IRTA

RECERCA I TECNOLOGIA
AGROALIMENTÀRIES

1 **Use of Ultra-High Pressure Homogenization processing in winemaking: control of**

2 **microbial populations in grape musts and effects in sensory quality.**

3

4 Iris LOIRA[1], Antonio MORATA[1]*, Mª Antonia BAÑUELOS[2], Anna PUIG-PUJOL[3],

5 Buenaventura GUAMIS[4], Carmen GONZÁLEZ[1], José Antonio SUÁREZ-LEPE[1],

6

7 [1]enotecUPM. Chemistry and Food Technology Department, ETSIAAB, Universidad

8 Politécnica de Madrid. Avenida Complutense S/N, 28040 Madrid, Spain.

9 [2]Dept. Biotecnología-Biología Vegetal, ETSIAAB, Universidad Politécnica de Madrid.

10 [3]Enological Research Department. Institut of Agrifood Research and Technology –

11 Catalan Institute of Vine and wine (IRTA-INCAVI). Plaça Àgora, 2. 08720 Vilafranca

12 del Penedès, Spain.

13 [4]Centre d'Innovació, Recerca i Transferència en Tecnologia dels Aliments (CIRTTA),

14 TECNIO, XaRTA, Departament de Ciència Animal i dels Aliments, Facultat de

15 Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain.

16

17

18

19 *Corresponding author. Tel.: +34 91 336 57 30; Fax: +34 91 336 57 46

20 E-mail address: antonio.morata@upm.es

21

Abstract

Ultra-high pressure homogenization (UHPH) is a fast and efficient technique that can sterilize fluid foods at low temperatures or even under cooling conditions. A white must (*Vitis vinifera* L.) was processed at 300 MPa (inlet temperature 20 ºC, in-valve temperature 98 ºC, outlet temperature 25 ºC, and time in valve 0.02 s) and their performance was compared with two untreated controls, a must that underwent a spontaneous fermentation (without SO_2 addition) and another must that was sulfited with 35 mg/L of total SO_2 and inoculated with the same *Saccharomyces cerevisiae* yeast as the UHPH-treated must. UHPH treatment led to the total elimination of grape microorganisms considering an initial population of $1x10^6$ CFU/mL in average of wild yeasts and fungi in must, and approximately $7x10^3$ CFU/mL of background bacteria. In a parallel assay, UHPH-processed must without yeast inoculation showed absence of fermentation for eight days at 18 ºC. The musts treated with UHPH showed a lighter appearance (10%) before fermentation compared to the control. The triangular test verified the existence of sensory differences between the wines obtained and the preference tests showed that the judges found the wine obtained from the UHPH-treated must more fruity (3.5/5 compared with 1.5-2 in controls) and with better aroma.

Industrial relevance

UHPH is an interesting way to process the must before fermentation allowing the reduction of sulfite addition while controlling wild and spoilage microorganisms.

1. Introduction

Pressurization technologies have been used to process must and crushed grapes to control wild grape microorganisms at low temperatures. High hydrostatic pressure (HHP) applied at 400-600 MPa for 10 min eliminates yeast counts of $1x10^4$ CFU/mL in grape must; however, it is less efficient in the elimination of lactic acid bacteria (Morata et al., 2017). It also shows some effects on skins that favor the extraction processes during grape maceration (Morata et al., 2015). However, the main drawback is that it is a batch process that is difficult to use in winemaking (Morata et al., 2017). Ultra-high pressure homogenization (UHPH) is also a high pressure technology, but operates in continuous mode. Antimicrobial effect is produced when fluid is pumped at 200-600 MPa and go through a "special valve" before expansion. During the process, microorganisms and colloidal particles suffer both strong shear forces and impact, which not only cause the complete destruction of living microorganisms but also spores (Amador Espejo, Hernández-Herrero, Juan, & Trujillo, 2014), thus producing sterilization. All particles are reduced to a range size of 100-300 nm (Zamora & Guamis, 2015). The mechanical effect due to the hypersonic speed reached in the valve and the subsequent depressurization produce an intense fragmentation of cells and particles. The only requirement for processing by UHPH is that the particles in the fluid must be less than 500 μm in size. Both HHP and UHPH can be referred to as cold pasteurization treatments that are sensory-protective because the processes do not affect covalent bonds; pigments, aroma (Bermúdez-Aguirre & Barbosa-Cánovas, 2011; Oey, Van der Plancken, & Van Loey, 2008). In UHPH, the exposure time to the peak process temperature is less than 0.2 seconds, and therefore, without significant thermal repercussion (Ypsicon, 2018).

69 Industrial UHPH equipment are currently available with capacities up to 50.000

70 liters/hour based on modular systems. Moreover, power consumption is approximately

71 50% lower than HPP (Ypsicon, 2018).

72 The use of UHPH or HHP in must processing is a clear alternative to the use of sulfites

73 to control wild spoilage microorganisms that can also affect the fermentation performance

74 and sensory quality of the wine (Morata et al., 2015; Puig, Olmos, Quevedo, Guamis, &

75 Mínguez, 2008). That is especially interesting when modern fermentation

76 biotechnologies are used such as fermentation with non-*Saccharomyces* yeasts or co-

77 inoculation using yeast and bacteria mixtures to perform simultaneous malolactic and

78 alcoholic fermentations (Bañuelos et al., 2016).

79 UHPH at 150 MPa has been also described as a way to accelerate biological ageing

80 processes like ageing on lees (Comuzzo et al., 2015, 2017). The homogenization effects

81 by high pressure produce the lysis and de-polymerization of yeast cell walls releasing

82 polysaccharides and mannoproteins that affect mouthfeel improving wine softness.

83 Moreover, UHPH can be used to modulate the autolytic capacity of yeast starters used to

84 age sparkling wines (Patrignani et al., 2013).

85 The aim of this work was to check the effectiveness of UHPH in the control of wild

86 microorganisms in grape musts and evaluate the enological and sensory parameters of the

87 wines obtained after fermentation with *Saccharomyces cerevisiae* compared to control

88 wines produced from sulfited must or spontaneously fermented must.

89

90 **2. Materials and Methods**

91 **2.1 Must preparation**

5

92   Grapes from *Vitis vinifera* L. variety "Hondarribi zuri" were pressed using a pneumatic

93   press and running must was settled at 4 ºC. Clean must was separated in three batches: i)

94   sulfited at 35 mg/L of total SO_2, ii) UHPH processed and iii) untreated. UHPH

95   sterilization was performed using a continuous device (150 L/h) patented by UAB

96   (EP2409583) and manufactured by Ypsicon Advance Technologies (Barcelona, Spain)

97   working at 300±3 MPa, an inlet temperature of 20°C, valve temperature of 98°C reached

98   at 0.02 s, and outlet temperature of 25°C (detailed temperatures and pressures included

99   as supplementary material). Initial must parameters are described in **Table 1**.

100

101   **2.2 Fermentations and microbial counts**

102   Fermentations were performed in 2-L flasks with 1.8 L of must in triplicate at 18 ºC.

103   Fermenters were inoculated with 50 mL starters of a 24-hours culture in YPD broth (10

104   g/L yeast extract, 20 g/L peptone, 20 g/L glucose. Supplied by Conda, Madrid, Spain)

105   containing $1x10^8$ CFU/mL. The population in the fermenters after inoculation was

106   checked by plating being $1x10^5$ CFU/mL. The yeast strain used was *Saccharomyces*

107   *cerevisiae* 7VA (enotecUPM, Spain).

108   As control, a parallel assay was performed in which another three batches of each

109   processing method (sulfited, UHPH and non-treated) were placed in 100-mL vials with

110   50 mL of must and allowed to ferment with the wild population. These flasks were sealed

111   with Müller valves and the fermentation development was monitored gravimetrically

112   recording weight losses by the release of CO_2. Each fermentation was performed in

113   triplicate and isothermally controlled at 18 ºC.

114   Microbiological analyses were performed in musts after UHPH treatments and in wines

115   at the end of fermentation. Serial decimal dilutions in saline peptone (0.85% NaCl with

116   peptone at 0.1%) were pour-plated (1 mL) in selective media for total aerobic bacteria

and lactic acid bacteria and 100 μL were spread-plated for yeasts. The media were: Glucose chloramphenicol agar (GCA) incubated aerobically during 4 days at 25 ℃ (yeast); synthetic lysine agar (Oxoid, Hampshire, UK) for non-*Saccharomyces* counts (Heard & Fleet, 1986); PCA supplemented with nystatin (50 mg/L) after sterilization, and incubated during 3 days at 30 ℃ (aerobic bacteria); MRS agar supplemented with nystatin (50 mg/l) after sterilization and incubated during 4 days at 30 ℃ in anaerobic conditions in a jar under CO_2 atmosphere (lactic acid bacteria). GCA and MRS media were purchased from Pronadisa (Barcelona, Spain).

2.3 Enological parameters by Infrared spectroscopy

The equipment OenoFoss™ (FOSS Iberia, Barcelona, Spain) using Fourier transform infrared spectroscopy (FTIR) was used to identify and quantify major compounds such as residual sugars, organic acids, total and volatile acidity (Urbano-Cuadrado, Luque De Castro, Pérez-Juan, García-Olmo, & Gómez-Nieto, 2004). This technique also determines pH value.

2.4. Analysis of organic acids and residual sugars

Lactic acid, malic acid and residual sugars were measured enzymatically (Peynaud, Blouin, & Lafon-Lafourcade, 1966) using an Y15 enzymatic autoanalyzer (Biosystems, Barcelona, Spain).

2.5. Ethanol quantification

Ethanol was analyzed by liquid chromatography with refractive index detection (LC-RI) using a Waters e2695 apparatus (Milford, Massachusetts, USA) equipped with a 2414 Refractive Index Detector. Analyses were performed using a Phenosphere XDB C18

142 column (4.6 x 150 mm, 5-µm particle size) (Phenomenex, Torrance, CA, USA). The

143 solvent was Milli-Q water (in isocratic mode) at 0.4 mL/min. The temperature was set at

144 30 ℃ both in the column and in the detector. Calibration was performed using an external

145 ethanol standard (Panreac, Barcelona, Spain). Samples were injected after filtration

146 through 0.45-µm cellulose methyl ester membrane filters (Tecknokroma, Barcelona,

147 Spain). The injection volume was 2 µL.

148

149 **2.6. Analysis of volatile compounds by gas chromatography with flame ionization**

150 **detection (GC-FID)**

151 Volatile compounds were determined using an Agilent Technologies 6850 gas

152 chromatograph (Network GC System) equipped with an integrated flame ionization

153 detector (GC-FID). A DB-624 column (60 m x 250 µm x 1.40 µm) was used. The

154 following compounds were used as external standards for calibration (r^2>0.999):

155 acetaldehyde, diacetyl, acetoin, methanol, 1-propanol, 1-butanol, 2-butanol, isobutanol,

156 2-methyl-1-butanol, 3-methyl-1-butanol, hexanol, 2-phenylethyl alcohol, 2-phenylethyl

157 acetate, 2,3-butanediol, ethyl acetate, isoamyl acetate, isobutyl acetate, ethyl butyrate and

158 ethyl lactate. 4-Methyl-2-pentanol was used as internal standard. All compounds were

159 purchased from Fluka (Sigma–Aldrich Corp., Buchs SG, Switzerland). The injector

160 temperature was 250 ℃, and the detector temperature 300 ℃. The column temperature

161 was 40 ℃ (5 min), rising linearly by 10 ℃/min until 250 ℃; this temperature was then

162 held for 5 min. Hydrogen was used as carrier gas. The injection split ratio was 1:10, the

163 in-column flow rate 2.2 L/min, and the detection limit 0.1 mg/L. One-hundred microliters

164 of internal standard (500 mg/L) were added to 1-mL test samples and filtered through

165 syringe membrane filters (pore size 0.45-µm) (Teknokroma, Barcelona, Spain). They

166   were then placed in 2-mL glass vials sealed with a PTFE/silicon septum. One microliter

167   of this filtrate was injected into the GC apparatus.

168

169   **2.7 Color measurements and phenols**

170   The color of wine has been determined by the use of a UV-visible (UV-Vis)

171   spectrophotometer 8453 from Agilent Technologies™ (Palo Alto, CA, USA) with a

172   photodiode array detector and the use of a 1-cm path length quartz cuvette. The absorption

173   at three different wavelengths (420 nm, 520 nm and 620 nm) was used to compare color

174   intensity and hue in all wines after fermentation was complete.

175   Total polyphenol index (TPI) was measured after dilution 1:10 with milli-Q water in 1-

176   cm path length quartz cuvette at 280 nm. Hydroxycinnamic acids were also estimated by

177   measuring the absorbance at 320 nm in the same conditions.

178

179   **2.8 Determination of Polyphenol oxidase (PPO) activity**

180   Polyphenol oxidase (PPO) activity was determined according to the method described by

181   Cano, Hernandez, & De Ancos (1997) with slight modifications. PPO determination

182   consisted on mixing 3 mL of a solution based on a 0.07 M catechol solution and 0.05 M

183   sodium phosphate buffer (pH 6.5) with 150 µL of the sample. The absorbance change

184   was spectrophotometrically monitored (UV2310, Dinko Instruments Ltd., Barcelona,

185   Spain) at 420 nm during 10 min at 25 ºC.

186

187   **2.9 Antioxidant activity: FRAP assay**

188   The reducing antioxidant power by the ferric reducing ability of plasma (FRAP) method

189   was used according to a modified version of Benzie & Strain (1996). A daily FRAP

190   reagent was prepared by mixing 25 mL of 0.3 mM acetate buffer (pH 3.6) with 2.5 mL

191   of 10 mm TPTZ solution in 40 mM HCl and 2.5 mL of 20 mM Ferric chloride

192   ($FeCl_3.6H_2O$). After heating the FRAP reagent to 37 ºC, 900 µL of the reagent were

193   allowed to react with 30 µL of sample and 90 µL of water. Readings were taken after 8

194   min at 37 ºC at the wavelength of 593 nm against an acetate buffer blank. Quantification

195   was based on the standard curve ranged from 0-1000 µM of Trolox. Antioxidant capacity

196   was expressed as mM of TE (Trolox Equivalents).

197

2.10 Sensory evaluation

199   A preference test was developed to assess the quality of the wines. A panel of nine

200   experienced tasters (age range: from 30 to 60 years old, 4 women and 5 men) evaluated

201   the wines. The blind tasting took place in the tasting room of Chemistry and Food

202   Technology Department, Universidad Politécnica de Madrid, provided with fluorescent

203   lighting and presenting samples in random order. The wines (20 mL/tasting glass) were

204   served at 20 ± 2 ºC in three different standard odor-free wine-tasting glasses. Briefly, the

205   panelists used a scale from 0 to 5 to rate the intensity of different attributes (0 = attribute

206   not perceptible, 5 = attribute strongly perceptible). Each panelist also provided an overall

207   impression of the wines produced, taking into account olfactory and taste features,

208   including any defect.

209

2.11 Statistical analysis

211   Means and standard deviations were calculated and differences examined using ANOVA

212   and the least significant difference (LSD) test. All calculations were made using PC

213   Statgraphics v.5 software (Graphics Software Systems, Rockville, MD, USA).

10

214   Significance was set at p<0.05. Fermentations of each treatment proceeded in triplicate

215   and chemical and microbiological analyses were done of each replicate. These data were

216   used in the statistical treatments.

217

218   **3. Results and discussion**

219   After must treatments, a higher limpidity with very low size colloidal particles in UHPH

220   processed must was observed. However, sulfited must showed bigger colloidal particles

221   and phase separation in a liquid fraction and pectin fragments in the bottom of the

222   fermenters. This is explained because of the intense homogenization by UHPH processing

223   producing nanoparticles lower than 300 nm of molecular size (Zamora & Guamis, 2015).

224

225   **3.1 Antimicrobial effect of UHPH**

226   UHPH has demonstrated higher efficiency in controlling spores (Amador Espejo et al.,

227   2014) and bacteria than discontinuous HHP processes because of the intense shear forces

228   to which the liquid is subjected when it crosses the valve and undergoes a strong

229   decompression. Microbial analyses were focused in wild microorganisms typically found

230   in grapes and must (fungi, *Saccharomyces* and non-*Saccharomyces* yeasts and lactic acid

231   bacteria). Yeast and bacteria counts were similar in sulfited and untreated musts.

232   *Saccharomyces* yeasts were in average 1×10^6 CFU/mL (**Figure 1a**), non-*Saccharomyces*

233   in lysine media in the same value, and the bacterial counts measured in PCA and MRS

234   were in average 7×10^3 CFU/mL. No yeasts were detected in the must processed by UHPH

235   (LOD 10 CFU/mL) (**Figure 1a**). Hence, at the end of fermentation, only the inoculated

236   yeast can be found in UHPH-processed must, with a total absence of non-*Saccharomyces*

237   (**Figure 1b**) which shows the effectiveness of the treatment. Conversely, non-

238   *Saccharomyces* wild yeasts can be found at 2 log CFU/mL in the sulfited must. Therefore,

239   the wild yeasts remain in the must throughout the entire fermentation. The presence of

240   non-*Saccharomyces* yeasts was more noticeable in the non-treated must, being higher

241   than 3 log CFU/mL at the end of fermentation. Vegetative bacteria populations were also

242   eliminated from the must by the UHPH treatment (LOD 1CFU/mL), and remained

243   undetected at the end of fermentation (**Figures 2a, b**). This result highlights the intense

244   antimicrobial effect of UHPH for all the microbial groups analyzed, even considering the

245   high initial microbial load in the must. HHP treatments have previously demonstrated

246   high efficiency in yeasts control at 400 MPa-10 min, but some residual populations of

247   bacteria still remain even at 550 MPa-10 min (Morata et al., 2015). The use of UHPH

248   allows winemakers to avoid the use of SO_2 to control apiculate yeasts and bacterial

249   populations, but also facilitates the implantation of non-*Saccharomyces* starters when the

250   use of these unconventional yeasts is desired to improve the sensory profile. This agrees

251   with the same application previously described for HHP processing (Bañuelos et al.,

252   2016). By using UHPH processing, it is possible to reduce the SO_2 doses only to the

253   suitable levels to control oxidations due to the total antimicrobial effect produced by

254   continuous pressurization. Moreover, the use of emerging antioxidants such as

255   glutathione (GSH) opens new possibilities to strongly reduce SO_2 in wines (Kritzinger,

256   Bauer, & Du Toit, 2013).

257   To evaluate how is the evolution of these wild populations over time, 50 mL of each must

258   (not inoculated) were left to ferment during 8 days. A fast evolution of the populations in

259   the sulfited must was observed, with strong fermentation on day 3 and reaching

260   approximately 10 %v/v of ethanol in 8 days (**Figure 3**). The untreated control showed a

261   slower fermentation, probably because the absence of sulfites promoted a greater

12

262   development of non-*Saccharomyces* yeasts with lower fermentative power, thus reaching

263   only 5 % v/v of ethanol in the same period. The fermentation did not occur in the UHPH-

264   treated must as the weight of the fermenters remained at the initial value during the 8 days

265   of the trial (**Figure 3**). The lack of fermentation for 8 days supports the absence of viable

266   but non-culturable yeasts that sometimes can be detected when microorganisms are

267   processed by discontinuous HHP (Lado & Yousef, 2002)

268

269   **3.2 Enological parameters in must before fermentation**

270   The sugar content in the musts is typical of Txacoli wines (**Table 1**), normally ranging

271   between 9-12 % v/v in alcoholic degree because of the early harvest that gives them their

272   distinctive sensory profile. Consequently, the acidity is high and, correspondingly, the pH

273   is quite low (3.2-3.3) in these musts. The levels of yeast assimilable nitrogen (YAN)

274   compounds, α-amino nitrogen and ammonia, were a little bit lower in the musts dosed

275   with SO_2, but the levels found were enough for a correct fermentation. It has been

276   described that 150 mg/L is a suitable YAN value to avoid sluggish or stuck fermentations

277   (Henschke & Jiranek, 1993). The absence of volatile acidity is considered an indicator of

278   grape health, because normally it increases when undesirable bacteria grow

279   uncontrollably before the alcoholic fermentation. In this case, the grape/must quality is

280   quite good (**Table 1**). Similarly, gluconic acid is not detected, which corroborates the

281   quality of the must. It is used as an indicator of fungal developments, since it is produced

282   by *Botrytis cinerea*'s metabolism (Cinquanta et al., 2015).

283

284   **3.3 Enological parameters in wine after fermentation**

285   After fermentation, wines from sulfited must reached an alcoholic strength of 10 % v/v

286   ethanol and about 9 % v/v in both the UHPH treatment and the untreated control, as

287   expected, according to the initial amount of sugars (**Table 2**). All fermentations finished

288   with very low levels of residual sugars (below 0.2 g/L). The concentrations of malic acid

289   (above 2 g/L) and the absence of lactic acid indicate that malolactic fermentation did not

290   occur. The low levels of acetic acid (0.2 g/L in UHPH and sulfited treatments) indicate

291   the purity of the alcoholic fermentation (Loira et al., 2014). However, in control

292   fermentation without SO_2, the values were a little higher than normal (**Table 2**), probably

293   because of the greater population of bacteria and non-*Saccharomyces* yeasts that remain

294   uncontrolled in absence of SO_2 (**Figures 1b and 2b**). This is a typical situation in

295   spontaneous fermentations with predominance of apiculate yeasts at the beginning of

296   fermentation (Fleet, 2003). The content of glycerol was higher in wines from sulfited

297   must because of the binding effect of SO_2 on acetaldehyde which delays its reduction to

298   ethanol and increases the production of glycerol (Wang, Zhuge, Fang, & Prior, 2001).

299

3.4 Fermentation volatiles by GC-FID

301   Sulfited wines showed the highest levels of volatile compounds (**Table 3**), mainly due to

302   the concentration of higher alcohols, which are undesirable because they normally

303   produce a winey aroma typical of low quality wines. Non-treated and especially UHPH-

304   processed wines had lower values of higher alcohols, allowing thus to show fruity or

305   varietal smells with better aromatic repercussion. Higher concentrations of esters can be

306   observed in wines from must processed by UHPH or untreated. These compounds

307   commonly produce fruity smells in wines and therefore increase the aromatic complexity.

308   Ethyl acetate is an ester that produces complexity but it can be defective at high

14

309   concentration. Normal values in wines are between 30-80 mg/L, but it produces spoilage

310   notes when present in concentrations higher than 150 mg/L (Zoecklein, Fugelsang,

311   Gump, & Nury, 1995). All techniques showed suitable values of ethyl acetate, but slightly

312   higher and with a bigger standard deviation in the wines from the untreated must,

313   probably because of the greater populations of bacteria and non-*Saccharomyces* yeasts.

314   Especially interesting were the values of isobutyl acetate and isoamyl acetate related to

315   fruits like pear and banana (Loira et al., 2015) that were undetected in the wines from the

316   sulfited must and with higher concentrations in the wines from the UHPH-treated must.

317   Similar behavior is showed by 2-phenylethyl acetate (rose petal smell) (Molina et al.,

318   2009) with higher concentrations in non-treated fermentations but also in UHPH

319   processed. In non-treated fermentations, the levels of volatile compounds can be favored

320   by the presence of non-*Saccharomyces* yeasts (Ciani, Comitini, Mannazzu, & Domizio,

321   2010; Viana, Belloch, Vallés, & Manzanares, 2011; Viana, Gil, Vallés, & Manzanares,

322   2009). Ethyl butyrate with fruity profile and ethyl lactate with toffee descriptors were also

323   found in higher concentrations in the wines obtained from the UHPH or non-treated

324   musts.

325   It is also remarkable that UHPH processed musts after fermentation showed a lower

326   concentration of hexanol than in the non-treated fermentations and especially in the

327   sulfited musts (**Table 3**). Hexanol is responsible for the herbaceous or grassy hints in

328   wine aroma (Peinado, Moreno, Bueno, Moreno, & Mauricio, 2004) which negatively

329   affect sensory quality. Concerning defective buttery notes, levels of acetoin were similar

330   in all fermentations regardless of the must treatment and within the suitable values.

331

3.5. Enzymatic and antioxidant activity

333   Enzymatic activities were measured in sulfited and UHPH-treated musts. A higher degree

334   of inactivation for PPO due to UHPH treatment was achieved compared to SO_2 must.

335   Considering 100 % of PPO activity the value given by sulfited must, UHPH sample

336   diminished up to 90 % their activity. Suárez-Jacobo et al., (2012) reported complete

337   inactivation of PPO in apple juice treated at 300 MPa. Grape juices containing PPO

338   enzymes are more prone to suffer oxidative reactions, trigger darkening reactions during

339   winemaking and thus decreasing the quality of the final wine (Hendrickx, Ludikhuyze,

340   Van den Broeck, & Weemaes, 1998).

341   Slight differences were found between sulfited and UHPH musts when determined

342   antioxidant activity by the FRAP assay. Sulfited samples reached values of 1.83 ± 0.36

343   mM of TE (Trolox Equivalents) while UHPH-treated must obtained 2.67 ± 0.41 mM of

344   TE. This indicated better antioxidant capacity in musts with UHPH treatment. The

345   antioxidant activity of a must or a wine is largely dependent on its phenolic content and

346   composition, as different compounds and their combinations exhibit varying degrees of

347   activity (Salaha, Kallithraka, Marmaras, Koussissi, & Tzourou, 2008). Although no

348   differences were observed in TPI data between must with SO_2 and UHPH-processed

349   (**Figure 4**), UHPH treatment could produce changes in the molecules of the matrix (amino

350   acids, peptides, sugars among others) that could affect this activity.

351   **3.6 Color, phenols and sensory evaluation**

352   Higher color intensity was measured in non-treated wine probably by browning oxidative

353   processes because of the absence of SO_2 (**Figure 4a**). As expected, the lowest values were

354   reached in the must processed with sulfites. UHPH wine showed intermediate values with

355   significant differences ($p<0.05$) regarding non-treated and sulfited wine. No significant

356   differences were found in the tonality of all the wines (**Figure 4a**). Sulfited wine and

357   UHPH-processed wine showed similar values of TPI and a slightly higher value was

358   found in the untreated wine (**Figure 4b**). Higher values of hydroxycinnamic acids

359   (HCAs) were observed in UHPH wine and lower concentrations in untreated samples,

360   probably by the mechanical effect of UHPH processing. Maybe due to inactivation of

361   PPO by UHPH.

362   As for the sensory analysis, UHPH wine was better evaluated in global quality, but

363   especially in aromatic profile, and described as fruitier by the panelists than either

364   untreated or sulfite added wines (**Figure 5**). This is in accordance with the higher values

365   of esters found in UHPH wines (**Table 3**) compared to sulfited wines. Tasters were able

366   to detect lower color intensity in the sulfited wine. However, no significant differences

367   ($p < 0.05$) were detected in tonality between UHPH and sulfited wines in agreement with

368   the spectrophotometric analysis. The sulfited wine was described as more herbaceous

369   than either untreated or UHPH-processed wines, what is also in accordance with the

370   higher hexanol concentrations observed by GC-FID (**Table 3**). The untreated wine

371   showed higher reduction hints.

372

373   **4. Conclusions**

374   UHPH is a fast and effective technique to remove wild microorganisms in grape must

375   facilitating the implantation of yeast starters and the use of new biotechnologies such as

376   the sequential fermentations with non-*Saccharomyces* yeasts. Moreover, compared with

377   previous reported results, UHPH shows better effectiveness against lactic acid bacteria

378   than traditional HHP. The processed must can be fermented with lower sulfite levels.

379

387 **Conflict of interest**

388 The authors declare that there is no conflict of interest.

389

390 **References**

391 Amador Espejo, G. G., Hernández-Herrero, M. M., Juan, B., & Trujillo, A. J. (2014).

392 Inactivation of Bacillus spores inoculated in milk by Ultra High Pressure

393 Homogenization. *Food Microbiology*, *44*, 204–210.

394 https://doi.org/10.1016/j.fm.2014.06.010

395 Bañuelos, M. A., Loira, I., Escott, C., Del Fresno, J. M., Morata, A., Sanz, P. D., …

396 Suárez-Lepe, J. A. (2016). Grape processing by high hydrostatic pressure: Effect

397 on use of non-Saccharomyces in must fermentation. *Food and Bioprocess*

398 *Technology*, *9*(10), 1769–1778. https://doi.org/10.1007/s11947-016-1760-8

399 Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a

400 measure of "Antioxidant Power": The FRAP assay. *Analytical Biochemistry*,

401 *239*(1), 70–76. https://doi.org/10.1006/ABIO.1996.0292

402   Bermúdez-Aguirre, D., & Barbosa-Cánovas, G. V. (2011). An update on high

403   hydrostatic pressure, from the laboratory to industrial applications. *Food*

404   *Engineering Reviews*, *3*(1), 44–61. https://doi.org/10.1007/s12393-010-9030-4

405   Cano, M. P., Hernandez, A., & De Ancos, B. (1997). High pressure and temperature

406   effects on enzyme inactivation in strawberry and orange products. *Journal of Food*

407   *Science*, *62*(1), 85–88. https://doi.org/10.1111/j.1365-2621.1997.tb04373.x

408   Ciani, M., Comitini, F., Mannazzu, I., & Domizio, P. (2010). Controlled mixed culture

409   fermentation: a new perspective on the use of non- *Saccharomyces*   yeasts in

410   winemaking. *FEMS Yeast Research*, *10*(2), 123–133.

411   https://doi.org/10.1111/j.1567-1364.2009.00579.x

412   Cinquanta, L., Albanese, D., De Curtis, F., Malvano, F., Crescitelli, A., & Di Matteo,

413   M. (2015). Rapid assessment of gray mold (*Botrytis cinerea*) infection in grapes

414   with a biosensor system. *American Journal of Enology and Viticulture*, *66*(4), 502–

415   508. https://doi.org/10.5344/ajev.2015.15029

416   Comuzzo, P., Calligaris, S., Iacumin, L., Ginaldi, F., Palacios Paz, A. E., & Zironi, R.

417   (2015). Potential of high pressure homogenization to induce autolysis of wine

418   yeasts. *Food Chemistry*, *185*, 340–348.

419   https://doi.org/10.1016/j.foodchem.2015.03.129

420   Comuzzo, P., Calligaris, S., Iacumin, L., Ginaldi, F., Voce, S., & Zironi, R. (2017).

421   Application of multi-pass high pressure homogenization under variable

422   temperature regimes to induce autolysis of wine yeasts. *Food Chemistry*, *224*, 105–

423   113. https://doi.org/10.1016/j.foodchem.2016.12.038

424   Fleet, G. H. (2003). Yeast interactions and wine flavour. *International Journal of Food*

425   *Microbiology*, *86*(1–2), 11–22. https://doi.org/10.1016/S0168-1605(03)00245-9

426   Heard, G. M., & Fleet, G. H. (1986). Evaluation of selective media for enumeration of

427   yeasts during wine fermentation. *Journal of Applied Bacteriology*, *60*(6), 477–481.

428   https://doi.org/10.1111/j.1365-2672.1986.tb01086.x

429   Hendrickx, M., Ludikhuyze, L., Van den Broeck, I., & Weemaes, C. (1998). Effects of

430   high pressure on enzymes related to food quality. *Trends in Food Science &*

431   *Technology*, *9*(5), 197–203. https://doi.org/10.1016/S0924-2244(98)00039-9

432   Henschke, P. A., & Jiranek, V. (1993). Yeasts - Metabolism of nitrogen compounds. In

433   G. H. Fleet (Ed.), *Wine Microbiology and Biotechnology* (pp. 77–164). Harword

434   Academic Publishers. Retrieved from https://ci.nii.ac.jp/naid/20001671587/

435   Kritzinger, E. C., Bauer, F. F., & Du Toit, W. J. (2013). Role of Glutathione in

436   Winemaking: A Review. *Journal of Agricultural and Food Chemistry*, *61*, 269–

437   277. https://doi.org/10.1021/jf303665z

438   Lado, B. H., & Yousef, A. E. (2002). Alternative food-preservation technologies:

439   efficacy and mechanisms. *Microbes and Infection*, *4*(4), 433–440.

440   https://doi.org/10.1016/S1286-4579(02)01557-5

441   Loira, I., Morata, A., Comuzzo, P., Callejo, M. J., González, C., Calderón, F., & Suárez-

442   Lepe, J. A. (2015). Use of *Schizosaccharomyces pombe* and *Torulaspora*

443   *delbrueckii* strains in mixed and sequential fermentations to improve red wine

444   sensory quality. *Food Research International*, *76*.

445   https://doi.org/10.1016/j.foodres.2015.06.030

446   Loira, I., Vejarano, R., Bañuelos, M. A., Morata, A., Tesfaye, W., Uthurry, C., …

447   Suárez-Lepe, J. A. (2014). Influence of sequential fermentation with *Torulaspora*

448   *delbrueckii* and *Saccharomyces cerevisiae* on wine quality. *LWT - Food Science*

449   *and Technology*, *59*(2), 915–922. https://doi.org/10.1016/J.LWT.2014.06.019

450   Molina, A. M., Guadalupe, V., Varela, C., Swiegers, J. H., Pretorius, I. S., & Agosin, E.

451   (2009). Differential synthesis of fermentative aroma compounds of two related

452   commercial wine yeast strains. *Food Chemistry*, *117*(2), 189–195.

453   https://doi.org/10.1016/J.FOODCHEM.2009.03.116

454   Morata, A., Loira, I., Vejarano, R., Bañuelos, M. A., Sanz, P. D., Otero, L., & Suárez-

455   Lepe, J. A. (2015). Grape processing by high hydrostatic pressure: Effect on

456   microbial populations, phenol extraction and wine quality. *Food and Bioprocess*

457   *Technology*, *8*(2). https://doi.org/10.1007/s11947-014-1405-8

458   Morata, A., Loira, I., Vejarano, R., González, C., Callejo, M. J., & Suárez-Lepe, J. A.

459   (2017). Emerging preservation technologies in grapes for winemaking. *Trends in*

460   *Food Science and Technology*, *67*. https://doi.org/10.1016/j.tifs.2017.06.014

461   Oey, I., Van der Plancken, I., & Van Loey, A. (2008). Does high pressure processing

462   influence nutritional aspects of plant based food systems? *Trends in Food Science*

463   *& Technology*, *19*(6), 300–308. https://doi.org/10.1016/J.TIFS.2007.09.002

464   Patrignani, F., Ndagijimana, M., Vernocchi, P., Gianotti, A., Riponi, C., Gardini, F., &

465   Lanciotti, R. (2013). High-Pressure Homogenization to Modify Yeast Performance

466   for Sparkling Wine Production According to Traditional Methods. *American*

467   *Journal of Enology and Viticulture*, *64*(2), 258–267.

468   https://doi.org/10.5344/ajev.2012.12096

469   Peinado, R. A., Moreno, J., Bueno, J. E., Moreno, J. A., & Mauricio, J. C. (2004).

470   Comparative study of aromatic compounds in two young white wines subjected to

471   pre-fermentative cryomaceration. *Food Chemistry*, *84*(4), 585–590.

472   https://doi.org/10.1016/S0308-8146(03)00282-6

473   Peynaud, E., Blouin, J., & Lafon-Lafourcade, S. (1966). Review of Applications of

474   Enzymatic Methods to the Determination of some Organic Acids in Wines.

475   *American Journal of Enology and Viticulture*, *17*(3).

476   Puig, A., Olmos, P., Quevedo, J. M., Guamis, B., & Mínguez, S. (2008).

477   Microbiological and sensory effects of musts treated by High-Pressure

478   Homogenization. *Food Science and Technology International*, *14*(5_suppl), 5–11.

479   https://doi.org/10.1177/1082013208094579

480   Salaha, M.-I., Kallithraka, S., Marmaras, I., Koussissi, E., & Tzourou, I. (2008). A

481   natural alternative to sulphur dioxide for red wine production: Influence on colour,

482   antioxidant activity and anthocyanin content. *Journal of Food Composition and*

483   *Analysis*, *21*(8), 660–666. https://doi.org/10.1016/J.JFCA.2008.03.010

484   Suárez-Jacobo, Á., Saldo, J., Rüfer, C. E., Guamis, B., Roig-Sagués, A. X., & Gervilla,

485   R. (2012). Aseptically packaged UHPH-treated apple juice: Safety and quality

486   parameters during storage. *Journal of Food Engineering*, *109*(2), 291–300.

487   https://doi.org/10.1016/J.JFOODENG.2011.09.007

488   Urbano-Cuadrado, M., Luque De Castro, M. D., Pérez-Juan, P. M., García-Olmo, J., &

489   Gómez-Nieto, M. A. (2004). Near infrared reflectance spectroscopy and

490   multivariate analysis in enology: Determination or screening of fifteen parameters

491   in different types of wines. *Analytica Chimica Acta*, *527*(1), 81–88.

492   https://doi.org/10.1016/j.aca.2004.07.057

493   Viana, F., Belloch, C., Vallés, S., & Manzanares, P. (2011). Monitoring a mixed starter

494   of *Hanseniaspora vineae–Saccharomyces cerevisiae* in natural must: Impact on 2-

495   phenylethyl acetate production. *International Journal of Food Microbiology*,

496   *151*(2), 235–240. https://doi.org/10.1016/J.IJFOODMICRO.2011.09.005

497   Viana, F., Gil, J. V., Vallés, S., & Manzanares, P. (2009). Increasing the levels of 2-

498   phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora*

499   *osmophila* and *Saccharomyces cerevisiae*. *International Journal of Food*

500   *Microbiology*, *135*(1), 68–74.

501   https://doi.org/10.1016/J.IJFOODMICRO.2009.07.025

502   Wang, Z., Zhuge, J., Fang, H., & Prior, B. A. (2001). Glycerol production by microbial

503   fermentation: A review. *Biotechnology Advances*, *19*(3), 201–223.

504   https://doi.org/10.1016/S0734-9750(01)00060-X

505   Ypsicon. (2018). Ultra High Pressure Homogenization/Sterilization. Retrieved August

506   29, 2018, from http://www.ypsicon.com

507   Zamora, A., & Guamis, B. (2015). Opportunities for Ultra-High-Pressure

508   Homogenisation (UHPH) for the Food Industry. *Food Engineering Reviews*, *7*(2),

509   130–142. https://doi.org/10.1007/s12393-014-9097-4

510   Zoecklein, B. W., Fugelsang, K. C., Gump, B. H., & Nury, F. S. (1995). Volatile

511   Acidity. In *Wine Analysis and Production* (pp. 192–198). Boston, MA: Springer

512   US. https://doi.org/10.1007/978-1-4757-6978-4_11

513

514 **Tables**

515

Table 1. Enological parameters of musts sulfited, processed by UHPH or untreated before fermentation. Values are means with standard deviations, n=3. Values with the same letter in the same row are not significantly different (p<0.05). Analyses were performed by FTIR.

	Must with SO$_2$	UHPH processed	Non treated
Sugars (g/L)	169.9 ± 1.1c	151.9 ± 0.3b	147.1 ± 2.2a
TSS (ºBrix)	16.1 ± 0.2c	14.6 ± 0.1b	14.2 ± 0.2a
Total acidity (g/L)	5.9 ± 0.1a	6.6 ± 0.1b	6.5 ± 0.2b
pH	3.2 ± 0.0a	3.3 ± 0.0a	3.3 ± 0.0a
Volatile acidity (g/L)	0.1 ± 0.0b	0.1 ± 0.0b	0.0 ± 0.0a
α-amino N (mg/L)	149.9 ± 4.5a	192.3 ± 6.1b	196.8 ± 6.1b
Ammonia (mg/L)	112.6 ± 1.3a	167.4 ± 11.8b	173.4 ± 9.9b
Gluconic acid (g/L)	nd	nd	0.1 ± 0.1

nd: Not detected

516
517
518
519
520
521
522
523

Table 2. Enological parameters of the wines produced from the musts sulfited, processed by UHPH or untreated. Values are means with standard deviations, n=3. Values with the same letter in the same row are not significantly different (p<0.05). Analysis of organic acids, residual sugars and glycerol were made by enzymatic tests. Ethanol was analyzed by LC-RID and pH with a pH-meter.

	Must with SO$_2$	UHPH processed	Non treated
Malic acid (g/L)	2.3 ± 0.0a	3.1 ± 0.0b	3.1 ± 0.0b
Lactic acid (g/L)	nd	nd	nd
Acetic acid (g/L)	0.2 ± 0.0a	0.2 ± 0.0a	0.6 ± 0.4a
Residual sugars (g/L)	0.1 ± 0.1a	0.2 ± 0.1a	0.1 ± 0.1a
Glycerol (g/L)	9.7 ± 0.1c	7.4 ± 0.1a	8.1 ± 0.1b
Ethanol (% v/v)	10.1 ± 0.1b	8.7 ± 0.1a	8.7 ± 0.1a
pH	3.1 ± 0.0a	3.1 ± 0.0a	3.1 ± 0.0a

nd: Not detected

524
525
526

527
528

Table 3. Fermentative metabolites analyzed by GC-FID produced in musts sulfited, processed by UHPH or untreated after fermentation with *S. cerevisiae* (7VA). Values are means with standard deviations, n=3. Values with the same letter in the same row are not significantly different (p<0.05). Concentrations in mg/L.

	Must with SO_2	UHPH processed	Non treated
Acetaldehyde	94.43 ± 19.94b	32.89 ± 3.18a	29.92 ± 8.72a
Diacetyl	nd	nd	nd
Acetoin	8.05 ± 0.46a	7.82 ± 0.59a	8.65 ± 1.21a
Methanol	52.86 ± 0.85b	31.30 ± 4.34a	32.68 ± 0.95a
1-Propanol	26.20 ± 0.54a	42.40 ± 3.45c	36.33 ± 0.63b
2-Butanol	nd	nd	nd
Isobutanol	79.88 ± 3.11c	62.27 ± 3.79b	55.91 ± 2.25a
1-Butanol	nd	4.01 ± 0.08	nd
2-Methyl-1-butanol	270.15 ± 9.79c	87.04 ± 4.52a	132.07 ± 0.64b
3-Methyl-1-butanol	68.61 ± 2.04c	22.48 ± 0.53a	30.22 ± 1.01b
Hexanol	7.41 ± 0.11c	5.01 ± 0.63a	6.03 ± 0.32b
2-Phenyl ethanol	49.58 ± 2.89c	22.64 ± 1.95a	27.91 ± 0.46b
2,3 butanediol	466.30 ± 3.17c	383.77 ± 17.16b	344.38 ± 5.39a
Ethyl acetate	27.62 ± 0.88a	61.24 ± 2.84b	69.56 ± 10.78b
Isoamyl acetate	nd	5.05 ± 0.25a	5.25 ± 0.90a
Isobutyl acetate	nd	1.25 ± 0.04a	0.85 ± 0.74a
Ethyl butyrate	nd	1.59 ± 0.05a	1.54 ± 0.01a
Ethyl lactate	4.21 ± 3.65a	6.33 ± 0.09a	6.27 ± 0.14a
2-Phenylethyl acetate	nd	5.52 ± 0.06b	5.37 ± 0.09a
Higher alcohols	501.84 ± 18.24c	245.85 ± 8.59a	288.48 ± 1.00b
Esters	31.84 ± 3.72a	80.98 ± 2.71b	88.84 ± 9.22b
Total volatiles	1,155.32 ± 36.33b	782.61 ± 24.45a	792.96 ± 11.64a

nd: Not detected

529

530

Figure captions

Figure 1. Wild yeast counts in musts sulfited, processed by UHPH or untreated before

fermentation (A) and 9 days after the beginning of fermentation (B). In B, the observed

yeasts are wild species together with the inoculated *S. cerevisiae*. Plating media used were

GCA for total yeasts and fungus, Lysine media for non-*Saccharomyces* yeasts. Values

are means with SD of 3 independent fermentations. Different letters indicate significant

differences between means ($p < 0.05$). nd: not detected, LOD 10 CFU/mL.

Figure 2. Wild bacteria counts in musts sulfited, processed by UHPH or untreated before

fermentation (A) and 9 days after the beginning of fermentation (B). Plating media used

were PCA for aerobic bacteria, and MRS for lactic acid bacteria. Values are means with

SD of 3 independent fermentations. Different letters indicate significant differences

between means ($p < 0.05$). nd: not detected, LOD 1 CFU/mL.

Figure 3. Fermentation kinetics in the musts that were sulfited, processed by UHPH or

untreated when evolved under fermentation by grape wild population without yeast

inoculation. Values are means with SD of 3 independent fermentations. Fermentation

evolution was represented by ethanol formed (% v/v) calculated from the CO_2 loses.
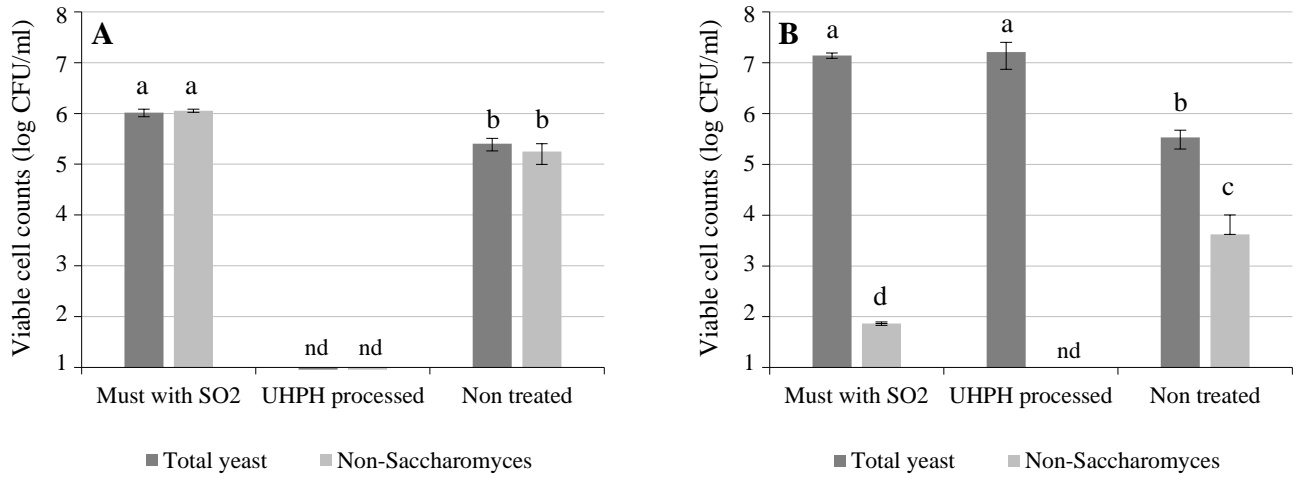
Figure 4. A. Wine color intensity and hue after fermentation of the musts sulfited,

processed by UHPH or untreated. **B.** Total polyphenol index (TPI) and hydroxycinnamic

acid index after fermentation of the musts sulfited, processed by UHPH or untreated.

553   Values are means with SD of 3 independent fermentations. Bars of the same parameter

554   with the same letter are not significantly different (p<0.05).
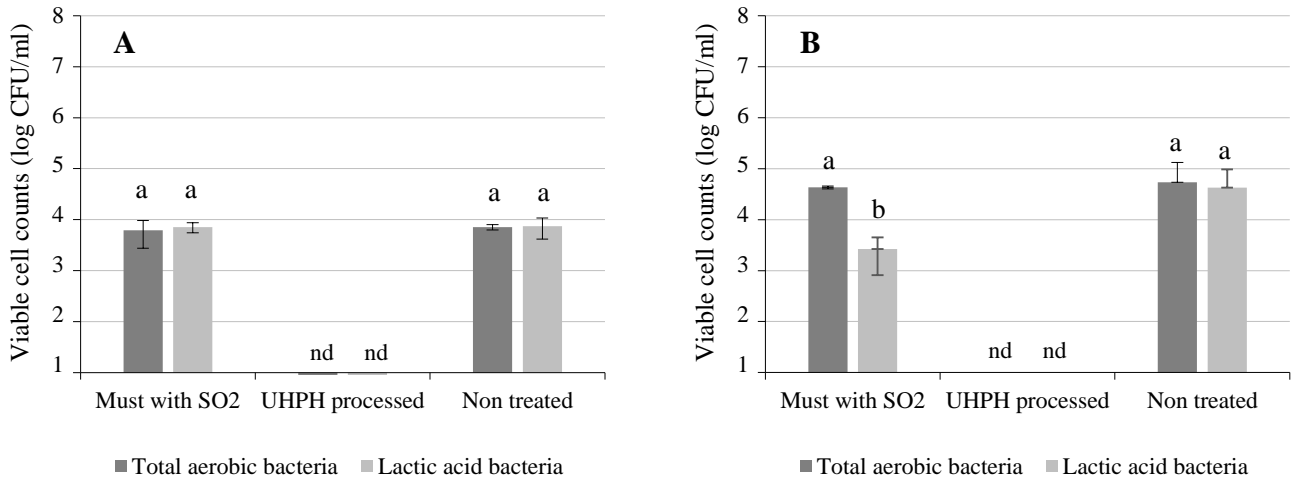
555

556   **Figure 5.** Sensory analysis of the wines made from the musts that were sulfited, processed

557   by UHPH or untreated. Values are means of 9 tasters. Means in the same axes with the

558   same letter are not significantly different (p<0.05).
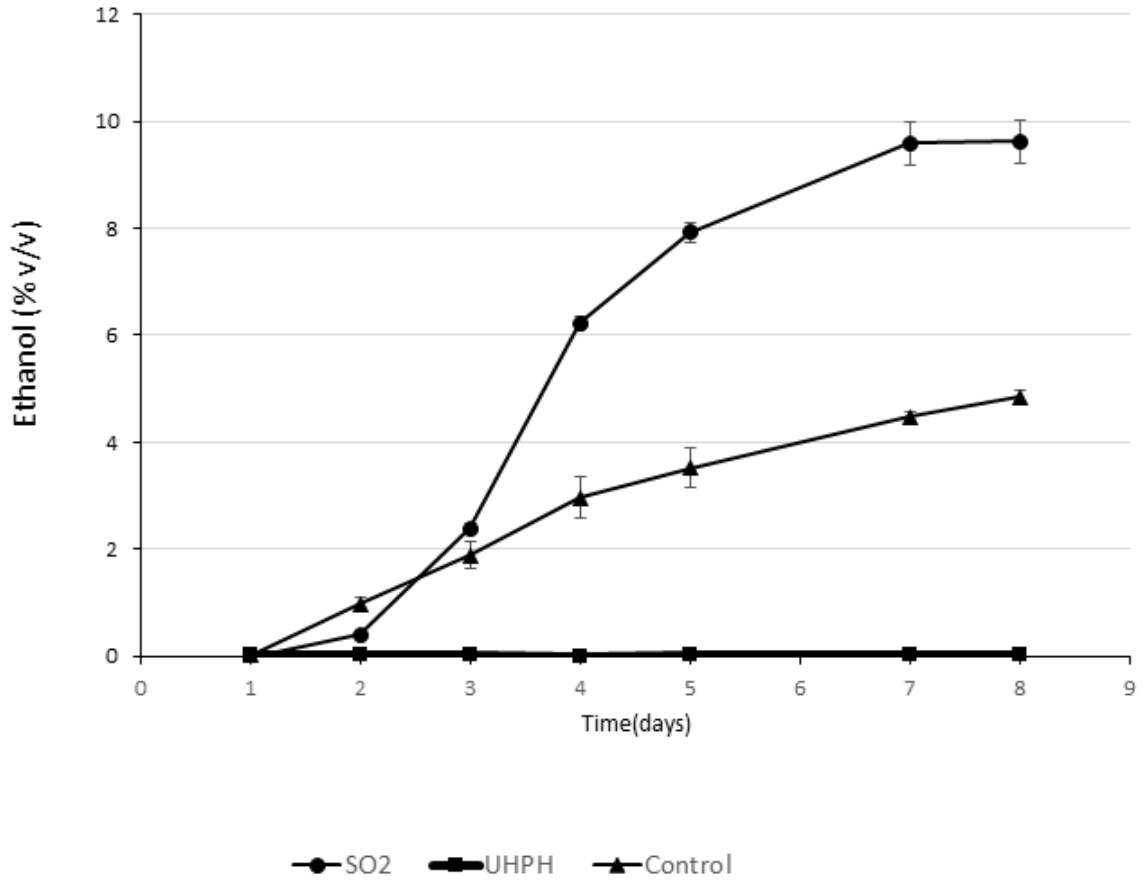
559 **Figure 1.**
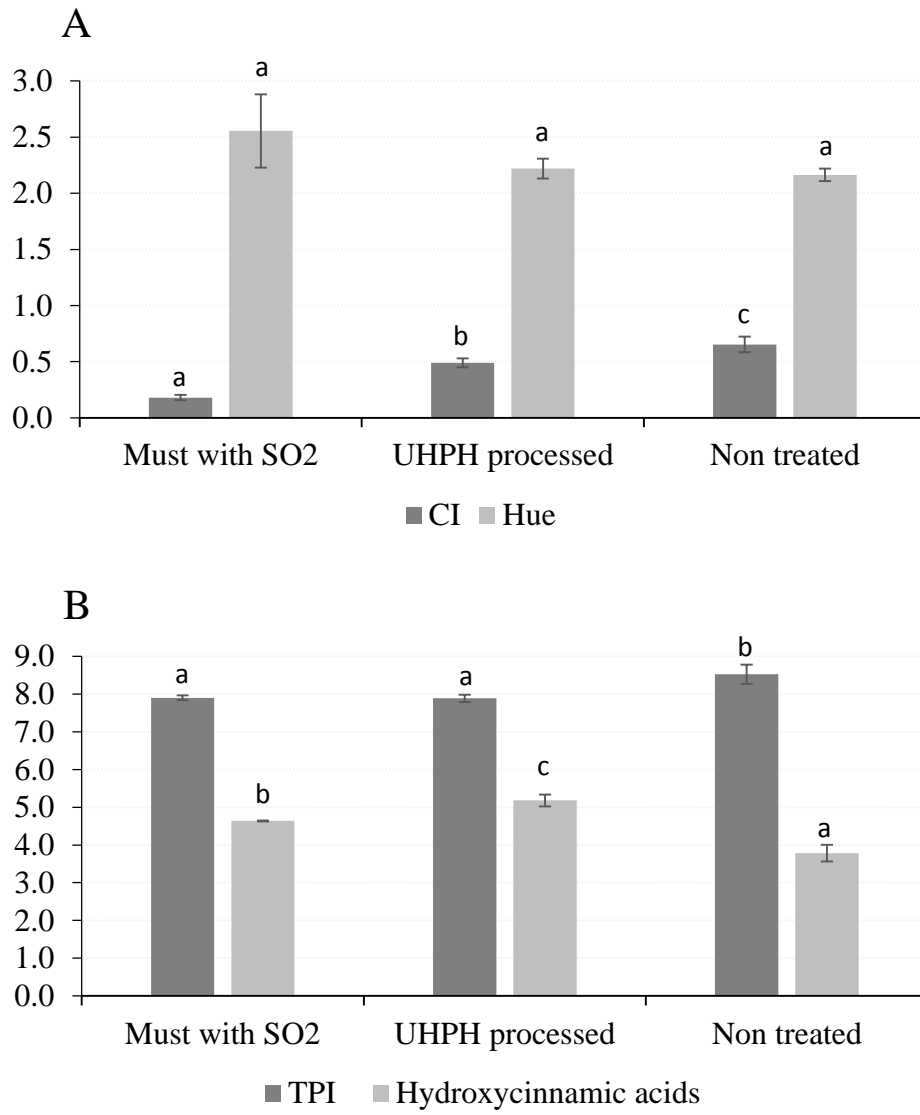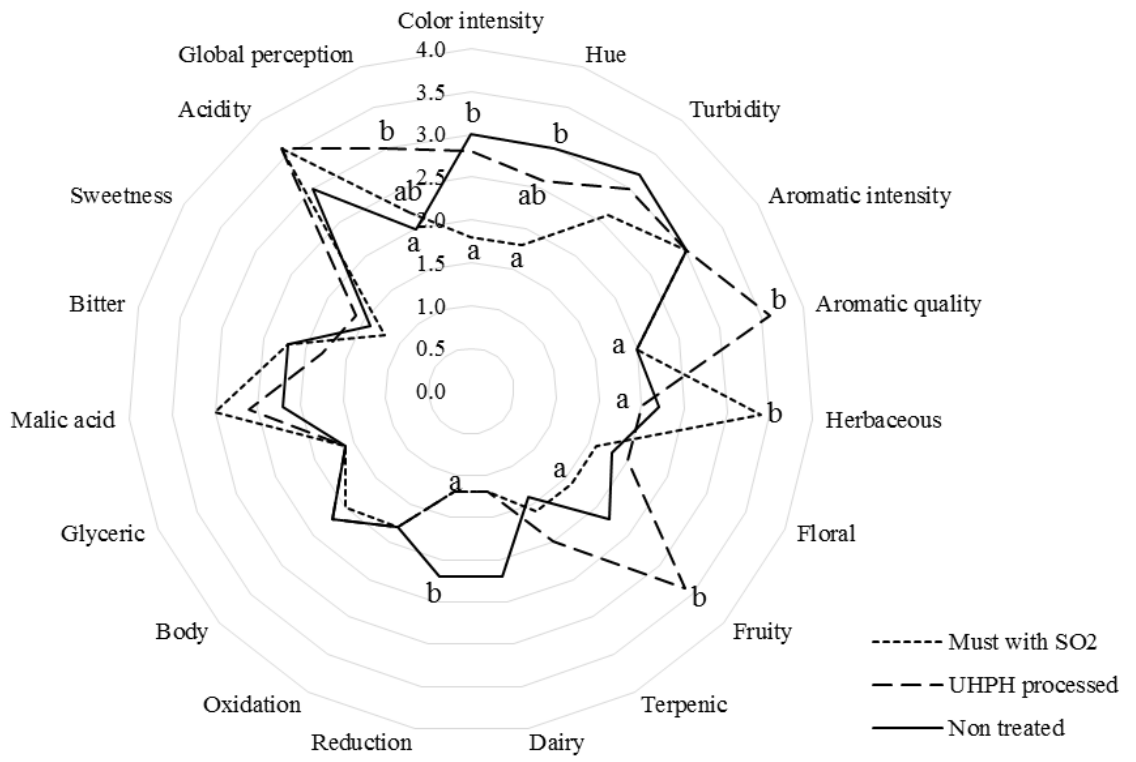

560

561

562 **Figure 2**

563

564

565   **Figure 3.**

566

Ethanol (% v/v) vs Time (days). Legend: SO2, UHPH, Control

567

568

569 **Figure 4.**

A

B

570

571

572   **Figure 5.**

Radar chart with axes: Color intensity, Hue, Turbidity, Aromatic intensity, Aromatic quality, Herbaceous, Floral, Fruity, Terpenic, Dairy, Reduction, Oxidation, Body, Glyceric, Malic acid, Bitter, Sweetness, Acidity, Global perception. Scale from 0.0 to 4.0. Legend: Must with SO2, UHPH processed, Non treated.
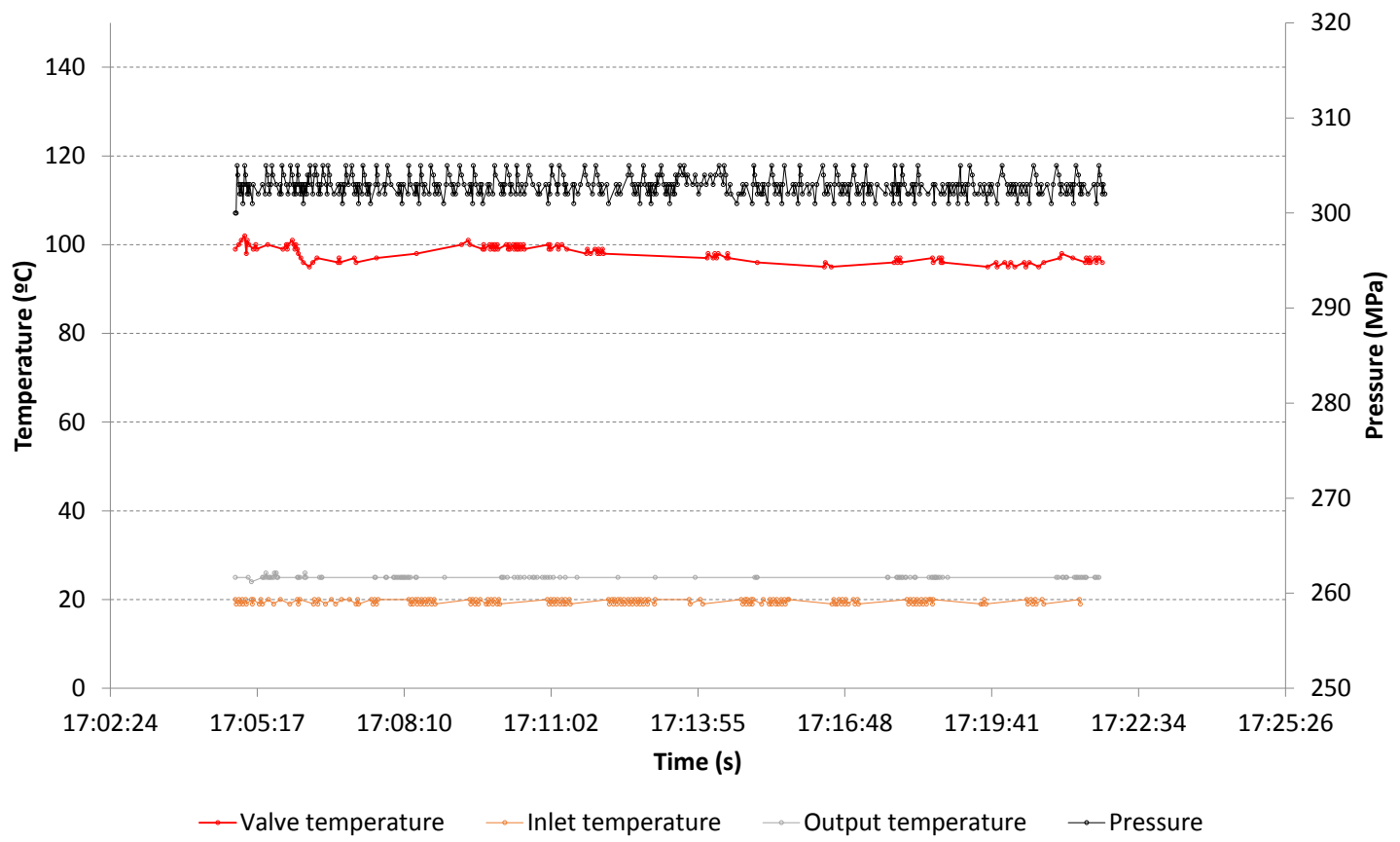
573

574

575

576   **Supplementary Figure**

577

579   Temperature (ºC) and pressure (MPa) in the valve during the process

580

581