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BIOACTIVE COMPOUNDS AND QUALITY CHANGES OF RED FRESH VEGETABLE SMOOTHIES DURING STORAGE

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Abstract

Changes in the polyphenoloxidase (PPO), peroxidase (POD), pectinmethylesterase (PME) and phenylalanine ammonia-lyase (PAL) activities, total phenolics content (TPC) and total antioxidant capacity (TAC) on two red fresh vegetables smoothies (R1 and R2) based on tomato, red pepper, broccoli and carrot were studied. A conventional thermal treatment of 3 min at 80 ºC was applied to extend shelf life. Changes of such quality parameters during storage at 5 and 20 ºC were monitored. The initial PPO, POD,

PME and PAL activities of R1/R2 smoothies (58/83, 0.023/0.020, 1.50/0.38 and 7.3/11.5 U kg^{-1} fresh weight, fw) were 100 % reduced after thermal treatment and maintained at zero levels during storage up to 40 and 58 days at 20 and 5 ºC, respectively. Initial PAL activities of R1/R2 smoothies of 7.3/11.5 µmol cinnamic acid formed kg^{-1} h^{-1} were reduced in a 65-70 % after thermal treatment. The initial TPC of R1/R2 smoothies were 404/462 mg GAE kg^{-1} fw and it was not significantly affected after the thermal treatment. No great TPC degradation during storage was observed either at 5 or 20 ºC. The initial TAC of R1/R2 smoothies were 301/373 mg of ascorbic acid equivalent kg^{-1} fw which was increased 62/77 % after the thermal treatment. The TAC showed a similar behaviour to TPC during storage being those two parameters well enough correlated (r^2=0.69-0.88). In conclusion, the thermal treatment inactivated the studied degradative-quality enzymes. Health-promoting compounds were well preserved during 58 days at 5 ºC and 40 days at 20 ºC in red fresh vegetable smoothies.

Keywords: polyphenoloxidase; peroxidase; pectinmethylesterase; phenylalanine ammonia-lyase; bioactive compounds; beverages.

1. INTRODUCTION

The Mediterranean diet has been particularly studied for its positive effects on the prevention of heart diseases and its potential to reduce the incidence of chronic degenerative diseases such as diabetes, high blood pressure and reduce the low density lipoprotein oxidation (Koloverou et al. 2014; Mattioli et al. 2013; Mitjavila et al. 2013). Epidemiological studies conducted by the PREDIMED (2015) suggest that most of those beneficial effects are derived from the phytochemical constituents of fruits, vegetables and olive oil which are the main components of this diet (Sofi et al. 2010;

51 Willett et al. 1995). Tomato, red pepper, carrot and broccoli have high contents of those
52 health-promoting phytochemicals such as carotenoids, phenolic compounds, vitamins C
53 and E, folates and glucosinolates, among others (Robins, 1997). However, the current
54 lifestyle does not allow the time needed for the preparation of these vegetables. Thus,
55 their consumption should be promoted through the development of ready-to-eat
56 products that should be processed with minimal non-aggressive treatments to preserve
57 as much as possible the quality parameters (Artés-Hernández et al. 2009).
58 Smoothies are no alcoholic beverages prepared from fresh or frozen fruit and/or
59 vegetables, which are blended and usually mixed with crushed ice to be immediately
60 consumed. Often, some smoothies may include other components like yogurt, milk, ice-
61 cream, lemon water or tea. They have a milk shake-like consistency that is thicker than
62 slush drinks. Accordingly, smoothies represent an excellent and convenient alternative
63 to promote the daily consumption of fruit and vegetables. The smoothie preparation
64 involves a breakdown of plant parenchyma which leads to a dispersed solution
65 consisting in a liquid phase (pectin and other soluble solids) and a solid phase composed
66 of insoluble solids (cell wall). The main issue of the smoothie processing is the limited
67 shelf life of these products since they are susceptible to spoilage (Palgan et al. 2012)
68 and quality degradation. Besides microbial spoilage, quality degradation due to
69 endogenous enzyme activity is also a factor of concern since smoothie preparation
70 offers enzymes to come in contact with substrates (Hendrickx et al. 1998). For that
71 reason, in order to increase the shelf life while keeping quality, mild thermal treatments
72 or equivalents must be used during processing (Di Cagno et al. 2011) in addition to low
73 storage temperature being recommended 5 ºC. However, the treatment should not be
74 much aggressive to preserve its nutritional and sensory quality. Oxidative enzymes like
75 polyphenoloxidase (PPO) and peroxidase (POD) are responsible for the deterioration of

colour, flavour and nutritional value (Liavoga and Matella 2012). The textural properties of smoothies can be greatly affected by enzymes like pectinmethylesterase (PME) which activity reduces the smoothie viscosity, consistency and cloud stability with modified colour and other organoleptic properties (Giovane et al. 2004). Thus, inactivation of these quality-degrading endogenous enzymes is needed for extension of smoothies shelf life (Chakraborty et al. 2014). Thermal treatment (generally in the range of 80 °C to 95 °C) is commercially applied for the inactivation of spoilage enzymes in fruit purées and juices. However, thermal treatments may reduce phytochemical contents of smoothies in detriment of related antioxidant properties. Furthermore, phenylalanine ammonia-lyase (PAL), the key enzyme of the polyphenol biosynthesis pathway, can be influenced by processing temperature, although this effect will depend on the magnitude and time of treatment (Tiwari and Cummins 2013). To the best of our knowledge, there is no information about the effects of thermal processing and subsequent storage on quality changes of fresh vegetable smoothies. For that reason, the aim of this work was to study the effect of a mild conventional pasteurization on PPO, POD, PME and PAL activities, total phenolic content (TPC) and total antioxidant capacity (TAC) changes of two red fresh vegetable smoothies throughout storage at 5 and 20 ºC.

2. MATERIALS AND METHODS

2.1 Reagents

Polyvinylpolypyrrolidone (PVPP), catechol, guaiacol, hydrogen peroxide, pectin (Poly-D-galacturonic acid methyl ester), L-phenyldiamine, gallic acid (GAE), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2 diphenyl-1-picrylhydrazil (DPPH), sodium chloride, borate sodium, mercaptoethanol, t-cinnamic acid, Folin

Ciocalteu reagent and ascorbic acid were purchased from Sigma-Aldrich (Spain). 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) and boric acid were purchased from Fluka (Germany) and Fisher Chemical (United Kingdom), respectively. Sodium phosphate, phosphoric acid, sodium hydroxide, methanol, sodium carbonate, sodium acetate trihydrate, acetic acid anhydrous, hydrochloride acid, Iron(III) chloride hexahydrate were purchased from Panreac (Spain).

2.2 Plant material and smoothie preparation

Fresh vegetables (tomato, red pepper, broccoli and carrot) were purchased at commercial maturity stage at a local supermarket from Cartagena (Spain) in September. All produce was firstly sanitized with 75 mg L^{-1} NaClO during 2 min and then rinsed with tap water during 1 min. Tomatoes and carrots were peeled and all vegetables were then cut and blended (MX2050 blender, Braun, Germany). Two different red smoothies (R1 and R2) were prepared. R1 was formulated as high tomato/low red pepper content and R2 for the opposite vegetable proportions. Table 1 presents the smoothie composition as well as the main initial quality attributes which corroborate the commercial maturity stage of purchased vegetables. Citric acid was added in order to low the pH to reduce the microbial growth during storage which microbial reaction products may alter the enzymatic activities and bioactive contents.

2.3. Thermal treatment and storage conditions

Smoothies were immediately placed in 15 mL polypropylene falcon tubes (2 mL head space after filling) after preparation and heat treated in an agitated water bath (J.P. Selecta, Barcelona, Spain). After 3 min of increasing temperature of the samples, when the core reached 80 ºC (measured with temperature probes in control tubes-not used for

126 further analyses- through the tube caps by a silicon septum), the treatment continued for
127 3 more min at such temperature by regulating the bath temperature. Heat treated
128 samples were immediately cooled up to 5 or 20 ºC in iced water and then stored in
129 darkness at 5 and 20 ºC. Fresh-blended samples non heated were used as control
130 (CTRL) which was just stored at 5 ºC. Sampling was conducted on processing day and
131 after 3, 7, 10, 14, 21, 24, 28, 35, 42, 49 and 58 days depending of the storage
132 temperature. Five replicates per treatment and sampling day (including processing day),
133 for each storage temperature (not applied on processing day), were prepared. The shelf
134 life of smoothies was established according to sensory evaluations for visual
135 appearance, flavour, texture, off-colours, off-odours, lumpiness, turbidity,
136 precipitation/phase separation and overall quality (Martínez-Hernández et al. 2013).
137 Panel test was performed by eight assessors according to international standards (ASTM
138 1986). Shelf life of untreated, and treated smoothies stored at 20 and 5 ºC were
139 established in 28, 40 and 58 days, respectively, being panellist scores on those days over
140 the limit of acceptance (3 from 5-point scale). Samples of each treatment were taken on
141 each sampling day and stored at -80 ºC until further analysis. Enzymatic and bioactive
142 compounds analyses of each of the five replicates per treatment and sampling day for
143 each storage temperature were analyzed by duplicate in order to reduce analytical error.
144

145 **2.4. Analyses and determinations**

146 **2.4.1. Enzymatic activity**

147 Polyphenoloxidase

148 For PPO and POD analyses, samples were extracted using the method of Sulaiman and
149 Silva (2013), but with slight modifications. A smoothie sample of 2.5 g was
150 homogenized (Ultra-turrax T-25, Ika-Labortechnik, Staufen, Germany) at low speed

151 with 3 mL of 0.2 M sodium phosphate buffer (pH 7.0) containing 40 g L^{-1} insoluble
152 polyvinylpolypyrrolidone (PVPP). The homogenate was filtered with 4-layers
153 cheesecloth and centrifuged at 14,000×g for 30 min at 4 °C. The supernatant was
154 collected and used as PPO and POD extracts. PPO was assayed by mixing 10 µL of
155 enzyme extract with 290 µL of substrate (5 mM catechol; 70 mM sodium phosphate
156 buffer; pH 5.8) in a 96 polystyrene flatbottom well plate (Grener Bio-one,
157 Frickenhausen, Germany). The increase in absorbance at 420 nm at 25 °C was recorded
158 for 10 min with a Multiscan plate reader (Tecan Infinite M200, Männedorf,
159 Switzerland). The same device will be used for the rest of absorbance measurements of
160 the other determinations. Water instead of the PPO assay solution was used as blank.
161 The enzyme activity ($\Delta A\ min^{-1}$) was estimated by the initial velocities method from the
162 linear portion of the curves. Accordingly, one PPO unit of activity (U) was defined as
163 an increase in absorbance of 1 min^{-1}. The PPO activity data, as well as POD and PME
164 are expressed relatively (%) to the enzyme activity of the respective CTRL smoothie on
165 day 0.
166
167 Peroxidase
168 The POD activity was determined according to Jung and Watkins (2011), but with
169 slight modifications. POD was assayed by mixing 24 µL of enzyme extract with 226 µL
170 of substrate (2.7 mM guaiacol and 4 mM hydrogen peroxide in 0.1 M sodium phosphate
171 buffer; pH 6.8) in a 96 polystyrene flatbottom well plate. The increase in absorbance at
172 470 nm at 25°C was recorded for 10 min with the Multiscan plate reader. Water instead
173 of the POD assay solution was used as blank. The $\Delta A\ min^{-1}$ was estimated by the initial
174 velocities method from the linear portion of the curves. Accordingly, one POD U was
175 defined as an increase in absorbance of 1 min^{-1}.

176 Pectinmethylesterase

177 PME activity was determined according to Ratner et al. (1969), but with slight
178 modifications. A sample of 2.5 g was homogenized with 10 mL of 0.2 M sodium
179 chloride cold solution. Subsequently, the pH of the PME extract was adjusted to 7.0
180 with 0.01 M NaOH. The reaction mixture consisted in 2.5 mL of PME extract and 15
181 mL of 1 % pectin (from citrus peel) solution containing 0.2 M NaCl (adjusted pH to
182 7.0). The pH decrease produced by the carboxyl groups released by the hydrolysis of
183 methyl esters of pectin was maintained at 7.0 by the addition of 0.01 N NaOH using an
184 automatic titrator (794 basic tritino, Metrohm, Switzerland). The consumption of NaOH
185 was recorded for 10 min. One PME U can be expressed as the amount of enzyme that
186 produces 1 nmol of acid per minute at pH 7.0 and 22 °C.
187
188 Phenylalanine ammonia-lyase

189 The PAL extraction was conducted according to Qin et al. (2003), but with slight
190 modifications. Briefly, a 2.5 g smoothie sample was homogenized with 5.5 mL of 0.05
191 M cold borate buffer (pH 8.5) containing 400 µL L^{-1} 2-mercaptoethanoland and 25 g L^{-1}
192 PVPP and homogenized. Homogenates were filtered through 4-layers of cheesecloth
193 and then centrifuged at 10,000×g at 2 °C for 20 min. The supernatants were collected
194 and used as PAL extracts. Two sets of UV-well plates containing 270 µL of PAL extract
195 were prepared for every sample and pre-incubated at 40 ºC for 5 min. Afterwards, 30 µL
196 of either water (blank) or 100 mM L-phenylalanine substrate solution (freshly prepared
197 before assay) were added to each of the well for every sample set. The absorbances of
198 the sample sets at 290 nm were measured at time 0 and after 1 h of incubation at 40 ºC.
199 The PAL activity was calculated as µmol of *t*-cinnamic acid synthesized kg^{-1} fresh
200 weight (fw) h^{-1} using a *t*-cinnamic acid standard curve (0.02-0.3 mmol L^{-1}).

201 **2.4.2. Total phenolic content**

202 Frozen samples of 0.5 g were placed in glass bottles and 3 mL of methanol was added.
203 The extraction was carried out in an orbital shaker (Stuart, Staffordshire, UK) for 1 h at
204 200×g in darkness inside a polystyrene box with an ice bed. The extracts were
205 transferred in eppendorf tubes and centrifuged at 15,000×g for 10 min at 4ºC. The
206 supernatant was used as TPC and TAC extracts. The TPC was determined based on
207 Singleton and Rossi (1965), but with modifications proposed by Martínez-Hernández et
208 al. (2011). Briefly, 19 µL of TPC extract was placed in a well plate and 29 µL of 1 N
209 Folin–Ciocalteu reagent was added. The mix was incubated for 3 min in darkness at
210 room temperature. Then, 192 µL of a solution containing Na_2CO_3 (0.4 %) and NaOH (2
211 %) was added. After 1 h of incubation at room temperature in darkness the absorbance
212 was measured at 750 nm. The TPC was expressed as mg gallic acid equivalents (GAE)
213 kg^{-1} fw.
214
215 **2.4.3. Total antioxidant capacity**
216 The TAC was determined by three different methods: free radical scavenging capacity
217 with 2,2-diphenyl-1-picrylhydrazil (DPPH) (Brand-Williams et al.1995), ferric reducing
218 antioxidant power (FRAP) (Benzie and Strain 1999) and 2,2′-azino-bis(3-
219 ethylbenzothiazoline-6-sulphonic acid (ABTS) (Cano et al. 1998). Briefly for DPPH, a
220 solution of 0.7 mM DPPH in methanol was prepared 2 h before the assay and adjusted
221 to 1.1 (nm) immediately before use. A 21 µL aliquot of the TAC extract was added to
222 194 µL of this solution. The mixture was incubated for 1 h at room temperature in
223 darkness. The TAC by DPPH was measured by the decrease in absorbance at 515 nm.
224 Briefly for ABTS, 14 mM ABTS and 4.9 mM de $K_2S_2O_8$ stock solutions were mixed
225 (1:1) and incubated for 16 h at room temperature in darkness. The absorbance of this

226 solution was adjusted to 0.7 nm. Then, 285 µL of the latter solution was added to 15 µL
227 of TAC extract in a well plate and incubated for 6 min at room temperature in darkness.
228 The TAC by ABTS was measured by the decrease in absorbance at 734 nm. Briefly for
229 FRAP, a daily reaction solution containing sodium acetate buffer (pH 3.6), 10 mM
230 TPTZ solution (in 40 mM HCl) and 20 mM $FeCl_3$ was prepared in a *v:v:v* proportion of
231 10:1:1 and incubated at 37 ºC for 2 h in darkness. Then, 6 µL of TAC extract was
232 allowed to react with 198 µL of the FRAP solution for 40 min at room temperature in
233 darkness. The TAC by FRAP was measured by the decrease in absorbance at 593 nm.
234 All TAC data were expressed as mg of ascorbic acid equivalents (AAE) equivalent kg^{-1}
235 fw.
236
237 **2.5. Statistical Analysis**
238 The experiment was a two-factor (treatment×storage time) design subjected to analysis
239 of variance (ANOVA) using Statgraphics Plus software (vs. 5.1, Statpoint Technologies
240 Inc, Warrenton, USA). Statistical significance was assessed at the level P=0.05, and
241 Tukey's multiple range test was used to separate means. Pearson correlation analysis
242 was performed to corroborate relationships between TPC and TAC.
243
244 **3. RESULTS AND DISCUSSION**
245 **3.1. Enzymatic activity**
246 3.1.1. Polyphenoloxidase
247 The initial PPO activity of CTRL-R1 and CTRL-R2 smoothies were 58±17 and 83±13
248 U kg^{-1} fw, respectively, which was assigned as 100 % residual PPO activities (Figure 1).
249 Refrigerated storage of CTRL samples affected their PPO enzyme activity (P≤0.5).
250 Accordingly, PPO activity of CTRL-R1 smoothie showed a progressive inactivation

reaching 3% residual activity after 28 days at 5ºC (Figure 1 A). Similarly, CTRL-R2 smoothie showed a progressive PPO inactivation reaching 3-18 % residual activity after 24-28 days at 5 ºC (Figure 1 B). However, CTRL-R2 smoothie showed an initial great PPO inactivation, which reached minimum values of 15 % after 10 days at 5 ºC followed by an increase up to 40 % residual activity. The effectiveness of several natural essential oils (clove, roomer, lemon, etc.) to reduce activity of browning-related enzymes activity, such as PPO, has been previously reported (Ponce et al. 2004). Accordingly, the oregano content of R2 could lead to the reported great initial PPO inactivation. The observed reduction of the PPO activity during storage has been previously observed in untreated fruit smoothie (composed by strawberry, apple, banana and orange) throughout storage at 4 ºC (Keenan et al. 2012). Latter authors attributed this enzymatic behaviour to the enzyme forming a temporary complex with an available substrate (phenols and anthocyanins) within the smoothie. The thermal treatment applied (3 min at 80 ºC) completely inactivated ($P \leq 0.05$) the PPO activity of both R1 and R2 smoothies generally remaining in that inactivation status throughout all storage period (Figure 1). Thermal treatments with higher temperature (100 ºC) but lower treatment time (1 min) only achieved a 65 % PPO reduction in spinach purée (Wang et al. 2012, 2013). Similarly, PPO strawberry puree showed to be highly thermostable since no significant inactivation was observed after 30 min at 100 ºC. Purees have a higher viscosity than smoothies. Accordingly, heat transmission of purees may be lower than that of smoothies remaining points of the product with incomplete PPO inactivation during thermal treatment. PPO is one of the major oxidative enzymes involved in browning reactions which greatly affects the colour and flavour of beverages. Accordingly, the thermal treatment used ensures the PPO inactivation avoiding browning development during storage of these smoothies.

276 3.1.2. Peroxidase

277 The initial POD activity of CTRL-R1 and CTRL-R2 smoothies were 0.023±0.001 U
278 kg^{-1} and 0.020±0.003 U kg^{-1} fw, respectively, which were assigned as 100 % residual
279 POD activities (Figure 2). Ortega-Ortiz et al. (2007) reported approximately 60 U g^{-1} fw
280 in tomatoes ('Rio Grande' variety). The higher POD activity of latter authors may be
281 attributed to the different tomato varieties used. POD have shown a great thermal
282 inactivation, higher than PPO, since a heat treatment at 70 ºC for less than 5 min was
283 enough to inactivate the activity of this enzyme in strawberry puree (Terefe et al. 2010).
284 Thermal treatment completely inactivated the POD activity in R1 and R2 samples
285 which remained in this inactivity status during storage at 5 and 20 ºC (Figure 2).
286 Morales-Blancas et al. (2002) reported that POD activity of broccoli was reduced by 95
287 % after thermal treatment of 80 ºC for 3 min. The POD activity of CTRL-R1 and
288 CTRL-R2 smoothies initially decreased reaching minimum residual POD activities of
289 80-90 % after 7-10 days due to the chilled shock during storage. However, when POD
290 enzymatic system was upregulated to refrigeration temperatures, the POD activities of
291 both CTR-R1 and CTR-R2 smoothies progressively increased registering residual POD
292 activities of 140-145 % after 28 days at 5 ºC. Accordingly, the thermal treatment used
293 was enough to inactivate POD, the enzyme involved together with PPO in the
294 deterioration of colour and flavour of beverages such as smoothies.

295

296 3.1.3. Pectinmethylesterase

297 PME is a target enzyme in fruit and vegetable juices and smoothies to avoid phase
298 separation of the colloidal suspension which is formed by two phases: serum and cloud
299 (Chakraborty et al. 2014). The initial PME activities of CTRL-R1 and CTRL-R2 were
300 1.50±0.08 and 0.38±0.05 U kg^{-1}fw, respectively, which were assigned as the 100 %

301 residual PME activities (Figure 3). PME activity of tomatoes have been reported to be 4
302 and 9-fold higher compared to that of broccoli and carrots while PME activity in red
303 peppers was not detectable (Espachs-Barroso et al. 2006; Castro et al. 2008; Houben et
304 al. 2014). Accordingly, the lower tomato proportion in the R2 formulation may explain
305 the lower initial PME activity of CTRL-R2 compared to CTRL-R1. After the thermal
306 treatment, PME residual activities of R1 and R2 smoothies were 6 and 34 %,
307 respectively. The thermal inactivation kinetics of PME in tomato juice was described
308 reasonably well by first order inactivation kinetics with an inactivation D value of 4 min
309 at 75 ºC (Terefe et al. 2009). Similarly, thermal treatments of 70-75 ºC for 5 min were
310 able to completely inactivate PME activities in tomato, carrot and broccoli purées
311 (Houben et al. 2013, 2014). Espachs-Barroso et al. (2006) found that activation energy
312 of PME thermal inactivation from tomatoes was approximately 10 % higher than that
313 for carrot. However, the PME activity and related thermal stability can greatly differ
314 among produce varieties and ripening stage (Barrett and González 1994; De Sio et al.
315 1995). Accordingly, the higher PME thermal stability of R2 may be attributed to the
316 presence of carrot with a high PME thermal stability in R2 smoothie. Houben et al.
317 (2014) attributed the higher PME stability of carrots compared to broccoli to a
318 protective effect of the carrot purée matrix as similarly observed Balogh et al. (2004).
319 During refrigerated storage, PME residual activity of CTRL-R1 and CTRL-R2 samples
320 decreased by 65 and 8 %, respectively, after 3 days at 5 ºC (Figure 3). The greater PME
321 decrease in CTRL-R1 smoothie can be owed to its greater tomato concentration and
322 presence of carrot which PME activities can be more sensible to the abiotic stress
323 provoked by the cold storage. Accordingly, after this initial PME decrease due to the
324 chilled shock, the PME was reactivated registering greater increases (up to 257 %
325 relative activity after 21 days) in CTRL-R2 smoothie due to the commented greater

326 stability during chilled storage. In general, PME activities of heat treated R1 and R2
327 samples did not significantly changed during storage at 5 and 20 ºC. However, the PME
328 activity of treated R2 samples stored at 5 ºC showed two maximum relative activities of
329 72 and 41 % after 7 and 35 days, although the activity at the end of shelf life was less
330 than 14 %. Last behaviour was similar to that of CTRL-R2 samples but was not
331 observed at 20 ºC since those PME activity peaks could be owed to the chilled shock as
332 explained before. In conclusion, thermal treatment was able to inactivate PME and
333 maintain low activity levels of this enzyme during storage at 5 and 20 ºC.
334
335 3.1.4. Phenylalanine ammonia-lyase
336 The initial PAL activities of CTRL-R1 and CTRL-R2 smoothies were 7.3±1.1 and
337 11.5±2.9 µmol cinnamic acid formed kg^{-1} h^{-1}, respectively, although no significant
338 ($P \leq 0.05$) differences were found between both smoothies (Figure 4 A). Bojórquez-
339 Gálvez et al. (2010) reported approximately 5 µmol cinnamic acid formed kg^{-1} h^{-1} of
340 PAL activity in tomato. The slightly higher PAL activity of our smoothies may be owed
341 to the red pepper content which has reported high PAL activities ranging from 9.4 to
342 23.4 µmol cinnamic acid formed kg^{-1} h^{-1} depending of the variety (Perucka and
343 Materska 2001). PAL activities were reduced in a 65-70 % after thermal treatment
344 without significant differences among both smoothies. Rees and Jones (1996) reported a
345 half-live of 1.4 min at 70 ºC for partially purified PAL preparations in water (pH 8.0).
346 The lower thermal stability of PAL found by those authors compared to our smoothies
347 may be attributed to a better heat distribution in that aqueous solution and protective
348 effects of the fibre particles of our smoothies.
349 The accumulation of phenolic compounds is a stress response caused by a change in
350 PAL activity, key enzyme in the phenylpropanoid pathway. That response depends on

several factors such as wounding intensity, storage temperature and atmospheric gas composition among other factors (Cisneros-Zevallos 2003). Throughout storage, PAL activities of CTRL samples did not show significant changes although a great enzyme activity increase was observed on days 10 and 24 for CTRL-R1 and CTRL-R2 smoothies, respectively (Figure 4A). That PAL activation of samples on days 10 and 24 may be owed to the abiotic stress response due to plant cell wounding implied in the smoothie preparation. The PAL activation delayed to 10-24 days may be owed to the low storage temperature and low pH reduced by the citric acid addition which slow down the sign needed to trigger the PAL activation. The longer delay of PAL peak in CTRL-R2 samples may be explained by the smoothie composition due to the lower content of tomato and/or presence of carrot compared to R1. The observed PAL increases of untreated samples were also observed in treated ones although due to the heat shock they were retarded to days 25 and 42 for heat-treated R1 and R2 samples, respectively, for both storage temperatures. PAL increases of R1 samples were approximately 2-fold higher than those of R2 at 5 ºC storage (treated and untreated) although the opposite behaviour was observed in those samples stored at 20 ºC. In general, the used thermal treatment was able to inactivate the quality degrading enzymes PPO, POD and PME but PAL activity was well kept. Accordingly, PAL activity during storage of smoothies allows the synthesis of phenolic compounds which may counteract the losses of these bioactive compounds throughout storage ensuring the related health-promoting properties of these smoothies.

3.2. Total phenolic content

The CTRL-R1 and CTRL-R2 smoothies showed initial TPC of 404±25 and 462±41 mg GAE kg^{-1} fw, respectively, without significant differences among them (Figure 5).

376 Similarly, Odriozola-Serrano et al. (2008) reported 311.0 mg GAE kg^{-1} fw in tomato
377 juice. In general, phenolic degradation is induced during food processing, by chemical
378 or enzymatic oxidation which can also lead to changes in bioavailability or biological
379 activity (Tomás-Barberán and Espín 2001). However, the thermal treatment hereby
380 applied did not induce significant changes in the TPC of the smoothies. In accordance
381 with our results, Patras et al. (2009a) and Odriozola-Serrano et al. (2008) did not find
382 significant changes of the TPC after thermal treatment of tomato juice (90 ºC for 60 s)
383 and tomato and carrot purées (70 ºC for 2 min).
384 The initial TPC of CTRL samples increased throughout storage up to 18 % after 10
385 days, keeping these levels without significant changes until the end of storage (Figure
386 5). PAL increments like those observed in CTRL-R1 samples after 10 days is
387 correspondent with the hereby found TPC increments of CTRL samples. On the other
388 side, TPC of T-R1 and T-R2 samples decreased during storage registering levels 19 and
389 13-18 % lower after 58 and 40 days at 5 and 20 ºC, respectively. Similarly, Odriozola-
390 Serrano et al. (2009) found 16-24 % TPC decreases in thermally treated (90 ºC for 60 s)
391 tomato juice after 56 days at 4 ºC. Since other enzymes apart from PAL are involved in
392 the phenylpropanoid metabolism, the thermal treatment could inactivate some of them
393 affecting the TPC biosynthesis observed in CTRL samples. Conclusively, thermal
394 treatments did not induce TPC changes although they were enough to inactivate PPO
395 and POD activities, leading to good TPC stability (<20 % degradation) during storage
396 either at 5 or 20 ºC.
397
398 **3.3. Total antioxidant capacity**
399 The initial total antioxidant capacities of CTRL-R1/R2 smoothies obtained by FRAP,
400 ABTS and DPPH were 301±35/373±12, 467±29/591±42 and 91±22/182±34 mg AAE

401 kg^{-1} fw, respectively. The initial total antioxidant capacities of T-R1/R2 smoothies
402 obtained by FRAP, ABTS and DPPH were 488±5/659±46, 527±42/637±40 and
403 233±24/287±24 mg AAE kg^{-1} fw, respectively. The low TAC by DPPH method may be
404 explained since one of the main maxim absorbance wavelengths for lycopene (Naviglio
405 et al. 2008), the main carotenoid of tomatoes, is close to that use for the DPPH method
406 which may lead to the observed reduced TAC by DPPH. Similarly to our results,
407 Keenan et al. (2010) found 3-fold higher TAC using FRAP method compared to DPPH
408 in fruit smoothies.
409 Phenolic compounds are the major contributors to the antioxidant properties of fresh
410 produce (Cisneros-Zevallos 2003). Antioxidant capacity of a food product may greatly
411 differ depending of the analytical method used (Prior et al. 2005). Accordingly, a
412 Pearson correlation using TPC and TAC data during storage was used to ascertain
413 which TAC method was better correlated to TPC (Table 2). In general, TAC was highly
414 correlated to TPC with r^2 ranging from 0.57 to 0.88. In general, FRAP method achieved
415 the best correlations (r^2=0.69-0.88) closely followed by ABTS (r^2=0.57-0.79).
416 Accordingly, FRAP data during storage is presented (Figure 6).
417 Contrary to the unchanged TPC data after thermal treatment, TAC increased after
418 thermal treatment registering FRAP method increments of 62 and 77 % in R1 and R2,
419 respectively, comparing to CTRL smoothies. Similar TAC increments have been
420 observed in tomato and carrot purées after thermal treatments (Patras et al. 2009b). It
421 has been hypothesized that latter behaviour is related to an increase in the extractability
422 of antioxidant components following thermal processing rather than an absolute
423 increase.
424 According to TPC data, TAC of CTRL samples increased during storage registering a
425 great increase of 51-55 % after 7 days compared to initial values being maintained (no

426 significant changes) those increased levels until the end of shelf life. However, and as
427 previously commented for TPC data, the TAC of treated samples progressively
428 decreased during storage at 5 and 20 ºC registering TAC levels 19-23 and 8-11 % lower
429 after 58 and 40 days, respectively. Similarly to our data on days 28 and 35, Keenan et
430 al. (2010) reported TAC (by FRAP) decreases of 19 % in heat-treated (70 ºC for 10
431 min) fruit smoothies after 30 days at 4 ºC.
432
433 **4. Conclusions**
434 A thermal treatment of red fresh vegetables smoothies of 3 min at 80 ºC inactivated
435 PPO, POD and PME which activities were minimal during subsequent storage either at
436 5 or 20 ºC. Moreover, the thermal treatment did not initially induce changes in the total
437 phenolics content of the samples, which was well correlated to PAL activity and total
438 antioxidant capacity, being these levels well preserved during shelf life even at 20 ºC.
439 Accordingly, this article presents two red fresh vegetables smoothies with stabilized
440 activities of degradative-quality enzymes and good levels of health-promoting
441 compounds during 58 and 40 days of storage at 5 and 20 ºC, respectively.
442
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617

618 **FIGURE AND TABLE CAPTIONS**

619

620 **Figure 1.** Polyphenoloxidase activity of untreated (CTRL) and heat-treated (T) red
621 smoothies R1 (A) and R2 (B) stored at 5 and 20 ºC (n=5±SD). Different letters denote
622 significant differences ($P \leq 0.05$) among sampling days for the same treatment.

623

624 **Figure 2.** Peroxidase activity of untreated (CTRL) and heat-treated (T) red smoothies
625 R1 (A) and R2 (B) stored at 5 and 20 ºC (n=5±SD). Different letters denote significant
626 differences ($P \leq 0.05$) among sampling days for the same treatment.
627
628 **Figure 3.**Pectinmethylesterase activity of untreated (CTRL) and heat-treated (T) red
629 smoothies R1 (A) and R2 (B) stored at 5 and 20 ºC (n=5±SD). Different capital letters
630 denote significant differences ($P \leq 0.05$) among treatments for the same sampling day.
631 Different lowercase letters denote significant differences ($P \leq 0.05$) among sampling
632 days for the same treatment.
633
634 **Figure 4.** Phenylalanine ammonia-lyase activity of untreated (stored at 5 ºC, A) and
635 heat-treated (stored at 5 ºC, B; stored at 20 ºC, C) red smoothies R1 and R2 (n=5±SD).
636 Different capital letters denote significant differences ($P \leq 0.05$) among treatments for
637 the same sampling day. Different lowercase letters denote significant differences
638 ($P \leq 0.05$) among sampling days for the same treatment.
639
640 **Figure 5.** Total phenolics content of untreated (stored at 5 ºC, A) and heat-treated
641 (stored at 5 ºC, B; stored at 20 ºC, C) red smoothies R1 and R2 (n=5±SD). Different
642 capital letters denote significant differences ($P \leq 0.05$) among treatments for the same
643 sampling day. Different lowercase letters denote significant differences ($P \leq 0.05$) among
644 sampling days for the same treatment.
645
646 **Figure 6.** Total antioxidant capacity of untreated (stored at 5 ºC, A) and heat-treated
647 (stored at 5 ºC, B; stored at 20 ºC, C) red smoothies R1 and R2 (n=5±SD). Different
648 capital letters denote significant differences ($P \leq 0.05$) among treatments for the same

649 sampling day. Different lowercase letters denote significant differences ($P \leq 0.05$) among
650 sampling days for the same treatment.
651
652 **Table 1.** Composition and main chemical quality parameters of red vegetable smoothies
653 (R1 and R2).
654
655 **Table 2.** Pearson correlation coefficients (r) between total phenolics content and
656 different total antioxidant capacity methods (FRAP, ABTS and DPPH) of red vegetable
657 smoothies, untreated (CTRL) and heat-treated, and stored at 5 or 20 ºC.
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