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Impact of high-pressure carbon dioxide on polyphenoloxidase activity and stability of fresh apple juice

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Abstract: Freshly-extracted apple juice was exposed to high pressure carbon dioxide (HP-CO<sub>2</sub>) treatment at 20, 35 and 45 °C at different pressure conditions (6.0, 12.0 and 18.0 MPa) for up to 30 min. Samples were analysed for residual enzymatic activity. The time needed for 90% enzyme inactivation (D<sub>p</sub>) decreased when CO<sub>2</sub> pressure increased, while the CO<sub>2</sub> pressure sensitivity of the enzyme (z<sub>p</sub>) showed no variation with temperature. The HP-CO<sub>2</sub> treatment at 12 MPa and 35 °C allowed the minimum residual enzyme activity (20%) to be reached in 10 min. Samples treated under these conditions showed lower polyphenoloxidase activity and higher microbial stability than untreated apple juice while presenting a sensory fresh-likelihood higher than thermally pasteurized apple juice.

Dear Editor,

We considered further comments and suggestions and modified the manuscript accordingly. We are thus sending you the revised work.

Kind regards,

Stella Plazzotta

**Answer to Editor's comments:**

Reference style: all authors (up to 6) should be given at the first citation of a publication (and in the subsequent citations 1st author et al, for more than 2 authors); if more than 6: 1st author et al also at first citation; in the reference list, according to APA "give surnames and initials for up to and including seven authors. When authors number eight or more, include the first six authors' names, then insert three ellipsis points, and add the last author's name". References were modified according to Editor's suggestion (lines 50, 52, 60, 62, 333, 401).

L167-168: Two experiments for each treatment but how many juice batches? How many replicates for the juice production?

Requested information was added in lines 79-83 and 174-175.

Express enzyme activity in katal

Enzyme activity was expressed in katal as requested (lines 141-147, 441).

As Figures 5 and 6 are all related to sensory analysis could you please group them in Figure 5 A and B

Figures 5 and 6 were grouped as suggested (lines 346, 349, 357)

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HP-CO<sub>2</sub> treatment increases microbial stability of apple juice

HP-CO<sub>2</sub> treatment does not impair apple juice fresh-likeness

1    **Impact of high-pressure carbon dioxide on polyphenoloxidase activity and stability of fresh**  
2    **apple juice**

3

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10

11    Key-words: dense-phase CO<sub>2</sub>; inactivation kinetics; colour; sensory properties

12

13    **Highlights**

14    HP-CO<sub>2</sub> treatment promotes partial inactivation of polyphenoloxidase in apple juice

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16    HP-CO<sub>2</sub> treatment does not impair apple juice fresh-likelihood

17

18    **Abstract**

19    Freshly-extracted apple juice was exposed to high pressure carbon dioxide (HP-CO<sub>2</sub>) treatment at  
20    20, 35 and 45 °C at different pressure conditions (6.0, 12.0 and 18.0 MPa) for up to 30 min.  
21    Samples were analysed for residual enzymatic activity. The time needed for 90% enzyme  
22    inactivation ( $D_p$ ) decreased when CO<sub>2</sub> pressure increased, while the CO<sub>2</sub> pressure sensitivity of the  
23    enzyme ( $z_p$ ) showed no variation with temperature. The HP-CO<sub>2</sub> treatment at 12 MPa and 35 °C  
24    allowed the minimum residual enzyme activity (20%) to be reached in 10 min. Samples treated  
25    under these conditions showed lower polyphenoloxidase activity and higher microbial stability than

untreated apple juice while presenting a sensory fresh-likelihood higher than thermally pasteurized apple juice.

28

## 29 **Introduction**

30 Consumption of unprocessed fruit juices has substantially risen over the last few years, mostly due  
31 to the increasing demand for good nutritional quality foods with fresh-like characteristics (Beuchat,  
32 1996; Raybaudi-Massilia, Mosqueda-Melgar, Soliva-Fortuny & Martín-Belloso, 2009). As a  
33 consequence of inappropriate manipulation and storage, both spoilage and pathogenic  
34 microorganisms can grow, leading to hygienic and quality issues. Enzymatic activity can also  
35 contribute to quality depletion, along with physical and chemical changes during the storage  
36 (Raybaudi-Massilia et al., 2009). To guarantee product safety and provide an adequate shelf-life,  
37 unpasteurized juices are generally distributed under refrigerated conditions. They are traditionally  
38 obtained by a combination of formulation strategies such as water activity reduction, nutrient  
39 restriction, acidification as well as use of antimicrobial additives (Davidson, 2001). These  
40 preservation strategies hardly fit with the current demand for fresh-like juices that are free from  
41 additives, generating the need for developing novel non-thermal treatments for juice stabilization.

42 High pressure carbon dioxide (HP-CO<sub>2</sub>) has been reported as a promising non-thermal technology  
43 for the stabilization of fresh products. During the treatment, food is in contact with pressurised CO<sub>2</sub>  
44 at temperature/pressure conditions that may be below or above the critical point (31.1 °C, 7.38  
45 MPa). Typical CO<sub>2</sub> pressure is generally within 4 and 30 MPa, rarely exceeding 50 MPa.  
46 Temperature is generally between 20 and 50 °C, low enough to maintain the freshlikelihood of  
47 treated products (Manzocco et al., 2016).

48 Significant lethal effects of HP-CO<sub>2</sub> on different microorganisms have been demonstrated in fruit  
49 juices (Spilimbergo & Bertucco, 2003; Damar & Balaban, 2006; Ferrentino, Bruno, Ferrari, Poletto  
50 & Balaban, 2009; Xu, Zhang, Wang, Bi, Buckow & Liao, 2011). In particular, the technology is  
51 known to promote up to 5 Log reductions in microbial counts, approaching those required for

pasteurization (Kincal, Hill, Balaban, Portier, Wei & Marshall, 2005; Ferrentino & Spilimbergo, 2011). The germicidal activity of HP-CO<sub>2</sub> is due to the combination of temperature, pressure and specific effects of HP-CO<sub>2</sub>. The treatment is associated with extracellular and intracellular acidification, destabilization of membranes and denaturation of microbial enzymes (Jones & Greenfield, 1982; Hutkins & Nannen, 1993; Bothun, 2004; Bothun, Knutson, Strobel & Nokes, 2005). More controversial is the effect of HP-CO<sub>2</sub> in inactivating fruit enzymes leading to juice quality decay. For instance, inactivation of polyphenoloxidase responsible for browning of fruit juices, depends on the nature of the enzyme and is strongly affected by CO<sub>2</sub> pressure, temperature and treatment time (Gui, Chen, Wu, Wang, Liao & Hu, 2006; Liao, Zhang, Bei, Hu & Wu, 2009; Zhou, Zhang, Hu, Liao & He, 2009; Spilimbergo, Komes, Vojvodic, Levaj & Ferrentino, 2013). The mechanisms involved in enzyme inactivation by HP-CO<sub>2</sub> include pH lowering (Balaban, Arreola, Marshall, Peplow, Wei & Cornell, 1991) and changes in the conformation of the secondary structure of the enzyme (Chen, Balaban, Wei, Marshall & Hsu, 1992; Manzocco et al., 2016). Based on these considerations, the present paper was addressed to investigate the impact of HP-CO<sub>2</sub> treatment on polyphenoloxidase activity and stability of fresh apple juice intended for refrigerated storage. To this aim, apple juice was exposed to HP-CO<sub>2</sub> treatments in a wide range of pressure, temperature and treatment time conditions. Apple juice was then submitted to the HP-CO<sub>2</sub> treatment leading to the minimum polyphenoloxidase activity at the mildest pressure/temperature combination and stored at 4 °C for up to 15 days. HP-CO<sub>2</sub> treated apple juice was monitored during storage for residual polyphenoloxidase activity, colour, microbial counts and sensory attributes. To verify the potential applicability of HP-CO<sub>2</sub> technology to produce fresh apple juice, data were compared to those relevant to an untreated apple juice. An apple juice submitted to conventional thermal pasteurization was also considered as additional control.

## 2. Material and methods



78    *2.1 Apple juice extract*

79    A 10 kg batch of fresh apples “Golden delicious” were purchased at the local market and stored at 4  
80    °C overnight. When the experiments were performed, apples had a dry matter content of  $164.7 \pm 1.6$   
81    g/kg, a soluble solid content of  $13.3 \pm 0.2$  °Brix, a pH of  $4.2 \pm 0.2$  and a titratable acidity of  
82     $4.6 \pm 0.3$  g/kg. Apple juice was prepared fresh for every trial from the same batch of fruits, to  
83    minimize sample variability. The juice was obtained by using a domestic juicer (Moulinex, mod.  
84    Vitae JU2000, Milan, Italy), filtered through two layers of cloth filter and centrifuged at 5000 g for  
85    5 min at 4 °C (Beckman, Avanti™ J-25, High performance centrifuge, Brea, USA). The supernatant  
86    was filtered again through two layers of cloth filter and the resulting clear juice was immediately  
87    treated.

88

89    *2.2 High-pressure CO<sub>2</sub> treatments*

90    HP-CO<sub>2</sub> inactivation process was carried out in a double-batch apparatus. The system consists of  
91    two identical stainless steel cylinders with a screwed cap and an internal volume of 150 mL,  
92    connected in parallel. Each reactor was connected to an on-off valve that can be used to  
93    depressurise it independently from the other. The two reactors were submerged in a thermostatic  
94    water bath (CB 8-30e, Heto, Allerød, Denmark). For more details, please refer to Manzocco et al.  
95    (2016). Before starting the pressurisation, the temperature of the sample was allowed to reach  
96    equilibrium. The time needed to reach the desired temperature (20, 35 or 45 °C) and the  
97    pressurisation time were lower than 3 min. After reaching the desired pressure (6, 12 and 18 MPa),  
98    the pump was switched off and valves connected to each vessel were tightly closed. After  
99    increasing treatment time up to 30 min, vessels were depressurised. In all experiments,  
100    depressurisation was completed within 10 min and the outlet flow was controlled using a digital  
101    flowmeter (PFM 750, SMC Italia S.p.A., Milan, Italy). Control samples were prepared by treating  
102    the apple juice in the vessels at atmospheric pressure (0.1 MPa) and thus at CO<sub>2</sub> partial pressure  
103    equal to 0.0039 MPa.

104

### 105 *2.3 Thermal treatment*

106 Aliquots of 100 mL of apple juice were placed in plastic pouches (PA/PE, 20 x 28 cm, Savonitti,  
107 Codroipo, Italy). A thin layer of sample was obtained, being the maximum thickness of the filled  
108 pouches lower than 0.5 cm. Pouches were heated in a water bath (IKA-Werke, Staufen, Germany)  
109 at 71.1 °C for 6 s (FDA, 2004). After thermal treatment, samples were quickly cooled under  
110 running water at room temperature.

111

### 112 *2.4 Apple juice storage*

113 Aliquots of 10 mL of apple juice were introduced in Eppendorf® vials of 10 mL capacity and  
114 stored for up to 15 days at 4 °C in a refrigerated cell. At increasing time during storage, samples  
115 were removed from the refrigerator, equilibrated at 22 °C and submitted to the analysis.

116

### 117 *2.5 Apple physical-chemical parameters*

118 Soluble solid content (SSC) was measured using a table refractometer (Unirefrax, Bertuzzi, Milan,  
119 Italy) calibrated with distilled water.

120 Dry matter content of apple samples was determined gravimetrically by recording difference in  
121 weight before and after drying at 70 °C, until a constant weight was achieved (M.U.A.C.V., 1989).

122 Titratable acidity was determined by titration with NaOH 0.1 mol/L and phenolphthalein as  
123 indicator (Sigma-Aldrich, Milan, Italy), accordingly to the official M.U.A.C.V. method (1989) and  
124 expressed as g of acids/kg of fresh product.

125 Analyses of SSC and TA were carried out on the solution obtained after homogenization (Polyton,  
126 Kinematica, Luzern, Switzerland) and filtration of apple cubes through filter paper (Whatman #1,  
127 Whatman International Ltd, Maidstone, UK).

128

### 129 *2.6 Temperature, pH*

130 During HP-CO<sub>2</sub> treatments and thermal pasteurization, temperature was measured by a  
131 thermocouple probe (Hanna Instruments, Tersid s.r.l., Milan, Italy); pH was assessed using a pH-  
132 meter (Mettler Toledo 355, Lou Analyzer, Halstead, England).

133

#### 134 *2.7 Polyphenoloxidase activity*

135 The polyphenoloxidase activity was assayed spectrophotometrically (Shimadzu UV-2501PC, UV-  
136 Vis recording spectrophotometer, Shimadzu Corporation, Kyoto, Japan) at 25 °C according to the  
137 methodology of Kahn (1995). The reaction was started by the addition of 500 µL of apple juice to 2  
138 mL of 0.1 mol/L potassium phosphate buffer pH 7 and 1.5 10<sup>-3</sup> mol/L L-Dopa (Carlo Erba, Milan,  
139 Italy). The absorbance at 420 nm was monitored each minute for 10 min. The changes in  
140 absorbance per min were calculated by linear regression, applying the pseudo zero order kinetic  
141 model. The eventual final stationary phase was excluded from regression data. The slope of the very  
142 first linear part of the reaction curve was used to determine polyphenoloxidase specific activity. The  
143 latter was defined as the amount of enzyme that produced 1 µmol of quinone per second (µkatal)  
144 (Lee et al., 2010). The average polyphenoloxidase activity in untreated juice was found to be 0.047  
145 µkatals. Polyphenoloxidase residual activity (RA%) upon treatments was calculated as the  
146 percentage ratio between enzymatic activity of the treated sample and that of the untreated one (de  
147 la Rosa et al., 2011; Niu et al., 2010; Xu et al., 2011).

148

#### 149 *2.8 Browning*

150 Browning was assessed spectrophotometrically (Shimadzu UV-2501PC, UV-Vis recording  
151 spectrophotometer, Shimadzu Corporation, Kyoto, Japan) measuring absorbance values at 420 nm  
152 and 25 °C of apple juice samples, diluted with water to obtain absorbance signals within the scale.

153

#### 154 *2.9 Microbiological analyses*

155 For microbiological analyses, appropriate aliquots (0.1 or 1 mL) of properly diluted apple juice  
156 were spread on agar plates. Plate Count Agar (Oxoid, Milan, Italy) and Man Ragosa Sharpe (MRS)  
157 (Oxoid, Milan, Italy) were used for enumeration of mesophilic and lactic acid bacteria respectively,  
158 and plates were incubated for 48 h at 37 °C. Oxytracycline-Glucose-Yeast Extract (OGYE) agar  
159 (Oxoid, Milan, Italy) was used for enumeration of yeasts and moulds, and plates were incubated for  
160 72 h at 28 °C.

161

#### 162 *2.10 Sensory analysis and off-odour perception*

163 A panel of 20 Italian assessors, not trained but expert in method, was selected. For sensory testing,  
164 10 mL apple juice was served in odourless plastic glasses at 6 °C. Water was used between samples  
165 for mouth rinsing. Samples were indicated by a three-digit code and served the panel paired with a  
166 just prepared control sample, identified as “reference”. The judges were asked to evaluate sample  
167 colour, apple flavour, cooked taste and acidity assigning each descriptor a score on a 9-point scale  
168 anchored with “reference” at point 5. Judges were also asked to indicate the possible perception of  
169 off-flavours. Off-flavour perception data were expressed as percentage of judges that identified the  
170 defect as respect to the reference. Sensory analysis requiring juice drinking was only carried out  
171 until all samples had total viable count lower than 5 Log CFU/g.

172

#### 173 *2.11 Data analysis*

174 Data reported in this work are expressed as mean ± S.D of at least three measurements carried out  
175 on two experiments replicated on different juice extraction batches.

176 Apparent inactivation rate constants of polyphenoloxidase were analysed by using a conventional  
177 first-order equation:

$$178 \quad \frac{d(RA\%)}{dt} = -k \cdot (RA\%) \quad (1)$$

179 Where  $RA\%$  is the polyphenoloxidase residual activity in the juice at time  $t$  (min) and  $k$  is the  
180 inactivation rate constant ( $\text{min}^{-1}$ ). The value of  $k$  was obtained as the slope of the regression of the  
181 decimal logarithm of  $RA\%$  vs.  $t$ .

182 The eventual final stationary phase was excluded from regression data.

183 The value of  $RA\%$  after 30 min was taken as an indicator of the minimum  $RA\%$  achievable by the  
184 treatment.

185 The kinetic parameter  $D_P$  was obtained using procedures analogous to that employed in thermal  
186 death time studies. In particular,  $D_P$  is the decimal reduction time, i.e. the treatment time needed for  
187 90% enzyme activity reduction at a given pressure and temperature.  $D_P$  was computed as the  
188 negative reciprocal of  $k$ .

189 The pressure increase needed for a 90% reduction of the  $D_P$  value was computed as  $z_p$  (MPa). The  
190 value of  $z_p$  was obtained by regressing the decimal logarithm of  $D_P$  versus pressure ( $P$ ):

191 
$$\log D_p = -\frac{P}{z_p} \quad (2)$$

192 The  $z_p$  was then derived as the negative reciprocal slope of the regression line.

193 The pressure dependence of the inactivation rate constants ( $k$ ) was expressed by the activation  
194 volume ( $\Delta V^\ddagger$ ,  $\text{cm}^3/\text{mol}$ ), according to the Eyring equation (Weemaes, Ludikhuyze, Van den Broeck  
195 & Hendrickx 1998):

196 
$$\ln k = \ln k_{atm} - \frac{\Delta V^\ddagger}{RT} \cdot (P - P_{atm}) \quad (3)$$

197 where  $P$  is pressure (MPa),  $k_{atm}$  is the inactivation rate constant at ambient pressure  $P_{atm}$  (0.1 MPa),  
198  $R$  is the gas constant ( $8.31 \text{ cm}^3 \text{ MPa K}^{-1} \text{ mol}^{-1}$ ) and  $T$  is temperature (K).  $\Delta V^\ddagger$  was estimated from  
199 the slope of the line obtained by the regression of the natural logarithm of  $k$  vs.  $P$ .

200 Goodness-of-fit was evaluated by means of the determination coefficients ( $R^2$ ). Analysis of  
201 variance (ANOVA and Tuckey test) were accomplished using the v. 3.1.1 of R software (The R  
202 foundation for statistical computing), to determine the significance at a 95% level.

203

### 204 3. Results and discussion

205

#### 206 3.1. Effect of high pressure carbon dioxide on apple juice polyphenoloxidase activity

207 Figure 1 shows the residual polyphenoloxidase activity of apple juice as a function of treatment  
208 time at different pressures at 20, 35 and 45 °C.

209 Control apple juice treated for increasing time at 20 °C under environmental conditions (0.1 MPa  
210 CO<sub>2</sub> pressure) showed a significant decrease in polyphenoloxidase activity. According to Le  
211 Bourvellec, Le Quéré, Sanoner, Drilleau & Guyot (2004), this effect is probably due to the  
212 formation of chemically oxidised polyphenols with anti-enzymatic activity upon contact of apple  
213 derivatives with oxygen. Exposure of apple juice to increasing CO<sub>2</sub> pressure resulted in  
214 progressively higher enzyme inactivation (Figure 1a). However, even applying 18 MPa for 30 min,  
215 the complete inactivation was not achieved. When apple juice was exposed to HP-CO<sub>2</sub> at 35 °C,  
216 polyphenoloxidase inactivation was more intense (Figure 1b). For instance, the minimum residual  
217 activity was reached upon few min of exposure to 18 MPa CO<sub>2</sub>. The effect of temperature on  
218 enzyme inactivation by HP-CO<sub>2</sub> was further confirmed by additional trials carried out at 45 °C  
219 (Figure 1c). In accordance with evidences from other Authors (Vamos-Vigyazo, 1981; Gui et al.,  
220 2007), these data demonstrate the existence of a negative relation between polyphenoloxidase  
221 activity and the increase in both CO<sub>2</sub> pressure and temperature, at least under the experimental  
222 conditions here tested.

223 Data shown in Figure 1 were analysed considering the minimum residual activity achievable by  
224 each treatment (Table 1). Due to the monotonic decrease of residual activity curves (Figure 1), the  
225 value after 30 min of juice treatment was taken as an indicator of the residual activity achievable at  
226 each temperature/pressure combination (Table 1). In particular, under the most intense CO<sub>2</sub>  
227 treatment conditions (18 MPa, 45 °C), a minimum residual activity (RA%) of 20% was still  
228 observed. It can be hypothesised that more intense treatments than those here performed are needed  
229 to reach complete inactivation. To this regard, contradictory information is reported in the literature.

230 In particular, Xu et al. (2011) found that polyphenoloxidase was completely inactivated by a  
231 treatment carried out at 22 MPa and 60 °C for 10 min. A similar effect was also observed by Niu et  
232 al. (2010) in apple slices treated at 20 MPa and 25 °C for 20 min. However, other authors reported  
233 that even applying CO<sub>2</sub> at 60 MPa and 55 °C for 60 min, a 40% minimum residual activity of  
234 polyphenoloxidase was still present (Gui et al., 2007). These different inactivation degrees can be  
235 attributed to many factors, including not only operative conditions, but also apple cultivar and  
236 derivative as well as equipment layout and operative parameters (Yemenicioğlu, Özkan,  
237 Cemeroğlu, Mehmet & Yemeniciog, 1997; Weemaes et al., 1998; Buckow, Weiss & Knorr, 2009;  
238 Xu et al., 2011).

239 The residual activity of polyphenoloxidase was regressed as a function of treatment time in the  
240 initial linear part of the curve (Figure 1) to obtain rate constants ( $k$ ) of polyphenoloxidase  
241 inactivation (Table 1). The latter were then used to calculate  $D_p$  values using procedures analogous  
242 to that employed in thermal death time studies (Table 1). In particular,  $D_p$  was defined as the  
243 treatment time needed for 90% enzyme activity reduction at a given pressure. The treatment at 6  
244 MPa and 20 °C led to a tenfold decrease of activity in *circa* 48 min. At the same temperature, this  
245 goal was achieved at 12 MPa in *circa* 34 min. On the other hand, keeping the pressure constant at 6  
246 MPa, inactivation was achieved at 35 or 45 °C in less than 25 or 12 min, respectively. These  $D_p$   
247 values suggest a lower resistance of apple juice polyphenoloxidase than that reported in the  
248 literature. To this regard, Gui et al. (2007) reported a 220 min  $D_p$  value for polyphenoloxidase of  
249 cloudy juice from *Fuji* apples upon exposure at 35 °C to 30 MPa CO<sub>2</sub>. These differences confirm  
250 the significant effect of processing conditions and *cultivar* on inactivation of apple  
251 polyphenoloxidase.

252  $D_p$  values (Table 1) were used to calculate the parameter  $z_p$ , describing the sensitivity of  
253 polyphenoloxidase to pressurised CO<sub>2</sub>. The decimal logarithmic values of  $D_p$  resulted well  
254 correlated ( $R^2 > 0.89$ ;  $p < 0.05$ ) with pressure for treatments carried out at 20, 35 and 45 °C (Table 2).

255 The  $z_p$  value, which represents the pressure range within which the  $D_p$  changes tenfold, resulted  
256 *circa* 20 MPa for treatments carried out at 20 °C. This indicates that an increase in pressure of 20  
257 MPa is necessary to get a 90 % decrease in  $D_p$  at this temperature. The increase in temperature from  
258 20 to 45 °C did not cause a significant decrease in  $z_p$  value, indicating a constant effect of pressure  
259 in inactivating the enzyme, at least within the temperature range here tested. To this regard,  
260 contradictory data are reported in the literature. Weemaes et al. (1998) detected antagonistic effects  
261 of pressure and temperature studying the effects of high static pressure combined with heating  
262 treatments on avocado polyphenoloxidase. On the other hand, when conditions similar to those here  
263 considered were applied on a polyphenoloxidase model system, a synergistic effect of pressure and  
264 temperature was observed (Manzocco et al., 2016). Values of  $z_p$  (Table 2) thus emphasise the  
265 critical role of enzyme origin and reaction media in determining its sensitivity to CO<sub>2</sub> pressure.

266 The effect of pressure on polyphenoloxidase inactivation was also expressed through the activation  
267 volume ( $\Delta V^\ddagger$ ) concept (Table 2). According to the transition state theory, the activation volume is a  
268 measure of the volume difference between the initial reactants and the activated complex at the  
269 transition state (Eyring, 1935). Data reported in Table 2 show that polyphenoloxidase inactivation is  
270 characterized by negative  $\Delta V^\ddagger$  with high absolute value ( $R^2 > 0.89$ ;  $p < 0.05$ ). This indicates that the  
271 increase in pressure strongly favoured the denaturation of the enzymatic protein (Ohmae,  
272 Murakami, Gekko & Kato, 2007). The values of activation volume for apple juice  
273 polyphenoloxidase resulted lower than that reported in the literature ( $-94.3 \text{ cm}^3 \text{ mol}^{-1}$  at 55 °C; Gui  
274 et al., 2007). This result indicates that, in our experimental conditions, polyphenoloxidase was more  
275 susceptible to CO<sub>2</sub> pressure variation. In addition, in agreement with  $z_p$  values, activation volume  
276 did not significantly decrease with the increase in temperature from 20 to 45 °C (Table 2). The  
277 increase in temperature promoted instead a significant increase in the pre-exponential or frequency  
278 factor ( $\ln k_{atm}$ ). The latter indicates how often the enzyme is properly oriented to undergo structural  
279 modifications leading to denaturation. In fact, the increase in temperature from 20 to 45 °C



enhanced the frequency factor (Table 2), indicating a higher frequency of steric conditions favouring denaturation.

### *3.2. Effect of high pressure carbon dioxide on apple juice quality during storage*

In the light of the previous results, it can be hypothesized that HP-CO<sub>2</sub> treatment could represent a non-thermal technological strategy to control enzymatic activity during storage of apple juice. In this context, HP-CO<sub>2</sub> could be proposed as a technology to stabilize refrigerated apple juice colour. To verify this hypothesis, a combination of processing conditions which could be potentially applicable on a larger scale to produce fresh refrigerated apple juice was selected. To this regard, treatments carried out at 20 °C were excluded since associated with residual activity higher than 30% even at the highest tested pressure (Table 1). The mildest pressure/temperature combination leading to the minimum residual activity (20%) was thus selected. As shown in Table 1, this combination corresponded to the treatment at 12 MPa and 35 °C. The juice was thus treated at these conditions for 10 min since longer treatment times did not promote further enzyme inactivation (Figure 1).

Even if similarly effective in terms of enzyme inactivation, treatments at temperature and pressure higher than 35 °C and 12 MPa respectively, were not considered since they are reasonably more energy-intensive and thus less sustainable from an environmental point of view.

Juice submitted to the selected treatment was then stored for up 15 days at 4 °C to simulate conventional distribution conditions of not thermally stabilized apple juice.

During storage, apple juice was analyzed for the evolution of polyphenoloxidase activity and browning. Microbial and sensory analyses were also performed to evaluate the hygienic level and the intensity of typical sensory attributes of the juice. Data were compared to those relevant to an untreated apple juice as well as a control thermal pasteurized apple juice (71 °C for 6 s). As expected, the latter presented no enzymatic activity during the entire storage time, in agreement with literature data (Golan-Goldhirsh, Whitaker & Kahn, 1984; McEvily, Iyengar & Otwell, 1992).

306 By contrast, HP-CO<sub>2</sub> treated and untreated apple juice showed different initial polyphenoloxidase  
307 activity, which progressively decreased during storage, approaching in both cases 5% after 10 days  
308 (Figure 2). These different inactivation degrees were probably associated with different evolution of  
309 browning (Whitaker, 1995; Yoruk & Marshall, 2006).

310 For this reason, juice browning was assessed spectrophotometrically at 420 nm (Figure 3).

311 Immediately after preparation, untreated, pasteurized and HP-CO<sub>2</sub> treated apple juices showed not  
312 significantly different browning. As expected, pasteurized juice did not show changes in browning  
313 during storage, due to the complete and irreversible inactivation of polyphenoloxidase upon thermal  
314 treatment. On the contrary, an increase in browning was detected in both untreated and HP-CO<sub>2</sub>  
315 treated samples. Beyond 3 days of storage, the latter showed browning values not significantly  
316 lower than those of the untreated sample, suggesting that the HP-CO<sub>2</sub> treatment here applied was  
317 not able to significantly reduce browning phenomena during storage.

318 To evaluate the ability of HP-CO<sub>2</sub> treatment to stabilize fresh apple juice against microbial spoilage,  
319 total viable and lactic acid bacteria, yeasts and moulds were determined during storage. Whilst  
320 pasteurized apple juice always presented microbial counts below the detection limit (data not  
321 shown), untreated and HP-CO<sub>2</sub> treated apple juice showed different evolution of these microbial  
322 populations during storage (Figure 4).

323 In the just prepared apple juice, lactic acid bacteria and moulds were below the detection limit and  
324 the total bacterial count was mainly represented by yeasts. These microorganisms are well known to  
325 be the main spoilage agents in fruit derivatives, due to their low pH (Raybaudi-Massilia et al.,  
326 2009). During the refrigerated storage, total bacterial count of untreated juice progressively  
327 increased up to 6 Log CFU/mL (Figure 4a) due to the growth of both yeasts and lactic acid bacteria  
328 (Figures 4b and c). HP-CO<sub>2</sub> treatment allowed to decrease the initial count of total viable bacteria  
329 and yeasts and to inhibit their growth during the storage (Figure 4a and 4b). In fact, after 15 days of  
330 refrigerated storage, total bacteria and yeast resulted about 4 and 1 Log lower than that of the  
331 untreated sample, respectively. On the contrary, lactic acid bacteria were below the detection limit

332 in HP-CO<sub>2</sub> treated juice, independently on storage time. Lactic acid bacteria have been actually  
333 reported to be more HP-CO<sub>2</sub> sensitive than yeasts (García-Gonzalez, Geeraerd, Elst, Van Ginneken,  
334 Van Impe & Devlieghere, 2009). As reported in the literature, the antimicrobial effects of HP-CO<sub>2</sub>  
335 are attributed not only to pressurization but also to media acidification (Balaban et al., 1991). To  
336 this regard, HP-CO<sub>2</sub> treatments were associated to a decrease in product pH due to the presence of  
337 residual carbonic acid after the treatment (Hong, Park & Pyun, 1997; Xu et al., 2011). However, in  
338 this study, the pH of the juice ( $4.2 \pm 0.2$ ) did not change upon the HP-CO<sub>2</sub> treatment and resulted  
339 analogous to that of pasteurized and untreated apple juice ( $p>0.05$ ). It is thus likely that CO<sub>2</sub>  
340 residues were removed from the juice during the depressurization of the reactor after the treatment.  
341 In order to evaluate the possible impact of HP-CO<sub>2</sub> treatment on sensory parameters of apple juice,  
342 the samples, stored for increasing time at 4 °C, were submitted to sensory evaluation. No significant  
343 changes in the evolution of the sensory attributes “acidity”, “fresh apple flavour” and “sweetness”  
344 were detected by the panelists ( $p>0.05$ ). In addition, judges did not detect any off-flavour in the  
345 samples. By contrast, significant changes in the scores of the sensory attributes “browning” and  
346 “cooked apple flavour” were noticed (Figure 5).

347 Due to complete polyphenoxidase inactivation, the browning sensory score of pasteurized sample  
348 resulted always significantly lower than that observed in untreated and HP-CO<sub>2</sub> treated juices,  
349 which showed progressively higher values (Figure 5a), mimicking the evolution of absorbance at  
350 420 nm during storage (Figure 3).

351 Immediately after the treatment, the pasteurized juice presented a high “cooked-apple flavour”  
352 score, confirming the well-known sensory quality depletion induced by thermal treatment (Aguilar-  
353 Rosas, Ballinas-Casarrubias, Nevarez-Moorillon, Martín-Belloso & Ortega-Rivas, 2007). After few  
354 days of storage, the intensity of this defect in the pasteurized juice decreased, possibly because of  
355 the evolution of the juice sensory profile. On the contrary, judges were not able to detect this defect  
356 in the HP-CO<sub>2</sub> treated juice. The latter also presented mean “cooked-apple flavour” values  
357 comparable to those of the untreated juice during the entire storage period ( $p>0.05$ ) (Figure 5b).

358 This result is certainly attributable to the low temperature (35 °C) experienced by the juice during  
359 the HP-CO<sub>2</sub> treatment.

360

#### 361 **4. Conclusions**

362 HP-CO<sub>2</sub> treatments at temperatures lower than 45 °C may allow partial inactivation of  
363 polyphenoloxidase in apple juice. The treatment time needed for reaching the minimum residual  
364 activity decreases with pressure and temperature but no further inactivation is obtained by  
365 increasing pressure and temperature beyond 12 MPa and 35 °C respectively. HP-CO<sub>2</sub> treatment  
366 could be applied under mild pressure/temperature conditions for short times to allow a significant  
367 microbial stabilisation of fresh refrigerated apple juice without impairing its fresh-likeness. Being  
368 HP-CO<sub>2</sub> treatment cheap and sustainable, these outcomes make it an interesting stabilisation  
369 technology for the production of fresh refrigerated apple juice.

370

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374

#### 375 **References**

376 Aguilar-Rosas, S. F., Ballinas-Casarrubias, M. L., Nevarez-Moorillon, G. V., Martin-Belloso,  
377 O., & Ortega-Rivas, E. (2007). Thermal and pulsed electric fields pasteurization of apple juice:  
378 Effects on physicochemical properties and flavour compounds. *Journal of Food Engineering*, 83(1),  
379 41-46.

380 Balaban, M. O., Arreola, A. G., Marshall, M. R., Peplow, A. Wei, C. I. & Cornell, J. (1991).  
381 Inactivation of pectinesterase in orange juice by supercritical CO<sub>2</sub>. *Journal of Food Science*, 56(3),  
382 743-750.

383 Beuchat, L. R. (1996). Pathogenic microorganisms associated with fresh produce. *Journal of*  
384 *Protection*, 59(13), 204-216.

385 Bothun, G. D. (2004). Pressurized solvents in whole-cell bioprocessing: metabolic and  
386 structural perturbations. PhD thesis, University of Kentucky, Lexington, Kentucky.

387 Bothun, G. D., Knutson, B. L., Strobel, H. J., & Nokes, S. E. (2005). Liposome fluidization  
388 and melting point depression by pressurized CO<sub>2</sub> determined by fluorescence anisotropy. *Langmuir*,  
389 21(2), 530-536.

390 Buckow, R., Weiss, U., & Knorr, D. (2009). Inactivation kinetics of apple polyphenol oxidase  
391 in different pressure-temperature domains. *Innovative Food Science & Emerging Technologies*,  
392 10(4), 441-448.

393 Chen, J. S., Balaban, M. O., Wei, C. I., Marshall, M. R., & Hsu, W. Y. (1992). Inactivation of  
394 polyphenol oxidase by high-pressure carbon dioxide. *Journal of Agricultural and Food Chemistry*,  
395 40(12), 2345-2349.

396 Damar, S., & Balaban, M. O. (2006). Review of dense phase CO<sub>2</sub> technology: microbial and  
397 enzyme inactivation and effects on food quality. *Journal of Food Science*, 71(1), 1-11.

398 Davidson, P. M. (2001). *Chemical preservatives and natural antimicrobial compounds*. In:  
399 Doyle, M. P., Beuchat, L. R., Montville, T. J., (Eds). Food microbiology: fundamentals and frontiers,  
400 2nd edition. Washington, D. C.: ASM Press. pp. 593-27.

401 de la Rosa, L. A., Alvarez-Parrilla, E., Moyers-Montoya, E., Villegas-Ochoa, M., Ayala-  
402 Zavala, J. F., Hernández, ... González-Aguilar, G. A. (2011). Mechanism for the inhibition of apple  
403 juice enzymatic browning by Palo Fierro (desert ironweed) honey extract and other natural  
404 compounds. *LWT - Food Science and Technology*, 44(1), 269-276.

405 Eyring, H. (1935). The Activated Complex in Chemical Reactions. *The Journal of Chemical*  
406 *Physics*, 3(2), 107-115.

407 FDA (2004). Guidance for Industry: Juice HACCP hazards and controls guidance, 1<sup>st</sup> ed.

408 Ferrentino, G., Bruno, M., Ferrari, G., Poletto, M., & Balaban, M. O. (2009). Microbial  
409 inactivation and shelf life of apple juice treated with high pressure carbon dioxide. *Journal of*  
410 *Biological Engineering*, 3(1), 3.

411 Ferrentino, G., & Spilimbergo, S. (2011). High pressure carbon dioxide pasteurization of solid  
412 foods: current knowledge and future trends. *Trends in Food Science and Technology*, 22, 427-441.

413 García-Gonzalez, L., Geeraerd, A. H., Elst, K., Van Ginneken, L., Van Impe, J. F. &  
414 Devlieghere, F. (2009). Influence of type of microorganism, food ingredients and food properties on  
415 high-pressure carbon dioxide inactivation of microorganisms. *International Journal of Food*  
416 *Microbiology*, 129(3), 253-263.

417 Golan-Goldhirsh, A., Whitaker, J. R., & Kahn, V. (1984). Relation between structure of  
418 polyphenol oxidase and prevention of browning. *Advances in Experimental Medicine and Biology*,  
419 177, 437-456.

420 Gui, F., Chen, F., Wu, J. Wang, Z., Liao, X., & Hu, X. (2006). Inactivation and structural  
421 change of horseradish peroxidase treated with supercritical carbon dioxide. *Food Chemistry*, 97(3),  
422 480-489.

423 Gui, F., Wu, J., Chen, F., Liao, X., Hu, X., Zhang, Z., & Wang, Z. (2007). Inactivation of  
424 polyphenol oxidases in cloudy apple juice exposed to supercritical carbon dioxide. *Food Chemistry*,  
425 100(4), 1678-1685.

426 Hong, S. I., Park, W. S., & Pyun, Y. R. (1997). Inactivation of *Lactobacillus* spp. from kimchi  
427 by high pressure carbon dioxide. *Food Science and Technology*, 64(4), 728-733.

428 Hutkins, R. W., & Nannen, N. L. (1993). pH homeostatis in lactic acid bacteria. *Journal of*  
429 *Dairy Science*, 76(8), 2354-2365.

430 Jones, R. P., & Greenfield, P. E. (1982). Effect of carbon dioxide on yeast and fermentation.  
431 *Enzyme and Microbial Technology*, 4(4), 210-223.

432 Kahn, V. (1995). Effect of proteins, protein hydrolysates and amino acids on o-  
 433 dihydroxyphenolase activity of polyphenoloxidase of mushrooms, avocado and banana. *Journal of*  
 434 *the Science of Food and Agriculture*, 6, 51-56.

435 Kincal, D., Hill, W. S., Balaban, M. O., Portier, K. M., Wei, C. I., & Marshall, M. R. (2005).  
 436 A continuous high – pressure carbon dioxide system for microbial reduction in orange juice.  
 437 *Journal of Food Science*, 70(5), 249-254.

438 Le Bourvellec, C., Le Quéré, J. M., Sanoner, P., Drilleau, J. F., & Guyot, S. (2004). Inhibition  
 439 of apple polyphenol oxidase activity by procyanidins and polyphenol oxidation products. *Journal of*  
 440 *Agricultural and Food Chemistry*, 52(1), 122-130.

441 Lee, M. R. F., Tweed, J. K. S., Cookson, A. & Sullivan, M. L. (2010). Immunogold labelling  
 442 to localize polyphenol oxidase (PPO) during wilting of red clover leaf tissue and the effect of  
 443 removing cellular matrices on PPO protection of glycerol-based lipid in the rumen. *Journal of the*  
 444 *Science of Food and Agriculture*, 90, 503–510.

445 Liao, X., Zhang, Y., Bei, J., Hu, X. & Wu, J. (2009). Alteration of molecular properties of  
 446 lipoxygenase induced by dense phase carbon dioxide. *Innovative Food Science and Emerging*  
 447 *Technologies*, 10(1), 47-53.

448 M.U.A.C.V. 1989. *Metodi ufficiali di analisi delle conserve vegetali*. D.M. 03.02.1989-  
 449 Gazzetta Ufficiale n.168 del 20.07. Titolo II 18.E.

450 Manzocco, L., Ignat, A., Valoppi, F., Burrafato, K. R., Lippe, G., Spilimbergo, S., & Nicoli,  
 451 M. C. (2016). Inactivation of mushroom polyphenoloxidase in model systems exposed to high-  
 452 pressure carbon dioxide. *The Journal of Supercritical Fluids*, 107, 669-675.

453 McEvily, A. J., Iyengar, R., & Otwell, W. S. (1992). Inhibition of enzymatic browning in  
 454 foods and beverages. *Critical Reviews in Food Science and Nutrition*, 32(3), 253-273.

455 Niu, S., Xu, Z., Fang, Y., Zhang, L., Yang, Y., Liao, X., & Hu, X. (2010). Comparative study  
 456 on cloudy apple juice qualities from apple slices treated by high pressure carbon dioxide and mild  
 457 heat. *Innovative Food Science and Emerging Technologies*, 11(1), 91-97.

458 Ohmae, E., Murakami, C., Gekko, K., & Kato, C. (2007). Pressure effects on enzyme  
459 functions. *International Journal of Biological Macromolecules*, 7, 23-29.

460 Raybaudi-Massilia, R. M., Mosqueda-Melgar, J., Soliva-Fortuny, R., & Martín-Belloso, O.  
461 (2009). Control of pathogenic and spoilage microorganisms in fresh-cut fruits and fruit juices by  
462 traditional and alternative natural antimicrobials. *Comprehensive Reviews in Food Science and*  
463 *Food Safety*, 8(3), 157-180.

464 Spilimbergo, S., & Bertucco, A. (2003). Non thermal microbial inactivation with dense CO<sub>2</sub>.  
465 *Biotechnology and Bioengineering*, 84(6), 627-638.

466 Spilimbergo, S., Komes, D., Vojvodic, A., Levaj, B., & Ferrentino, G. (2013). High pressure  
467 carbon dioxide pasteurization of fresh-cut carrot. *The Journal of Supercritical Fluids*, 79, 92-100.

468 Vamos-Vigyazo, L. (1981). Polyphenol oxidase and peroxidase in fruits and vegetables.  
469 *Critical Reviews in Food Science and Nutrition*, 15(1), 49-127.

470 Weemaes, C., Ludikhuyze, L., Van den Broeck, I., & Hendrickx, M. (1998). Kinetics of  
471 combined pressure-temperature inactivation of avocado polyphenoloxidase. *Biotechnology and*  
472 *Bioengineering*, 60(3), 292-300.

473 Whitaker, J. R. (1995). *Polyphenol oxidase*. In: Wong, D.W.S. (Ed). Food enzymes structure  
474 and mechanism. New York: Chapman and Hall. pp. 271-307

475 Xu, Z., Zhang, L., Wang, Y., Bi, X., Buckow, R., & Liao, X. (2011). Effects of high pressure  
476 CO<sub>2</sub> treatments on microflora, enzymes and some quality attributes of apple juice. *Journal of Food*  
477 *Engineering*, 104(4), 577-584.

478 Yemenicioğlu, A., Özkan, M., Cemeroğlu, B., Mehmet, O., & Yemeniciog, A. (1997). Heat  
479 inactivation kinetics of apple polyphenoloxidase and activation of its latent form. *Journal of Food*  
480 *Science*, 62(3), 508-510.

481 Yoruk, R., & Marshall, M. R. (2006). Physicochemical properties and function of plant  
482 polyphenol oxidase: A review. *Journal of Food Biochemistry*, 27(5), 361-422.



483           Zhou, L., Zhang, Y., Hu, X., Liao, X., & He, J. (2009). Comparison of the inactivation  
484   kinetics of pectin methylesterase from carrot and peach by high-pressure carbon dioxide. *Food*  
485   *Chemistry*, 115(2), 449-455.

486 Table 1. Minimum residual activity ( $RA\%$ ) of polyphenoloxidase, apparent inactivation rate  
 487 constants ( $k$ ), relevant determination coefficient ( $R^2$ ) and  $D_p$  values following HP-CO<sub>2</sub> treatments of  
 488 apple juice at increasing pressures (0.1, 6.0, 12.0 and 18.0 MPa) at 20, 35 and 45 °C.

Temperature (°C)	Pressure (MPa)	Minimum $RA\%$	$k$ (min <sup>-1</sup> )	$R^2$	$D_p$ (min)
20	0.1	69.0 ± 2.2 <sup>a</sup>	-0.0053 ± 0.0008 <sup>b</sup>	0.94	188.8 ± 26.8 <sup>a</sup>
	6	55.3 ± 1.0 <sup>b</sup>	-0.0207 ± 0.0024 <sup>b</sup>	0.97	48.4 ± 5.6 <sup>bc</sup>
	12	48.0 ± 1.8 <sup>c</sup>	-0.0297 ± 0.0053 <sup>b</sup>	0.94	33.7 ± 6.0 <sup>bc</sup>
	18	30.2 ± 1.5 <sup>de</sup>	-0.0452 ± 0.0063 <sup>b</sup>	0.96	22.1 ± 3.1 <sup>c</sup>
35	0.1	35.5 ± 0.6 <sup>d</sup>	-0.0133 ± 0.0019 <sup>b</sup>	0.94	75.1 ± 10.5 <sup>b</sup>
	6	24.6 ± 0.8 <sup>e</sup>	-0.0394 ± 0.0107 <sup>b</sup>	0.87	25.4 ± 6.9 <sup>bc</sup>
	12	18.0 ± 1.2 <sup>f</sup>	-0.0660 ± 0.0180 <sup>b</sup>	0.84	15.2 ± 4.1 <sup>c</sup>
	18	18.0 ± 0.6 <sup>f</sup>	-0.1228 ± 0.0530 <sup>b</sup>	0.97	8.1 ± 3.5 <sup>c</sup>
45	0.1	32.5 ± 1.3 <sup>de</sup>	-0.0465 ± 0.0055 <sup>b</sup>	0.97	21.5 ± 2.5 <sup>c</sup>
	6	19.0 ± 1.2 <sup>f</sup>	-0.0803 ± 0.0141 <sup>b</sup>	0.92	12.5 ± 2.2 <sup>c</sup>
	12	19.3 ± 0.8 <sup>f</sup>	-0.4107 ± 0.1034 <sup>a</sup>	0.89	2.4 ± 0.6 <sup>c</sup>
	18	20.3 ± 1.1 <sup>f</sup>	-0.6005 ± 0.1051 <sup>a</sup>	0.94	1.7 ± 0.3 <sup>c</sup>

489

490

491

492 Table 2:  $z_p$  (MPa),  $\Delta V^\ddagger$  (cm<sup>3</sup> mol<sup>-1</sup>) and  $\ln k_{atm}$  (min<sup>-1</sup>) values of apple juice polyphenoloxidase  
 493 inactivation by HP-CO<sub>2</sub> treatments carried out at increasing temperature (20, 35 and 45 °C).  
 494 Coefficients of determination ( $R^2$ ) are also shown.

Temperature (°C)	$z_p$ (MPa)	$\Delta V^\ddagger$ (cm <sup>3</sup> mol <sup>-1</sup> )	$\ln k_{atm}$ (min <sup>-1</sup> )	$R^2$
20	20.3 ± 5.0 <sup>a</sup>	-276.7 ± 68.9 <sup>a</sup>	-4.94 ± 0.14 <sup>b</sup>	0.89
35	19.2 ± 2.4 <sup>a</sup>	-307.9 ± 38.4 <sup>a</sup>	-4.17 ± 0.17 <sup>ab</sup>	0.97
45	14.8 ± 2.6 <sup>a</sup>	-412.3 ± 73.8 <sup>a</sup>	-3.13 ± 0.31 <sup>a</sup>	0.94

495

Figure

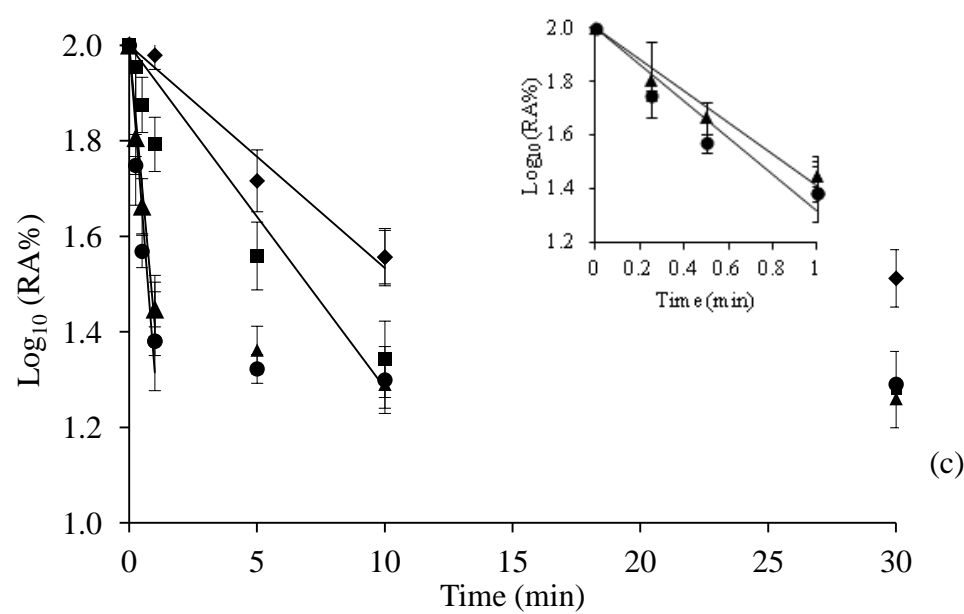
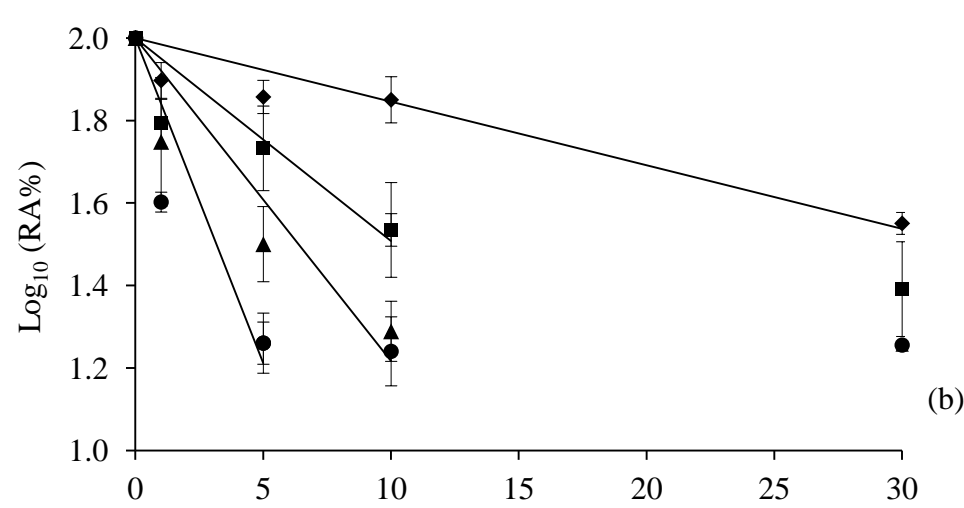
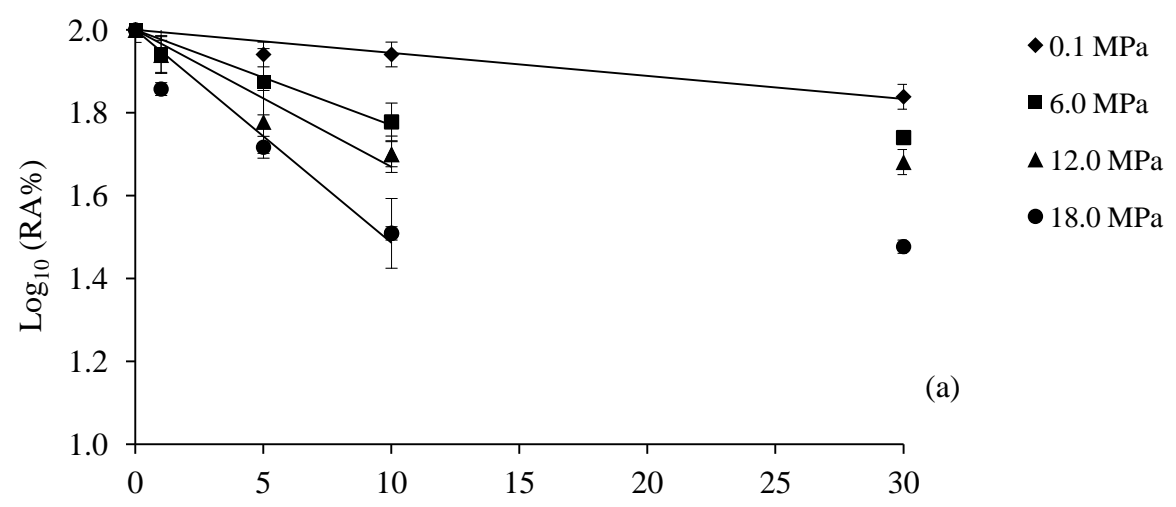


Figure 1: Polyphenoloxidase residual activity ( $RA\%$ ) of apple juice as a function of exposure time to increasing  $CO_2$  pressures (6.0, 12.0 and 18.0 MPa) at 20 (a), 35 (b) and 45 °C (c). Samples treated at environmental pressure (0.1 MPa) were considered as control. Symbols: experimental data. Solid lines: regression lines obtained in the linear part of the curve. Inset of figure 1c: magnification of  $RA\%$  in the 0-1 min time range.

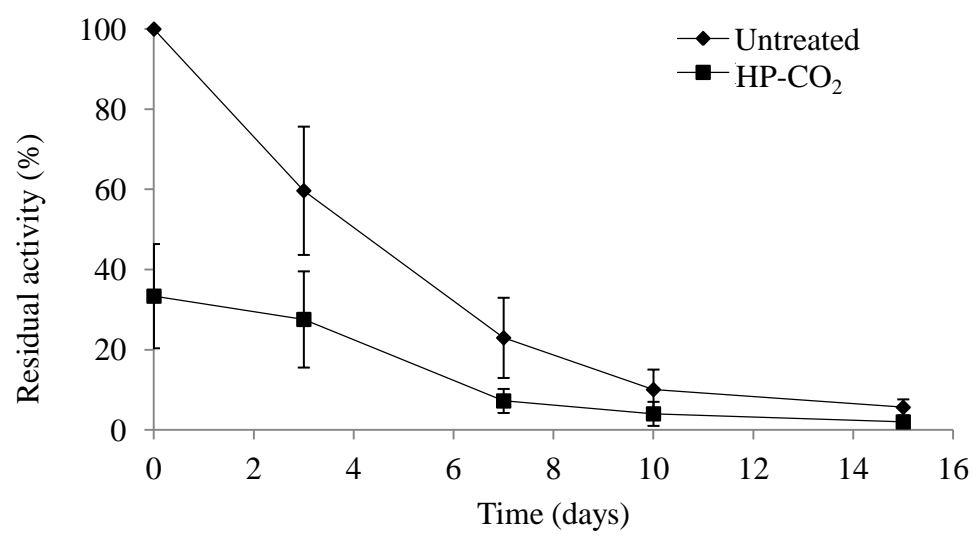


Figure 2. Polyphenoloxidase residual activity (%) during storage at 4 °C of HP-CO<sub>2</sub> treated and untreated apple juice.

Figure

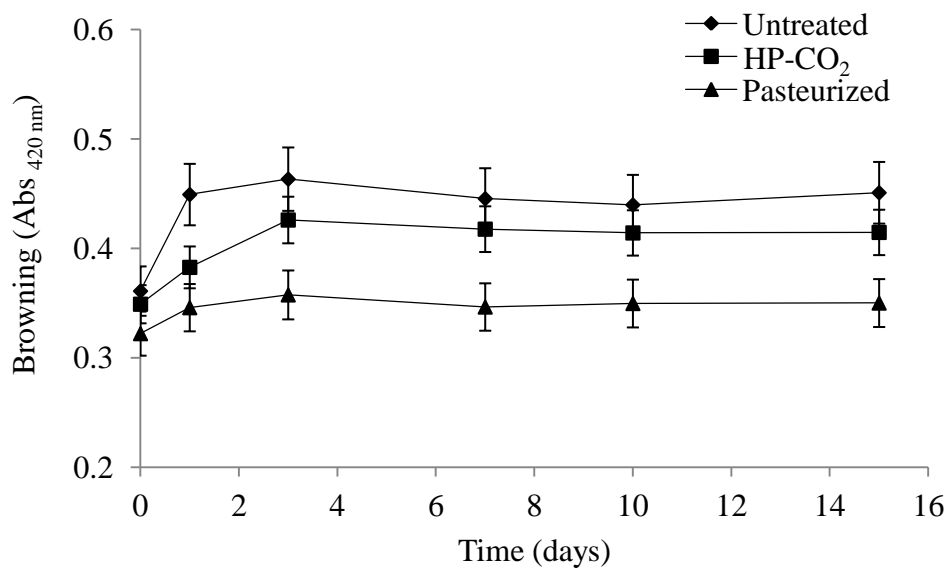


Figure 3. Absorbance at 420 nm of pasteurized, untreated and HP-CO<sub>2</sub> treated apple juices, during refrigerated storage.

Figure

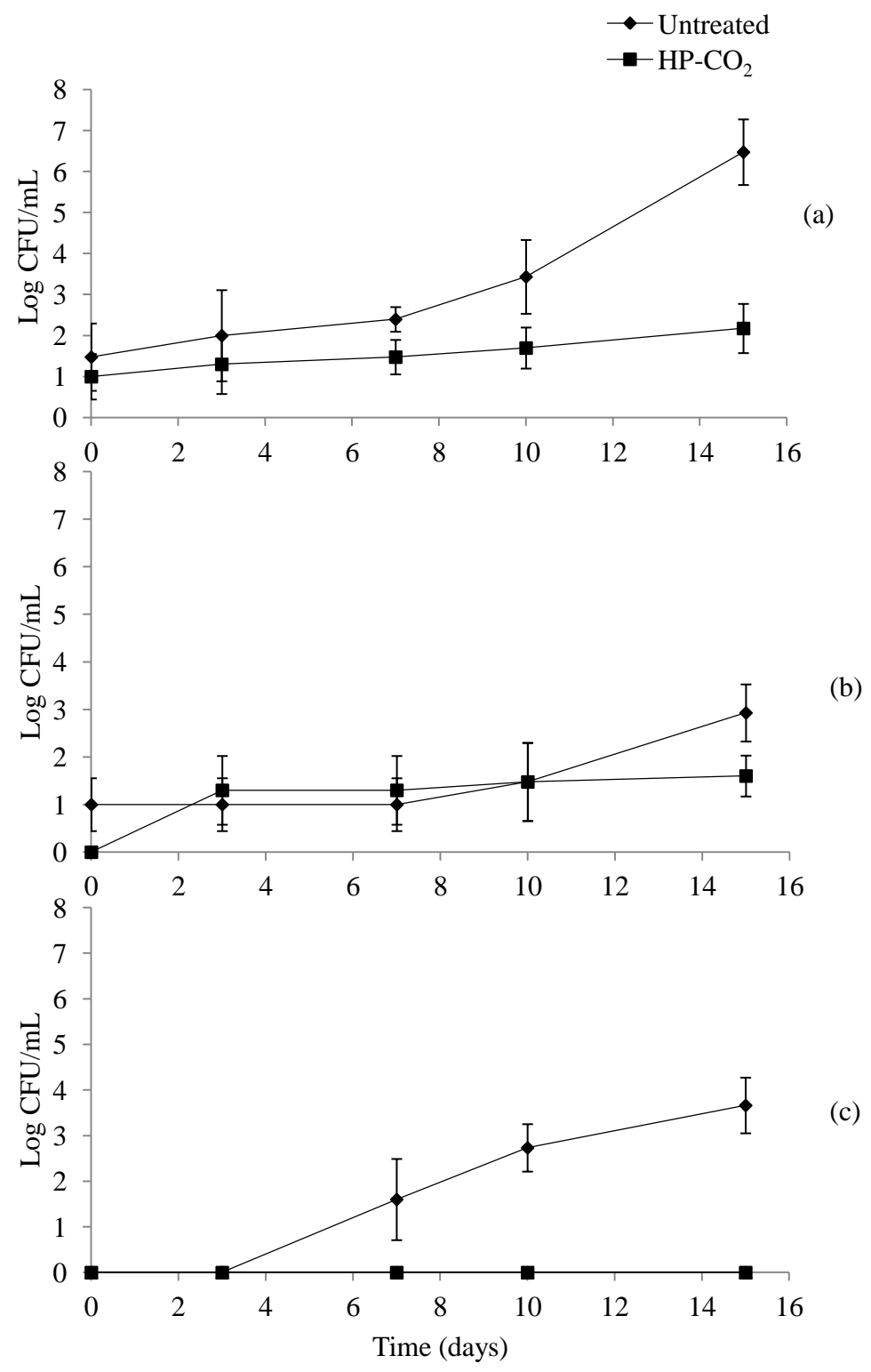


Figure 4. Total viable bacteria (a), yeast (b) and lactic acid bacteria (c) counts of untreated and HP-CO<sub>2</sub> treated apple juice, during refrigerated storage.

Total viable bacteria and yeast detection limit: 10 CFU/mL

Lactic acid bacteria detection limit: 1 CFU/mL

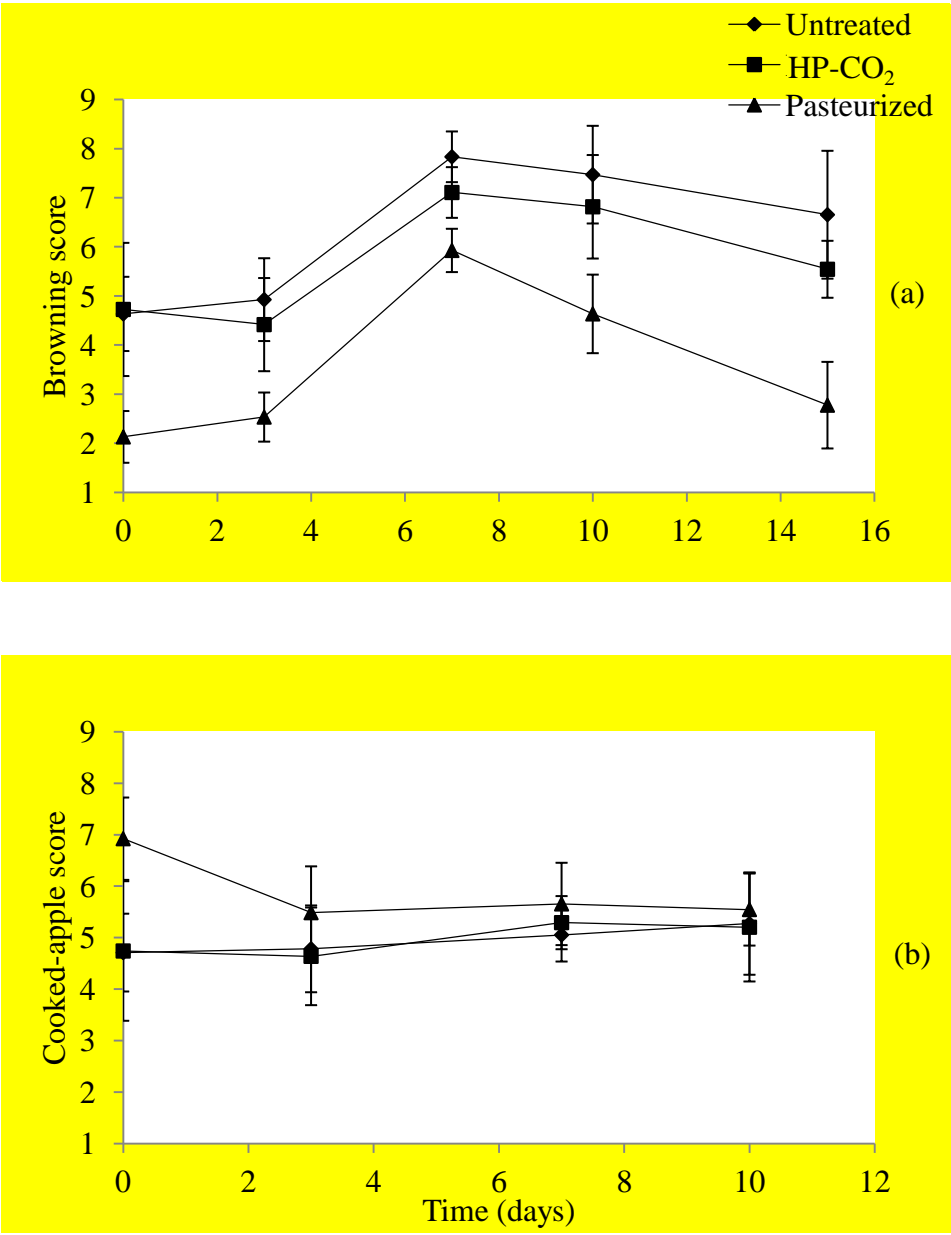


Figure 5. Browning (a) and cooked-apple flavour (b) sensory scores of pasteurized, untreated and HP-CO<sub>2</sub> treated apple juice, during refrigerated storage.