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Highlights

Long aged sparkling wines were produced with free and immobilized yeast

Eleven volatile compounds contribute with more than 70% to the overall aroma

Volatiles associated with the immobilization format allows wine classification by PCA

Immobilized yeasts could be an alternative to improve quality of long-aged cava wines

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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1 **ABSTRACT.**

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

13

14 **1. INTRODUCTION**

15 Spanish sparkling wine (Cava) (Certified Brand of Origin) elaborated following the traditional
16 method (analogous to the Champenoise method used to produce Champagne in France) undergo a
17 second fermentation in closed bottles of base wine, followed by period of aging during at least 9
18 months in the presence of yeast lees (EC Regulation N° 479/2008). The second fermentation is
19 carried out by adding to the base wine “liquer de tirage” composed mainly by sucrose, selected
20 yeasts and grape must or wine to produce the desired CO₂ pressure. Moreover, small amounts of
21 bentonite and sulfur dioxide are usually added in order to simplify the lees removal and also to
22 prevent oxidative effects and biological degradation (Torresi, Frangipane, & Anelli, 2011). During
23 aging yeast autolysis is produced and different compounds are released into the wine which together
24 with chemical and biochemical changes affecting both the foam characteristics and the final quality
25 of sparkling wines (Alexandre & Guilloux-Benatier, 2006).

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

30 Other than foam characteristics, aroma can be considered one of the most important attributes in the
31 quality of sparkling wines. The second fermentation and the aging on lees can lead to important
32 changes in volatile composition of the wines obtained by the traditional method (Riu-Aumatell,
33 Bosch-Fusté, López-Tamames, & Buxaderas, 2006; Torrens, Riu-Aumatell, Vichi, López-Tamames,
34 & Buxaderas, 2010). During aging on lees, the sorption mechanism of the yeast cell walls can
35 change the volatile profile over time (Gallardo-Chacón, Vichi, López-Tamames, & Buxaderas,
36 2010). This fact will determine the oenological characteristics and the type and amount of the
37 volatile compounds present in the sparkling wine (Gallardo-Chacón, et al., 2010; Riu-Aumatell, et
38 al., 2006).

39 Yeast immobilization consists in the physical confinement of yeasts usually by using an external
40 support (Karel, Libicki, & Robertson, 1985). One of the most critical requirements for successful
41 immobilization of cells is the use of an appropriate material as support. Immobilization supports
42 suitable for the wine industry must have additional prerequisites such as food-grade purity, low cost,
43 abundance, non-degradable nature and suitability for low-temperature fermentation (Torresi, et al.,
44 2011).

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

55 A new immobilization method using two microorganisms in absence of external supports namely
56 yeast biocapsules have shown promising results in the area of alcoholic fermentation and wine
57 production (García-Martínez, et al., 2013; García-Martínez, Moreno, Mauricio, & Peinado, 2015;
58 López de Lerma, García-Martínez, Moreno, Mauricio, & Peinado, 2012; Peinado, et al., 2006). As
59 with other immobilization methods, the use of bioimmobilized yeast make it possible to complete
60 riddling in less than two minutes, resulting in a decrease in manual labor and thus making sparkling
61 wine manufacturing more profitable (Puig-Pujol, et al., 2013). Although immobilized yeast cells are
62 confined in a space and wrapped in an external support, during aging there is a release of compounds
63 from their metabolism and autolysis through the porous network of the immobilizing matrix. Some

64 studies have determined the effect of using these formats in the composition and quality of sparkling
65 wines (Bozdogan & Canbas, 2011). However, these studies focus on changes in the level of nitrogen
66 compounds linked to the quality of the foam. To our knowledge, there are no studies comparing the
67 volatile composition of long-aged sparkling wines produced with the same yeast strain under
68 conventional technology (free cells) or immobilized with or without an external support.
69 In this paper, the influence on the volatile composition of sparkling wines aged during 32 months of
70 two yeast strains used immobilized in calcium alginate beads, as biocapsules and as free cells has
71 been analyzed.

72 **2. MATERIALS AND METHODS**

73 **2.1. Chemicals and standards**

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

76 **2.2. Yeast strains and Filamentous fungus**

77 Two industrial *Saccharomyces cerevisiae* strains were used in this study: *S. cerevisiae* P29 (Spanish
78 Type Culture Collection, CECT 11770), a wine yeast isolated in the Catalan Institute of Vine and
79 Wine (Vilafranca del Penedés, Spain) from Catalonia vineyards, and *S. cerevisiae* Enoferm QA23
80 (Lallemand, Montreal, Canada). Both yeast strains were selected on the basis of their working ability
81 under the absence of oxygen and from media with low availability of assimilable nitrogen as are the
82 conditions for producing sparkling wines (Puig-Pujol, et al., 2013). The filamentous fungus used for
83 cell immobilization was *Penicillium chrysogenum* strain H3 isolated from the environment by the
84 Viticulture and Enology research group from the University of Cordoba and identified by the
85 Spanish Type Culture Collection (CECT).

86 **2.3 Cell immobilizations**

87 Calcium alginate beads of the two yeast strains were made in the laboratory from the department of
88 Enological Research (Institute of Agrifood Research and Technology–Catalan Institute of Vine and

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

104 **2.4. Sparkling wine elaboration**

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

113 14°C in the dark. Four bottles of each batch were riddled and disgorged for performing all the
114 analysis.

115 **2.5. Enological parameters**

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

125 **2.6. Aroma compounds.**

126 **2.6.1. Extraction.**

127 The extraction was carried out according to (Tredoux, et al., 2008) with minor changes. The wine
128 sample was diluted in a proportion 1:10 with a hydro ethanolic solution containing 12% ethanol (v/v)
129 and which was previously adjusted to pH 3.5 with 2.6 g/L tartaric acid and 2.2 g/L potassium
130 bitartrate. A stir bar (0.5 mm film thickness, 10 mm length, Gerstel GmbH, Mulheim an der Ruhr,
131 Germany) coated with PDMS was placed in a 10 mL glass headspace vial containing 10 mL of the
132 diluted sample and 0.1 mL of a solution of ethyl nonanoate (0.45 mg/L) as internal standard. The vial
133 was sealed with a Teflon-coated crimp cap. The stir bar was stirred at 1500 rpm at 25 °C for 100
134 min. After removal from the wine sample, the stir bar was gently dried with a lint-free tissue and
135 then transferred into a glass thermal desorption tube for GC-MS analysis.

136 **2.6.2. Determination.**

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

144 The GC was fitted with an Agilent-19091S capillary column 30 m×0.25 mm i.d., 0.25-µm film
145 thickness. Helium was used as carrier gas with a column flow rate of 1 mL min⁻¹. The GC oven
146 temperature was programmed as follows: 50 °C for 2 min, ramped at 4 °C min⁻¹ to 190 °C, held for
147 10 min. The mass detector was used in the scan mode and the studied mass range spanned values
148 from 39 to 300 m/z. Retention times, spectral libraries supplied by Wiley (version 7 N) and pure
149 chemical compounds were used for identification and confirmation of the volatile compounds. Each
150 compound was quantified from its calibration curve, which was obtained by using standard solutions
151 of known concentrations previously subjected to the same treatment as the samples in conjunction
152 with the target and qualifier ions selected for each compound by the Hewlett– Packard Chemstation
153 (Palo Alto, CA).

154 **2.7. Calculation of aromatic series.**

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

162 series. The same compound can be included in one or several aromatic series, in agreement with its
163 aromatic descriptors.

164 **2.8. Sensory analysis**

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

176 **2.9. Statistical analysis.**

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

186 **3. RESULTS AND DISCUSSION**

187 **3.1. Influence of immobilized yeasts on the composition of aged sparkling wines.**

188 Table 1 shows the values obtained for the most important variables of sparkling wines produced by
189 free yeast, yeasts immobilized in alginate beds and biocapsules after 32 months of aging under lees.
190 Only the QA23 *S. cerevisiae* strain immobilized in alginate beds showed significant differences in
191 the ethanol concentration. The alginate format also showed the highest reducing sugar content in
192 both yeast strains highlighting the QA23 *S. cerevisiae* strain. This fact was yet observed when
193 sparkling wine was analyzed after 10 months of aging (Puig-Pujol, et al., 2013). These authors also
194 detected high sugar content in wines obtained with the QA23 strain used as biocapsules in one of the
195 experimental conditions. Both events, as is our case, were attributed to different fermentation
196 efficiencies among the strains and their inoculums format. In our study, after 36 months of aging, all
197 wines can be categorized as dry wines according to the residual sugar content. In addition, the low
198 volatile acidity of the wines and the sensory analysis carried out show that there were not stability
199 problems in any of the analyzed wines. No differences were observed for pH, total acidity and malic
200 acid among the different batches of wine.

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

206 Regarding to the different forms of nitrogen, it has been observed that sparkling wines produced with
207 cells immobilized in alginate showed the highest concentration of yeast available nitrogen (YAN,
208 sum of ammonium ion and free amino nitrogen). This characteristic is related with the highest
209 foamability (Hm) value found in these batches (Coelho, Rocha, & Coimbra, 2011). It has also been
210 described that the addition of a clarifying agent, such as bentonite, to facilitate the riddling process

211 when free yeast cells are used, decreases the content of nitrogen due to an absorption phenomenon
212 which reduces the quality of the foam in the resulting wine (Vanrell, et al., 2007).

213 **3.2. Volatile aroma compounds and multivariate analysis of variance.**

214 Table 2 shows the concentration of the volatile aroma compounds analyzed in the sparkling wine
215 (Cava). The concentration of a given compound could be dependent on the yeast strain, on their way
216 of use (free, bioimmobilized and immobilized in alginate) or on both factors. For these reason a
217 multivariate analysis of variance has been carried out and the results are also showed in Table 2. Of
218 importance were octanoic acid, decanoic acid and hexanol with concentrations above 1mg/L.

219 Amongst the chemical families, short chain fatty acids shows the highest concentrations, ranging
220 from 21 to 30 mg/L although this concentration is conditioned by the high amount of octanoic acid.
221 These compounds are released to the wine during aging due to the yeast lysis (Buxaderas & López-
222 Tamames, 2010). Furthermore, a high number of esters were detected. These compounds have
223 pleasant aromas and usually low perception threshold (Table 2), so their contribution to wine aroma
224 could be relevant. Esters are also the family with the highest number of volatile compounds and their
225 concentration have been described to change with the aging time (Alexandre & Guilloux-Benatier,
226 2006). Also, depending on the physicochemical characteristic of the volatile compound and on the
227 less cell wall, yeast can absorb some kinds of esters (Gallardo-Chacón, et al., 2010). Most of ethyl
228 esters depend on the yeast strain and on the way of use (Table 2). Wines produced with the P29
229 strain show an overall ester concentration higher than those obtained with the QA23 strain. Although
230 no differences due to the immobilization method were observed in wines produced with the P29
231 strain this was not the case with the QA23 strain.

232 Two C-13 norisoprenoids, namely vitispirane and TDN (1,1,6-trimethyl-1,2-dihydro naphthalene)
233 were detected in Cava wines. Vitispirane has a megastigme precursor and is linked to a sugar
234 molecule. TDN is produced by the degradation of carotene (Moreno & Peinado, 2012). These
235 compounds appear in long aged sparkling wines (Riu-Aumatell, et al., 2006) and depend on the way

236 the yeast is used, and the wines obtained with bioimmobilized yeast showed the highest
237 concentration. TDN concentration also depended on the yeast strain, showing the highest values the
238 Cava obtained with the P29 strain.

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

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

246 **3.3 Aromatic series.**

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

261 comprise the same volatile compounds. In addition, this procedure greatly reduces the number of
262 variables to be processed and allows changes during the winemaking of sparkling wine to be
263 assessed in terms of several odor descriptors.

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

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

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

279 **3.4. Statistical treatment.**

280 **3.4. 1. Multivariate analysis.**

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

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

293 **3.4.2. Principal component analysis.**

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

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

304 **3.5. Sensory analysis**

305 The sensory analysis of the sparkling wines obtained by two different *S. cerevisiae* strains and three
306 inoculum formats was performed by evaluating the global organoleptic quality through a descriptive
307 test to define differences among the samples. Figure 3 shows the radar diagrams for each strain. It
308 can be observed that the tasters evaluated similarly the three batches of Cava wines fermented with
309 the strain P29, regardless of whether it was used immobilized into a support or with free form. In the
310 case where *S. cerevisiae* QA23 was used, the results showed a significant difference ($p < 0.05$) in the

311 aroma quality descriptor among wines made with immobilized yeasts and those made with free cells
312 when ANOVA was performed. Although no differences were detected in the remaining attributes, it
313 is interesting to note that the best scores for odor descriptors were for sparkling wines elaborated
314 with immobilized yeasts. These compounds form part of the bouquet of the wine and were positively
315 appreciated by the tasters. These sparkling wines also had the lowest yeasty aroma, a negative
316 descriptor when it reaches high values.

317 **3. 6. Conclusions.**

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

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338 **5. CONFLICT OF INTEREST.**

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

341

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- 447

448 **Figure legends**

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

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

454 **Figure 3.** Sensory analysis of sparkling wines made with *S. cerevisiae strain* P29 (a) or *S. cerevisiae*
455 strain QA23 (b) obtained by the mean of the scores given by the panellists for each descriptor. Red
456 line: bioimmobilized. Blue lines immobilized in alginate. Green line: free cells.

457 (*) Significant differences among free cells and immobilized formats: alginate and biocapsules at
458 $p < 0.05$.

459

460

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

Table 2. Volatile aroma compounds ($\mu\text{g/L}$, 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 Way of use	<i>S. cerevisiae</i> P29			<i>S. cerevisiae</i> QA23			Effect			OPT	Aroma descriptor	AS
	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, caramelize	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<0.05; 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, 2016); 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	<i>S. cerevisiae</i> P29			<i>S. cerevisiae</i> QA23			Effect		
	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 $p < 0.05$; ns: no significant interaction.

Figure 1.

Figure 1a.

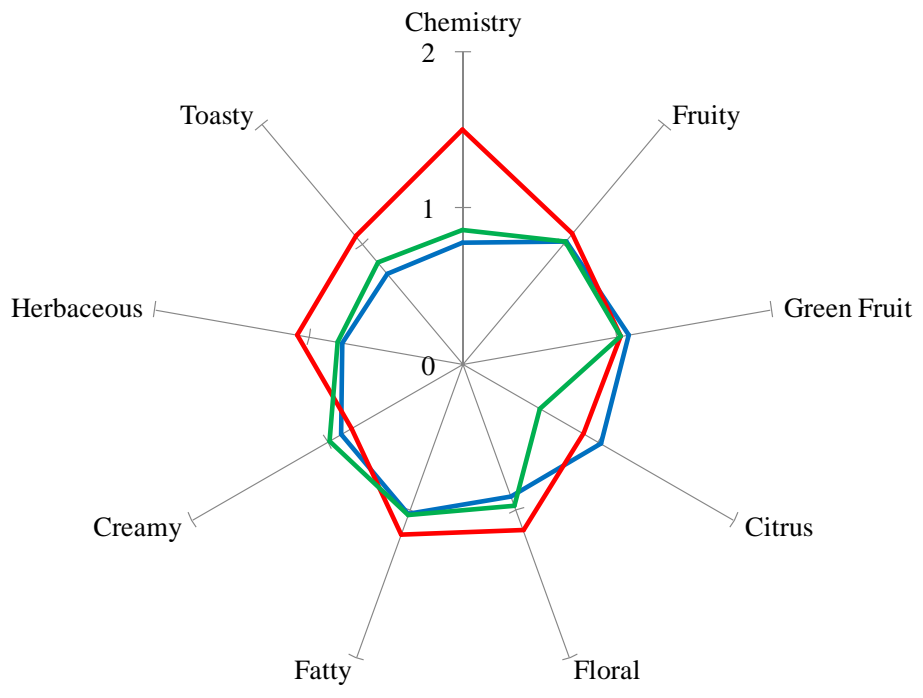


Figure 1b

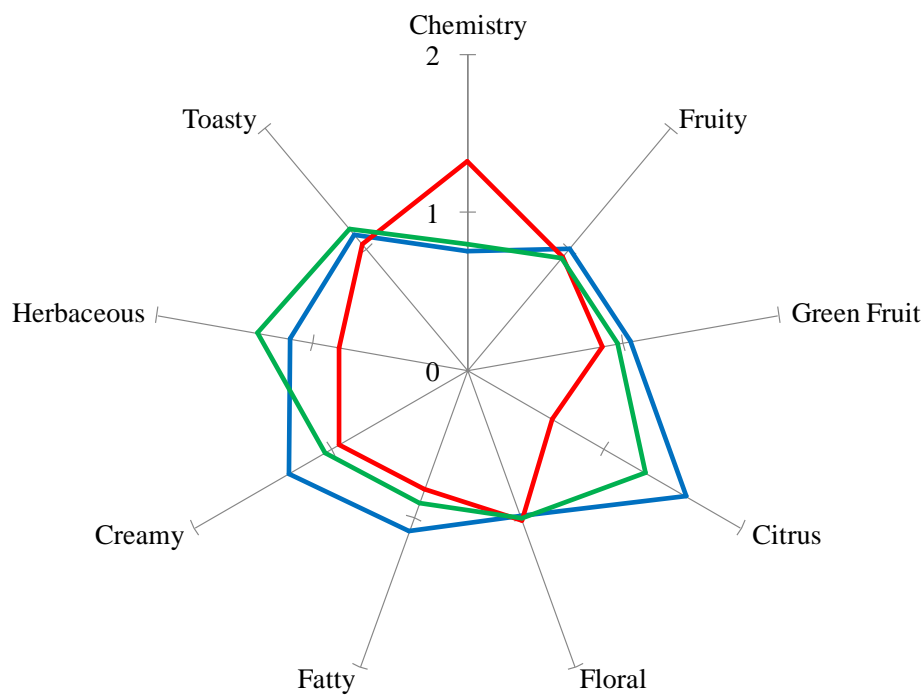


Figure 2.

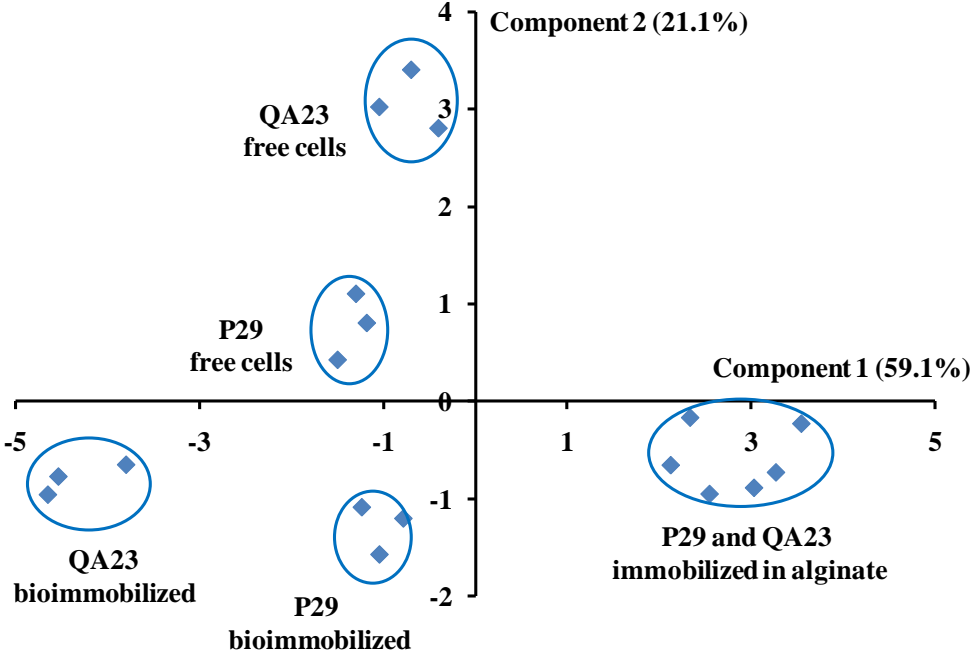


Figure 3.

Figure 3a.

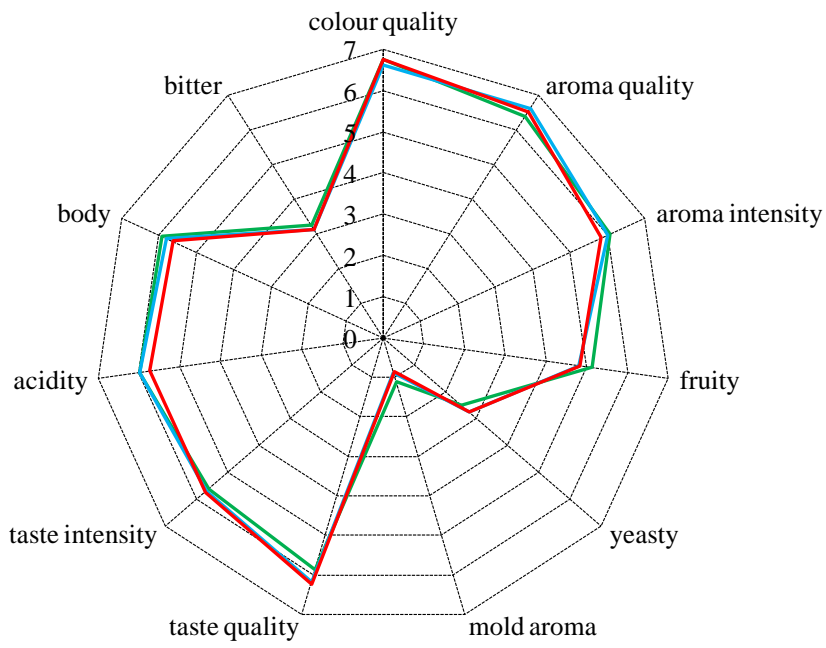


Figure 3b.

