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

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Title: 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-CO₂) 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_p) decreased when CO₂ pressure increased, while the CO₂ pressure sensitivity of the enzyme (z_p) showed no variation with temperature. The HP-CO₂ 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 katals

Enzyme activity was expressed in katals 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)

***Highlights (for review)**

HP-CO₂ treatment promotes partial inactivation of polyphenoloxidase in apple juice

HP-CO₂ treatment increases microbial stability of apple juice

HP-CO₂ 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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5

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10

11 Key-words: dense-phase CO₂; inactivation kinetics; colour; sensory properties

12

13 **Highlights**

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18 **Abstract**

19 Freshly-extracted apple juice was exposed to high pressure carbon dioxide (HP-CO₂) 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 CO₂ pressure increased, while the CO₂ pressure sensitivity of the
23 enzyme (z_p) showed no variation with temperature. The HP-CO₂ 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

26 untreated apple juice while presenting a sensory fresh-likelihood higher than thermally pasteurized
27 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-CO₂) 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 CO₂
44 at temperature/pressure conditions that may be below or above the critical point (31.1 °C, 7.38
45 MPa). Typical CO₂ 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-CO₂ 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

52 pasteurization (Kincal, Hill, Balaban, Portier, Wei & Marshall, 2005; Ferrentino & Spilimbergo,
53 2011). The germicidal activity of HP-CO₂ is due to the combination of temperature, pressure and
54 specific effects of HP-CO₂. The treatment is associated with extracellular and intracellular
55 acidification, destabilization of membranes and denaturation of microbial enzymes (Jones &
56 Greenfield, 1982; Hutkins & Nannen, 1993; Bothun, 2004; Bothun, Knutson, Strobel & Nokes,
57 2005). More controversial is the effect of HP-CO₂ in inactivating fruit enzymes leading to juice
58 quality decay. For instance, inactivation of polyphenoloxidase responsible for browning of fruit
59 juices, depends on the nature of the enzyme and is strongly affected by CO₂ pressure, temperature
60 and treatment time (Gui, Chen, Wu, Wang, Liao & Hu, 2006; Liao, Zhang, Bei, Hu & Wu, 2009;
61 Zhou, Zhang, Hu, Liao & He, 2009; Spilimbergo, Komes, Vojvodic, Levaj & Ferrentino, 2013).
62 The mechanisms involved in enzyme inactivation by HP-CO₂ include pH lowering (Balaban,
63 Arreola, Marshall, Peplow, Wei & Cornell, 1991) and changes in the conformation of the secondary
64 structure of the enzyme (Chen, Balaban, Wei, Marshall & Hsu, 1992; Manzocco et al., 2016).
65 Based on these considerations, the present paper was addressed to investigate the impact of HP-CO₂
66 treatment on polyphenoloxidase activity and stability of fresh apple juice intended for refrigerated
67 storage. To this aim, apple juice was exposed to HP-CO₂ treatments in a wide range of pressure,
68 temperature and treatment time conditions. Apple juice was then submitted to the HP-CO₂ treatment
69 leading to the minimum polyphenoloxidase activity at the mildest pressure/temperature combination
70 and stored at 4 °C for up to 15 days. HP-CO₂ treated apple juice was monitored during storage for
71 residual polyphenoloxidase activity, colour, microbial counts and sensory attributes. To verify the
72 potential applicability of HP-CO₂ technology to produce fresh apple juice, data were compared to
73 those relevant to an untreated apple juice. An apple juice submitted to conventional thermal
74 pasteurization was also considered as additional control.

75

76 **2. Material and methods**

77

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 ± 1.6
81 g/kg, a soluble solid content of 13.3 ± 0.2 °Brix, a pH of 4.2 ± 0.2 and a titratable acidity of
82 4.6 ± 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 CO₂ treatments*

90 HP-CO₂ 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 CO₂ 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-CO₂ 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 10⁻³ 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:

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

179 Where $RA\%$ is the polyphenoloxidase residual activity in the juice at time t (min) and k is the
180 inactivation rate constant (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 (ΔV^\ddagger , cm^3/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). ΔV^\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 CO₂ 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 CO₂ 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-CO₂ 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 CO₂. The effect of temperature on
218 enzyme inactivation by HP-CO₂ 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 CO₂ 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 CO₂
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 CO₂ 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 Cemeroglu, 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 CO₂. 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 CO₂. 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 CO₂ pressure.

266 The effect of pressure on polyphenoloxidase inactivation was also expressed through the activation
267 volume (ΔV^\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 ΔV^\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 CO₂ 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

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

282

283 *3.2. Effect of high pressure carbon dioxide on apple juice quality during storage*

284 In the light of the previous results, it can be hypothesized that HP-CO₂ treatment could represent a
285 non-thermal technological strategy to control enzymatic activity during storage of apple juice. In
286 this context, HP-CO₂ could be proposed as a technology to stabilize refrigerated apple juice colour.
287 To verify this hypothesis, a combination of processing conditions which could be potentially
288 applicable on a larger scale to produce fresh refrigerated apple juice was selected. To this regard,
289 treatments carried out at 20 °C were excluded since associated with residual activity higher than
290 30% even at the highest tested pressure (Table 1). The mildest pressure/temperature combination
291 leading to the minimum residual activity (20%) was thus selected. As shown in Table 1, this
292 combination corresponded to the treatment at 12 MPa and 35 °C. The juice was thus treated at these
293 conditions for 10 min since longer treatment times did not promote further enzyme inactivation
294 (Figure 1).

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

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

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

306 By contrast, HP-CO₂ 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-CO₂ 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-CO₂
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-CO₂ treatment here applied was
317 not able to significantly reduce browning phenomena during storage.

318 To evaluate the ability of HP-CO₂ 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-CO₂ 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-CO₂ 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-CO₂ treated juice, independently on storage time. Lactic acid bacteria have been actually
333 reported to be more HP-CO₂ sensitive than yeasts (García-Gonzalez, Geeraerd, Elst, Van Ginneken,
334 Van Impe & Devlieghere, 2009). As reported in the literature, the antimicrobial effects of HP-CO₂
335 are attributed not only to pressurization but also to media acidification (Balaban et al., 1991). To
336 this regard, HP-CO₂ 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 ± 0.2) did not change upon the HP-CO₂ treatment and resulted
339 analogous to that of pasteurized and untreated apple juice ($p > 0.05$). It is thus likely that CO₂
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-CO₂ 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-CO₂ 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-CO₂ 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-CO₂ treatment.

360

361 **4. Conclusions**

362 HP-CO₂ 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-CO₂ 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-CO₂ 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

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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-CO₂ 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 ⁻¹)	R^2	D_p (min)
20	0.1	69.0 ± 2.2 ^a	-0.0053 ± 0.0008 ^b	0.94	188.8 ± 26.8 ^a
	6	55.3 ± 1.0 ^b	-0.0207 ± 0.0024 ^b	0.97	48.4 ± 5.6 ^{bc}
	12	48.0 ± 1.8 ^c	-0.0297 ± 0.0053 ^b	0.94	33.7 ± 6.0 ^{bc}
	18	30.2 ± 1.5 ^{de}	-0.0452 ± 0.0063 ^b	0.96	22.1 ± 3.1 ^c
35	0.1	35.5 ± 0.6 ^d	-0.0133 ± 0.0019 ^b	0.94	75.1 ± 10.5 ^b
	6	24.6 ± 0.8 ^e	-0.0394 ± 0.0107 ^b	0.87	25.4 ± 6.9 ^{bc}
	12	18.0 ± 1.2 ^f	-0.0660 ± 0.0180 ^b	0.84	15.2 ± 4.1 ^c
	18	18.0 ± 0.6 ^f	-0.1228 ± 0.0530 ^b	0.97	8.1 ± 3.5 ^c
45	0.1	32.5 ± 1.3 ^{de}	-0.0465 ± 0.0055 ^b	0.97	21.5 ± 2.5 ^c
	6	19.0 ± 1.2 ^f	-0.0803 ± 0.0141 ^b	0.92	12.5 ± 2.2 ^c
	12	19.3 ± 0.8 ^f	-0.4107 ± 0.1034 ^a	0.89	2.4 ± 0.6 ^c
	18	20.3 ± 1.1 ^f	-0.6005 ± 0.1051 ^a	0.94	1.7 ± 0.3 ^c

489

490

491

492 Table 2: z_p (MPa), ΔV^\ddagger (cm³ mol⁻¹) and $\ln k_{atm}$ (min⁻¹) values of apple juice polyphenoloxidase
 493 inactivation by HP-CO₂ 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)	ΔV^\ddagger (cm ³ mol ⁻¹)	$\ln k_{atm}$ (min ⁻¹)	R^2
20	20.3 ± 5.0 ^a	-276.7 ± 68.9 ^a	-4.94 ± 0.14 ^b	0.89
35	19.2 ± 2.4 ^a	-307.9 ± 38.4 ^a	-4.17 ± 0.17 ^{ab}	0.97
45	14.8 ± 2.6 ^a	-412.3 ± 73.8 ^a	-3.13 ± 0.31 ^a	0.94

495

Figure

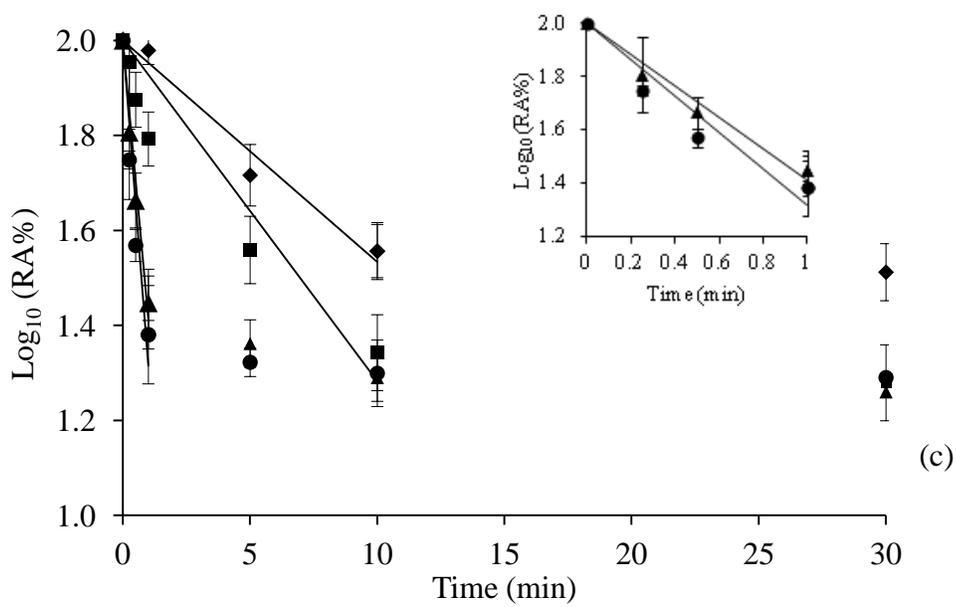
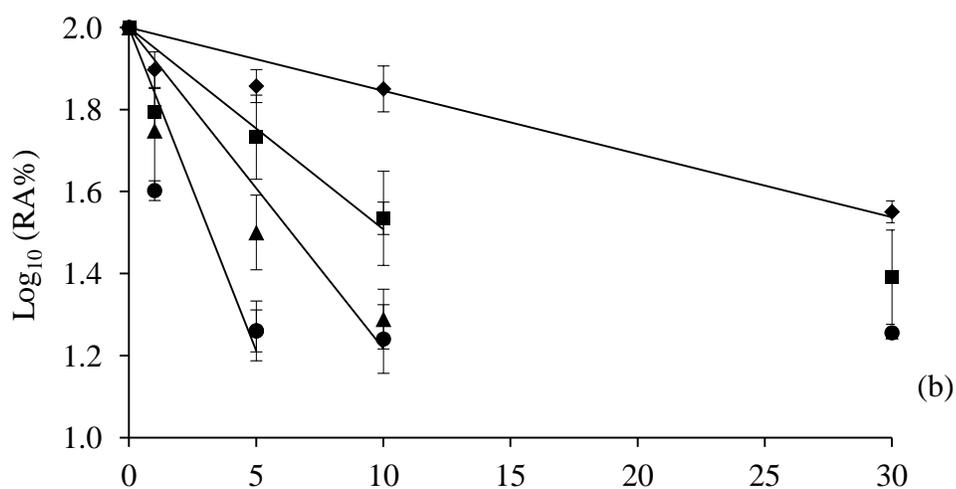
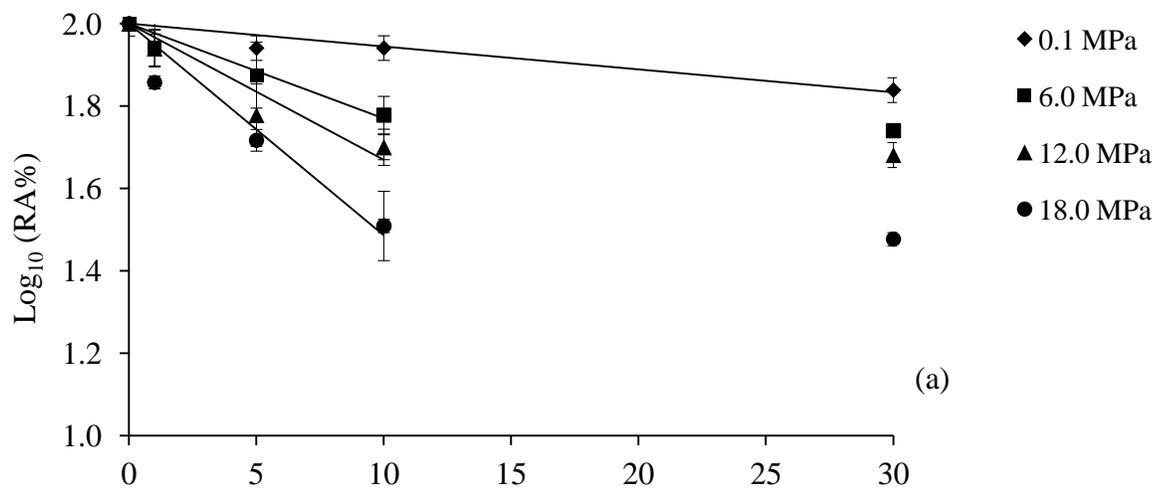


Figure 1: Polyphenoloxidase residual activity ($RA\%$) of apple juice as a function of exposure time to increasing CO_2 pressures (6.0, 12.0 and 18.0 MPa) at 20 (a), 35 (b) and 45 °C (c). Samples treated at environmental pressure (0.1 MPa) were considered as control. Symbols: experimental data. Solid 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.

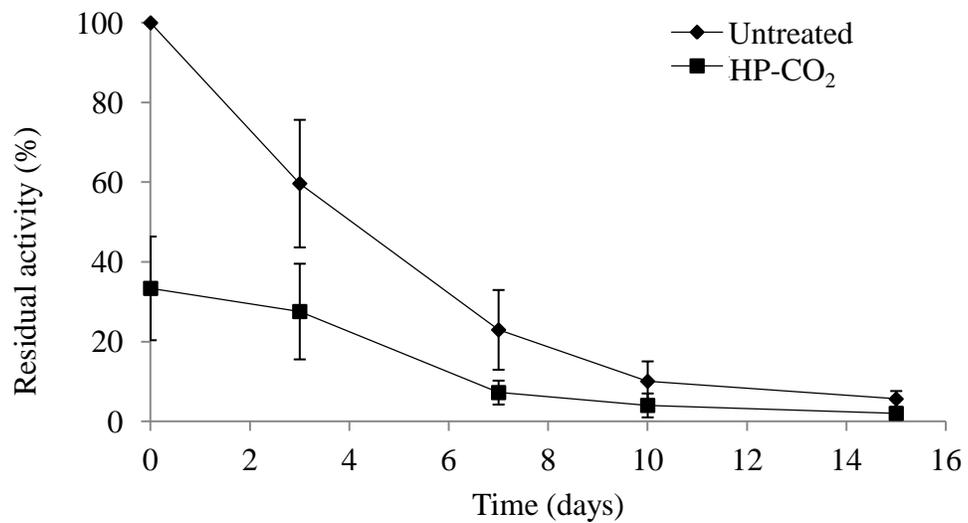


Figure 2. Polyphenoloxidase residual activity (%) during storage at 4 °C of HP-CO₂ treated and untreated apple juice.

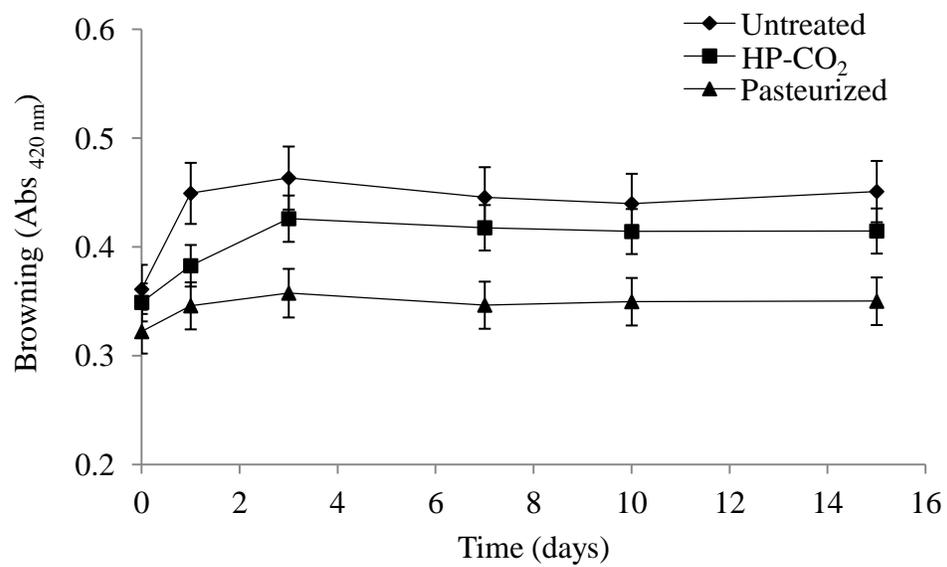


Figure 3. Absorbance at 420 nm of pasteurized, untreated and HP-CO₂ treated apple juices, during refrigerated storage.

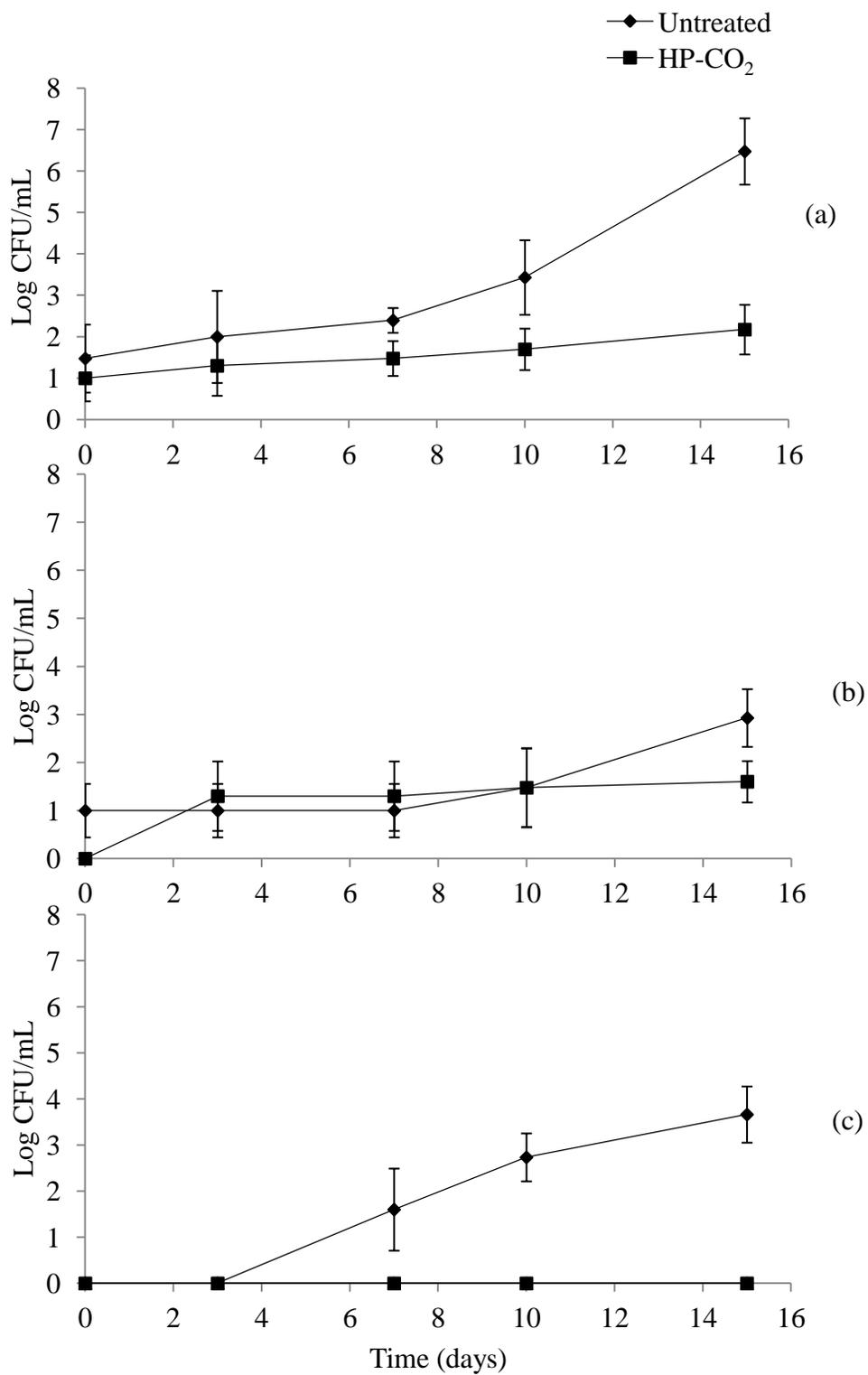


Figure 4. Total viable bacteria (a), yeast (b) and lactic acid bacteria (c) counts of untreated and HP-CO₂ treated apple juice, during refrigerated storage.

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

Lactic acid bacteria detection limit: 1 CFU/mL

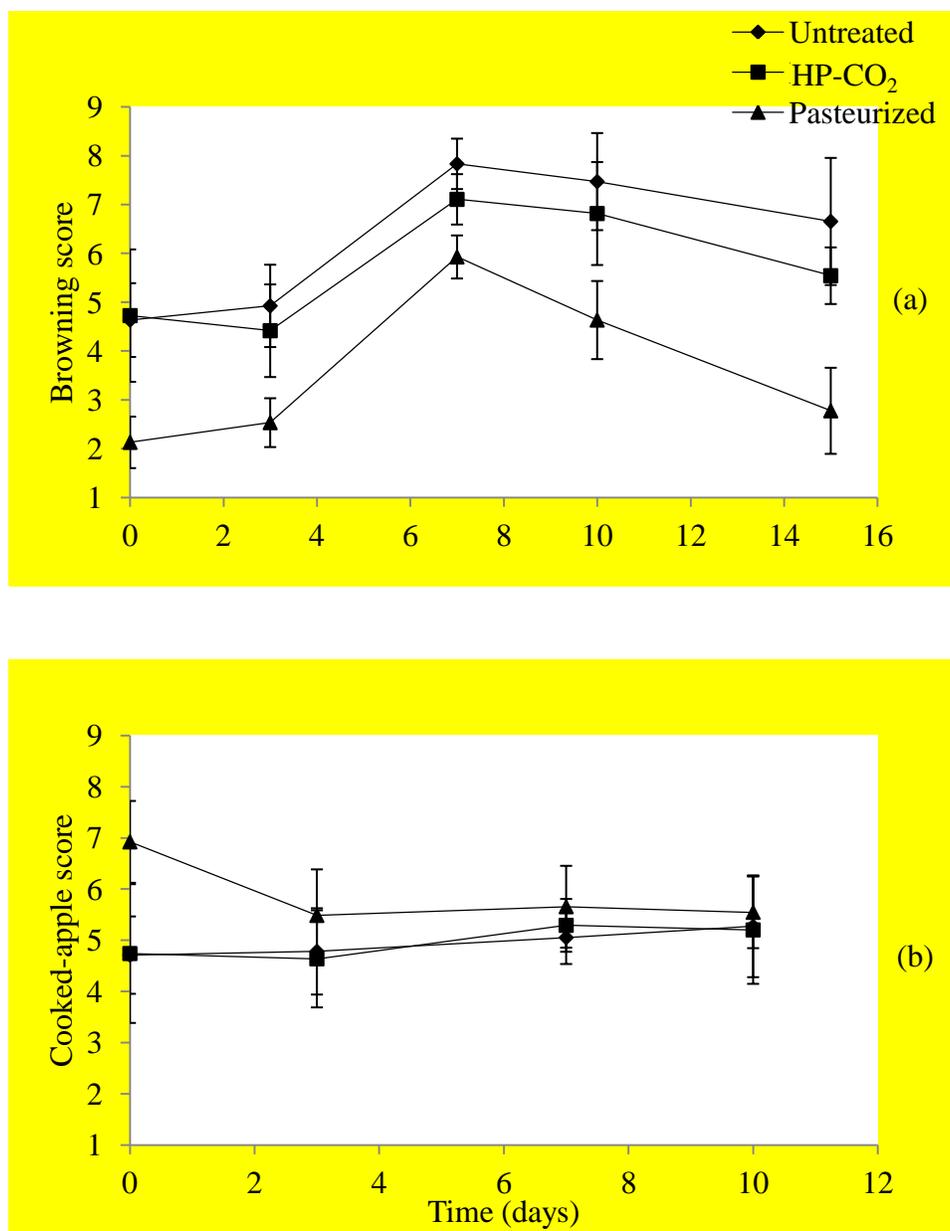


Figure 5. Browning (a) and cooked-apple flavour (b) sensory scores of pasteurized, untreated and HP-CO₂ treated apple juice, during refrigerated storage.