc}	0.1	0.5^{b}	0.1	0.7^{a}_{b}	0.1	0.2_{c}	0.1	0.2_{c}	0.0	1.0_{a}	0.2

a, b, c, d – for each compound (*i.e.* each row) values with the same letters mean no significant difference at 95 % confidence level; superscript letters compare essays FC to B7; subscript letters compare essays B7 to B10.

Immobilized cell fermentations from batches 1 to 7 presented slightly higher levels of methanol (57.7 mg/L to 189.0 mg/L) than those observed for white wines produced with cells immobilized on grape pomace (Genisheva et al., 2012) and on quince (Kourkoutas, Douma, et al., 2002). Nevertheless, all the assays contained methanol in concentrations below its limit permitted for consumption of 250 mg/L (OIV, 2012b). Methanol results from the pectins of the skin of the grapes that undergoes an enzymatic conversion (Ribéreau-Gayon et al., 2006). Since the fermenting must was in contact with the grape skins for long and repeated time periods, high amounts of methanol could be found in the product. However, no differences were found between the wines produced with immobilized cells and with free cells.

Respecting to higher alcohols, all the produced wines present similar levels of these compounds, around 300 mg/L, although FC and B10 assays seemed to have lower concentrations. Although higher alcohols, individually, do not give pleasant notes to the wine (except 2-phenylethanol), together they can positively contribute to the overall aroma (Rapp & Versini, 1995).

Individually, 2-methyl-1-propanol, 3-methyl-1-butanol and 2-phenylethanol were present in the wines in concentrations above their perception thresholds; 2-methyl-1-propanol and 3-methyl-1-butanol may contribute to the "spirituous", "fusel" and "nail polish" odor notes of wines (Siebert et al., 2005), mainly for assays B3 to B7. Moreover, the presence of 2-phenylethanol in the samples (19.8 mg/L to 52.9 mg/L), above its perception threshold, may give "rose" and "sweetish" nuances to the wines (Siebert et al., 2005).

The storage of the support with immobilized cells and the application of higher doses of SO_2 did not influence the production of major volatile compounds, with the exception of acetaldehyde and methanol. Acetaldehyde concentration diminished after the previous storage of the immobilized support, as well as with the higher concentrations of SO_2 added.

3.4. Minor volatile compounds

Table 3 shows a total of 24 minor volatile compounds that were identified in the wines, which belong to different chemical groups including ethyl esters, acetates, terpenols, C_{13}-norisoprenoids, volatile phenols and volatile fatty acids.

Ethyl butyrate, ethyl hexanoate and ethyl octanoate were found in concentrations markedly above their perception thresholds in all the produced wines. Similar fact occurred for ethyl decanoate, but only for some samples. Under these conditions, these four compounds may bring "fruity" (apple, papaya) and "sweetish" notes to the wines (Escudero et al., 2004; Meilgaard, 1975). Also isoamyl acetate and 2-phenylacetate may bring "banana" and "roses/flowery" notes to the overall aroma of the wines (Escudero et al., 2004; Genisheva et al., 2012; Meilgaard, 1975). Wines produced with free cells had the lowest total concentration of acetates when compared to wines produced with immobilized cells. Moreover, the total concentration of acetates seemed to increase from batches 1 to 6.

Terpenols were found in similar concentrations in all the wines. This could be explained by the fact that all assays were carried out with the same grape must, and terpenols are part of the varietal aroma of grapes (Genisheva & Oliveira, 2009; Oliveira, Oliveira, Baumes, & Maia, 2008). In all produced wines, linalool was in concentrations above its perception threshold, thus bringing "flower" and "lavender" notes to the wines (Chaves et al., 2007). Also Geraniol, only in free cell assays, may contribute with "flower" notes (Ugliano & Moio, 2008).

The levels of C_{13}-norisoprenoids are comparable, except for B9 assay. Similarly, β-damascenone did not show significant

Table 3
Mean concentrations (C), confidence limits ($p = 0.05$) and aroma perception threshold (PT) of the major and minor volatile compounds at the end of alcoholic fermentation.

	Major volatile compounds																													
	FC			B1			B2			B3			B4			B5			B6			B7			B8			B9			B10			PT
	C/(mg/L)	±		C/(mg/L)	±		C/(mg/L)	±		C/(mg/L)	±		C/(mg/L)	±		C/(mg/L)	±		C/(mg/L)	±		C/(mg/L)	±		C/(mg/L)	±		C/(mg/L)	±		mg/L
Acetaldehyde	26.5^{a}	20.5		15.0^{bc}	8.2		23.7^{ab}	4.5		15.3^{bc}	9.8		12.5^{bc}	5.2		10.8^{c}	3.4		25.2^{ab}	3.4		20.1$^{abc}_{a}$	5.0		11.8$_{b}$	1.7		9.9$_{b}$	5.9		12.4$_{b}$	2.7		10^{A}
Ethyl acetate	31.8^{bc}	13.2		35.6^{abc}	8.2		30.7^{c}	9.8		35.9^{abc}	6.2		36.1^{abc}	2.8		43.5^{ab}	18.8		41.1^{abc}	10.4		45.6$^{a}_{a}$	7.4		40.4$_{a}$	14.6		29.7$_{a}$	21.7		36.8$_{a}$	22.4		12.3^{B}
Methanol	96.9^{ab}	55.2		131.4^{ab}	42.7		145.5^{ab}	111.2		137.6^{ab}	76.7		189.0^{a}	150.3		145.7^{ab}	137.8		159.6^{ab}	154.3		57.7$^{b}_{c}$	26.7		75.1$_{b}$	12.9		77.3$_{b}$	6.7		162.2$_{a}$	4.9		668^{A}
Higher alcohols																																		
1-propanol	26.5^{a}	9.5		32.7^{a}	13.6		31.7^{a}	10.8		34.7^{a}	15.7		35.2^{a}	19.5		38.2^{a}	19.1		36.8^{a}	16.0		35.8$^{a}_{a}$	30.7		25.7$_{a}$	11.5		22.4$_{a}$	9.7		26.3$_{a}$	16.3		830^{A}
2-methyl-1-propanol	33.7^{c}	3.9		34.6^{c}	10.7		33.4^{c}	8.9		45.9^{bc}	11.8		53.1^{abc}	15.6		70.7^{a}	8.2		54.2^{abc}	15.2		60.0$^{ab}_{a}$	44.3		71.2$_{a}$	23.3		61.8$_{a}$	26.3		61.9$_{a}$	32.8		40^{A}
2-methyl-1-butanol	35.8^{ab}	1.5		45.3^{a}	9.2		37.0^{ab}	10.4		33.0^{ab}	4.3		29.5^{b}	10.3		31.3^{b}	17.3		26.6^{b}	9.2		25.8$^{b}_{a}$	21.6		39.5$_{a}$	15.0		29.9$_{a}$	14.9		26.2$_{a}$	12.8		
3-methyl-1-butanol	147.6^{a}	12.8		159.5^{a}	28.1		148.9^{a}	41.0		155.4^{a}	39.0		153.0^{a}	49.5		182.0^{a}	58.6		148.2^{a}	58.3		149.2$^{a}_{a}$	109.5		194.7$_{a}$	75.1		159.2$_{a}$	81.7		149.2$_{a}$	76.7		30^{A}
2-phenylethanol	38.6^{ab}	27.0		42.3^{ab}	36.7		52.9^{a}	12.4		31.0^{ab}	24.3		26.6^{b}	19.6		19.8^{b}	8.7		22.1^{b}	14.2		30.0$^{ab}_{a}$	16.8		38.6$_{a}$	27.3		27.0$_{a}$	16.3		23.5$_{a}$	7.1		14^{C}
Total	**282.2**	31.6		**314.4**	50.2		**303.9**	46.2		**300.0**	50.2		**297.4**	59.7		**342.0**	65.1		**287.9**	64.6		**300.8**	125.1		**369.7**	85.4		**300.3**	89.2		**287.1**	86.2		

	Minor volatile compounds																													
	FC			B1			B2			B3			B4			B5			B6			B7			B8			B9			B10			PT
	C/(μg/L)	±		C/(μg/L)	±		C/(μg/L)	±		C/(μg/L)	±		C/(μg/L)	±		C/(μg/L)	±		C/(μg/L)	±		C/(μg/L)	±		C/(μg/L)	±		C/(μg/L)	±		C/(μg/L)	±		μg/L
Ethyl esters																																		
Ethyl butyrate	95.0^{ab}	16.7		89.6^{b}	17.5		139.8^{ab}	51.3		187.2^{a}	197.5		102.9^{ab}	26.8		100.5^{ab}	40.9		120.3^{ab}	7.9		120.2$^{ab}_{a}$	91.0		141.5$_{a}$	19.7		68.4$_{b}$	6.8		121.2$_{a}$	10.7		20^{D}
Ethyl hexanoate	358.0^{a}	112.5		366.0^{a}	37.1		415.5^{a}	86.7		475.5^{a}	132.2		402.9^{a}	43.2		379.2^{a}	209.9		482.6^{a}	14.2		433.4$^{a}_{ab}$	191.7		344.6$_{bc}$	28.3		254.9$_{c}$	34.1		488.9$_{a}$	47.7		14^{C}
Ethyl octanoate	350.4^{a}	39.8		311.4^{a}	28.2		365.5^{a}	120.8		449.0^{a}	98.3		426.3^{a}	56.2		380.3^{a}	182.0		431.3^{a}	132.0		357.1$^{a}_{a}$	205.8		252.6$_{ab}$	13.8		196.0$_{b}$	29.7		349.3$_{a}$	50.9		5^{C}
Ethyl decanoate	270.7^{ab}	118.9		169.5^{b}	65.9		206.8^{ab}	117.8		244.4^{ab}	59.8		282.5^{a}	44.2		244.2^{ab}	89.2		251.1^{ab}	29.0		182.7$^{ab}_{a}$	135.2		122.0$_{ab}$	16.7		78.0$_{b}$	28.6		124.3$_{ab}$	42.9		200^{C}
Total	**1074.1**	169.3		**936.5**	82.6		**1127.6**	196.5		**1356.1**	264.0		**1214.6**	87.7		**1104.2**	294.6		**1285.3**	136.1		**1093.4**	325.1		**860.7**	40.7		**597.3**	53.9		**1083.7**	82.6		
Acetates																																		
Isoamyl acetate	517.9^{c}	174.9		698.8^{bc}	79.1		744.8^{bc}	113.5		889.6^{abc}	339.1		887.6^{abc}	181.9		972.4^{ab}	452.9		1209.5^{a}	40.2		1092.1$^{a}_{ab}$	586.0		871.2$_{bc}$	12.6		630.3$_{c}$	118.6		1398.7$_{a}$	337.0		30^{A}
Hexyl acetate	27.2^{c}	8.6		28.6^{c}	8.5		45.3^{abc}	11.6		48.9^{ab}	11.1		61.6^{a}	27.2		48.2^{ab}	30.8		61.3^{a}	8.5		40.4$^{bc}_{ab}$	12.4		42.7$_{a}$	9.0		25.9$_{b}$	11.8		40.7$_{ab}$	16.5		1000^{E}
2-phenylethyl acetate	497.4^{a}	214.4		578.4^{a}	20.7		711.3^{a}	327.3		599.0^{a}	105.7		695.8^{a}	318.5		579.0^{a}	233.2		638.2^{a}	133.8		532.2$^{a}_{ab}$	271.9		508.8$_{b}$	103.0		479.1$_{b}$	72.6		708.0$_{a}$	154.7		250^{A}
Total	**1042.5**	276.8		**1305.8**	82.2		**1501.4**	346.6		**1537.5**	355.4		**1645.0**	367.8		**1599.6**	510.3		**1909.0**	140.0		**1664.7**	646.1		**1422.7**	104.2		**1135.3**	139.6		**2147.4**	371.2		
Terpenols																																		
Linalool	104.6^{a}	13.3		74.4^{c}	11.0		91.1^{abc}	2.9		93.2^{abc}	3.2		96.5^{ab}	4.8		81.3^{bc}	36.3		90.3^{abc}	8.5		79.7$^{bc}_{ab}$	34.8		86.8$_{ab}$	2.9		69.1$_{b}$	5.1		94.1$_{a}$	5.1		25.2^{C}
HO-trienol	24.4^{a}	8.6		23.4^{a}	3.0		20.9^{a}	5.3		18.1^{a}	0.4		18.2^{a}	1.1		16.4^{a}	6.8		17.9^{a}	1.8		16.6$^{a}_{ab}$	8.6		15.1$_{ab}$	2.6		13.9$_{b}$	3.5		19.9$_{a}$	0.5		110^{F}
α-terpineol	40.0^{a}	12.7		28.0^{b}	3.7		39.2^{a}	5.6		38.4^{ab}	1.5		40.8^{a}	5.0		33.9^{ab}	14.2		36.9^{ab}	3.5		34.0$^{ab}_{b}$	14.8		38.5$_{ab}$	3.9		30.7$_{b}$	2.9		42.6$_{a}$	3.9		250^{C}
Citronellol	12.6^{a}	6.2		6.9^{b}	0.5		8.5^{b}	0.8		7.1^{b}	1.8		8.2^{b}	0.7		6.7^{b}	1.5		5.9^{b}	1.6		5.7$^{b}_{c}$	2.8		16.1$_{a}$	1.9		11.3$_{b}$	0.3		11.6$_{b}$	1.4		100^{A}
Nerol	4.4^{a}	2.1		3.9^{a}	0.8		4.3^{a}	2.1		3.7^{a}	0.7		4.0^{a}	0.4		3.2^{a}	1.1		3.4^{a}	0.3		3.1$^{a}_{c}$	1.2		5.1$_{a}$	0.9		3.1$_{bc}$	0.1		4.0$_{b}$	0.7		400^{G}
Geraniol	37.1^{a}	11.1		27.3^{ab}	10.2		33.6^{a}	8.0		34.5^{a}	18.3		18.3^{b}	2.5		16.1^{b}	7.0		32.2^{a}	5.8		30.3$^{a}_{b}$	10.2		29.7$_{b}$	5.0		24.5$_{b}$	0.8		38.6$_{a}$	10.3		36^{B}
Total	**223.1**	24.0		**163.9**	15.8		**197.6**	11.7		**195.0**	18.7		**186.0**	7.5		**157.6**	40.2		**186.6**	11.1		**169.4**	40.2		**191.3**	7.7		**152.6**	6.9		**210.8**	12.2		
C_{13}-norisoprenoids																																		
β-damascenone	2.9^{a}	1.1		2.2^{a}	1.0		2.3^{a}	1.1		2.7^{a}	1.4		3.2^{a}	1.9		2.1^{a}	1.7		2.6^{a}	1.5		1.9$^{a}_{b}$	0.6		2.6$_{a}$	0.6		1.4$_{b}$	0.2		1.8$_{b}$	0.6		0.05^{D}
3-hydroxy-β-damascone	5.9^{ab}	0.9		7.1^{a}	2.6		7.7^{a}	2.6		6.0^{ab}	0.7		6.8^{a}	0.8		4.7^{bc}	0.9		4.9^{bc}	1.0		3.5$^{c}_{b}$	2.2		3.6$_{b}$	1.2		2.6$_{b}$	0.9		5.2$_{a}$	0.6		
3-oxo-α-ionol	5.9^{ab}	4.7		8.1^{a}	1.9		7.0^{ab}	1.1		4.7^{bc}	0.4		5.5^{abc}	2.3		3.1^{c}	2.4		6.2^{ab}	2.1		4.8$^{bc}_{b}$	1.8		5.2$_{a}$	1.1		1.4$_{bc}$	0.2		4.5$_{b}$	0.4		
Total	**14.7**	4.9		**17.4**	3.4		**17.0**	3.0		**13.4**	1.6		**15.5**	3.1		**9.9**	3.1		**13.7**	2.8		**10.2**	6.4		**11.4**	1.7		**5.4**	0.9		**11.5**	0.9		
Volatile phenols																																		
4-vinylguaiacol	404.7^{a}	163.6		72.2^{b}	5.8		128.0^{b}	12.2		133.6^{b}	11.3		142.2^{b}	17.7		131.2^{b}	42.9		141.1^{b}	26.1		122.6$^{b}_{a}$	49.4		75.5$_{b}$	8.1		69.0$_{b}$	11.3		102.6$_{a}$	4.7		130^{H}
4-vinylphenol	353.6^{a}	141.2		79.2^{c}	5.3		136.8^{bc}	21.0		133.7^{bc}	13.1		153.6^{b}	49.1		136.3^{bc}	51.2		146.7^{bc}	37.2		130.5$^{bc}_{a}$	57.7		75.4$_{b}$	9.4		72.0$_{b}$	14.3		109.4$_{a}$	6.4		180^{H}
Total	**758.3**	216.1		**151.4**	7.9		**264.8**	24.3		**267.3**	17.3		**295.8**	52.2		**267.5**	66.8		**287.8**	45.4		**253.1**	76.0		**150.9**	12.4		**141.0**	18.2		**212.0**	7.9		
Volatile fatty acids																																		
Butanoic acid	92.2^{a}	38.2		14.7^{b}	10.7		9.2^{b}	0.6		8.1^{b}	4.0		9.7^{b}	3.2		8.1^{b}	1.9		10.4^{b}	5.2		8.7$^{b}_{b}$	4.6		21.8$_{a}$	2.1		9.6$_{b}$	1.9		8.9$_{b}$	0.2		173^{C}
Hexanoic acid	308.5^{a}	111.8		348.0^{a}	69.7		398.1^{a}	74.5		353.3^{a}	65.3		412.4^{a}	130.3		316.6^{a}	118.9		394.2^{a}	106.4		331.9$^{a}_{a}$	159.8		266.1$_{ab}$	10.3		177.8$_{b}$	36.6		348.9$_{a}$	72.1		420^{C}
Octanoic acid	1367.9^{b}	126.6		927.2^{c}	267.5		1519.1^{ab}	222.1		1521.6^{ab}	229.0		1785.4^{a}	81.2		1394.2^{ab}	512.0		1748.4^{ab}	353.2		1508.8$^{ab}_{a}$	624.7		751.8$_{b}$	143.1		623.3$_{b}$	88.1		1336.9$_{a}$	265.9		500^{C}
Decanoic acid	1267.4^{a}	484.2		167.4^{c}	60.9		355.0^{bc}	153.4		474.5^{bc}	83.8		667.0^{b}	179.5		494.6^{bc}	164.7		682.7^{b}	115.6		616.1$^{b}_{a}$	262.3		92.4$_{c}$	25.7		115.3$_{c}$	17.5		282.8$_{b}$	120.4		1000^{C}
2+3-methyl-butanoic acids	91.8^{b}	28.5		161.0^{a}	16.9		95.9^{b}	41.6		59.6^{cd}	10.1		70.9^{bc}	40.9		48.4^{cd}	14.8		47.8^{cd}	12.0		40.3$^{d}_{c}$	18.5		127.1$_{a}$	17.6		60.6$_{b}$	12.4		55.6$_{bc}$	11.5		33.4^{C}
Total	**3127.8**	515.0		**1618.3**	283.8		**2377.3**	283.1		**2417.1**	252.7		**2945.4**	239.7		**2261.9**	551.0		**2883.5**	386.8		**2505.8**	696.4		**1259.2**	146.8		**986.6**	97.8		**2033.1**	300.9		

a, b, c, d – for each compound (*i.e.* each row) values with the same letters mean no significant difference at 95 % confidence level between fermentation assays; superscript letters compare assays FC to B7; subscript letters compare assays B7 to B10.
A – Moreno et al., 2005; B – Escudero et al., 2004; C – Ferreira, López, & Cacho, 2000; D – Guth, 1997; E – Chaves et al., 2007; F – Simpson, 1979; G – Ribéreau-Gayon et al., 2006; H – Boidron et al., 1988.

differences, but it was always above its perception, thus bringing "sweet" and "apple" notes to the wines (Escudero et al., 2004).

Wines produced with immobilized cells (B1 to B7) did not show statistically significant differences ($p < 0.05$) regarding the concentration of volatile phenols. Nevertheless, the assay with free cells showed to be different, recording the highest concentration for 4-vinylguaiacol, 404.7 μg/L. Furthermore, this phenol was found in concentrations above its perception threshold in several assays using immobilized cells, bringing "spice" and "wood" characteristic to the wines (Ugliano & Moio, 2008). Free cell fermentations also showed the highest value of 4-vinylphenol, and this compound was found in concentrations above its perception threshold of 180 μg/L (Boidron, Chatonnet, & Pons, 1988). Assays with free cells had two to three times higher total concentrations of volatile phenols than assays with immobilized cells. The volatile phenols 4-vinylguaiacol and 4-vinylphenol are produced during fermentation by the ability of *S. cerevisiae* to decarboxylate hydroxycinnamic acids (Chatonnet, Dubourdieu, Boidron, & Lavigne, 1993). The lower concentrations of volatile phenols in the assays with immobilized cells might be explained by the hypothetically modifications of the cell metabolism.

Wines produced from free cells recorded the highest total concentrations of volatile fatty acids, particularly decanoic acid. Octanoic acid and 2+3-methylbutanoic acids seemed to have decisive influence on the aroma of all wines, bringing "cheese" and "rancid" notes (Escudero et al., 2004; Genovese, Gambuti, Piombino, & Moio, 2007). Moreover, hexanoic acid may influence the aroma of the produced wines since the determined concentrations are near the perception threshold.

The storage of the support with immobilized cells seemed to have negatively influenced the production of ethyl esters, acetates, fatty acids and volatile phenols. In contrast, terpenic compounds and C_{13}-norisoprenoids had higher concentrations in wines produced with previously stored immobilized supports (B8). The high concentrations of SO_2 present in the grape must (60 mg/L or 90 mg/L) did not have a strong influence over the minor volatile compounds, except for esters and acetates. For the majority of minor volatile compounds, the recorded concentrations for B9 wines were lower than those determined in B10 wines. These results demonstrate the adaptation of immobilized cells to the more elevated concentrations of SO_2 present in the grape must.

3.5. Color analysis

Color analysis of the wines (not performed for B8, B9 and B10 assays) was carried out using the CIELab method, with the determination of the coordinates L^*, a^* and b^*. In order to compare the wines, variation in lightness, ΔL^*, and variation in saturation, ΔC^*, were also determined (Table 4). The results obtained for the coordinates L^*, a^* and b^*, as well as for saturation C^*, showed significant differences between wines in terms of the color parameters. Wines produced with free cells had higher values of brightness L^* and lower values of saturation C^*, revealing lower color intensity. Moreover, the parameter a^* had higher values, while parameter b^* had lower values, which indicate a yellowy-greenish color. According to the color parameters, wines produced in batches 6 and 7 were more similar to wines produced with free cells, than to the others. The wines from batch 1 had the highest color intensity (lower values of L^*) as well as increased color saturation (highest values of C^*), compared to the other produced wines.

Table 4
CIELab coordinates (L^*, a^* and b^*) and saturation of color (C^*), including confidence limits ($p = 0.05$).

	L^*	±	a^*	±	b^*	±	C^*	±
FC	94.0^{a}	0.4	-0.7^{a}	0.0	9.2^{a}	0.3	9.2^{d}	0.3
B1	88.0^{e}	1.5	-2.3^{d}	0.2	33.6^{a}	5.3	33.7^{a}	5.3
B2	89.1^{e}	0.6	-1.0^{bc}	0.2	15.4^{b}	0.7	15.4^{b}	0.7
B3	92.6^{bcd}	0.6	-0.9^{abc}	0.1	11.7^{c}	0.7	11.7^{c}	0.7
B4	92.1^{cd}	1.6	-0.9^{abc}	0.2	11.8^{c}	0.5	11.9^{c}	0.5
B5	91.6^{d}	1.7	-0.8^{ab}	0.3	11.5^{cd}	1.0	11.5^{cd}	1.0
B6	93.5^{ab}	0.9	-1.0^{abc}	0.2	10.3^{cd}	1.5	10.4^{cd}	1.5
B7	93.4^{abc}	1.1	-1.2^{c}	0.6	10.1^{cd}	1.1	10.1^{cd}	1.0

a, b, c, d – for each parameter (*i.e.* each column) values with the same letters mean no significant difference at 95 % confidence level.

Figure 1 shows the differences in color of the produced wines, using a graphical representation of the variation in lightness, ΔL^*, as function of variation in saturation, ΔC^*, which reduces the CIELab coordinates into a two-dimensional color space (Almela et al., 1995). Thus, the deviations in the color of the wines produced by the immobilized cells, compared to those produced with free cells, could be observed. It was found that the wines produced in the first batch of fermentation with immobilized cells (B1) had darker color than those produced in the second fermentation (B2), due to the lower values of ΔL^*. In general the values of ΔL^* increased from batches 1 to 7, showing that wines became brighter in that direction. As the number of successive batch fermentations increased the colored compounds released from the grape skins diminished and the color of the wines tended to stabilize becoming more similar of those produced with free cells. This fact was previously reported by Genisheva et al. (2012).

3.6. Sensory analysis

An experienced panel performed the sensory characterization of the wines produced in this study (FC and B1 to B7). The panel generated a total of 29 descriptors from wines: 20 for aroma and 9 for taste; additionally, a global value was attributed to each sample. Then, the geometric mean (*GM*) was determined in order to reduce the number of descriptors. Accordingly, Figure 2 shows the selected descriptors (10 for aroma and 8 for taste), *i.e.* those with $GM > 15$ %. The used QDA methodology permitted to take into account descriptors that were rarely mentioned but which are very important in terms of the perceived intensity, and descriptors with a low perceived intensity but which are mentioned often (Dravnieks & Bock, 1978). Respecting aroma, ANOVA showed significant differences for the following descriptors: intensity, toast bread, apple and

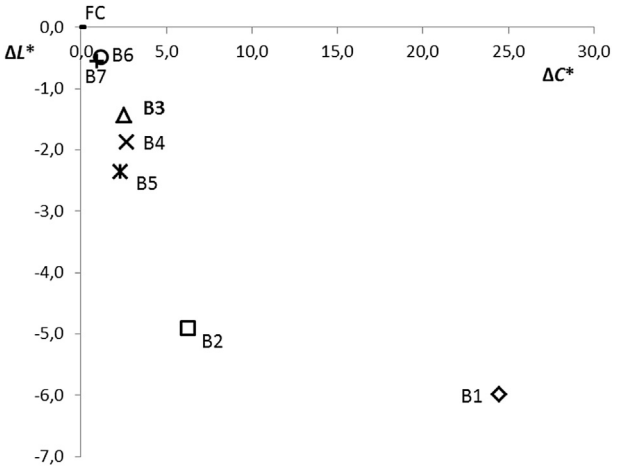

Fig. 1. Variation of saturation, ΔC^*, and variation of lightness, ΔL^*, of wines produced using immobilized yeasts (batch series B1 to B7) and with free cells (FC).

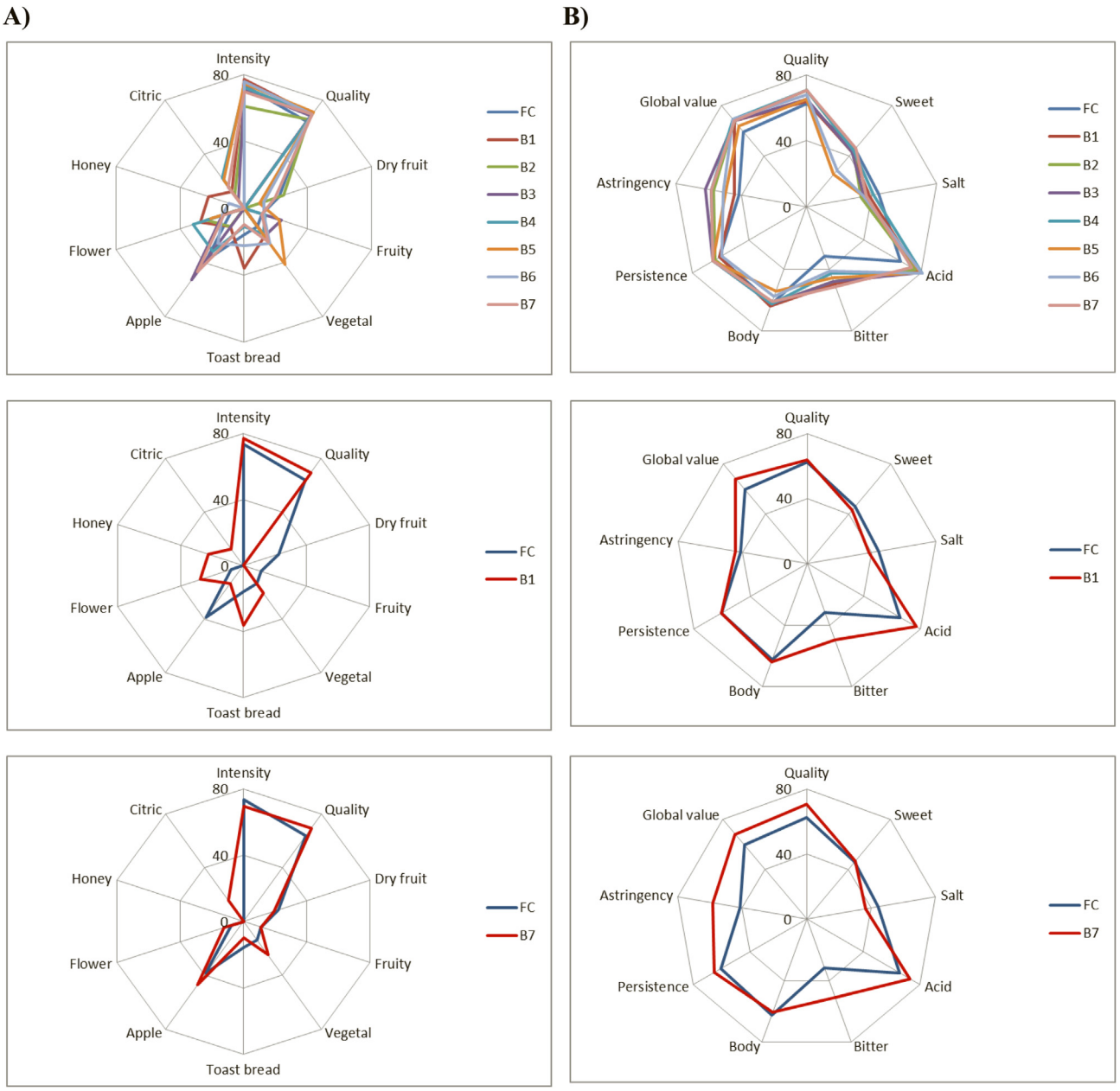

Fig. 2. Sensory profiles of wines obtained with immobilized and free cells, represented by the geometric mean of the selected descriptors ($GM > 15$ %). Aroma profile (**A**) and taste profile and global value (**B**).

honey. Taste descriptors and the global value were not affected by the type of wine elaboration.

Sensory profiles of wines (only for selected attributes), representing the geometric mean for aroma and taste as well as global value, are shown in Figure 2. Firstly, a comparison was made involving all wines: those produced with free cells (FC) and those produced in consecutive batch fermentations with immobilized cells (B1 to B7). Then, in order to check the evolution of the quality of wines produced in consecutive batch fermentations, a comparison was carried out involving only the two extremes, *i.e.* FC *vs.* B1 and FC *vs.* B7. In general, the profiles of different wines, respecting taste and global value, did not represent obvious differences. However, respecting the aroma profiles, some differences could be perceived, particularly when comparing FC *vs.* B1.

The sensory dissimilarities of wines produced from free and immobilized cells (FC *vs.* B1 and FC *vs.* B7) were also checked by a triangle test. Considering the number of assessors (22), the minimum number of correct responses required to consider a perceptible difference between the samples ($\alpha = 0.05$) was 12. In our study, for wine aroma evaluation, only 9 assessors (FC *vs.* B1) or 6 (FC *vs.* B7) correctly identified the samples, representing 40.0 % and 27.3 % respectively. In the same way, for taste analysis, only 7 (FC *vs.* B1) and 4 (FC *vs.* B7) responses were correct, representing 31.8 % and 18.2 %, respectively. Although no perceptible differences could be statistically attributed to wines, the panelists were able to better differentiate the pair FC–B1 than the pair FC–B7, indicating greater dissimilarities between wines produced in the first batch (B1) than those produced in the last batch of the series (B7), when compared

to conventional FC wines. On the other hand, the taste analysis was more inconclusive. These results are in agreement with sensory profile of wines performed with the expert panel.

4. Conclusion

Grape skins were found to be an appropriate long-term use support for *S. cerevisiae* immobilization to carry out the alcoholic fermentation in a winemaking process. The immobilized yeasts could be stored at least one month, at 4 °C, without losing its biological activity and operational stability. Furthermore, yeasts were not inhibited by the presence of SO_2 in amounts three times higher than the usual concentration.

After an adaptation period, *i.e.* after three successive batches, immobilized cells on grape skins were able to carry out the complete alcoholic fermentation in 4 d against the 7 d needed for the traditional free cells system. Moreover, the overall quality of the produced wines with both systems became identical.

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