Food Chemistry 250 (2018) 22–29

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Influence of two yeast strains in free, bioimmobilized or immobilized with alginate forms on the aromatic profile of long aged sparkling wines

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ARTICLE INFO

Keywords:
Aroma compounds
Bioimmobilization
Cava
Chemometrics
Immobilization
Sparkling wine

ABSTRACT

Production of sparkling wines involve a second alcoholic fermentation and contact with yeast less over an extended period of time, which influences the aroma composition and sensory quality of the resulting wines. Sparkling wines obtained with two yeast strains inoculated as free cells, immobilized in alginate bed and bioimmobilized as biocapsules, were aged during 32 months. Among the volatile compounds, high Odor Activity Values were obtained with isoamyl acetate, ethyl propanoate, ethyl butanoate, ethyl 3-methylbutanoate, ethyl hexanoate, ethyl octanoate, hexanol, 2-methoxy-4-vinylphenol, decanal, octanoic acid, decanoic acid and TDN. Taken together these contribute more than 70% of the overall aromatic series value. Although some results rely more on the yeast strain than the inoculation format, specific aroma compounds were associated with the immobilization format, allowing the classification of sparkling wines by PCA. As a result the aroma quality of sparkling wines could be improved using immobilized yeasts.

1. Introduction

Spanish sparkling wine (Cava) (Certified Brand of Origin) elaborated following the traditional method (analogous to the Champenoise method used to produce Champagne in France) undergo a second fermentation in closed bottles of base wine, followed by period of aging during at least 9 months in the presence of yeast lees (EC Regulation N° 479/2008). The second fermentation is carried out by adding to the base wine "liquer de tirage" composed mainly by sucrose, selected yeasts and grape must or wine to produce the desired CO_2 pressure. Moreover, small amounts of bentonite and sulfur dioxide are usually added in order to simplify the lees removal and also to prevent oxidative effects and biological degradation (Torresi, Frangipane, & Anelli, 2011). During aging yeast autolysis is produced and different compounds are released into the wine which together with chemical and biochemical changes affecting both the foam characteristics and the final quality of sparkling wines (Alexandre & Guilloux-Benatier, 2006).

One of the final steps of the Cava production is known as riddling, whose purpose is to slowly collect the lees into the neck of the bottle. Lees removal is a very labour-intensive and time-consuming process and the use of immobilized yeast is an expanding research area because this can reduce and simplify the riddling process.

Other than foam characteristics, aroma can be considered one of the most important attributes in the quality of sparkling wines. The second fermentation and the aging on lees can lead to important changes in volatile composition of the wines obtained by the traditional method (Riu-Aumatell, Bosch-Fusté, López-Tamames, & Buxaderas, 2006; Torrens, Riu-Aumatell, Vichi, López-Tamames, & Buxaderas, 2010). During aging on lees, the sorption mechanism of the yeast cell walls can change the volatile profile over time (Gallardo-Chacón, Vichi, López-Tamames, & Buxaderas, 2010). This fact will determine the oenological characteristics and the type and amount of the volatile compounds present in the sparkling wine (Gallardo-Chacón et al., 2010; Riu-Aumatell et al., 2006).

Yeast immobilization consists in the physical confinement of yeasts usually by using an external support (Karel, Libicki, & Robertson, 1985). One of the most critical requirements for successful immobilization of cells is the use of an appropriate material as support. Immobilization supports suitable for the wine industry must have additional prerequisites such as food-grade purity, low cost, abundance, non-degradable nature and suitability for low-temperature fermentation (Torresi et al., 2011).

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https://doi.org/10.1016/j.foodchem.2018.01.036
Received 23 August 2017; Received in revised form 18 December 2017; Accepted 3 January 2018
Available online 04 January 2018

Calcium alginate is currently the major carrier used in biocatalysts for bottle fermentation. The use of calcium alginate beds shows a number advantageous since they are easily prepared and allow the incorporation of yeast beds under mild conditions. When a bottle containing immobilized yeast is inverted, the beads quickly settle into the neck of the bottle and they can be easily removed which greatly simplify the riddling procedure. In addition, the uses of immobilized yeast have technical and economic advantages compared to the conventional free cell system (Kourkoutas, Manojlović, & Nedović, 2010). Although slight differences have been reported between wines produced with immobilized yeast or those obtained with free cells (Yokotsuka, Yajima, & Matsudo, 1997), some studies have shown that the use of calcium alginate beds increase calcium and sodium ions in the finished wine (Puig-Pujol et al., 2013).

A new immobilization method using two microorganisms in absence of external supports namely yeast biocapsules have shown promising results in the area of alcoholic fermentation and wine production (García-Martínez, Moreno, Mauricio, & Peinado, 2015; García-Martínez et al., 2013; López de Lerma, García-Martínez, Moreno, Mauricio, & Peinado, 2012; Peinado et al., 2006). As with other immobilization methods, the use of bioimmobilized yeast make it possible to complete riddling in less than two minutes, resulting in a decrease in manual labor and thus making sparkling wine manufacturing more profitable (Puig-Pujol et al., 2013). Although immobilized yeast cells are confined in a space and wrapped in an external support, during aging there is a release of compounds from their metabolism and autolysis through the porous network of the immobilizing matrix. Some studies have determined the effect of using these formats in the composition and quality of sparkling wines (Bozdogan & Canbas, 2011). However, these studies focus on changes in the level of nitrogen compounds linked to the quality of the foam. To our knowledge, there are no studies comparing the volatile composition of long-aged sparkling wines produced with the same yeast strain under conventional technology (free cells) or immobilized with or without an external support.

In this paper, the influence on the volatile composition of sparkling wines aged during 32 months of two yeast strains used immobilized in calcium alginate beads, as biocapsules and as free cells has been analyzed.

2. Materials and methods

2.1. Chemicals and standards

To identify and quantify the volatiles, commercial standards were purchased from Merck, Sigma–Aldrich, Riedel de Haën, and Fluka. The standard used as those reported in Table 2.

2.2. Yeast strains and filamentous fungus

Two industrial *Saccharomyces cerevisiae* strains were used in this study: *S. cerevisiae* P29 (Spanish Type Culture Collection, CECT 11,770), a wine yeast isolated in the Catalan Institute of Vine and Wine (Vilafranca del Penedés, Spain) from Catalonia vineyards, and *S. cerevisiae* Enoferm QA23 (Lallemand, Montreal, Canada). Both yeast strains were selected on the basis of their working ability under the absence of oxygen and from media with low availability of assimilable nitrogen as are the conditions for producing sparkling wines (Puig-Pujol et al., 2013). The filamentous fungus used for cell immobilization was *Penicillium chrysogenum* strain H3 isolated from the environment by the Viticulture and Enology research group from the University of Cordoba and identified by the Spanish Type Culture Collection (CECT).

2.3. Cell immobilizations

Calcium alginate beads of the two yeast strains were made in the laboratory from the department of Enological Research (Institute of Agrifood Research and Technology–Catalan Institute of Vine and Wine; IRTA-INCAVI) according to specifications described elsewhere (Hidalgo, 2010). The number of beads to be introduced in each bottle was calculated as indicated in the same protocol, to establish an inoculum equivalent to 1×10^6 cells/mL of base wine. Biocapsules of P29 and QA23 strains were obtained in the laboratory from the department of Microbiology (University of Cordoba), according to previous methods (Peinado et al., 2006). The biocapsules formation medium consisting of Yeast Nitrogen Base without amino acids (YNB, Difco, Becton Dickinson and Company, Sparks, MD) buffered at pH 7 with sodium and potassium phosphate and containing 5 g/L of gluconic acid (Sigma-Aldrich, St. Louis, MO) as carbon source. This medium was divided in 250 mL Erlenmeyer flasks and were sterilized previously to the inoculation with 7.5×10^6 viable yeast cells/mL and spores of *P. chrysogenum* strain H3. The flasks were thermostated at 28 °C and shaken at 150 rpm on an orbital shaker from New Brunswick Scientific (Edison, NJ, USA) for 7 days. In this way, spontaneous bioimmobilization in the absence of an external support was accomplished and as a result yeast biocapsules were obtained. Yeast cell counts in biocapsules were carried out according to (García-Martínez, Puig-Pujol, Peinado, Moreno, & Mauricio, 2012), and base wine bottles were inoculated with an equivalent to 1×10^6 cells/mL.

2.4. Sparkling wine elaboration

Base wine obtained by blending autochthonous *Vitis vinifera* of the Penedés winemaking region, north east of Spain, (30% Macabeo, 40% Parellada and 30% Xarel·lo) was used to produce six series of Cava (Spanish sparkling wine). The sparkling wine was obtained by the traditional method. Secondary fermentation took place in standard 750 mL sparkling wine bottles filled with base wine plus sugar at 22 g/L which contained 1×10^6 cells/mL. For each yeast strain three inoculation methods were used: yeast immobilized in calcium alginate beads, yeast immobilized as biocapsules and free yeast cells. Yeasts in their different forms were introduced in bottles that were closed with plastic lid and overcap metal. Sparkling wines were kept in the basement of a cellar for 32 months at 14 °C in the dark. Four bottles of each batch were riddled and disgorged for performing all the analysis.

2.5. Enological parameters

The common enological variables of sparkling wines such as ethanol content (% v/v), total acidity, pH and volatile acidity, were analyzed by Infrared Spectroscopy (FT-IR) in a Winescan 120 FOSS, (Rellingen, Germany), according to the Official Methods established by the European Union. The ammonium ion, free amino nitrogen, yeast available nitrogen (YAN), sugar and malic acid were analyzed by enzymatic reaction with a multiparametric analyzer Lisa 200 (Hycel Diagnostics, Tecnología Difusión Ibérica, Barcelona, Spain). Calcium ion was measured by flame atomic absorption spectrometry in a Perkin-Elmer 280 (Madrid, Spain) device determined in accordance with European regulations. The foam characteristics (Hm and Hs) of sparkling wines were measured using the Mosalux procedure (Poinsaut, 1991).

2.6. Aroma compounds

2.6.1. Extraction

The extraction was carried out according to (Tredoux et al., 2008) with minor changes. The wine sample was diluted in a proportion 1:10 with a hydro ethanolic solution containing 12% ethanol (v/v) and which was previously adjusted to pH 3.5 with 2.6 g/L tartaric acid and 2.2 g/L potassium bitartrate. A stir bar (0.5 mm film thickness, 10 mm length, Gerstel GmbH, Mulheim an der Ruhr, Germany) coated with PDMS was placed in a 10 mL glass headspace vial containing 10 mL of the diluted sample and 0.1 mL of a solution of ethyl nonanoate

Table 1
Enological variables in long aged sparkling wines obtained with the P29 and QA23 *Saccharomyces cerevisiae* strains used as free cells, immobilized in alginate or bioimmobilized (Bio).

Variable	*S. cerevisiae* P29			*S. cerevisiae* QA23		
	Alginate	Bio	Free cells	Alginate	Bio	Free cells
Ethanol (% v/v)	$12.2^{a} \pm 0.1$	$12.3^{a} \pm 0.1$	$12.3^{a} \pm 0.1$	$11.9^{b} \pm 0.1$	$12.1^{a} \pm 0.1$	$12.2^{a} \pm 0.1$
Sugar (g/L)	$2.5^{b} \pm 0.4$	$0.7^{c} \pm 0.1$	$0.9^{c} \pm 0.1$	$5.9^{a} \pm 0.2$	$0.2^{d} \pm 0.1$	$0.1^{d} \pm 0.1$
Total acidity	$5.5^{b} \pm 0.1$	$5.5^{b} \pm 0.1$	$5.5^{b} \pm 0.1$	$5.8^{a} \pm 0.1$	$5.7^{a} \pm 0.1$	$5.6^{ab} \pm 0.1$
Volatile acidity	$0.23^{a} \pm 0.02$	$0.20^{b} \pm 0.01$	$0.21^{b} \pm 0.01$	$0.23^{a} \pm 0.01$	$0.20^{b} \pm 0.01$	$0.17^{c} \pm 0.01$
pH	$3.08^{a} \pm 0.01$	$3.06^{a} \pm 0.01$	$3.06^{a} \pm 0.01$	$3.11^{a} \pm 0.01$	$3.09^{a} \pm 0.01$	$3.09^{a} \pm 0.01$
Malic acid (g/L)	$1.5^{a} \pm 0.1$	$1.5^{a} \pm 0.1$	$1.5^{a} \pm 0.1$	$1.6^{a} \pm 0.1$	$1.6^{a} \pm 0.1$	$1.6^{a} \pm 0.1$
Ca (mg/L)	$62^{a} \pm 1$	$49^{b} \pm 1$	$49^{b} \pm 1$	$60^{a} \pm 1$	$51^{b} \pm 2$	$50^{b} \pm 1$
$NH4^{+}$ (mg/L)	$11^{b} \pm 1$	$8^{b} \pm 1$	$8^{b} \pm 1$	$12^{a} \pm 1$	$9^{b} \pm 1$	$8^{b} \pm 1$
FAN	$62^{a} \pm 2$	$60^{a} \pm 1$	$57^{b} \pm 1$	$61^{a} \pm 1$	$59^{ab} \pm 1$	$57^{b} \pm 1$
YAN	$73^{a} \pm 2$	$68^{ab} \pm 1$	$65^{b} \pm 1$	$73^{a} \pm 1$	$68^{ab} \pm 1$	$65^{b} \pm 1$
Hm (mm)	$24^{a} \pm 3$	$15^{b} \pm 4$	$14^{b} \pm 4$	$20^{a} \pm 2$	$18^{ab} \pm 1$	$17^{ab} \pm 1$
Hs (mm)	$16^{a} \pm 2$	$12^{ab} \pm 2$	$10^{b} \pm 1$	$10^{b} \pm 2$	$11^{b} \pm 2$	$16^{a} \pm 1$

Total acidity as g of tartaric acid per liter; Volatile acidity as g of acetic acid per liter; FAN: Free amino nitrogen (mg/L); YAN: Yeast available nitrogen (mg/L); Hm: foamability expressed by the height in mm; Hs: foam persistence expressed by the height in mm.
Different letters denote significant differences at 95% confidence level.

(0.45 mg/L) as internal standard. The vial was sealed with a Teflon-coated crimp cap. The stir bar was stirred at 1500 rpm at 25 °C for 100 min. After removal from the wine sample, the stir bar was gently dried with a lint-free tissue and then transferred into a glass thermal desorption tube for GC–MS analysis.

2.6.2. Determination

The glass thermal desorption tube is introduced into Gerstel TDS 2 thermodesorption system which is attached to the GC–MS model. The stir bar was heated to release and transfer the extracts into a cooled injection system/programmed temperature vaporizer (CIS 4 PTV) containing a tenax adsorption tube. The thermal desorption was carried out with a temperature program from 35 °C, ramped at 120 °C min^{-1} to 280 °C and held for 10 min; the helium flow rate was 3 mL/min. The CIS injector was held at 25 °C for the total desorption time and then ramped at 12 °C s^{-1} in splitless mode to 280 °C and held for 7 min.

The GC was fitted with an Agilent-19091S capillary column 30 m × 0.25 mm i.d., 0.25-μm film thickness. Helium was used as carrier gas with a column flow rate of 1 mL min^{-1}. The GC oven temperature was programmed as follows: 50 °C for 2 min, ramped at 4 °C min^{-1} to 190 °C, held for 10 min. The mass detector was used in the scan mode and the studied mass range spanned values from 39 to 300 *m/z*. Retention times, spectral libraries supplied by Wiley (version 7 N) and pure chemical compounds were used for identification and confirmation of the volatile compounds. Each compound was quantified from its calibration curve, which was obtained by using standard solutions of known concentrations previously subjected to the same treatment as the samples in conjunction with the target and qualifier ions selected for each compound by the Hewlett–Packard Chemstation (Palo Alto, CA).

2.7. Calculation of aromatic series

The perception threshold is defined as the minor concentration of a substance capable of producing a detectable sensation at least for 50% of the members of a tasting panel (Cutzach, Chatonnet, & Dubourdieu, 2000). In addition, the contribution of a volatile compound to the wine aroma can be evaluated qualitatively by its aroma descriptor, and quantitatively by its odorant activity value (OAV). This OAV is obtained by dividing the concentration of each compound by its perception threshold. An aromatic series is defined as a group of volatile compound with similar aroma descriptors and its value is obtained as the sum of the OAVs of the compounds that make up the series. The same compound can be included in one or several aromatic series, in agreement with its aromatic descriptors.

2.8. Sensory analysis

The sparkling wines were evaluated by a panel of eight winery tasters with extensive experience in sparkling wine sensory evaluation. The aim of the analysis was to establish the organoleptic profile of the sparkling wines obtained with different inoculation methods and to identify differences among products in relation with the descriptors previously selected (UNE 87-017-92). A descriptive analysis of each wine was performed in a room set in accordance with ISO 8589 (2007). Colour, Odor and taste descriptors were evaluated by the panelists, assigning a value ranging from 1 (no intensity) to 9 (maximum intensity). The sensory attributes used for this analysis were color quality, aroma quality, aroma intensity, fruity, yeasty and mold aroma. In terms of the taste, intensity and quality and the gustatory attribute acid, body and bitter. Samples were tasted in a randomized order. Sparkling wines were presented to the panelists in tasting glasses marked with two-digit random numbers. Tasting was performed at 20–22 °C, and water was provided to rinse the palate between tastings.

2.9. Statistical analysis

The statistical treatment of the data was performed using Statgraphics Centurion XVI of StatPoint Technologies Inc. (Warrenton, Virginia). Data reported are the means of three repetitions (three different bottles of the same batch). Volatile aroma compounds and the aromatic series were processed using multivariate analysis of variance (MANOVA) to study the effect of the different yeast strains and the way of use (free, biocápsulas or immobilized in alginate). Also homogeneous group analysis was carried out to analyze the effect of the different treatment on enological parameters. Multivarate analysis was carried out to get a footprint of the wines analyzed. Lastly, principal components analysis was also carried out to analyze the differences among the yeast strains and the way of use.

3. Results and discussion

3.1. Influence of immobilized yeasts on the composition of aged sparkling wines

Table 1 shows the values obtained for the most important variables of sparkling wines produced by free yeast, yeasts immobilized in alginate beds and biocapsules after 32 months of aging under lees.

Table 2
Volatile aroma compounds (μg/L, except where indicated) quantified in long aged sparkling wines obtained with the P29 and QA23 *Saccharomyces cerevisiae* strains used as free cells, immobilized in alginate or bioimmobilized (Bio). Multivariate analysis of variance: Y: yeast; W: way of use; I: interaction between factors. Odor perception threshold (OPT), aroma descriptor and aromatic series (AS) assigned to the volatile aroma compounds. 1. Chemical; 2: Ripe fruit; 3. Green Fruit; 4: Floral; 5: Fatty; 6: Creamy; 7: Toasty; 8: Herbaceous; 9: Citrus.

Yeast	*S. cerevisiae* P29			*S. cerevisiae* QA23			Effect			OPT	Aroma descriptor	AS
Way of use	Alginate	Bio	Free cells	Alginate	Bio	Free cells	Y	W	I			
Acetates	68 ± 2	55 ± 1	58 ± 2	76 ± 3	42 ± 2	53 ± 3	s	s	ns			
Methyl acetate	10.4 ± 0.7	7.3 ± 0.3	10 ± 1	17 ± 1	9.7 ± 0.4	11.1 ± 0.9	s	s	s	470[I]	Solvent-like, fruity	1,2
Isoamyl acetate	51 ± 2	40 ± 1	40 ± 1	50 ± 1	27 ± 2	35 ± 3	ns	s	ns	30[II]	Banana	2
Hexyl acetate	1.9 ± 0.2	1.4 ± 0.2	1.46 ± 0.07	3.3 ± 0.2	1.35 ± 0.08	1.79 ± 0.09	ns	ns	ns	2[III]	Apple, pear	3
2-Ethylhexyl acetate	2.0 ± 0.2	1.4 ± 0.2	1.46 ± 0.07	3.3 ± 0.2	1.35 ± 0.08	1.79 ± 0.09	s	s	s	12[XVI]	Herbal	8
2-Phenylethyl acetate	4.2 ± 0.1	4.3 ± 0.4	4.4 ± 0.3	4.3 ± 0.3	2.9 ± 0.1	4.1 ± 0.2	ns	ns	ns	250[IV]	Fruity, floral, rose	4
Ethyl Esters	1769 ± 11	1760 ± 54	1684 ± 7	1706 ± 20	1501 ± 21	1586 ± 17	s	s	s			
Ethyl propanoate	221 ± 19	165 ± 11	170 ± 5	207 ± 11	128 ± 5	180 ± 8	ns	s	s	45 [V]	Fruity	2
Ethyl isobutanoate	3.5 ± 0.2	1.3 ± 0.1	0.7 ± 0.1	3.9 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	s	s	ns	15[II]	Apple, strawberry	2
Ethyl butanoate	465 ± 12	454 ± 7	451 ± 14	441 ± 3	355 ± 9	429 ± 7	s	ns	s	20[II]	Fruity, tutti frutti	2
Ethyl 2-methyl butanoate	7.2 ± 0.6	4.9 ± 0.2	4.1 ± 0.2	6.4 ± 0.5	3.1 ± 0.3	4.1 ± 0.3	s	s	s	18[II]	Fruity, estery, berry	2
Ethyl 3-methyl butanoate	19.9 ± 0.6	19.3 ± 0.8	17 ± 1	19.4 ± 0.2	15.3 ± 0.7	16.9 ± 0.7	s	s	s	3[II]	Green pineapple	3
Ethyl hexanoate	639 ± 16	608 ± 15	614 ± 10	618 ± 3	512 ± 10	574 ± 4	s	s	s	14[II]	Pineapple, green banana	2,3
Ethyl furoate	24 ± 2	24 ± 1	22 ± 1	26 ± 4	20 ± 1	26 ± 1	ns	ns	s	1000[I]	Floral balsamic	4
Ethyl heptanoate	0.31 ± 0.03	0.39 ± 0.02	0.31 ± 0.03	0.34 ± 0.04	0.36 ± 0.01	0.39 ± 0.01	s	ns	s	2,2[VI]	Fruity pineapple	2
Ethyl benzoate	0.53 ± 0.02	0.65 ± 0.04	0.66 ± 0.03	0.64 ± 0.03	0.58 ± 0.03	0.66 ± 0.03	ns	s	ns	575[II]	Medicinal, fruity, wintergreen	1,2,4
Ethyl octanoate	368 ± 16	436 ± 16	385 ± 15	364 ± 9	392 ± 12	332 ± 12	s	s	s	5[II]	Pineapple, floral	2
Ethyl decanoate	14 ± 1	43 ± 3	15 ± 1	12 ± 1	67 ± 3	15 ± 1	ns	s	ns	200[II]	Fruity, sweet apple, grape	2
Ethyl laurate	1.85 ± 0.02	2.12 ± 0.02	1.89 ± 0.05	1.87 ± 0.05	2.2 ± 0.1	1.9 ± 0.03	s	s	ns	2000[VI]	Creamy, floral	4,6
Ethyl mirystate	2.09 ± 0.09	2.00 ± 0.01	2.04 ± 0.09	2.02 ± 0.07	2.1 ± 0.1	2.07 ± 0.08	ns	ns	ns	2000 [VI]	Creamy, waxy, violet	4, 6
Ethyl palmitate	2.3 ± 0.1	2.8 ± 0.4	2.51 ± 0.06	2.05 ± 0.02	3.8 ± 0.2	3.6 ± 0.3	s	s	s	2000[VI]	Fruity, creamy, milky	2,6
Other esters	7.6 ± 0.3	8.1 ± 0.3	7.8 ± 0.1	7.8 ± 0.2	8.1 ± 0.1	7.8 ± 0.2	s	s	ns			
Hexyl hexanoate	3.1 ± 0.2	3.3 ± 0.1	3.2 ± 0.1	3.2 ± 0.2	3.4 ± 0.1	3.3 ± 0.1	s	s	s	14 [X]	Green, fruity, tropical	3,8
2-Phenylethyl isobutyrate	0.69 ± 0.04	0.78 ± 0.05	0.74 ± 0.03	0.72 ± 0.03	0.77 ± 0.02	0.68 ± 0.01	ns	s	ns	150 [X]	Rose, floral	4
2-Phenylethyl butyrate	1.05 ± 0.05	1.20 ± 0.06	1.03 ± 0.08	1.16 ± 0.08	1.18 ± 0.02	1.13 ± 0.04	ns	s	ns	200 [X]	Floral, musty	4
2-Phenylethyl octanoate	2.76 ± 0.03	2.8 ± 0.3	2.79 ± 0.09	2.79 ± 0.01	2.77 ± 0.07	2.70 ± 0.09	ns	ns	ns	500[X]	Sweet, creamy, caramelise	6
Alcohols	1461 ± 70	1384 ± 47	1370 ± 82	1656 ± 49	1355 ± 52	1479 ± 26	ns	ns	s			
Furanmethanol	258 ± 17	219 ± 16	223 ± 12	432 ± 13	283 ± 7	311 ± 16	ns	ns	s	2000[IV]	Burnt, coffee	7
Hexanol	1165 ± 76	1099 ± 48	1099 ± 75	1170 ± 40	1010 ± 41	1092 ± 37	ns	ns	ns	2500[VII]	Grass	8
Guaiacol	9.6 ± 0.6	13.1 ± 0.8	6.7 ± 0.3	10.5 ± 0.5	15.0 ± 0.6	18.1 ± 0.7	s	ns	s	75[VIII]	Medicine, smoke	1,7
4-vinylguaiacol	28 ± 2	53 ± 2	41 ± 2	43 ± 1	47 ± 3	57 ± 2	s	ns	s	40[IX]	Clove, woody	7
Lactones	723 ± 43	600 ± 22	784 ± 29	1167 ± 46	736 ± 42	881 ± 42	s	ns	s			
Crotonolactone	712 ± 42	587 ± 23	771 ± 29	1154 ± 46	721 ± 42	867 ± 42	s	ns	s	1000[X]	Buttery, toasty	6,7
Caprolactone	1.5 ± 0.1	2.2 ± 0.1	2.4 ± 0.2	3.9 ± 0.2	3.9 ± 0.2	4.4 ± 0.4	s	ns	ns	13[X]	Freshly mown hay, vanilla, tobacco	7,8
γ-Nonalactone	4.2 ± 0.2	5.1 ± 0.2	4.8 ± 0.2	4.1 ± 0.1	4.9 ± 0.3	4.2 ± 0.2	ns	ns	ns	30[II]	Creamy, coconut	2,6
γ-Decalactone	4.7 ± 0.1	5.4 ± 0.5	5.4 ± 0.4	5.0 ± 0.2	5.4 ± 0.2	5.1 ± 0.2	ns	ns	ns	47 [V]	Peach, milky	2,6
Carbonilics	519 ± 6	561 ± 19	430 ± 51	674 ± 34	467 ± 23	425 ± 18	ns	s	s			
Furfural	467 ± 9	498 ± 20	419 ± 50	610 ± 34	452 ± 22	406 ± 18	ns	s	s	770[XI]	Burned almonds, fusel alcohol	1,7
5-Methyl furfural	46 ± 3	45 ± 2	8 ± 1	57 ± 3	8 ± 1	15 ± 1	ns	ns	ns	350[XI]	Bitter almond, cherry, smoked	1,2,7
Benzaldehyde	1.7 ± 0.1	17.0 ± 0.8	1.4 ± 0.1	0.3 ± 0.1	5.5 ± 0.4	0.8 ± 0.1	s	s	s	1100[III]	Caramel	7
Octanal	0.88 ± 0.03	0.58 ± 0.03	0.59 ± 0.05	1.7 ± 0.2	0.86 ± 0.04	1.04 ± 0.07	s	s	s	2.5[XIV]	Citrus	9
Nonanal	1.17 ± 0.05	0.75 ± 0.03	0.90 ± 0.07	1.7 ± 0.2	0.72 ± 0.05	1.06 ± 0.05	s	s	s	2.5 [XIV]	Citrus	9
Decanal	1.24 ± 0.08	0.35 ± 0.06	0.43 ± 0.04	2.2 ± 0.3	0.49 ± 0.03	0.84 ± 0.05	s	s	s	1.25 [XIV]	Citrus	9
Terpenes	1.4 ± 0.1	9.0 ± 0.4	1.5 ± 0.1	1.60 ± 0.06	9.8 ± 0.2	1.65 ± 0.05	ns	s	s			
Limonene	1.4 ± 0.1	9.0 ± 0.4	1.5 ± 0.1	1.60 ± 0.06	9.8 ± 0.2	1.65 ± 0.05	ns	s	s	10 [XV]	Citrus, herbal	8,9
C-13 Norisoprenoids	226 ± 6	313 ± 15	250 ± 7	239 ± 4	282 ± 5	250 ± 8	ns	s	s			
Vitispirane	211 ± 6	272 ± 12	292 ± 5	229 ± 5	247 ± 8	235 ± 9	ns	s	s	800[XIII]	Floral	4
TDN	15 ± 1	41 ± 3	20 ± 2	10 ± 1	35 ± 2	15 ±	s	s	ns	20[XIII]	Camphor, kerosene	1
Acids (mg/L)	27 ± 1	30 ± 2	27 ± 1	29 ± 1	21 ± 1	24 ± 1	ns	s	s			
Butanoic	96 ± 4	84 ± 3	83 ± 4	71 ± 6	66 ± 4	72 ± 2	ns	s	ns	173[II]	Rancid, cheese	5
Hexanoic	171 ± 9	80 ± 9	132 ± 10	177 ± 7	168 ± 5	131 ± 10	ns	ns	s	420[II]	Rancid, fatty, soapy	5
Octanoic (mg/L)	25 ± 1	29 ± 2	25 ± 1	26 ± 1	20 ± 1	22 ± 1	ns	s	s	500[II]	Cheese	5
Decanoic(mg/L)	2.1 ± 0.1	1.0 ± 0.1	1.7 ± 0.1	2.8 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	s	s	s	1000[II]	Rancid fat, plastician	5
Lauric	62 ± 2	85 ± 3	82 ± 4	115 ± 4	94 ± 6	107 ± 5	s	ns	s	6100[XII]	Fatty	5
Palmitic	65 ± 4	60 ± 2	65 ± 4	82 ± 4	66 ± 5	73 ± 3	s	ns	ns	100000[XII]	Waxy, fatty	5

s: significant interaction $p < .05$; ns: no significant interaction.; nd: not detected.
I (Etievant, 1991); II (Ferreira, López, & Cacho, 2000); III (Abraham, Sánchez-Moreno, Cometto-Muñiz, & Cain, 2012); IV (Gómez-Míguez, Cacho, Ferreira, Vicario, & Heredia, 2007); V (Culleré, Ferreira, & Cacho, 2011); VI (http://www.leffingwell.com/esters.htm); VII (Lopez de Lerma, Bellincontro, Mencarelli, Moreno, & Peinado, 2012); VII (Rocha, Rodrigues, Coutinho, Delgadillo, & Coimbra, 2004); IX (López, Aznar, Cacho, & Ferreira, 2002); X. Determined by the authors in hydro-ethanol solution at 13%, pH = 3.5.; XI (Peinado, Moreno, Bueno, Moreno, & Mauricio, 2004); XII (Dragone, Mussatto, Oliveira, & Teixeira, 2009); XIII (Simpson, 1978); XIV (Culleré et al., 2011); XV (Buttery, Seifert, Guadagni, & Ling, 1971); XVI (Guadagni, Buttery, & Harris, 1966)

Table 3
Values of the aromatic series in long aged sparkling wines obtained with the P29 and QA23 *Saccharomyces cerevisiae* strains used as free cells, immobilized in alginate or bioimmobilized (Bio). Multivariate analysis of variance: Y: yeast; W: way of use; I: interaction between factors.

Yeast	*S. cerevisiae* P29			*S. cerevisiae* QA23			Effect		
Way of use	Alginate	Bio	Free cells	Alginate	Bio	Free cells	Y	W	I
Chemical	1.5 ± 0.1	2.9 ± 0.2	1.7 ± 0.2	1.5 ± 0.1	2.6 ± 0.1	1.5 ± 0.1	s	s	ns
Fruity	150 ± 4	159 ± 5	149 ± 2	146 ± 2	137 ± 4	135 ± 3	s	ns	s
Green fruit	53 ± 1	50 ± 1	50 ± 1	51 ± 1	43 ± 2	47 ± 1	s	s	s
Floral	0.32 ± 0.01	0.40 ± 0.01	0.34 ± 0.01	0.34 ± 0.01	0.36 ± 0.01	0.34 ± 0.01	ns	ns	ns
Fatty	52 ± 2	59 ± 3	53 ± 2	55 ± 1	41 ± 1	46 ± 1	ns	s	s
Creamy	0.95 ± 0.05	0.87 ± 0.02	1.05 ± 0.03	1.40 ± 0.04	1.00 ± 0.04	1.11 ± 0.04	s	ns	s
Herbaceous	1.61 ± 0.07	2.22 ± 0.06	1.67 ± 0.03	2.36 ± 0.06	1.70 ± 0.07	2.78 ± 0.08	s	ns	s
Toasty	1.73 ± 0.05	2.47 ± 0.07	1.96 ± 0.01	2.58 ± 0.08	2.41 ± 0.01	2.70 ± 0.01	s	ns	s
Citrus	1.95 ± 0.09	1.71 ± 0.09	1.09 ± 0.08	3.07 ± 0.03	1.19 ± 0.06	2.49 ± 0.06	s	ns	s

s: significant interaction $p < .05$; ns: no significant interaction.

Only the QA23 *S. cerevisiae* strain immobilized in alginate beds showed significant differences in the ethanol concentration. The alginate format also showed the highest reducing sugar content in both yeast strains highlighting the QA23 *S. cerevisiae* strain. This fact was yet observed when sparkling wine was analyzed after 10 months of aging (Puig-Pujol et al., 2013). These authors also detected high sugar content in wines obtained with the QA23 strain used as biocapsules in one of the experimental conditions. Both events, as is our case, were attributed to different fermentation efficiencies among the strains and their inoculums format. In our study, after 36 months of aging, all wines can be categorized as dry wines according to the residual sugar content. In addition, the low volatile acidity of the wines and the sensory analysis carried out show that there were not stability problems in any of the analyzed wines. No differences were observed for pH, total acidity and malic acid among the different batches of wine.

Yeasts used in alginate beds showed significant higher concentration of calcium ion. This result is in agreement with previous studies (Puig-Pujol et al., 2013) who observed an increase of calcium ion in sparkling wines when alginate was used for yeast immbolization. The presence of a high concentration of calcium ions give rise to insoluble tartrates which could negatively affect the foam characteristic of the Cava wines (Moreno & Peinado, 2012).

Regarding to the different forms of nitrogen, it has been observed that sparkling wines produced with cells immobilized in alginate showed the highest concentration of yeast available nitrogen (YAN, sum of ammonium ion and free amino nitrogen). This characteristic is related with the highest foamability (Hm) value found in these batches (Coelho, Rocha, & Coimbra, 2011). It has also been described that the addition of a clarifying agent, such as bentonite, to facilitate the riddling process when free yeast cells are used, decreases the content of nitrogen due to an absorption phenomenon which reduces the quality of the foam in the resulting wine (Vanrell et al., 2007).

3.2. Volatile aroma compounds and multivariate analysis of variance

Table 2 shows the concentration of the volatile aroma compounds analyzed in the sparkling wine (Cava). The concentration of a given compound could be dependent on the yeast strain, on their way of use (free, bioimmobilized and immobilized in alginate) or on both factors. For these reason a multivariate analysis of variance has been carried out and the results are also showed in Table 2. Of importance were octanoic acid, decanoic acid and hexanol with concentrations above 1 mg/L. Amongst the chemical families, short chain fatty acids shows the highest concentrations, ranging from 21 to 30 mg/L although this concentration is conditioned by the high amount of octanoic acid. These compounds are released to the wine during aging due to the yeast lysis (Buxaderas & López-Tamames, 2010). Furthermore, a high number of esters were detected. These compounds have pleasant aromas and usually low perception threshold (Table 2), so their contribution to wine aroma could be relevant. Esters are also the family with the highest number of volatile compounds and their concentration have been described to change with the aging time (Alexandre & Guilloux-Benatier, 2006). Also, depending on the physicochemical characteristic

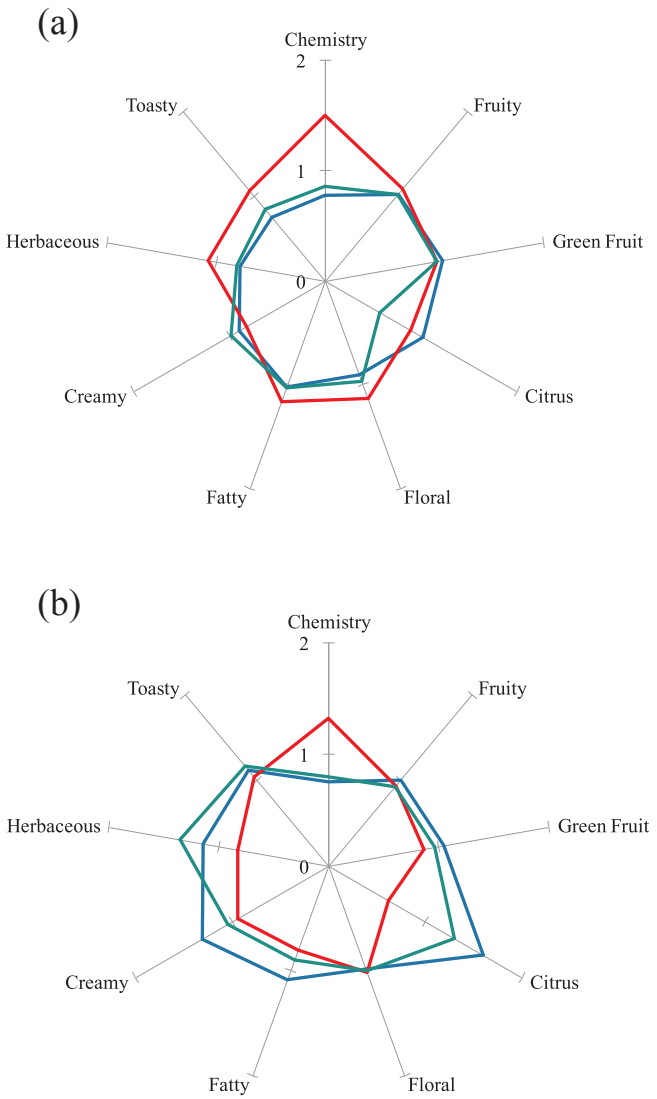

Fig. 1. Footprints obtained by multivariate data analysis of aroma compounds grouped in aromatic series of the long aged sparkling wines obtained with P29 strains (a) and QA23 (b) yeast strains. Red line: bioimmobilized. Blue lines immobilized in alginate. Green line: free cells.

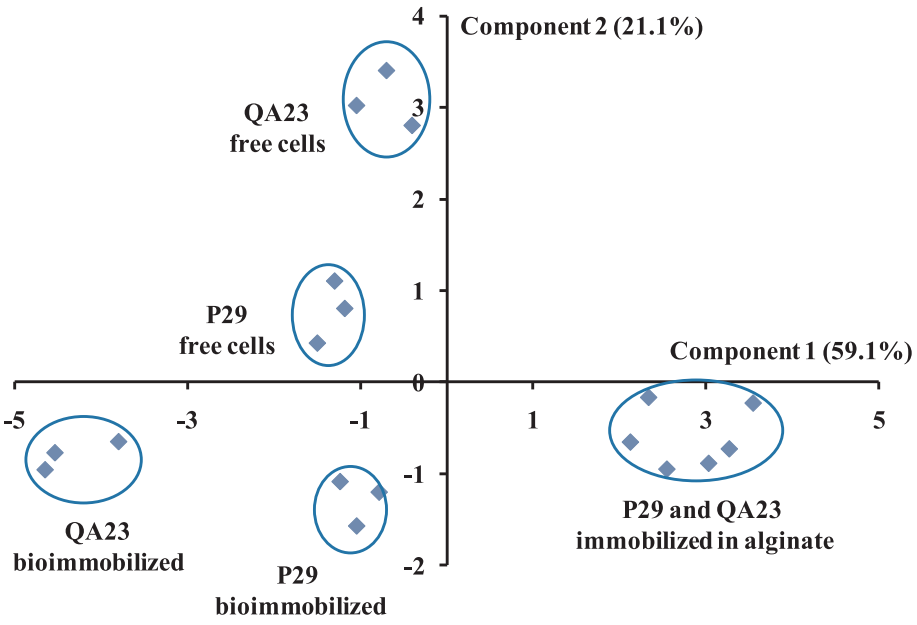

Fig. 2. Principal component analysis using as classifying variables the volatile aroma with odor activity values above the unity in at least one of the long aged sparkling wine.

of the volatile compound and on the less cell wall, yeast can absorb some kinds of esters (Gallardo-Chacón et al., 2010). Most of ethyl esters depend on the yeast strain and on the way of use (Table 2). Wines produced with the P29 strain show an overall ester concentration higher than those obtained with the QA23 strain. Although no differences due to the immobilization method were observed in wines produced with the P29 strain this was not the case with the QA23 strain.

Two C-13 norisoprenoids, namely vitispirane and TDN (1,1,6-trimethyl-1,2-dihydro naphthalene) were detected in Cava wines. Vitispirane has a megastigme precursor and is linked to a sugar molecule. TDN is produced by the degradation of carotene (Moreno & Peinado, 2012). These compounds appear in long aged sparkling wines (Riu-Aumatell et al., 2006) and depend on the way the yeast is used, and the wines obtained with bioimmobilized yeast showed the highest concentration. TDN concentration also depended on the yeast strain, showing the highest values the Cava obtained with the P29 strain.

Other compounds such as alcohols and terpenes are also released to the wine during yeast autolysis. Limonene showed the higher concentration in wines obtained with bioimmobilized yeast, whereas major alcohols neither depend on the yeast strain nor the way of use.

Lastly, octanal, nonanal and decanal have citrus aroma (Table 2). Although these were present at low concentrations their odor thresholds are also low, so it is expected that contributes with citric aromas to the wine flavor. The wines obtained with yeast immobilized in alginate showed the highest values of these compounds.

3.3. Aromatic series

Wine aroma and flavor consist of a large quantity of aroma-active compounds, interacting with each other and resulting in masking or suppressing effects as well as additive interactions for compounds (Hein, Ebeler, & Heymann, 2009). Odor activity values (OAVs) are often used to point out which volatile compounds contribute substantially to the wine aroma (Francis & Newton, 2005). This value is obtained as the ratio between the concentration of an individual compound and its odor perception threshold. Table 2 lists the odor perception threshold of the volatile compounds determined in cava wines. In addition, OAVs could be used to identify the potential aroma contribution/impact odorant of a wine. To this end, volatile compounds are classified into aroma series according to their odor descriptors. The OAV for a given series is obtained as the sum of the odor activity values of the volatile compounds that the aromatic series comprises. In this respect, fingerprints of the sparkling wines can be obtained by classifying aroma compounds into nine aroma series (Table 3). The addition of the individual OAVs to calculate the value of an aromatic series should not be interpreted as an arithmetical addition of odor sensations. Anyway, this method is useful to compare the aromatic profile of wines produced by different methods because the aromatic series always comprise the same volatile compounds. In addition, this procedure greatly reduces the number of variables to be processed and allows changes during the winemaking of sparkling wine to be assessed in terms of several odor descriptors.

Of importance were the values of the fruity series in all wines, whereas the floral have a low impact because showed values below unity in all cases (Table 3). With the exception of the floral series, all the aromatic series depended on both factor studied and in general terms immobilized cells shows higher values than free yeast independently of the strain used.

The sum of the aromatic series values reach the highest values in the Cava wine produced with the P29 strain used in bioimmobilized form. QA23 and P29 yeast strains immobilized in alginate showed similar values in both wines.

Regarding to the individual aroma compounds, isoamyl acetate, ethyl propanoate, ethyl butanoate, ethyl 3-methylbutanoate, ethyl hexanoate, ethyl octanoate, hexanol, 2-methoxy-4-vinylphenol, decanal, octanoic acid, decanoic acid and TDN show OAV above the unity in at least one of the wines. Among these compounds highlight qualitatively the esters and quantitatively, ethyl butanoate, ethyl hexanoate, ethyl octanoate and 2-methoxy-4-vinylphenol. These compounds contribute to the sum of the aromatic series with more than 70% and their aroma descriptors have fruity and toasty aromas. Lastly, the yeast used in bioimmobilized forms shows the higher contribution to the total value of the aromatic series.

3.4. Statistical treatment

3.4.1. Multivariate analysis

Multivariate analysis applied to the compounds grouped in aromatic series results in a footprint for each wine (Fig. 1). The values of the aromatic series were standardized to obtain 9 rays of the same length. The unity represents the median value of a given aromatic series. Values above the unity indicates that these wine show higher values for such series than the median. The opposite is also true for values below the unity. Fig. 1a shows that P29 bioimmobilized strain differs from the

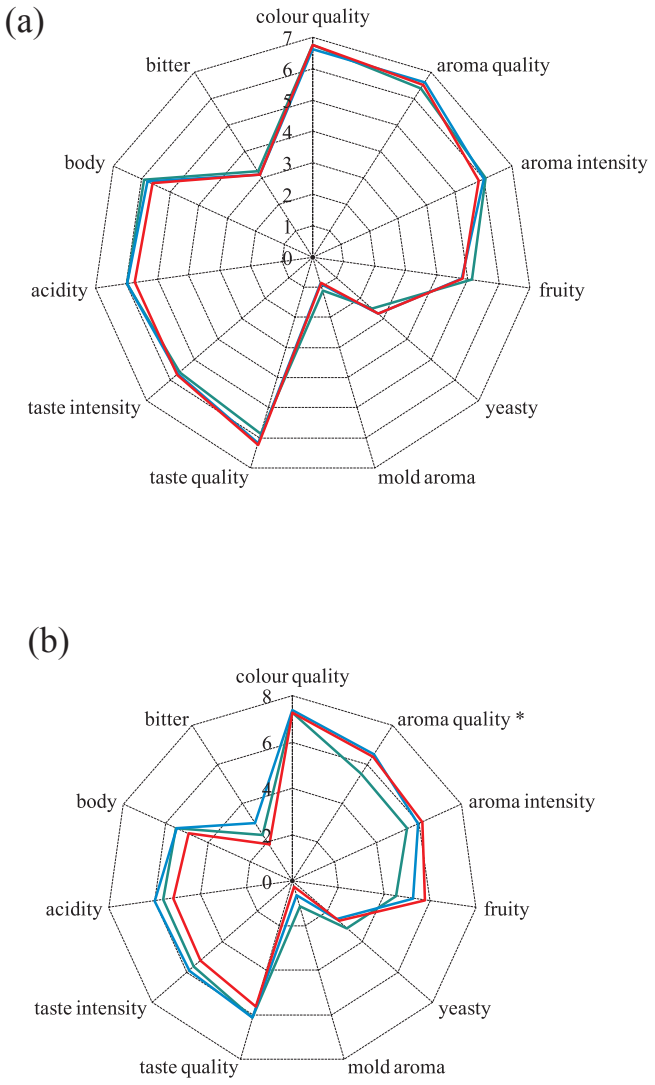

Fig. 3. Sensory analysis of sparkling wines made with *S. cerevisiae strain* P29 (a) or *S. cerevisiae* strain QA23 (b) obtained by the mean of the scores given by the panelists for each descriptor. Red line: bioimmobilized. Blue lines immobilized in alginate. Green line: free cells. (*) Significant differences among free cells and immobilized formats: alginate and biocapsules at $p < .05$.

rest by its higher values in the chemistry, toasty, floral, herbaceous and fatty series. Wines obtained with free cells or with yeast immobilized in alginate show a similar footprint. On the other hand, QA23 strain immobilized in alginate shows higher values than the median in the toasty, creamy, herbaceous, fatty and citrus series (Fig. 1b). Citrus series also highlights in the cava wine obtained with free yeast. The use of this statistical methodology allows associating cause and effect in a graphical and useful way and has been used recently by a number of authors (Martínez-García, García-Martínez, Puig-Pujol, Mauricio, & Moreno, 2017).

3.4.2. Principal component analysis

With the aim to classify the Cava wines according to the different winemaking treatment a principal component analysis was carried out using as classifying variables those volatile aroma compounds that show OAVs above the unity (Fig. 2).

Two principal components have been selected than explain 59.1% and 21.1% of the total variability of the data. The first principal component is mainly influenced by the esters, with the exception of ethyl octanoate, and differentiates among the wines produced with both immobilization systems although no differences can be made among the wine obtained with P29 and QA23 strains immobilized in alginate. The second principal component differentiates among wine produced with free cells and those produced with both immobilization systems and it is mainly influenced by ethyl octanoate, hexanol, 2-methoxy-4-vinylphenol, octanoic acid, decanoic acid and TDN.

3.5. Sensory analysis

The sensory analysis of the sparkling wines obtained by two different *S. cerevisiae* strains and three inoculums formats was performed by evaluating the global organoleptic quality through a descriptive test to define differences among the samples. Fig. 3 shows the radar diagrams for each strain. It can be observed that the tasters evaluated similarly the three batches of Cava wines fermented with the strain P29, regardless of whether it was used immobilized into a support or with free form. In the case where *S. cerevisiae* QA23 was used, the results showed a significant difference ($p < .05$) in the aroma quality descriptor among wines made with immobilized yeasts and those made with free cells when ANOVA was performed. Although no differences were detected in the remaining attributes, it is interesting to note that the best scores for odor descriptors were for sparkling wines elaborated with immobilized yeasts. These compounds form part of the bouquet of the wine and were positively appreciated by the tasters. These sparkling wines also had the lowest yeasty aroma, a negative descriptor when it reaches high values.

3.6. Conclusions

This work shows the first characterization of the volatile compounds and sensorial quality of sparkling wines obtained with a new yeast immobilization method such as biocapsules. According to the obtained results, both the yeast strain and the way of use have a great impact in the volatile composition of the resulting Cava wines. Among the aroma compounds eleven compounds shows OAVs higher than the unity in the analyzed wines, and among then ethyl butanoate, ethyl hexanoate, ethyl octanoate and 2-methoxy-4-vinylphenol, contribute with more than 70% to the sum of aromatic series. Comparing both immobilization systems, the P29 bioimmobilized strain produced wines with a higher concentration of aroma compounds than the P29 used in alginate. The opposite is true when was used the strain QA23. This fact has been established by the footprint of the Cava wines. Both immobilization systems produce wines with the highest values in the aromatic series, so it can be expected that these wines have a more complex aroma. The only imperfection of the immobilization in alginate, under the studied conditions, is that this system releases a higher concentration of calcium ions which could produce insoluble tartaric salts and hence the foam characteristic of the Cava wines could be affected. Anyway, both cell immobilization systems provide an effective way to perform the second fermentation of sparkling wines and can be a real alternative to the use of free yeasts to obtain wines with better aroma quality.

Acknowledgements

The authors wish to acknowledge co-funding of this work by Spain's Ministry of Economy and Competitiveness (MINECO-INIA-CCAA) and the European Fund of Regional Development (FEDER): Grant No. RTA2014-00016-C03-03.**Conflict of interest**

There is no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations.

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