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Application of multi-pass high pressure homogenization under variable temperature regimes to induce autolysis of wine yeasts

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Abstract: The effects of the number of passes and processing temperature management (controlled vs uncontrolled) were investigated during high pressure homogenization-induced autolysis of *Saccharomyces bayanus* wine yeasts, treated at 150 MPa. Both variables were able to affect cell viability, and the release of soluble molecules (free amino acids, proteins and glucidic colloids), but the effect of temperature was averagely more evident. *S. bayanus* cells were completely inactivated in 10 passes without temperature control (corresponding to a processing temperature of 75 °C). The two processing variables also affected the volatile composition of the autolysates produced: in particular, higher temperatures led to a lower concentration of volatile compounds. The management of the operating conditions, may allow to modulate the compositional characteristics of the products obtained, making them suitable for different winemaking applications.

Cover Letter

Yeast derivatives (YDs) are perhaps the most used enological products in the wineries, after active dry yeasts. They are basically inactive dry yeasts or yeast autolysates, promoted by the suppliers for a wide series of applications: fermentation enhancers, flavor and aroma modulators, mouthfeel enhancers, scavengers against undesired compounds and antioxidant preparations.

Despite their wide utilization, very few commercial YDs are specifically tailored for winemaking use and winemakers are often forced to use formulations developed for other food products. One of the most critical points is that, in food industry, these additives are generally used as flavoring and aromatizing agents, so that they may release off-flavors when they are added into the wine. These off-flavors are mostly connected with the thermal treatments that occur during manufacturing (e.g. compounds from Maillard reaction or oxidative breakdown of lipids). For this reason, the development of non-thermal alternative processes for the obtainment of such products may open new opportunities for the production of commercial preparations suitable for winemaking use.

In a previous paper, we have demonstrated the potential of high pressure homogenization (HPH) for inducing autolysis of wine yeasts. This manuscript aims to investigate the effects of the number of passes and processing temperature management (controlled vs not controlled) on yeast autolysis induction, as well as on the characteristics of the yeast autolysates produced (e.g. release of free amino acids, colloids, and volatile compounds).

HPH demonstrated to be an interesting technique from this point of view, because it may allow different winemaking applications, such as the production of specific preparations of yeast derivatives, to be used either as yeast nutrient supplement, or for wine storage and ageing, depending on the processing conditions. Moreover, HPH appeared also a promising tool, for the direct application in the wineries, with the purpose of shortening the time needed for *sur lie* maturation. As far as we know, this is currently one of the few papers dealing with these aspects.

Highlights

Number of passes and process temperature affect HPH-induced autolysis of wine yeasts

S. bayanus cells were inactivated in 10 passes without temperature control at 75 °C

Temperature control more effective than multi-pass for release of soluble molecules

Lower temperatures led to higher concentration of ethyl esters and aroma compounds

Different operating conditions allow to modulate the composition of the autolysates

1 **Application of multi-pass high pressure homogenization**
2 **under variable temperature regimes to induce autolysis of**
3 **wine yeasts**

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15

16 **Abbreviated running title**

17 Yeast autolysis by different high pressure homogenization conditions

18

19 **Abstract**

20 The effects of the number of passes and processing temperature management (controlled vs
21 uncontrolled) were investigated during high pressure homogenization-induced autolysis of
22 *Saccharomyces bayanus* wine yeasts, treated at 150 MPa. Both variables were able to affect
23 cell viability, and the release of soluble molecules (free amino acids, proteins and glucidic
24 colloids), but the effect of temperature was averagely more evident. *S. bayanus* cells were
25 completely inactivated in 10 passes without temperature control (corresponding to a
26 processing temperature of 75 °C). The two processing variables also affected the volatile
27 composition of the autolysates produced: in particular, higher temperatures led to a lower
28 concentration of volatile compounds. The management of the operating conditions, may allow
29 to modulate the compositional characteristics of the products obtained, making them suitable
30 for different winemaking applications.

31

32 **KEYWORDS: high pressure homogenization; *Saccharomyces*; autolysis; wine; inactive**
33 **dry yeasts**

34

35 1 Introduction

36 Inactive dry yeasts (IDY) and yeast autolysates (also known as yeast derivatives) are a group
37 of important winemaking additives, used for different purposes, including their ability to
38 provide substances which are naturally released into the wine, during the traditional practice
39 of *sur-lie* maturation (Pozo-Bayón, Andújar-Ortiz, & Moreno-Arribas, 2009). As well known,
40 during ageing on the lees, natural yeast autolysis occurs and wine progressively enriches in
41 important compounds, such as peptides, sterols, amino acids and mannoproteins (Pérez-
42 Serradilla & Luque de Castro, 2008). Despite these interesting modifications, *sur-lie*
43 maturation is a slow process and during the several months required for wine evolution, wine
44 itself may be subjected to non-negligible risks of microbiological spoilage and sensory
45 modifications (Pozo-Bayón et al., 2009).

46 The ability of yeast derivatives to increase wine colloidal content (Pozo-Bayón et al., 2009),
47 their capacity to interact with aroma compounds modulating their volatility (Comuzzo, Tat,
48 Fenzi, Brotto, Battistutta, & Zironi, 2011) and their claimed antioxidant capacity (Rodríguez-
49 Bencomo et al., 2014; Comuzzo, Battistutta, Vendrame, Páez, Luisi, & Zironi, 2015b) suggest
50 the use of these products as potential alternatives of *sur-lies* ageing. Unfortunately,
51 commercial IDYs may often negatively affect wine sensory characters, by releasing off-
52 flavors (Comuzzo, Tat, Tonizzo, & Battistutta, 2006), formed because of the high
53 temperatures to which they are subjected during manufacturing (Münch, Hofmann, &
54 Schieberle, 1997). For this reason, a process allowing to speed up yeast autolysis, reducing
55 the heating during manufacturing, may be helpful for the production of yeast derivatives
56 specifically tailored for winemaking use and to limit the release of such off-flavors into the
57 wine.

58 High pressure homogenization (HPH) is a mechanical processing method, used since different
59 decades to extract intracellular components (i.e. proteins and enzymes) from

60 microorganisms, including *Saccharomyces* cells (Follows, Hetherington, Dunnill, & Lilly,
61 1971; Hetherington, Follows, Dunnill, & Lilly, 1971). It is known that HPH may induce cell
62 disruption, through cavitation, shear and turbulence phenomena that occur when yeast
63 suspension is forced through the narrow gap of the homogenizer valve (Popper & Knorr,
64 1990). Despite the ability of HPH in promoting *Saccharomyces* cell breakdown is well
65 documented, very few publications deal with the effects of such technology in inducing the
66 autolysis of wine yeasts (Patrignani et al., 2013), or with its use for the production of yeast
67 derivatives (Comuzzo, Calligaris, Iacumin, Ginaldi, Palacios Paz, & Zironi, 2015a).

68 In a recent paper (Comuzzo et al., 2015a), the potential of HPH for the production of
69 autolysates for winemaking use, was examined. In particular, the study focused on the effects
70 of pressure level (from 0 to 150 MPa) on the viability of yeast cells, the release of soluble
71 molecules and colloids in wine-like solution (indices of autolysis occurrence), and the volatile
72 composition of the autolysates produced. Results showed the good potentialities of this
73 technique to obtain yeast derivatives with interesting volatile composition. At the same time,
74 the tested conditions did not allow the complete inactivation of yeast cells leading to the need
75 of a processing optimization.

76 In the present research, the effects of the number of HPH passes at 150 MPa and processing
77 temperature (controlled vs not controlled) on the characteristics of a yeast autolysate were
78 investigated. In particular, cell viability, the content of glucidic colloids (e.g. mannoproteins),
79 free amino acids and soluble proteins as well as the volatile composition of the autolysates
80 obtained were determined, with the purpose of finding evidences on how HPH processing
81 variables may potentially affect the composition and the performances in wine of the products
82 obtained.

83 **2 Materials and Methods**

84 2.1 *Reagents and materials*

85 Bacteriological peptone and Malt Extract Agar were purchased from Oxoid (Basingstoke,
86 UK). Sodium hydroxide, boric acid, sodium chloride, copper sulfate pentahydrate, sodium
87 tartrate, sodium carbonate, tartaric acid and ethanol (96 % v/v) were from Carlo Erba
88 Reagents (Milan, Italy). Bovine serum albumin (BSA) fraction V, *o*-phthaldialdehyde, *N*-
89 acetyl-L-cysteine, Folin-Ciocalteu's phenol reagent and HPLC grade isoleucine (Ile) were
90 obtained from Sigma-Aldrich (St. Louis, MO, USA). Milli Q grade water was produced by a
91 Milli-Q Advantage A10 apparatus (Merck Millipore, Billerica, MA, USA) and microfiltered at 0.2
92 µm before use. Commercial *Saccharomyces bayanus* (Mycoferm Cru-05 active dry yeast
93 preparation) was from EverIntec (Pramaggiore, VE, Italy).

94 2.2 *High pressure homogenization treatments*

95 Aliquots of 40 g of active dry yeast preparation (ADY) were suspended in 400 ml of Milli Q
96 grade water, in sterile Erlenmeyer flasks. After 10 min, to allow the yeast rehydration, the
97 samples were processed by using a two stage high pressure homogenizer (Panda PLUS 2000,
98 Gea Niro Soavi, Parma, Italy) provided with two cylindrical tungsten carbide homogenizing
99 valves. The first one, was set at 150 MPa, whereas the second was fixed at 5 MPa. Samples
100 (400 ml) were homogenized at 150 MPa and a flow rate of 10.0 l h⁻¹, *via* 1, 4, 6 and 10 passes.
101 Two different sets of experiments were carried out: the first one was without any temperature
102 control, whereas in the second, the homogenizer inlet and outlet were connected to a heat
103 exchanger (Julabo F70, Julabo GmbH, Seelbach, Germany) set at 4 °C, to avoid the heating of
104 the suspensions during processing. Sample temperature was measured before and after
105 homogenization, by a copper-constantan thermocouple (Ellab, Hillerød, Denmark) connected
106 to a portable data logger (mod. 502A1, Tersid, Milan, Italy). All the experiments were carried
107 out in three repetitions.

108 Before each homogenization treatment, the equipment was subjected to three different
109 washing steps in order to avoid the presence of residual (viable) yeast cells and reduce the risk
110 of cross contamination between the different samples. The first washing cycle was a rinsing
111 with microfiltered Milli Q grade water (1 l), then a mixture of water: ethanol 1:1 v/v (1 l) was
112 circulated, and subsequently, a final rinsing with microfiltered Milli Q water (1 l) was carried
113 out. The operating conditions used for such three washing steps have been determined to not
114 have any viable cell detectable in the water collected after the final rinse.

115 After homogenization, 10 ml of each treated suspension were collected in sterile Falcon tubes
116 and subjected to microbiological analysis as reported below. The remaining part of the
117 suspension was collected in food-grade aluminum trays (approx. in a 1 cm layer), frozen at -
118 20 °C, and freeze-dried by using a pilot plant model Mini Fast 1700 (Edwards Alto Vuoto,
119 Milan, Italy). At the end of the process, the samples were finally ground in a ceramic mortar
120 and stored in 50 ml sterile Falcon tubes (0/+4 °C), until chemical and GC-MS analyses. As a
121 reference sample, the active dry yeast preparation used for the trials, was subjected to the
122 same analytical determinations reported below.

123 *2.3 Microbiological analyses*

124 One (1) ml of each sample was mixed with 9 ml of saline-peptone water (8 g l⁻¹ sodium
125 chloride, 1 g l⁻¹ bacteriological peptone) in a sterile Falcon tube and vortexed for 1.5 min
126 using a VWR vortex mixer (International PBI, Milan, Italy). Additional decimal dilutions
127 were made in the same solution, and yeast cells were plated in three repetitions on Malt
128 Extract Agar. Plates were then incubated at 25°C for 48-72 h under aerobic conditions and
129 colonies were counted.

130 2.4 *Glucidic colloids*

131 The glucidic colloids soluble in wine-like medium were assessed by ethanol precipitation, by
132 a modified version of the method published by Usseglio-Tomasset & Castino (1975); 1.00 g
133 of freeze-dried powder was suspended in 10 ml of hydroalcoholic-tartaric buffer (12.00 % v/v
134 ethanol in 33 mM tartaric acid, pH 3.20). After 10 min, the suspension was centrifuged (5000
135 rpm for 10 min) and 5 ml of the supernatant was mixed with 25 ml of ethanol 96 % v/v and
136 kept for 24 h at 0/+4 °C. The precipitate of glucidic colloids was separated by vacuum
137 filtration on a 0.45 µm nylon membrane (Albet-Hahnemühle, Barcelona, Spain) and then
138 determined by weighing, after complete evaporation of ethanol (carried out at 50 °C, until
139 constant weight). Results were expressed in mg of total colloids per g of freeze-dried powder.

140 2.5 *Soluble proteins*

141 Soluble proteins were analyzed according to the Lowry assay as reported by Regenstein &
142 Regenstein (1984). One (1.00) g of freeze-dried powder was suspended in 100 ml of
143 hydroalcoholic-tartaric buffer (12.00 % v/v ethanol in 33 mM tartaric acid, pH 3.20). After 10
144 min, the suspension was centrifuged (5000 rpm for 10 min) and 400 µl of the limpid
145 supernatant was transferred in a 10 mm optical path length glass cuvette. A mixture of 2 %
146 w/v sodium carbonate in 0.1 M sodium hydroxide solution (1.96 ml), 1 % w/v copper sulfate
147 pentahydrate (20 µl) and 2 % w/v sodium tartrate (20 µl) was added, and the sample was
148 carefully homogenized. After 10 min at ambient temperature, 200 µl of Folin-Ciocalteu's
149 reagent was introduced and the content of the cuvette was further mixed. After additional 30
150 min, the absorbance of the sample was measured at 750 nm, reading against a blank obtained
151 by replacing the supernatant with 400 µl of Milli Q grade water. Results were given in mg g⁻¹
152 of freeze-dried powder, according to a calibration line prepared with increasing amounts of
153 bovine serum albumin.

154 2.6 *Free amino acids*

155 Free amino acids were determined by OPA derivatization, according to the method published
156 by Dukes & Butzke (1998). Two aliquots (50 μ l each) of the same supernatant used for the
157 analysis of soluble proteins (see Section 2.5) was subdivided in two 10 mm optical path
158 length quartz cuvettes. The first one (cuvette A) was diluted in 3 ml of a solvent mix
159 containing ethanol (100 ml l⁻¹), sodium hydroxide (3.837 g l⁻¹), boric acid (8.468 g l⁻¹) and *N*-
160 acetyl-L-cysteine (0.816 g l⁻¹). The second (cuvette B) was mixed with the same solvent
161 buffer, additionally containing 0.671 g l⁻¹ of *o*-phthaldialdehyde. After 10 min, the absorbance
162 of both cuvettes was measured at 335 nm, reading against Milli Q grade water. The net
163 absorbance of the sample was calculated by subtracting the absorbance of cuvette A,
164 (reference) from that of cuvette B (derivatized sample); the results were expressed in mg g⁻¹
165 of freeze-dried powder, on the basis of a calibration line obtained with isoleucine (Ile).

166 2.7 *SPME-GC-MS analyses*

167 The characterization of the volatile composition of the headspace of the freeze-dried powders
168 was carried out by solid-phase microextraction and gas chromatography. The equipment used
169 was a GC-17A gas chromatograph, coupled with a QP-5000 mass spectrometer (both from
170 Shimadzu, Kyoto, Japan). Two (2.00) g of each freeze-dried sample were introduced in 50 ml
171 amber glass vials and sealed with PTFE/silicone septa. SPME was carried out by a 2 cm
172 length 50/30 μ m DVB/Carboxen/PDMS fiber (from Supelco, Bellefonte, PA, USA), at 40 °C
173 for 15 min. To allow the thermal equilibration of the sample, the vials were pre-conditioned
174 for 15 min before microextraction.

175 GC separation was carried out on a J&W DB-Wax capillary column, 30 m x 0.25 mm i.d.,
176 0.25 μ m film thickness (Agilent Technologies Inc., Santa Clara, CA, USA), according to the
177 conditions reported by Comuzzo et al. (2015a). Electron impact mass spectra were recorded at
178 70 eV and the identification of volatile compounds was carried out by comparison of their₈

179 mass spectra and retention times with those of standard compounds, or by comparison of
180 mass spectrum, with those reported in the mass spectrum libraries Wiley 6 and NIST 107.
181 Linear retention indices were also calculated on the basis of the retention times of *n*-alkanes,
182 and compared with those reported in literature.

183 *2.8 Statistical analyses*

184 Statistical elaborations were carried out by using the specific software Statistica for Windows,
185 Version 8.0 (StatSoft, Tulsa, OK, USA). The values collected for the different parameters
186 analyzed and the absolute areas of the volatile compounds detected in the headspace of the
187 freeze-dried powders were processed by One-way ANOVA and Tukey HSD Test. Significant
188 differences between the samples were assessed at $p < 0.05$. Additionally, concerning SPME-
189 GC-MS analyses, aroma compounds were grouped, sample by sample, according to their
190 chemical class and total absolute areas obtained for each chemical group were analyzed by
191 Principal Component Analysis (PCA).

192 **3 Results and Discussion**

193 *3.1 Effect of HPH treatment on yeast viability*

194 The effects of the different operating conditions on yeast viability are reported in Fig. 1. The
195 number of passes affected the microbial population in both controlled and uncontrolled
196 temperature conditions. However, when the temperature remained below 32°C, the number of
197 viable microorganisms was slightly reduced, and more than 6.0 Log CFU g⁻¹ of viable cells
198 was detected after 10 passages of the yeast suspension into the homogenizer (Fig. 1a). In such
199 conditions, the reduction of yeast population may be attributed mainly to the mechanical
200 stress suffered during the passage of the suspensions through the homogenization valves. The
201 resistance of *Saccharomyces* cells towards high pressure treatments is in agreement with the
202 data reported by Patrignani et al. (2013) and Comuzzo et al. (2015a).

203 Contrary, an intense and progressive reduction of viable yeasts was evident when the
204 temperature was not controlled during processing and increased up to 75 °C (Fig. 1b). It is
205 well known that HPH treatments may provoke an increase of the temperature, in the products
206 subjected to homogenization (Popper & Knorr, 1990). The temperature raise was able to
207 promote an intense cell death, and as it reached values close to 70 °C (6 and 10 passes), the
208 number of viable cells became lower than 2 Log CFU g⁻¹. This correspond to the maximum
209 value recommended by the World Organization of Vine and Wine (O.I.V.), for what concerns
210 the presence of viable cells in the yeast autolysate preparations for winemaking use
211 (Resolution Oeno 496/2013, 2013).

212 Considering the flow rate of the process (10 l h⁻¹), the volumes treated (200 ml) and the
213 number of passes (up to 10), the extent of the heating carried out in the present experiment
214 (75 °C as maximum value for the processing with 10 passes) was, anyway, less intense and
215 prolonged than those normally applied in other thermolytic processes, during which
216 temperatures higher than 75-80 °C were held for some hours (Peat, 1961; Halasz & Lasztity,
217 1991; de la Torre, Flores, & Chong, 1994). The reduction of processing temperature is
218 expected to improve the quality characteristics of the autolysates, which might be less
219 subjected to thermal degradation and production of off-odors deriving from Maillard reaction
220 and lipid oxidation (Ames & McLeod, 1985; Nagodawithana, 1992).

221 Due to the negligible impact of the management conditions (controlled vs uncontrolled) on
222 product temperature, after processing the suspensions via single pass (Fig. 1), the analytical
223 determinations described below were carried out, for samples 1P, only on the three repetitions
224 obtained without temperature conditioning.

225 *3.2 Release of proteins and free amino acids in wine-like solution*

226 To study the performances of HPH-yeast autolysates after processing, they were freeze-dried

227 and suspended in wine-like solution (ethanol 12.00 % v/v, pH 3.20), to assess the release of
228 soluble compounds.

229 Figure 2 reports the average values of soluble proteins and free amino acids, released by the
230 freeze-dried autolysates; as control, data regarding ADY were also reported. In comparison
231 with the levels detected in the ADY preparation, the amount of proteins released in the
232 medium (Fig. 2a) increased significantly, just after the first HPH pass. A further increase of
233 the number of passes, did not determined any statistical difference on protein solubilization,
234 independently on the type of applied temperature regime. Anyway, the samples derived from
235 no temperature controlled process showed a content in soluble proteins, slightly lower than
236 the corresponding samples obtained with temperature control. This can be attributed to the
237 higher extent of heating (Fig. 1), which might have determined a more intense protein
238 denaturation. In terms of concentration, however, the values reported in Fig 2a are quite low,
239 and this might be positive for what concerns the potential use of these autolysates in
240 winemaking, because proteins are reported to bind wine aroma compounds, reducing their
241 volatility and perception (Voilley, Beghin, Charpentier, & Peyron, 1991).

242 The behavior of free amino acids (FAA) reflects that of proteins (Fig. 2b), with some minor
243 differences. The amount of FAA released in model wine increased from a value close to 2 mg
244 g⁻¹ in the ADY preparation, to about 4-6 mg g⁻¹ in the samples processed without temperature
245 control, with no significant effects given by the number of passes. Contrary, in the products
246 obtained with thermal conditioning, the number of passes caused a significant increase of
247 FAA release above the fourth passage through the homogenizer valve. This approximately
248 double concentration of soluble amino acids detected in the autolysates produced in controlled
249 temperature conditions, might be linked with their minor involvement in Maillard reaction, as
250 a consequence of the lower extent of heating registered for this set of samples (Ashoor &
251 Zent, 1984). This observation is interesting, because it seems clear that the opportunity to
252 control or not the processing temperature may affect the composition of the yeast

253 autolysates obtained by HPH: for instance, when the IDY preparation is requested as nutrient
254 supplement to be used during alcoholic fermentation, the processing with temperature control
255 might be advisable for increasing FAA content. Contrary, a high level of free amino acids
256 might be negative in the attempt of using IDYs during wine ageing, because such molecules
257 might become substrate for the growth of unwanted microorganisms, such as wild lactic acid
258 bacteria strains or *Brettanomyces* spp.

259 *3.3 Release of glucidic colloids in wine-like solution*

260 The release of soluble glucidic colloids (SGC) reflects the same general behavior found for
261 free amino acids and proteins (Fig. 3). As previously reported, this group of macromolecules
262 represents basically yeast glycoproteins (Comuzzo, Tat, Liessi, Brotto, Battistutta, & Zironi,
263 2012), and they are considered to positively contribute to wine structure and composition.
264 SGC increased from less than 50 mg g⁻¹ in the ADY preparation, to values higher than 150
265 mg g⁻¹ after one homogenization passage, confirming the capacity of HPH processing to
266 promote cell autolysis (Comuzzo et al. 2015a). The increase of the number of passes
267 determined a progressive increment of the SGC solubilized, particularly in conditions of
268 controlled temperature. Anyway, differently respect to what observed for amino acids and
269 soluble proteins (where the effects of heating seemed more efficient than the number of
270 passes in promoting solubilization), the role of temperature conditioning, on the release of
271 glucidic colloids, appeared less important, and the increase of the number of passages showed
272 a non-negligible effect in promoting the solubilization of these macromolecules. As shown in
273 Fig. 3, the highest concentration detected was higher than 200 mg g⁻¹; this means that,
274 considering the amounts normally used for inactive dry yeast supplementation in winemaking
275 (200-400 mg l⁻¹), the yeast derived product manufactured by HPH in the present conditions,
276 might be able to increase wine colloidal content of 40-80 mg l⁻¹.

277 *3.4 Volatile composition of the freeze-dried powders*

278 Fifty volatile compounds were tentatively identified in the headspace of the autolysates
279 obtained by multi-pass HPH, under different temperature regimes (Table 1).

280 Concerning their origin, some of them are well known yeast metabolites (e.g. alcohols, short-
281 chain fatty acids and ethyl esters); others may arise from the thermal and oxidative
282 degradation of the lipid fraction (γ - and δ -lactones, hexanal, nonanal and certain carboxylic
283 acids) (Nawar, 1969; Grosch, 1987). However, it should be pointed out that the development
284 of Maillard reaction between sugar and proteins is expected to be the main critical factor able
285 to affect the formation of odor compounds in yeast derivatives (Ames & McLeod, 1985;
286 Münch et al., 1997). In effects, some of the compounds reported in Table 1 (furaldehyde,
287 pyrazines and 2-methyl-thiazolidine) may derive exactly from Maillard reaction
288 (Nagodawithana, 1992). Aldehydes, such as 2- and 3-methylbutanal, may be produced from
289 the Strecker degradation of isoleucine and leucine, respectively. Such aldehydes may be
290 oxidized to 3-methylbutanoic and 2-methylpropanoic acid (Ames & McLeod, 1985), which
291 have been detected as some of the most abundant compounds in the volatile composition of
292 yeast derivatives, and may be responsible of off-flavors perceivable in wine (Comuzzo et al.,
293 2006).

294 The results of Principal Components Analysis (PCA), carried out after grouping all the aroma
295 compounds on the basis of their chemical class, are reported in Fig. 4. The projections of
296 variables and cases highlight that ADY preparation was different respect to the yeast
297 autolysates obtained after HPH. Moreover, among high pressure treated samples, it was
298 possible to distinguish two different groups, depending on the mode of temperature
299 management. In particular, the powders obtained under controlled temperature conditions
300 (4P_TC, 6P_TC, 10P_TC) and the sample 1P, appeared richer in alkyl-pyrazines, alcohols
301 and esters. Contrary, samples processed without thermal control (4P, 6P, 10P) had a less
302 intense volatile profile, closer to that shown by the original ADY preparation, and this₁₃

303 might be due to a more intense volatilization connected to the higher extent of heating
304 registered for these samples.

305 ANOVA results confirm these considerations (Table 2); the main differences among the
306 treatments were related for the most, to alcohols, ethyl esters, alkyl-pyrazines, fatty acids and
307 carbonyl compounds. Higher Alcohols were not present in significant concentrations in the
308 active dry yeast, whereas their content increased even after a single-pass treatment. This is
309 likely connected with the ability of HPH to induce autolysis (Comuzzo et al., 2015a),
310 promoting in such a way the release of these compounds. ANOVA and Tukey HSD Test well
311 highlighted the differences among the samples. In particular, the content of alcohols showed a
312 tendential decrease, as the temperature and the number of passes increased, and the powders
313 obtained without thermal conditioning had a lower concentration of such molecules in their
314 headspace. This might be due to volatilization phenomena, or to their thermal degradation,
315 both induced by heating. Exceptions to this trend were represented by 1-hexanol and 2-ethyl-
316 1-hexanol, whose concentration appeared to be greater in ADY and in some of the samples
317 processed without thermal control.

318 Ethyl esters were tentatively identified in the products obtained by single-pass treatment,
319 confirming that these compounds may be released during the autolytic process (Alexandre
320 and Guilloux-Benatier, 2006). The samples obtained with temperature control did not show
321 any significant difference from the sample 1P, while the powders produced without thermal
322 conditioning were characterized by a statistically lower content of esters. According to this
323 trend, the esters concentration appeared to be more affected by heating, rather than by the
324 number of homogenization passes, even if a certain reduction of the average values was
325 observed, as the number of cycles increased (Table 2).

326 The concentrations found for alkyl-pyrazines also highlighted some minor differences among
327 the samples. Pyrazines were not detected in ADY preparation, while they were found in HPH
328 treated samples, for most of them, even after one single pass. The increase of the number of

329 passes did not significantly modify their amount, while the effect of processing temperature
330 on such heterocyclic compounds was more evident, and it was similar to that observed for
331 alcohols and esters. In fact, even if without broad statistical confirmations, pyrazines were
332 averagely present in greater extent in the samples produced with thermal conditioning, and in
333 lower amounts in those obtained without temperature control. This behavior, became
334 statistically significant in the case of 2,3,5-trimethylpyrazine (the most abundant, tentatively
335 identified in the former group of samples but not in the latter), while it was not found for 3-
336 ethyl-2,5-dimethylpyrazine, the sole among such compounds which did not follow this trend.
337 The lower presence of pyrazines in the samples not thermally controlled might be connected
338 with the fact that they might be further involved in subsequent steps of Maillard reaction itself
339 during processing. The same phenomena did not occur in the samples produced by controlling
340 temperature.

341 Few differences were found concerning fatty acids composition. From the statistical point of
342 view, no significant differences were marked among the samples, for the most of the
343 compounds detected, neither as a function of the number of passes, nor depending on the way
344 of temperature management during the process. Unlike, significant differences among the
345 autolysates produced with and without temperature conditioning, were found in the case of 2-
346 ethylhexanoic and octanoic acid. These two compounds were both present in high
347 concentration in the samples obtained with thermal control probably due to the effect of
348 heating on the degradation of lipid components. This trend was found also for hexanoic acid,
349 even if without any statistical confirmation.

350 The carbonyl compounds detected in the powders can be divided in two subgroups: the first
351 one is represented by acetoin, a well known yeast metabolite, while the second includes
352 compounds that are reported as oxidation products of lipids (e.g. hexanal, heptanal or
353 nonanal) (Grosh, 1982) or breakdown derivatives of carotenoids (e.g. 6-methyl-5-heptene-2-
354 one) (Schreier, Drawert, & Junker, 1977). Acetoin level did not show any significant

355 modification induced by varying the processing variables, and the compound was found to be
356 present in higher amounts in the ADY preparation. Contrary, significant differences were
357 found for all the compounds included in the second group of volatiles, highlighting, for all of
358 them, a greater concentration in the powders obtained without temperature conditioning. The
359 number of passes, instead, did not modify substantially the content of such molecules.

360 **4 Conclusions**

361 In conclusion, high pressure homogenization confirmed its ability to induce autolysis of wine
362 yeasts and its potential to be applied in the production of inactive dry yeasts for winemaking.
363 Both the number of passes and processing temperature were able to affect yeast viability and
364 the release of soluble molecules (glucidic colloids, proteins and free amino acids), but the
365 latter appeared more powerful for tailoring the compositional characteristics of the IDYs
366 produced.

367 In the conditions of the present experiment, suitable levels of viable cell inactivation (in
368 compliance with the limits recommended by the O.I.V. in inactive dry yeasts and yeast
369 autolysates for winemaking – Resolution Oeno 496/2013, 2013), were achieved with 6-10
370 homogenization passes, under uncontrolled temperature regime, with a temperature of the
371 yeast suspension after processing, which raised up to 70-75 °C. Such conditions are quite
372 difficult to be scaled-up at industrial level, due to the high number of homogenization steps
373 required, but these results suggest that HPH may anyhow be used in the manufacturing of
374 inactive dry yeasts (e.g. in combination with natural or added enzymes), for making the
375 natural autolytic process faster and more rational.

376 The management of processing temperature could also be a suitable tool for tailoring the
377 composition of the yeast derivatives produced by HPH. An interesting observation, connected
378 to the opportunity to control or not the processing temperature, is related to the release of
379 soluble macromolecules (glucidic colloids in particular) and free amino acids. Being equal

380 the number of passes, higher temperatures only slightly affected the levels of soluble colloids,
381 but the heating led to reduced concentrations of soluble amino acids in the autolysates. This
382 information may be important for the production of IDYs to be used as fermentation
383 enhancers, for which a higher content of soluble amino acids is required: for such application,
384 a suitable temperature control might be recommended.

385 Finally, temperature and number of passes also affected the volatile composition of the yeast
386 derivatives produced; even in this case, the former variable gave effects that were averagely
387 more evident. The autolysates obtained without cooling were less characterized from the point
388 of view of their volatile composition; such minor concentration in aroma molecules could be
389 interesting when IDY supplementation is required for increasing wine colloidal content (e.g.
390 during ageing), without affecting wine sensory profile with the release of exogenous aromas.
391 Contrary, the higher concentration in ethyl esters, detected in the samples obtained with
392 temperature control, may represent an interesting opportunity to simulate the natural
393 enrichment of wine itself in these molecules, during ageing on the lees.

394 Further investigations will be required to assess the potential effects of such yeast autolysates
395 produced by HPH on the colloidal and volatile composition of the wines, in comparison with
396 traditionally produced IDY preparations as well as with commercial IDY products.

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508 **Figure Captions**

509 **Fig. 1.** Viable yeasts in the active dry yeast preparation (ADY) and in the samples obtained by HPH
510 treatment (150 MPa), as a function of the number of passes (1P, 4P, 6P, 10P), in controlled (a) and
511 not controlled temperature conditions (b). Different letters mark significant differences among yeast
512 counts, according to ANOVA and Tukey HSD test ($p < 0.05$). Vertical bars represent standard
513 deviation. For the sample 10P, data are reported in CFU g⁻¹ (<10 CFU g⁻¹).

514

515 **Fig. 2.** Soluble proteins (a) and free amino acids (b) in the active dry yeast preparation (ADY) and
516 in the freeze-dried powders obtained by HPH treatment (150 MPa), as a function of the number of
517 passes (1P, 4P, 6P, 10P), in controlled and not controlled temperature conditions. Different letters
518 mark significant differences according to ANOVA and Tukey HSD test, at $p < 0.05$. Vertical bars
519 represent standard deviations.

520

521 **Fig. 3.** Soluble glucidic colloids in the active dry yeast preparation (ADY) and in the freeze-dried
522 powders obtained by HPH treatment (150 MPa), as a function of the number of passes (1P, 4P, 6P,
523 10P), in controlled and not controlled temperature conditions. Different letters mark significant
524 differences according to ANOVA and Tukey HSD test, at $p < 0.05$. Vertical bars represent standard
525 deviations.

526

527 **Fig. 4.** Results of PCA carried out on the absolute areas of the volatile compounds detected by
528 SPME-GC-MS, and grouped basing on their chemical class. The projection of both cases (samples)
529 (a) and variables (chemical groups) (b) on the factor plane is reported. ADY: active dry yeast; 1P,
530 4P, 6P, 10P: HPH treatments (1, 4, 6 and 10 passes at 150 MPa) without temperature control;
531 4P_Tc, 6P_Tc, 10P_Tc: HPH treatments (4, 6 and 10 passes at 150 MPa) with temperature control.

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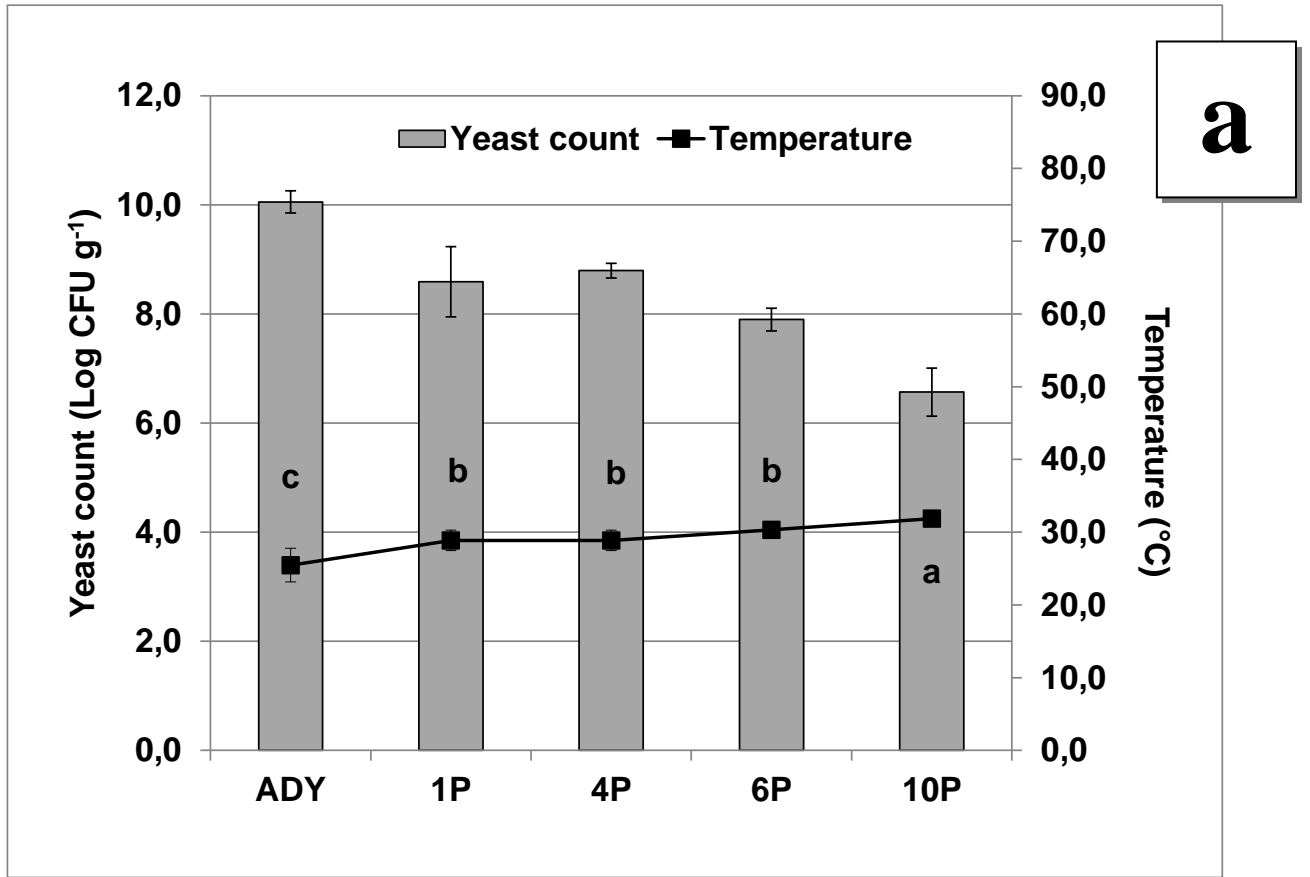
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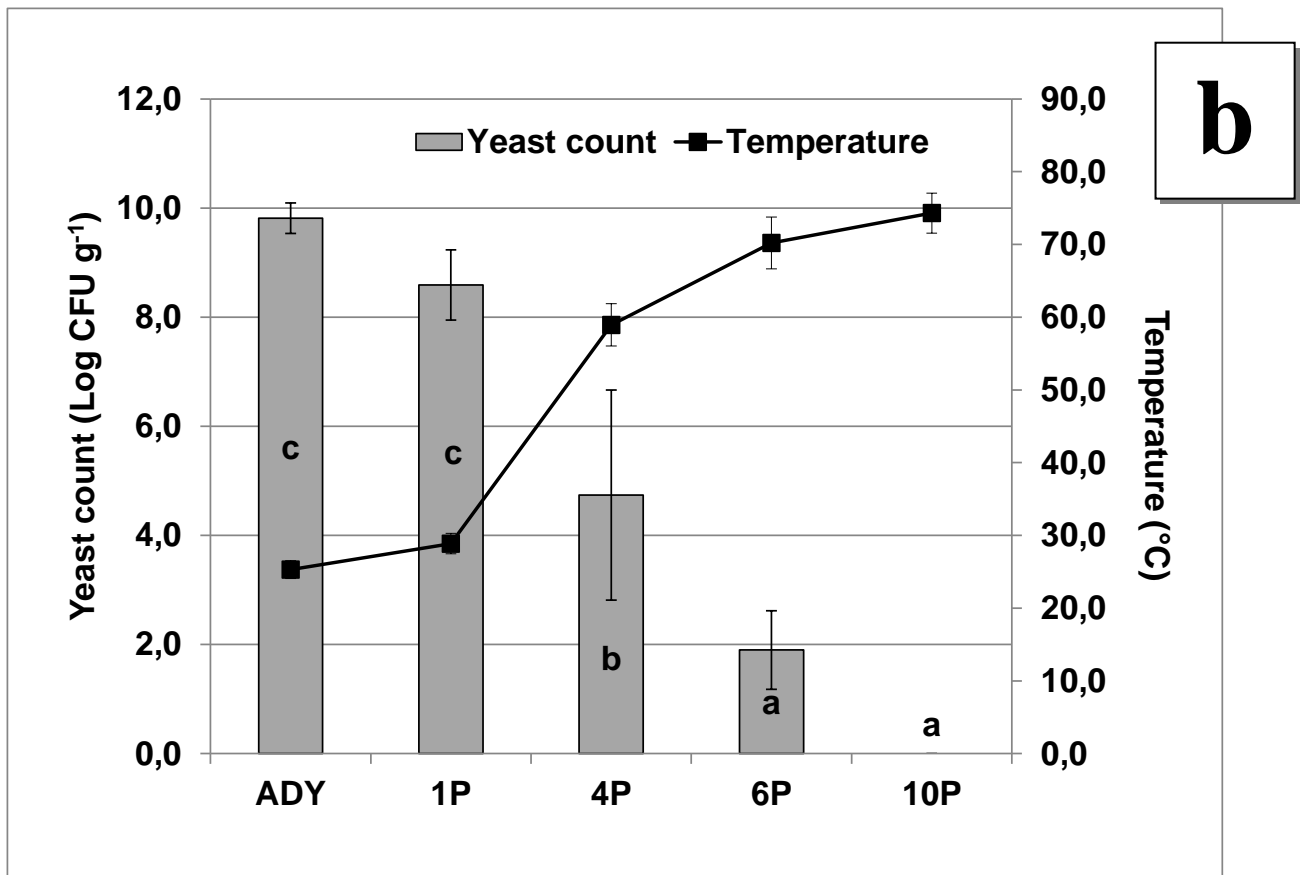
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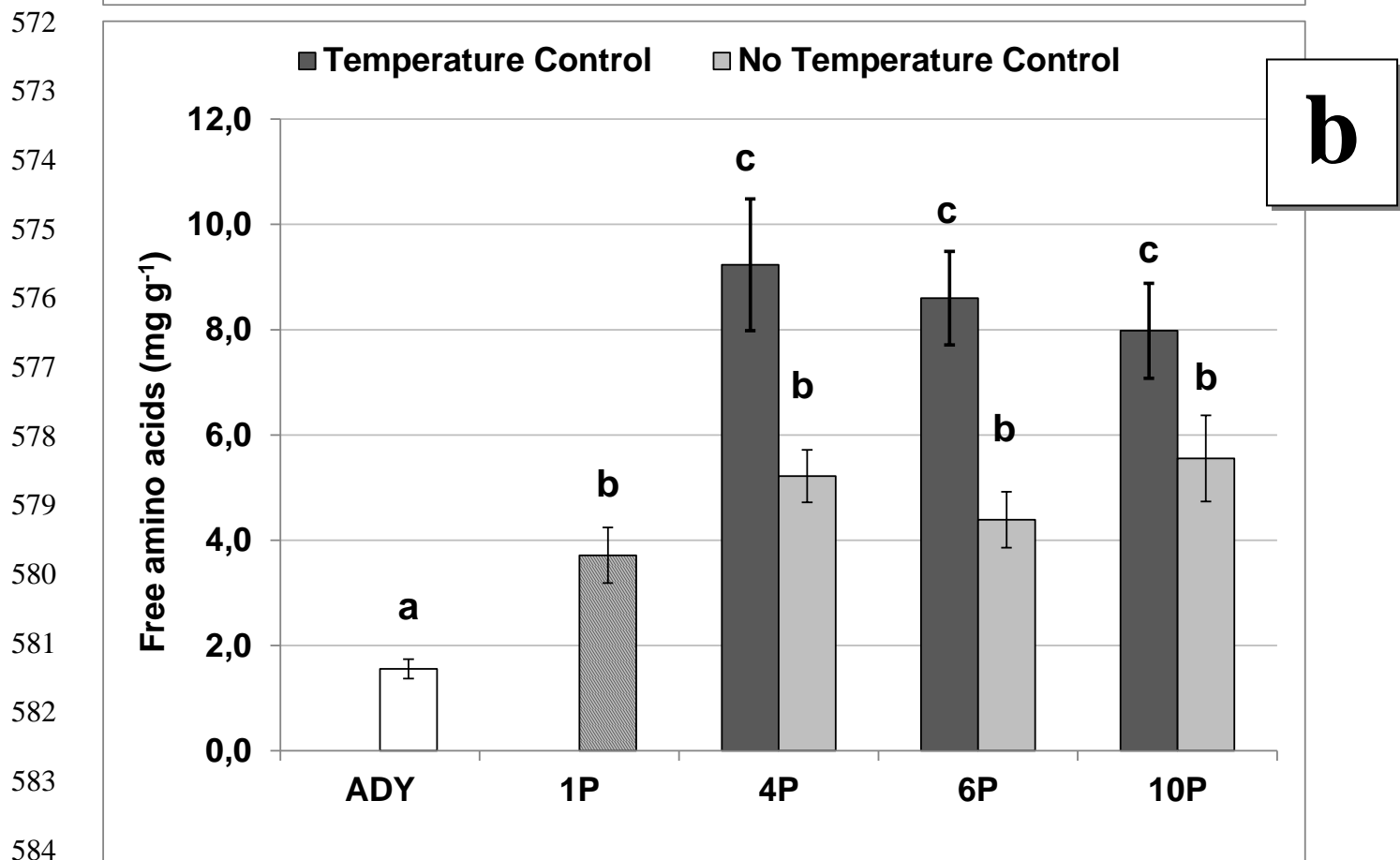
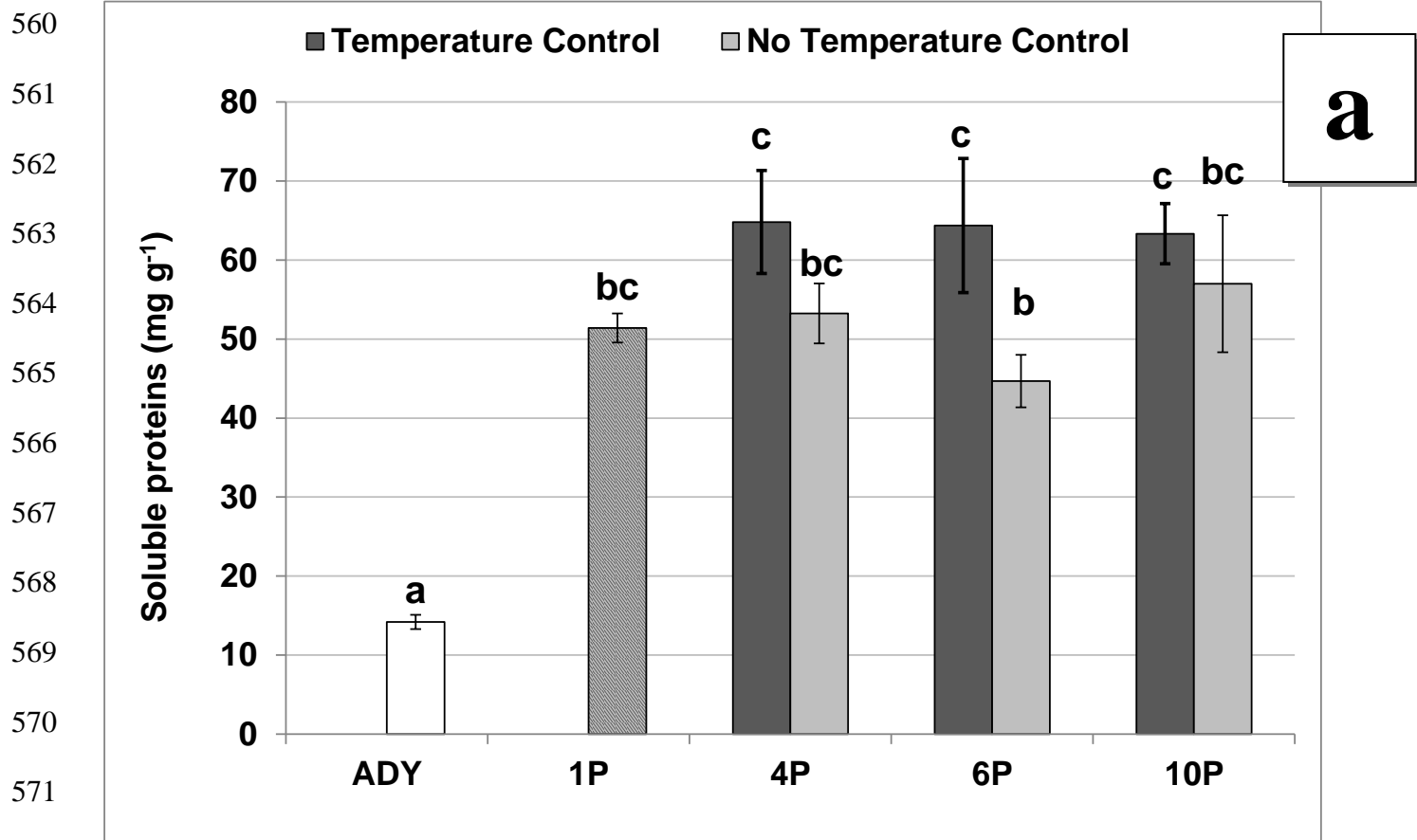
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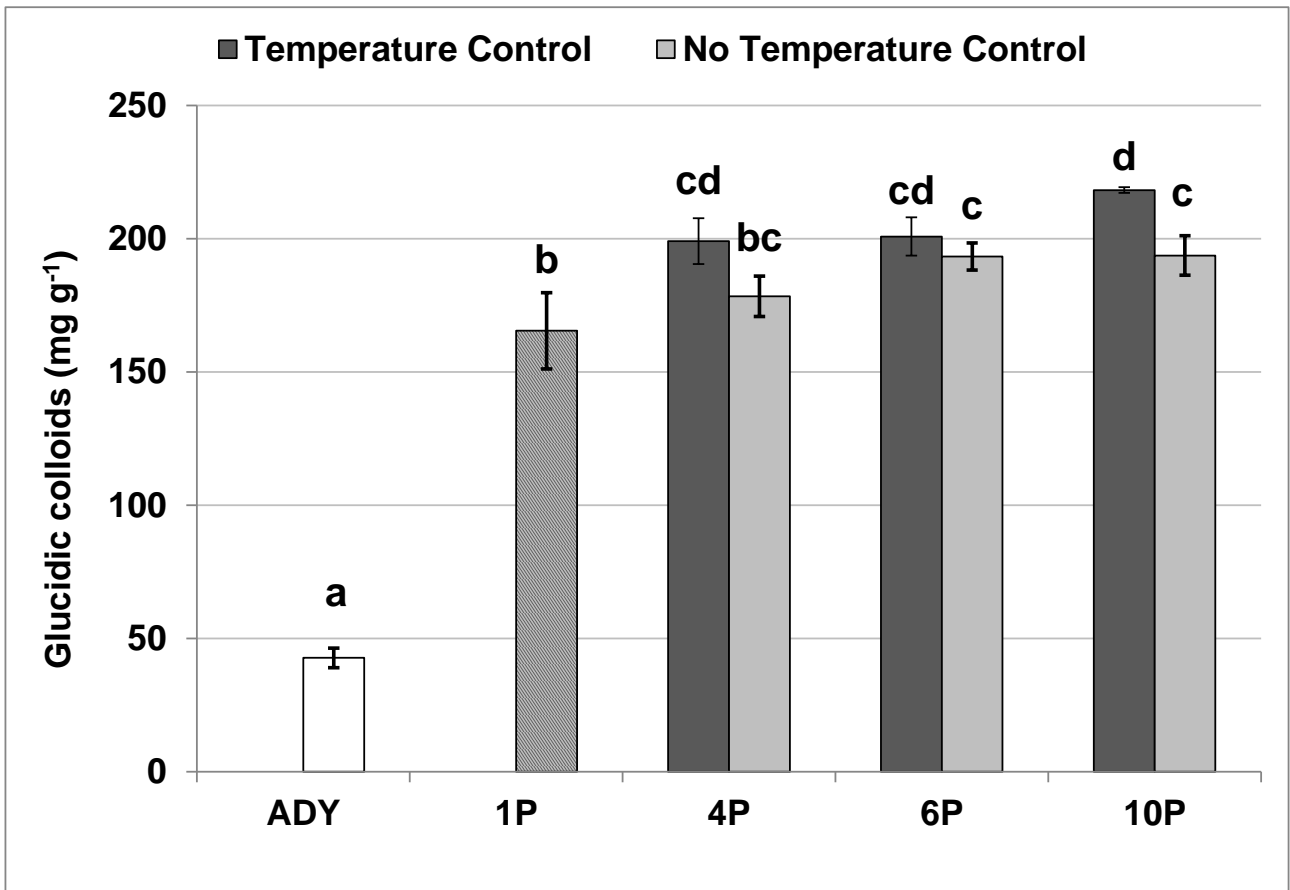
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Fig. 1



585 **Fig. 2**

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Fig. 3

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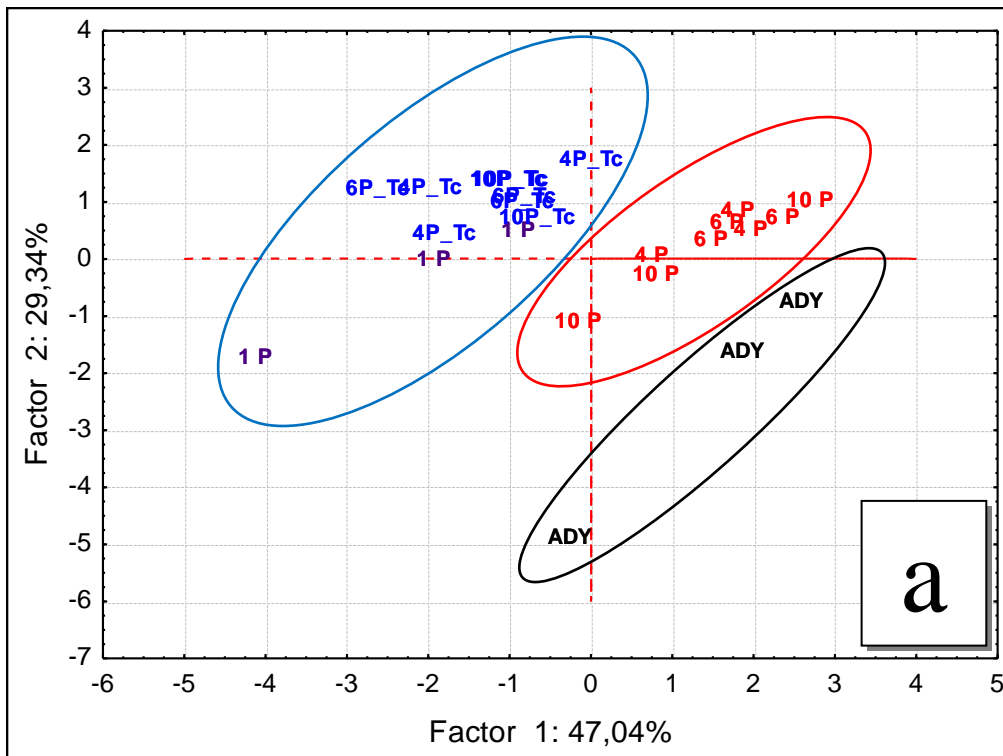
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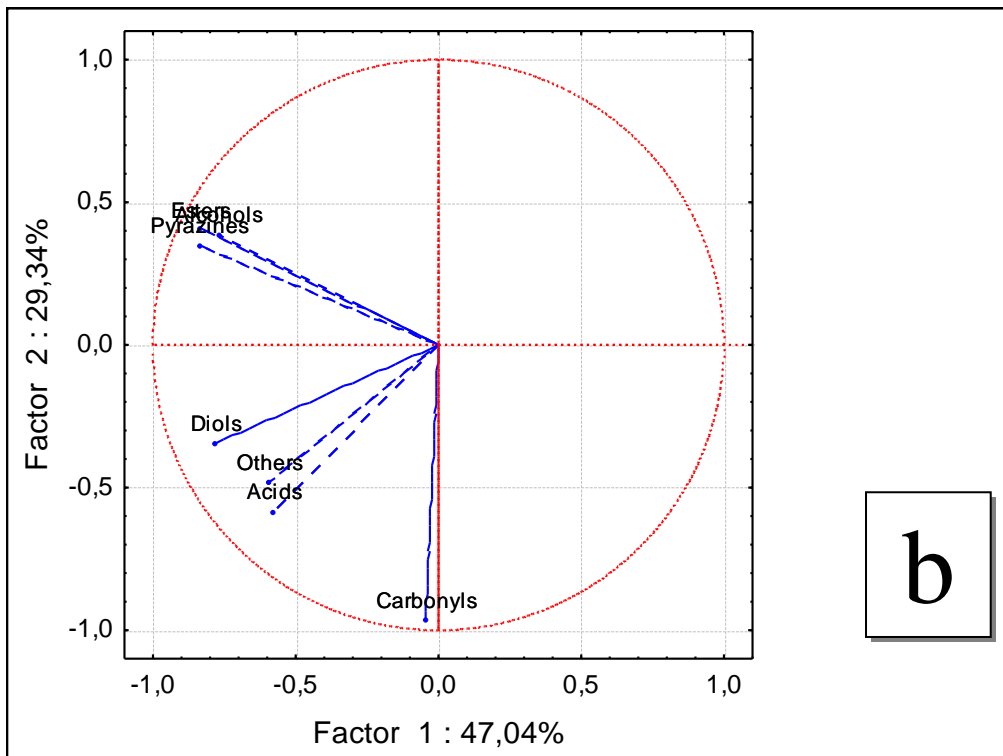
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Fig. 4

621 **Table 1**

622 Volatile compounds tentatively identified by SPME-GC-MS, in the headspace of the samples
 623 analyzed.

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Compound	Ir ^a	Ir _(lit) ^b	Ref. ^c	IM ^d
1 ethyl acetate				MS
2 ethanol	936	929	Acree & Arn, 2016	MS, RI
3 ethyl butanoate	1.038	1.034	Baek & Cadwallader, 1999	MS, RI
4 ethyl 2-methylbutanoate	1.053	1.051	Baek & Cadwallader, 1999	MS, RI
5 ethyl 3-methylbutanoate	1.069	1.082	Cullere et al., 2004	MS, RI
6 hexanal	1.080	1.084	Jennings & Shibamoto, 1980	MS, RI, S
7 2-methyl-1-propanol	1.093	1.096	Lopez et al., 1999	MS, RI
8 1-butanol	1.149	1.145	Baek & Cadwallader, 1999	MS, RI, S
9 β-myrcene	1.162	1.166	Davies, 1990	MS, RI
10 heptanal	1.182	1.184	Goodner, 2008	MS, RI
11 2- and 3-methyl-1-butanol	1.212	1.210	Baek & Cadwallader, 1999	MS, RI, S
12 ethyl hexanoate	1.237	1.234	Baek & Cadwallader, 1999	MS, RI, S
13 unknown	1.252			
14 3-hydroxy-2-butanone (acetoin)	1.281	1.290	Baek & Cadwallader, 1999	MS, RI, S
15 2,6-dimethyl-pyrazine	1.326	1.325	Jennings & Shibamoto, 1980	MS, RI
16 6-methyl-5-hepten-2-one	1.333	1.336	Comuzzo et al., 2015	MS, RI
17 ethyl heptanoate	1.333	1.325		MS, S
18 ethyl lactate	1.341	1.358	Acree & Arn, 2016	MS, RI
19 1-hexanol	1.359	1.359	Lopez et al., 1999	MS, RI, S
20 2-ethyl-6-methylpyrazine	1.382	1.384	Comuzzo et al., 2006	MS, RI
21 nonanal	1.388	1.382	Jennings & Shibamoto, 1981	MS, RI
22 2-butoxyethanol	1.400			MS
23 2,3,5-trimethylpyrazine	1.401	1.408	Comuzzo et al., 2006	MS, RI
24 2-methyl-thiazolidine	1.420	1.415	Yeo & Shibamoto, 1991	MS, RI
25 ethyl octanoate	1.432	1.435	Baek & Cadwallader, 1999	MS, RI, S
26 3-ethyl-2,5-dimethylpyrazine	1.445	1.464	Comuzzo et al., 2006	MS, RI
27 acetic acid	1.448	1.451	Baek & Cadwallader, 1999	MS, RI, S
28 2-furaldehyde (furfural)	1.452	1.475	Lopez et al., 1999	MS, RI, S
29 2,3,5,6-tetramethylpyrazine	1.474	1.458	Ames & McLeod, 1985	MS, RI
30 2-ethyl-1-hexanol	1.493	1.490	Madruza & Mottram, 1998	MS, RI, S
31 benzaldehyde	1.542	1.528	Baek & Cadwallader, 1999	MS, RI, S
32 ethyl 3-hydroxybutanoate	1.514			MS
33 propanoic acid	1.530	1.528	Münch et al., 1997	MS, RI, S
34 2,3-butandiol	1.545	1.545	Baek & Cadwallader, 1999	MS, RI
35 2-methylpropanoic acid	1.567	1.548	Münch et al., 1997	MS, RI, S
36 1,2-propandiol	1.582	1594	Wong & Bernhard, 1988	MS, RI
37 dihydro-5-methyl-2(3H)-furanone (γ-valerolactone)	1.595	1617	Jennings & Shibamoto, 1981	MS
38 dihydro-2(3H)-furanone (γ-butyrolactone)	1.618	1.632	Jennings & Shibamoto, 1981	MS, RI, S
39 butanoic acid	1.627	1.612	Münch et al., 1997	MS, RI, S
40 ethyl decanoate	1.635	1.634	Lopez et al., 1999	MS, RI, S
41 2-furanmethanol	1.655	1.673	Comuzzo et al., 2006	MS, RI
42 3-methylbutanoic acid	1.669	1.672	Baek & Cadwallader, 1999	MS, RI, S
43 diethyl succinate	1.676	1.642	Jennings & Shibamoto, 1980	MS

	Compound	Ir^a	Ir_(lit)^b	Ref.^c	IM^d
44	5,6-dihydro-2 <i>H</i> -pyran-2-one	1.688			<i>MS</i>
45	3-(methylthio)-1-propanol (methionol)	1.711	1714	Lopez et al., 1999	<i>MS, RI</i>
46	hexanoic acid	1.848	1854	Lopez et al., 1999	<i>MS, RI, S</i>
47	2-phenylethanol	1.902	1.922	Baek & Cadwallader, 1999	<i>MS, RI, S</i>
48	1,4-butandiol	1.924	1861	Jennings & Shibamoto, 1980	<i>MS</i>
49	2-ethylhexanoic acid	1.947	1974	Welke et al., 2012	<i>MS, RI</i>
50	octanoic acid	2.059	2060	Lopez et al., 1999	<i>MS, RI, S</i>

a Ir: Retention index

b Ir_(lit): Retention index from literature

c Ref.: bibliographic reference

d IM: *S* comparison of mass spectra and retention time with those of standard compounds; *RI* comparison of order of elution with those reported in literature; *MS* comparison of mass spectra with those reported in Wiley 6 and NIST 107 mass spectrum libraries

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629 Effect of HPH treatments (150 MPa) on the volatile composition of the freeze-dried powders. ADY: active dry yeast; 1P, 4P, 6P, 10P: HPH treatments (1, 4, 6
630 and 10 passes) without temperature control; 4P_Tc, 6P_Tc, 10P_Tc: HPH treatments (4, 6 and 10 passes) with temperature control. Different letters mark
631 significant differences according to ANOVA and Tukey HSD test, at $p < 0.05$. SD: standard deviation.

Compound	Rt ^a	Absolute area / 1000																															
		ADY			1P			4P_Tc			6P_Tc			10P_Tc			4P			6P			10P										
		Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD								
<i>Acids</i>																																	
acetic acid	16,8	181798	±	7266	b	58372	±	29767	a	98544	±	53522	ab	83105	±	30705	a	58724	±	21995	a	56272	±	10607	a	41548	±	9280	a	73617	±	49072	a
2-methylpropanoic acid	20,5	118098	±	113510	a	177846	±	76090	a	130535	±	38677	a	136560	±	30376	a	128831	±	26163	a	144347	±	52402	a	129336	±	38840	a	182951	±	116161	a
butanoic acid	22,2	27063	±	26176	a	16997	±	11016	a	9653	±	5902	a	12725	±	4531	a	11645	±	3266	a	9970	±	4509	a	8803	±	3585	a	16040	±	12987	a
3-methylbutanoic acid	23,5	66043	±	77127	a	149490	±	87686	a	109432	±	60460	a	131037	±	41831	a	111916	±	35724	a	88601	±	44662	a	75083	±	35185	a	146391	±	115190	a
hexanoic acid	28,4	3235	±	3239	a	3405	±	2122	a	1976	±	259	a	1393	±	1224	a	2433	±	786	a	0	±	0	a	0	±	0	a	606	±	688	a
2-ethylhexanoic acid	31,0	0	±	0	a	0	±	0	a	711	±	631	ab	1554	±	283	b	1085	±	969	ab	0	±	0	a	0	±	0	a	0	±	0	a
octanoic acid	33,8	0	±	0	a	14960	±	11716	b	0	±	0	a	1680	±	2910	a	787	±	1363	a	0	±	0	a	0	±	0	a	132	±	229	a
<i>Alcohols</i>																																	
ethanol	3,3	122445	±	28115	a	1217247	±	96244	e	970939	±	39987	cd	946836	±	8793	cd	932973	±	14120	bcd	811894	±	25910	b	837218	±	39074	bc	845959	±	59638	bc
2-methyl-1-propanol	6,2	31173	±	53992	a	361173	±	122000	c	299241	±	33400	bc	174116	±	28157	ab	127623	±	20666	a	112491	±	43891	a	101455	±	26006	a	106295	±	50009	a
2- and 3-methyl-1-butanol	9,5	208380	±	360925	a	1620151	±	244887	c	1633470	±	75973	c	1330683	±	83097	bc	1179959	±	109112	bc	1029730	±	211711	bc	983472	±	181846	b	934591	±	302713	b
1-hexanol	14,0	5402	±	2924	bc	8489	±	702	c	1048	±	393	a	1037	±	899	a	2177	±	749	ab	2078	±	330	ab	1820	±	256	ab	2390	±	2176	ab
2-ethyl-1-hexanol	18,3	8058	±	902	b	3272	±	1402	a	1566	±	586	a	1919	±	557	a	3036	±	1416	a	2437	±	919	a	3134	±	283	a	3935	±	2796	a
2-phenylethanol	29,9	5070	±	2563	a	53863	±	32661	bc	58792	±	20303	c	32896	±	12835	abc	24147	±	9953	abc	8905	±	4417	a	7595	±	3892	a	13933	±	8554	ab
<i>Carbonyls</i>																																	
hexanal	5,9	0	±	0	a	5596	±	1503	b	129	±	223	a	0	±	0	a	0	±	0	a	4197	±	1105	b	4370	±	1456	b	4055	±	2451	b
heptanal	8,6	0	±	0	a	14272	±	2434	c	0	±	0	a	0	±	0	a	0	±	0	a	6494	±	2398	b	6929	±	2819	b	8230	±	2124	b
3-hydroxy-2-butanone (acetoin)	11,6	95462	±	62134	b	30859	±	24727	ab	20374	±	6536	a	9843	±	3682	a	4894	±	1933	a	7837	±	3816	a	7383	±	1663	a	8816	±	6410	a
6-methyl-5-hepten-2-one	13,2	0	±	0	a	0	±	0	a	0	±	0	a	0	±	0	a	1717	±	2973	ab	6239	±	1015	c	5151	±	902	bc	3618	±	2105	abc
nonanal	15,0	0	±	0	a	6211	±	1133	bcd	3650	±	631	b	4372	±	1114	bc	3729	±	596	b	6816	±	1161	cde	7364	±	1405	de	9390	±	1506	e
<i>Heterocyclic compounds</i>																																	
2-methyl-thiazolidine	16,0	0	±	0	a	0	±	0	a	6222	±	9679	a	3363	±	3355	a	0	±	0	a	0	±	0	a	0	±	0	a	0	±	0	a
dihydro-5-methyl-2(3H)-furanone (γ-valerolactone)	21,4	0	±	0	a	7722	±	4214	a	3322	±	4557	a	7069	±	1234	a	6038	±	1314	a	3438	±	5954	a	1349	±	2336	a	6424	±	5589	a
dihydro-2(3H)-furanone (γ-butyrolactone)	22,0	30431	±	10759	b	10780	±	5016	a	8349	±	2512	a	8443	±	1482	a	7959	±	1074	a	8551	±	3344	a	7957	±	1498	a	9990	±	4655	a
5,6-dihydro-2H-pyran-2-one	24,1	2390	±	2073	ab	4053	±	1194	b	0	±	0	a	0	±	0	a	659	±	1142	a	994	±	239	a	867	±	266	a	541	±	503	a

Table 2. Continued

Compound	Rt ^a	Absolute area / 1000																															
		ADY			IP			4P_Tc			6P_Tc			10P_Tc			4P			6P			10P										
		Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD								
<i>Diols</i>																																	
2,3-butandiol	19,8	22793	±	14276	a	22037	±	11445	a	21827	±	2950	a	16581	±	3363	a	10856	±	1600	a	8403	±	1818	a	7418	±	2850	a	13595	±	5020	a
1,2-propandiol	20,9	9553	±	6907	ab	12755	±	5017	abc	13930	±	4052	abc	22285	±	2986	c	18070	±	4449	bc	7892	±	2741	ab	1692	±	586	a	6394	±	5526	ab
1,4-butandiol	30,4	0	±	0	a	0	±	0	a	1211	±	168	b	1212	±	280	b	894	±	452	ab	1103	±	110	b	1534	±	284	b	686	±	795	ab
<i>Esters</i>																																	
ethyl acetate	2,8	0	±	0	a	0	±	0	a	169376	±	60706	d	111520	±	17077	cd	72708	±	26615	bc	26011	±	7142	ab	30946	±	5759	ab	15428	±	4701	ab
ethyl butanoate	4,9	0	±	0	a	76065	±	4671	b	66828	±	28331	b	69142	±	21649	b	51437	±	15886	bc	0	±	0	a	0	±	0	a	0	±	0	a
ethyl 2-methylbutanoate	5,3	0	±	0	a	17092	±	202	b	16281	±	8888	b	14378	±	13225	b	2808	±	4864	b	0	±	0	a	0	±	0	a	0	±	0	a
ethyl 3-methylbutanoate	5,6	0	±	0	a	12311	±	6100	b	7955	±	4209	b	8515	±	4657	b	6295	±	3190	b	0	±	0	a	0	±	0	a	0	±	0	a
ethyl hexanoate	10,2	0	±	0	a	55528	±	10108	b	98161	±	29681	b	72522	±	14668	b	73791	±	29887	b	1245	±	1467	a	0	±	0	a	570	±	987	a
ethyl heptanoate	13,2	0	±	0	a	16211	±	1618	b	12898	±	3690	b	12141	±	1792	b	8624	±	7847	ab	0	±	0	a	0	±	0	a	0	±	0	a
ethyl lactate	13,5	0	±	0	a	16623	±	6946	b	5560	±	2847	a	5357	±	3939	a	4830	±	3792	ab	0	±	0	a	0	±	0	a	0	±	0	a
ethyl octanoate	16,3	13870	±	5378	a	1042294	±	83438	b	1038839	±	237474	b	931907	±	301368	b	935959	±	359645	b	43077	±	17782	a	15083	±	5607	a	23177	±	26642	a
ethyl 3-hydroxybutanoate	18,9	0	±	0	a	2375	±	1077	a	606	±	1050	a	1204	±	1383	a	747	±	772	a	0	±	0	a	219	±	379	a	2565	±	3806	a
ethyl decanoate	22,5	8902	±	2604	a	93992	±	12175	b	102659	±	30056	b	92958	±	26716	b	96502	±	41188	b	2388	±	784	a	985	±	516	a	2249	±	2262	a
diethyl succinate	23,7	0	±	0	a	75487	±	40743	b	14778	±	2450	b	6749	±	569	b	8755	±	7963	b	0	±	0	b	0	±	0	b	0	±	0	b
<i>Pyrazines</i>																																	
2,6-dimethyl-pyrazine	13,0	0	±	0	a	2987	±	2024	a	1773	±	263	a	6452	±	6440	a	7572	±	5665	a	983	±	992	a	1118	±	564	a	1093	±	1893	a
2-ethyl-6-methylpyrazine	14,8	0	±	0	a	3376	±	1987	a	1505	±	1501	a	2880	±	1587	a	2099	±	1116	a	742	±	516	a	656	±	568	a	1188	±	1280	a
2,3,5-trimethylpyrazine	15,4	0	±	0	a	10609	±	5485	b	10949	±	2852	b	10941	±	4430	b	7929	±	3456	ab	0	±	0	a	0	±	0	a	0	±	0	a
3-ethyl-2,5-dimethylpyrazine	16,7	0	±	0	a	0	±	0	a	1071	±	1855	ab	1873	±	1683	ab	3165	±	223	b	2732	±	848	ab	1939	±	630	ab	2394	±	1113	ab
2,3,5,6-tetramethylpyrazine	17,7	0	±	0	a	0	±	0	a	268	±	465	a	523	±	688	a	1221	±	1058	a	979	±	1288	a	0	±	0	a	250	±	432	a
<i>Others</i>																																	
β-myrcene	8,0	0	±	0	a	177243	±	28048	b	4112	±	1998	a	10701	±	765	a	6701	±	2535	a	9834	±	8019	a	9918	±	6492	a	4550	±	1737	a
unknown	10,6	102094	±	19610	a	40652	±	3264	a	36154	±	62620	a	46507	±	18478	a	75214	±	39600	a	10617	±	2328	a	35146	±	42167	a	34396	±	40136	a
benzaldehyde	18,7	0	±	0	a	13408	±	12939	a	1892	±	1903	a	2971	±	1531	a	2576	±	957	a	2046	±	580	a	2031	±	540	a	4019	±	2892	a
3-(methylthio)-1-propanol (methionol)	24,7	0	±	0	a	3459	±	2405	bc	5828	±	1014	c	3661	±	1371	bc	2324	±	1012	ab	711	±	423	ab	579	±	198	ab	1016	±	1002	ab

^a Rt: retention time