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

Elsevier Editorial System(tm) for Food Chemistry

Manuscript Draft

Manuscript Number:

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

Article Type: Research Article (max 7,500 words)

Keywords: high pressure homogenization; Saccharomyces; autolysis; wine; inactive dry yeasts

Corresponding Author: Dr. Piergiorgio Comuzzo, Ph.D.

Corresponding Author's Institution: Università degli Studi di Udine

First Author: Piergiorgio Comuzzo, Ph.D.

Order of Authors: Piergiorgio Comuzzo, Ph.D.; Sonia Calligaris; Lucilla Iacumin; Federica Ginaldi; Sabrina Voce; Roberto Zironi

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

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

Piergiorgio Comuzzo*, Sonia Calligaris, Lucilla Iacumin, Federica Ginaldi, Sabrina Voce, Roberto Zironi

Università degli Studi di Udine, Dipartimento di Scienze Agroalimentari, Ambientali ed Animali, via Sondrio 2/A, 33100 Udine – Italy

* Corresponding Author
Piergiorgio Comuzzo
Tel: + 39 0432 55 8166
Fax: + 39 0432 55 8130
e-mail: piergiorgio.comuzzo@uniud.it

Abbreviated running title

Yeast autolysis by different high pressure homogenization conditions

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.

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

1 Introduction

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

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

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

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

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

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

2 Materials and Methods

2.1 Reagents and materials

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

2.2 High pressure homogenization treatments

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

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

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

2.3 Microbiological analyses

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

2.4 Glucidic colloids

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

2.5 Soluble proteins

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

2.6 Free amino acids

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

2.7 SPME-GC-MS analyses

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

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

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

2.8 Statistical analyses

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

3 Results and Discussion

3.1 Effect of HPH treatment on yeast viability

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

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

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

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

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

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

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

3.3 Release of glucidic colloids in wine-like solution

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

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

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

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

The concentrations found for alkyl-pyrazines also highlighted some minor differences among the samples. Pyrazines were not detected in ADY preparation, while they were found in HPH 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

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

4 Conclusions

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

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

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

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

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

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

References

Acree, T., & Arn, H. (2016). Flavornet and human odor space. Gas chromatography - olfactometry (GCO) of natural products. URL http://www.flavornet.org/flavornet.html, Accessed 19.04.2016.

Alexandre, A., & Guilloux-Benatier, M. (2006). Yeast autolysis in sparkling wine. A review. *Australian Journal of Grape and Wine Research*, *12*, 119-127.

Ames, J.M., & McLeod, G.M. (1985). Volatile components of a yeast extract composition. *Journal of Food Science*, *50*, 125-135.

405 Ashoor, S.H., & Zent, J.B. (1984). Maillard reaction of common amino acids and sugars.
406 *Journal of Foof Science*, *49*, 1206-1207.
407 Baek, H.H., & Cadwallader, K.R. (1999). Contribution of free and glycosidically bound
408 volatile compounds to the aroma of muscadine grape juice. *Journal of Food Science*, *64*, 441-
409 444.
410 Comuzzo, P., Tat, L., Tonizzo, A., & Battistutta, F. (2006). Yeast derivatives (extracts and
411 autolysates) in winemaking: Release of volatile compounds and effects on wine aroma
412 volatility. *Food Chemistry*, *99*, 217-230.
413 Comuzzo, P., Tat, L., Fenzi, D., Brotto, L., Battistutta, F., & Zironi, R. (2011). Interactions
414 between yeast autolysates and volatile compounds in wine and model solution. *Food*
415 *Chemistry*, *127*, 473-480.
416 Comuzzo, P., Tat, L., Liessi, A., Brotto, L., Battistutta, F., & Zironi, R. (2012). Effect of
417 different lysis treatments on the characteristics of yeast derivatives for winemaking. *Journal*
418 *of Agricultural and Food Chemistry*, *60*, 3211-3222.
419 Comuzzo, P., Calligaris, S., Iacumin, L., Ginaldi, F., Palacios Paz, A.E., & Zironi, R. (2015a).
420 Potential of high pressure homogenization to induce autolysis of wine yeasts. *Food*
421 *Chemistry*, *185*, 340-348.
422 Comuzzo, P., Battistutta, F., Vendrame, M., Páez, M.S., Luisi, G., & Zironi, R. (2015b).
423 Antioxidant properties of different products and additives in white wine. *Food Chemistry*,
424 *168*, 107–114.
425 Culleré, L., Escudero, A., Cacho, J., & Ferreira, V. (2004). Gas chromatography-olfactometry
426 and chemical quantitative study of aroma of six premium quality spanish aged red wines.
427 *Journal of Agricultural and Food Chemistry*, *52*, 1653-1660.
428 Davies, N.W. (1990). Gas chromatographic retention indices of monoterpenes and
429 sesquiterpenes on methyl silicone and Carbowax 20M phases. *Journal of Chromatography*,
430 *503*, 1-24.

de la Torre, M., Flores, L.B., & Chong, E. (1994). High cell density yeast production: process synthesis and scale-up. In E. Galindo, & O.T. Ramirez (Eds.), *Advances in Bioprocess Engineering* (pp. 67-74). Dordrecht, The Nederlands: Springer Science + Business Media.

Dukes, B.C., & Butzke, C.E. (1998). Rapid determination of primary amino acids in grape juice using an o-phtaldialdehyde / N-acetyl-L-cysteine spectrophotometric assay. *American Journal of Enology and Viticulture*, *49*, 125-134.

Follows, M., Hetherington, P.J., Dunnill, P., & Lilly, M.D. (1971). Release of enzymes from bakers' yeast by disruption in an industrial homogenizer. *Biotechnology and Bioengineering*, *13*, 549-560.

Gonzalez-Rios, O., Suarez-Quiroz, M.L., Boulanger, R., Barel, M., Guyot, B., Guiraud, J.P., & Schorr-Galindo, S. (2007). Impact of "ecological" post-harvest processing on coffee aroma: II. Roasted coffee. *Journal of Food Composition and Analysis*, *20*, 297-307.

Goodner, K.L. (2008). Practical retention index models of OV-101, DB-1, DB-5, and DB-Wax for flavor and fragrance compounds. *LWT - Food Science and Technology*, *41*, 951-958.

Grosch, W. (1987). Reactions of hydroperoxides-products of low molecular weight. In H.W.-S. Chan, *Autoxidation of unsaturated lipids* (pp. 95-140). London: Academic Press.

Halasz, A., & Lasztity, R. (1991). *Use of yeast biomass in food production*. Boca Raton, USA: CRC Press, (Chapter 9).

Hetherington, P.J., Follows, M., Dunniil, P., & Lilly, M.D. (1971). Release of protein from baker's yeast *(Saccharomyces cerevisiae)* by disruption in an industrial homogeniser. *Transactions of the Institution of Chemical Engineers*, *49*, 142-148.

Jennings, W., & Shibamoto, T. (1980). *Qualitative analysis of flavor and fragrance volatiles by glass capillary gas chromatography*. New York, USA: Academic Press.

Lopez, R., Ferreira, V., Hernandez, P., & Cacho, J. (1999). Identification of impact odorants of young red wines made with Merlot, Cabernet Sauvignon and Grenache grape varieties: a comparative study. *Journal of the Science of Food and Agriculture*, *79*, 1461-1467.

Madruga, M.S., & Mottram, D.S. (1998). The effect of pH on the formation of volatile compounds by heating a model system containing 5'-IMP and cysteine. *Journal of Brazilian Chemical Society, 9,* 261-271.

Münch, P., Hofmann, T., & Schieberle, P. (1997). Comparison of key odorants generated by thermal treatment of commercial and self-prepared yeast extracts: influence of the amino acid composition on odorant formation. *Journal of Agricultural and Food Chemistry, 45*, 1338-1344.

Nagodawithana, T. (1992). Yeast-derived flavors and flavor enhancers and their probable mode of action. *Food Technology, 46,* 138-144.

Nawar, W.W. (1969). Thermal degradation of lipids: a review. *Journal of Agricultural and Food Chemistry, 17*, 18-21.

Organisation Internationale de la Vigne et du Vin, O.I.V. (2013). *Monograph on yeast autolysates*. Resolution Oeno, 496/2013.

Patrignani, F., Ndagijimana, M., Vernocchi, P., Gianotti, A., Riponi, C., Gardini, F., & Lanciotti, R. (2013). High-pressure homogenization to modify yeast performance for sparkling wine production according to traditional methods. *American Journal of Enology and Viticulture, 64,* 258-267.

Peat, S., Whelan, W.J., & Edwards, T.E. (1961). Polysaccharide of baker's yeast. Part 4. Mannan. *Journal of the Chemical Society (London), 1*, 28-35.

Pérez-Serradilla, J.A., & Luque de Castro, M.D. (2008). Role of lees in wine production: A review. *Food Chemistry, 111*, 447–456.

Popper, L., & Knorr, D. (1990). Application of high pressure homogenization for food preservation. *Food Technology, 44*, 84–89.

Pozo-Bayón, M.Á., Andújar-Ortiz, I., & Moreno-Arribas, M.V. (2009). Scientific evidences beyond the application of inactive dry yeast preparations in winemaking. *Food Research International, 42*, 754-761.

Regenstein, J.M., & Regenstein, C.E. (1984). *Food protein chemistry*. New York, USA: Academic Press.

Rodríguez-Bencomo, J.J., Andujar-Ortiz, I., Moreno-Arribas, M.V., Simò, C., Gonzales, J., Chana, A., Davalos, J., & Pozo-Bayon, M.A. (2014). Impact of glutathione-enriched inactive dry yeast preparations on the stability of terpenes during model wine aging. *Journal of Agricultural and Food Chemistry*, *62*, 1373-1383.

Schreier, P., Drawert, F., & Junker, A. (1977). The quantitative composition of natural and technologically changed aromas of plants. IV. Enzymic and thermal reaction products formed during the processing of tomatoes. *Zeitschrift fuer Lebensmittel-Untersuchung und-Forschung*, *165*, 23-27.

Usseglio-Tomasset, L., & Castino, M. (1975). I colloidi solubili di natura glucidica dei mosti e dei vini. Parte I. *Rivista di Viticoltura ed Enologia*, *28*, 374-391.

Voilley, A., Beghin, V., Charpentier, C., & Peyron, D. (1991). Interaction between aroma substances and macromolecules in a model wine. *Lebensmittel-Wissenschaft und -Technologie*, *24*, 469-472.

Welke, J.E., Manfroi, V., Zanus, M., Lazarotto, M., & Alcaraz Zini, C. (2012). Characterization of the volatile profile of Brazilian Merlot wines through comprehensive two dimensional gas chromatography time-of-flight mass spectrometric detection. *Journal of Chromatography A*, *1226* (124-139).

Wong, J.M., & Bernhard, R.A. (1988). Effect of nitrogen source on pyrazine formation. *Journal of Agricultural and Food Chemistry*, *36*, 123-129.

Yeo, H.C.H., & Shibamoto, T. (1991). Microwave-induced volatiles of the Maillard model system under different pH conditions. *Journal of Agricultural and Food Chemistry*, *39*, 370-373.

Figure Captions

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

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

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

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

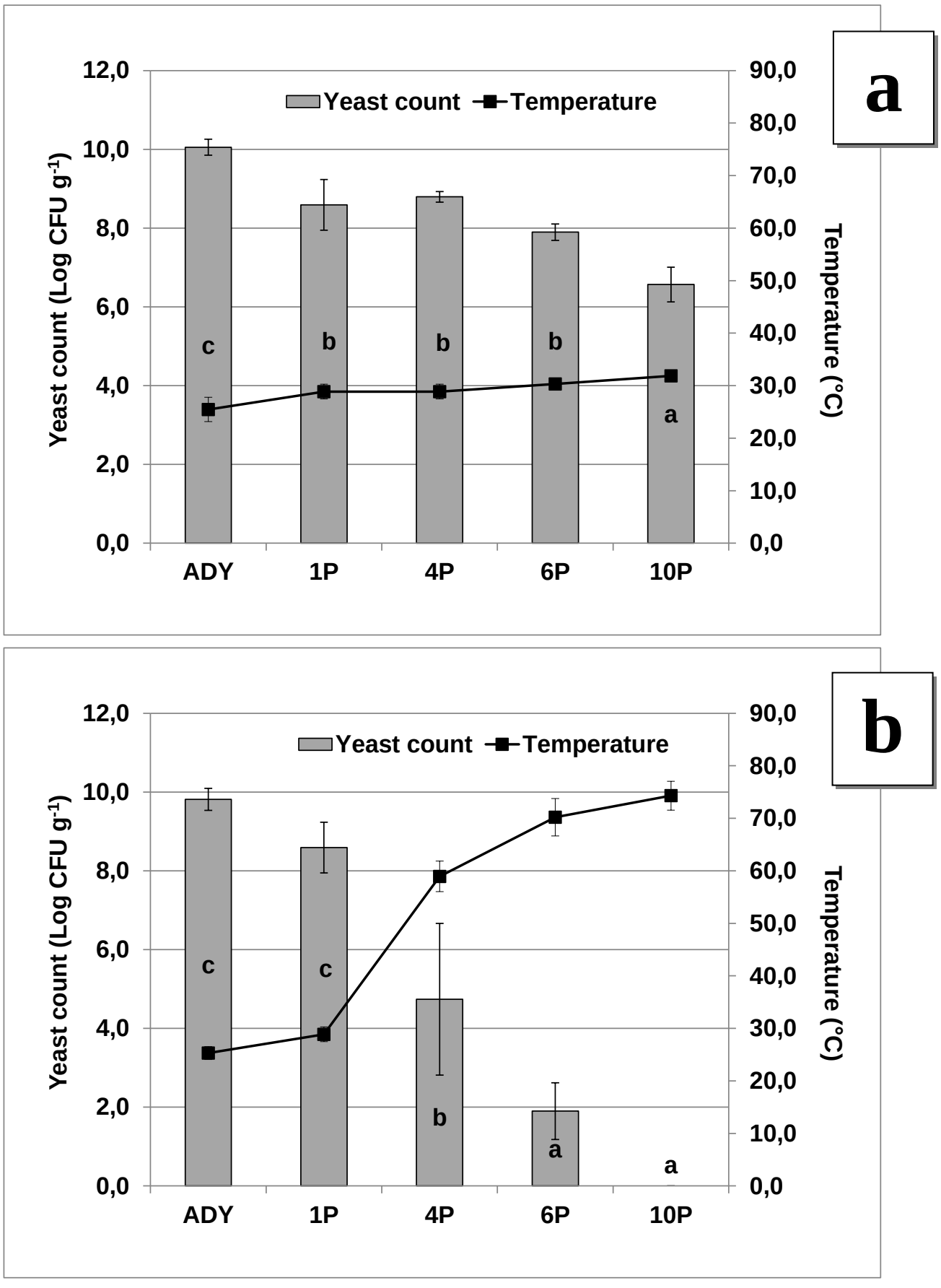
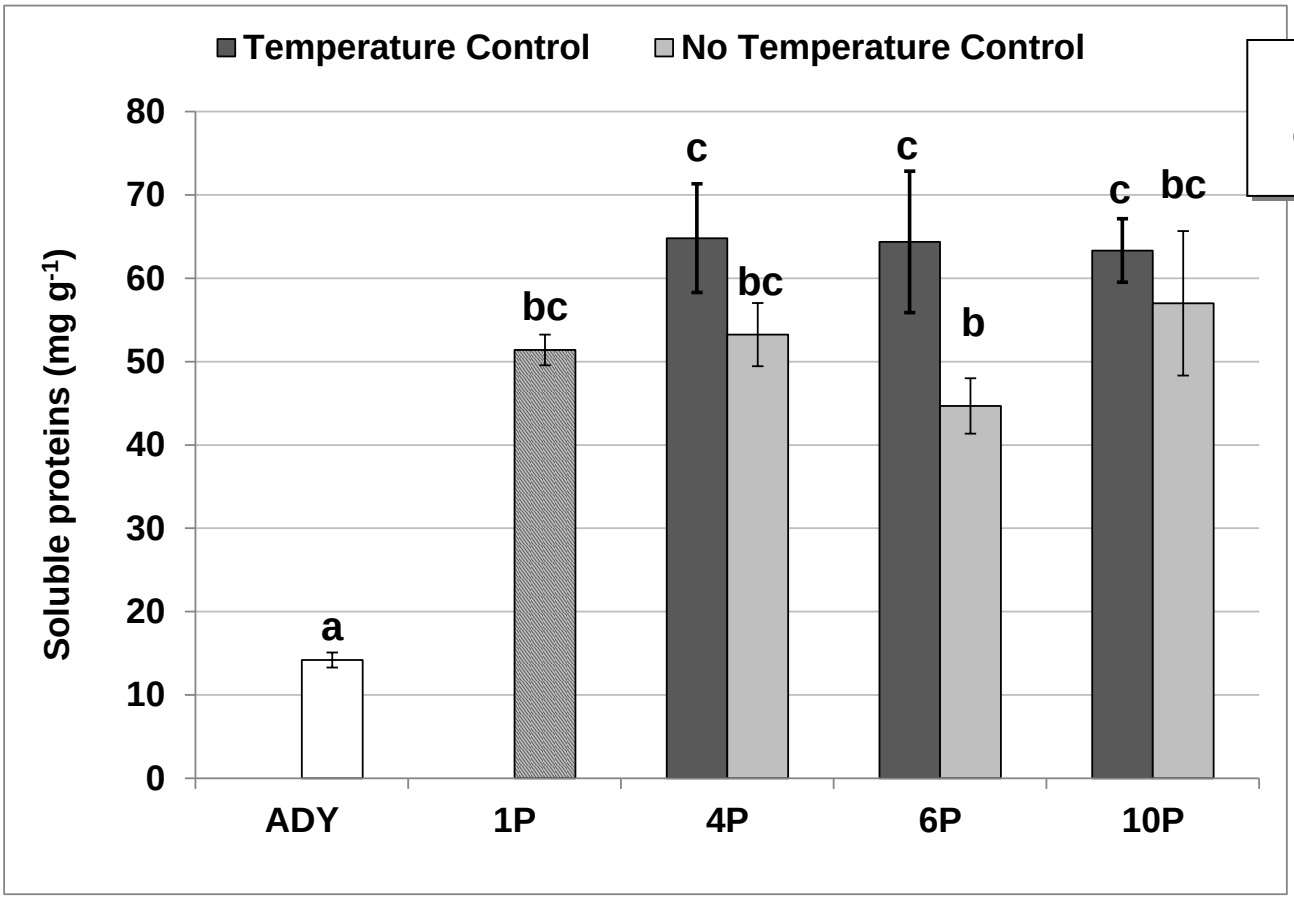

a
Yeast count
Temperature
Yeast count (Log CFU g-1)
Temperature (°C)
12,0
10,0
8,0
6,0
4,0
2,0
0,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
c
b
b
b
a
ADY
1P
4P
6P
10P
b
Yeast count
Temperature
Yeast count (Log CFU g-1)
Temperature (°C)
c
c
b
a
a
ADY
1P
4P
6P
10P

Fig. 1


Temperature Control
No Temperature Control
a
Soluble proteins (mg g-1)
80
70
60
50
40
30
20
10
0
a
bc
c
bc
c
b
c
bc
ADY
1P
4P
6P
10P

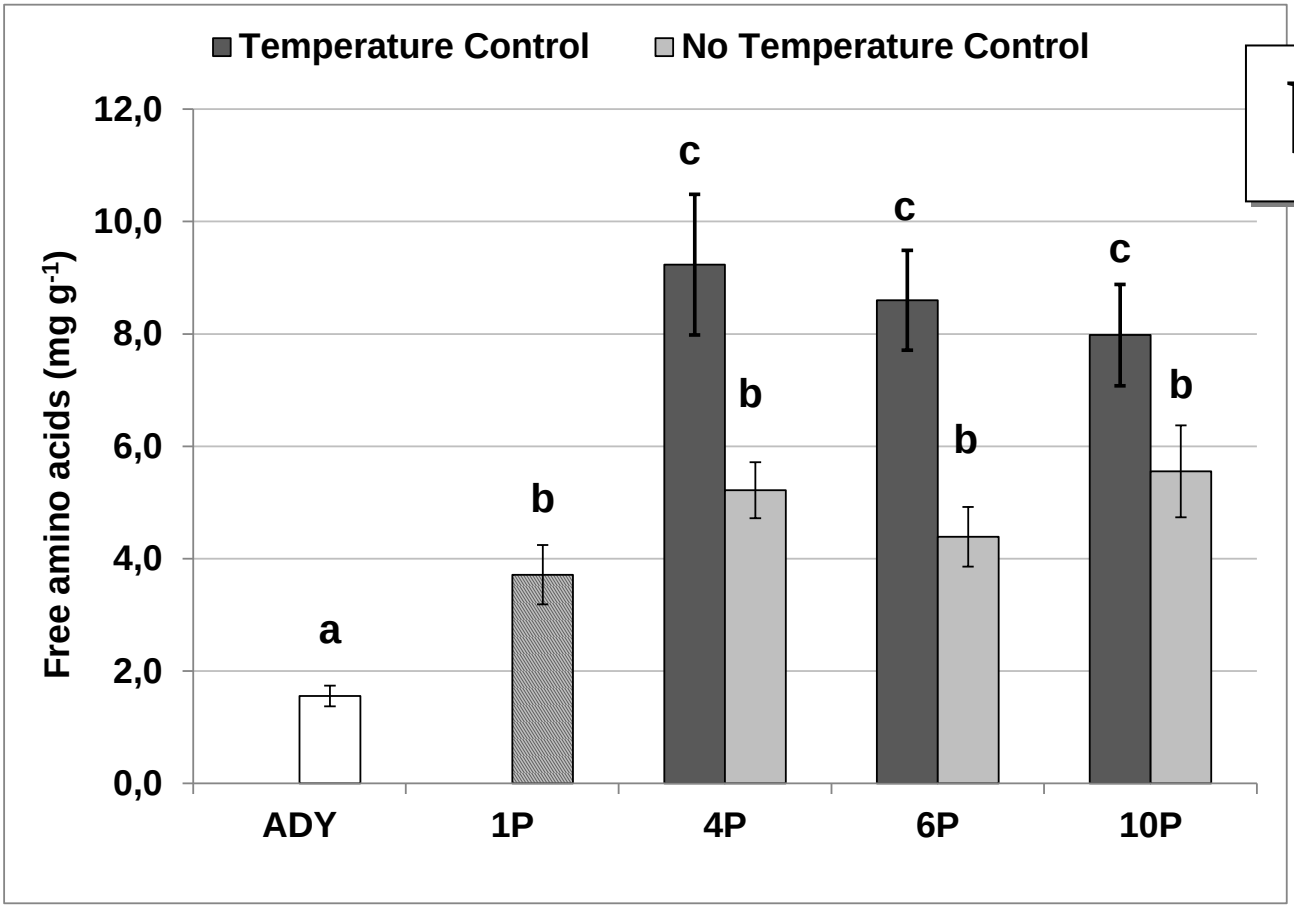

Temperature Control
No Temperature Control
b
Free amino acids (mg g-1)
12,0
10,0
8,0
6,0
4,0
2,0
0,0
a
b
c
b
c
b
c
b
ADY
1P
4P
6P
10P

Fig. 2

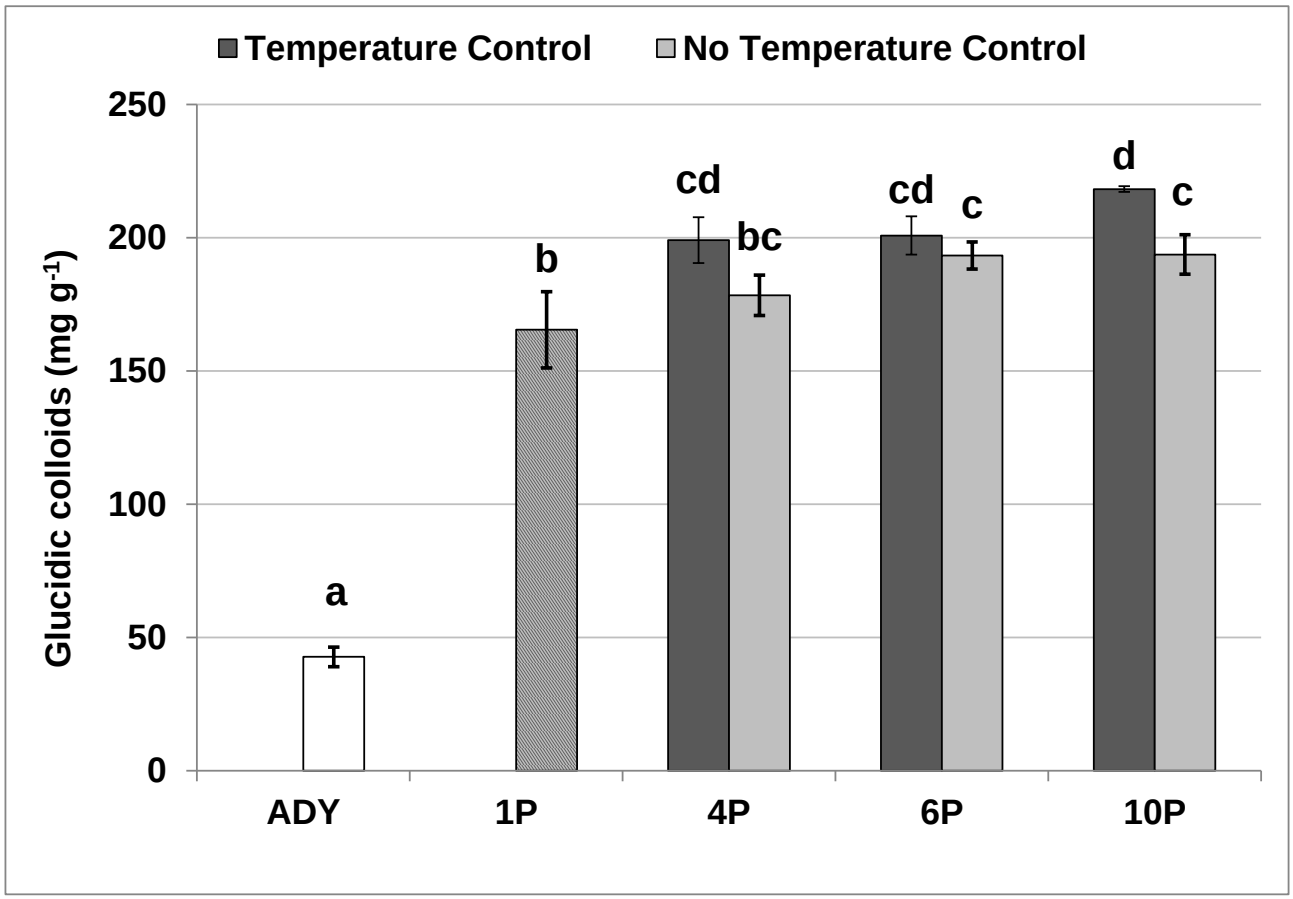

Temperature Control
No Temperature Control
Glucidic colloids (mg g-1)
250
200
150
100
50
0
a
b
cd
bc
cd
c
d
c
ADY
1P
4P
6P
10P

Fig. 3

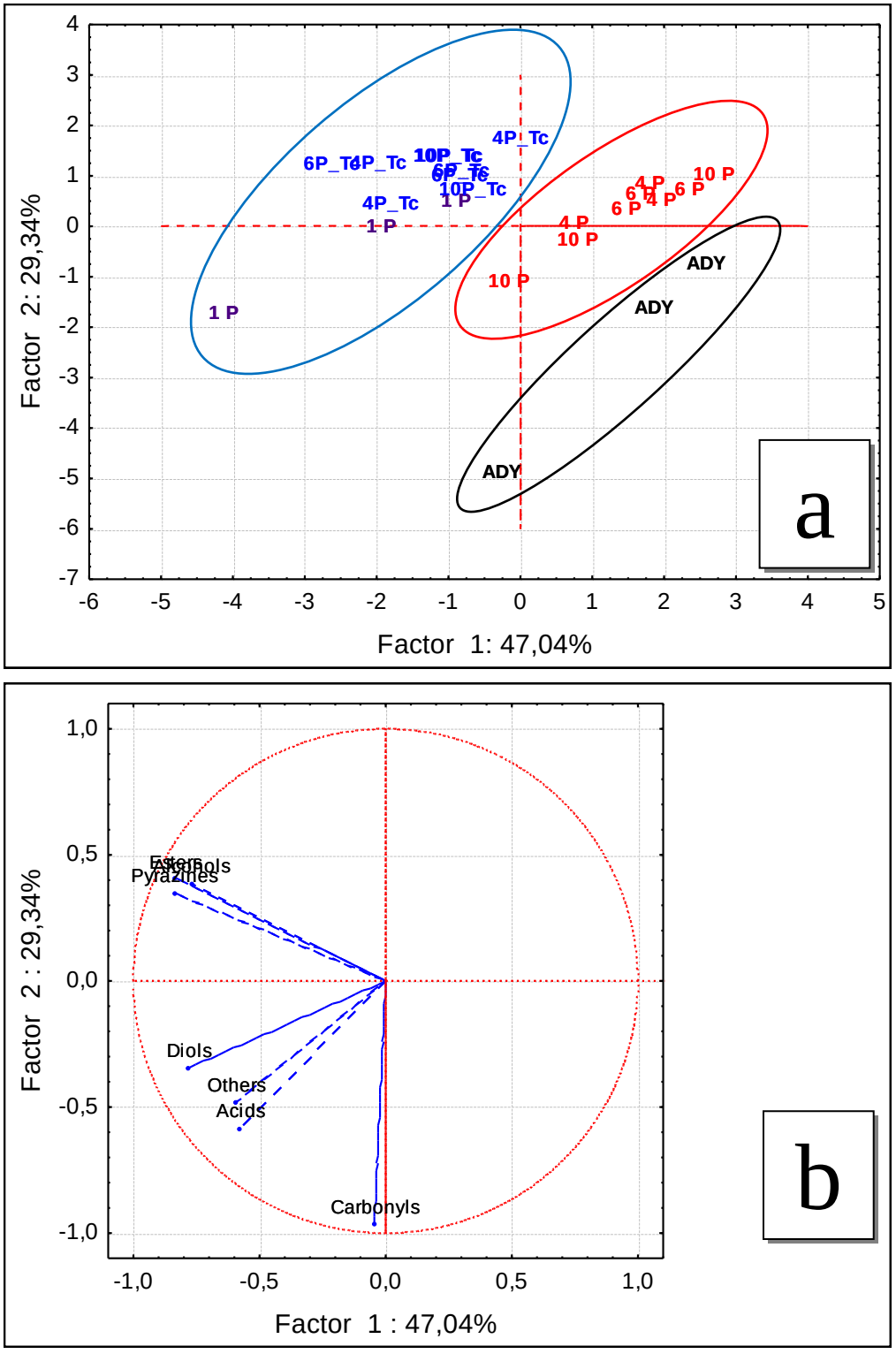

Factor 2: 29,34%
Factor 1: 47,04%
4P_Tc
6P_Tc
10P_Tc
1 P_Tc
4P_Tc
1 P
1 P
4 P
10 P
6 P
4 P
10 P
10 P
ADY
ADY
ADY
a
Factor 2 : 29,34%
Factor 1 : 47,04%
Esters
Alcohols
Pyrazines
Diols
Others
Acids
Carbonyls
b

Fig. 4

621 **Table 1**

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

624

	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	Madruga & 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(3*H*)-furanone (γ-valerolactone)	1.595	1617	Jennings & Shibamoto, 1981	*MS*
38	dihydro-2(3*H*)-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*

625 **Table 1** Continued

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

a Ir: Retention index

b $Ir_{(lit)}$: Retention index from literature

c Ref.: bibliografic 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

626

627

Table 2

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 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 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
Acids																													
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
Alcohols																													
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
Carbonyls																													
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
Heterocyclic compounds																													
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(3*H*)-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(3*H*)-furanone (γ -butyrrolactone)	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-2*H*-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				1P				4P_Tc				6P_Tc				10P_Tc				4P				6P			
		Mean	±	SD		Mean	±	SD		Mean	±	SD		Mean	±	SD		Mean	±	SD		Mean	±	SD		Mean	±	SD	
Diols																													
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
Esters																													
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
Pyrazines																													
2,6-dimethyl-pyrazyne	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-methylpyrazyne	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-trimethylpyrazyne	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-dimethylpyrazyne	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-tetramethylpyrazyne	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
Others																													
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