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 °C was applied to extend shelf life. Changes of such quality parameters during storage at 5 and 20 °C 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 °C, 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 °C. 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 (r²=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 °C and 40 days at 20 °C 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;
Tomato, red pepper, carrot and broccoli have high contents of those health-promoting phytochemicals such as carotenoids, phenolic compounds, vitamins C and E, folates and glucosinolates, among others (Robins, 1997). However, the current lifestyle does not allow the time needed for the preparation of these vegetables. Thus, their consumption should be promoted through the development of ready-to-eat products that should be processed with minimal non-aggressive treatments to preserve as much as possible the quality parameters (Artés-Hernández et al. 2009).

Smoothies are no alcoholic beverages prepared from fresh or frozen fruit and/or vegetables, which are blended and usually mixed with crushed ice to be immediately consumed. Often, some smoothies may include other components like yogurt, milk, ice-cream, lemon water or tea. They have a milk shake-like consistency that is thicker than slush drinks. Accordingly, smoothies represent an excellent and convenient alternative to promote the daily consumption of fruit and vegetables. The smoothie preparation involves a breakdown of plant parenchyma which leads to a dispersed solution consisting in a liquid phase (pectin and other soluble solids) and a solid phase composed of insoluble solids (cell wall). The main issue of the smoothie processing is the limited shelf life of these products since they are susceptible to spoilage (Palgan et al. 2012) and quality degradation. Besides microbial spoilage, quality degradation due to endogenous enzyme activity is also a factor of concern since smoothie preparation offers enzymes to come in contact with substrates (Hendrickx et al. 1998). For that reason, in order to increase the shelf life while keeping quality, mild thermal treatments or equivalents must be used during processing (Di Cagno et al. 2011) in addition to low storage temperature being recommended 5 ºC. However, the treatment should not be much aggressive to preserve its nutritional and sensory quality. Oxidative enzymes like 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
further analyses—through the tube caps by a silicon septum), the treatment continued for 3 more min at such temperature by regulating the bath temperature. Heat treated samples were immediately cooled up to 5 or 20 °C in iced water and then stored in darkness at 5 and 20 °C. Fresh-blended samples non heated were used as control (CTRL) which was just stored at 5 °C. Sampling was conducted on processing day and after 3, 7, 10, 14, 21, 24, 28, 35, 42, 49 and 58 days depending of the storage temperature. Five replicates per treatment and sampling day (including processing day), for each storage temperature (not applied on processing day), were prepared. The shelf life of smoothies was established according to sensory evaluations for visual appearance, flavour, texture, off-colours, off-odours, lumpiness, turbidity, precipitation/phase separation and overall quality (Martínez-Hernández et al. 2013). Panel test was performed by eight assessors according to international standards (ASTM 1986). Shelf life of untreated, and treated smoothies stored at 20 and 5 °C were established in 28, 40 and 58 days, respectively, being panellist scores on those days over the limit of acceptance (3 from 5-point scale). Samples of each treatment were taken on each sampling day and stored at -80 °C until further analysis. Enzymatic and bioactive compounds analyses of each of the five replicates per treatment and sampling day for each storage temperature were analyzed by duplicate in order to reduce analytical error.

2.4. Analyses and determinations

2.4.1. Enzymatic activity

Polyphenoloxidase

For PPO and POD analyses, samples were extracted using the method of Sulaiman and Silva (2013), but with slight modifications. A smoothie sample of 2.5 g was homogenized (Ultra-turrax T-25, Ika-Labortechnik, Staufen, Germany) at low speed
with 3 mL of 0.2 M sodium phosphate buffer (pH 7.0) containing 40 g L\(^{-1}\) insoluble polyvinylpolypyrrolidone (PVPP). The homogenate was filtered with 4-layers cheesecloth and centrifuged at 14,000×g for 30 min at 4 °C. The supernatant was collected and used as PPO and POD extracts. PPO was assayed by mixing 10 μL of enzyme extract with 290 μL of substrate (5 mM catechol; 70 mM sodium phosphate buffer; pH 5.8) in a 96 polystyrene flatbottom well plate (Greiner Bio-one, Frickenhausen, Germany). The increase in absorbance at 420 nm at 25 °C was recorded for 10 min with a Multiscan plate reader (Tecan Infinite M200, Männedorf, Switzerland). The same device will be used for the rest of absorbance measurements of the other determinations. Water instead of the PPO assay solution was used as blank. The enzyme activity (ΔA min\(^{-1}\)) was estimated by the initial velocities method from the linear portion of the curves. Accordingly, one PPO unit of activity (U) was defined as an increase in absorbance of 1 min\(^{-1}\). The PPO activity data, as well as POD and PME are expressed relatively (%) to the enzyme activity of the respective CTRL smoothie on day 0.

Peroxidase

The POD activity was determined according to Jung and Watkins (2011), but with slight modifications. POD was assayed by mixing 24 μL of enzyme extract with 226 μL of substrate (2.7 mM guaiacol and 4 mM hydrogen peroxide in 0.1 M sodium phosphate buffer; pH 6.8) in a 96 polystyrene flatbottom well plate. The increase in absorbance at 470 nm at 25°C was recorded for 10 min with the Multiscan plate reader. Water instead of the POD assay solution was used as blank. The ΔA min\(^{-1}\) was estimated by the initial velocities method from the linear portion of the curves. Accordingly, one POD U was defined as an increase in absorbance of 1 min\(^{-1}\).
Pectinmethylesterase

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

Phenylalanine ammonia-lyase

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

Frozen samples of 0.5 g were placed in glass bottles and 3 mL of methanol was added. The extraction was carried out in an orbital shaker (Stuart, Staffordshire, UK) for 1 h at 200×g in darkness inside a polystyrene box with an ice bed. The extracts were transferred in eppendorf tubes and centrifuged at 15,000×g for 10 min at 4°C. The supernatant was used as TPC and TAC extracts. The TPC was determined based on Singleton and Rossi (1965), but with modifications proposed by Martinez-Hernández et al. (2011). Briefly, 19 µL of TPC extract was placed in a well plate and 29 µL of 1 N Folin–Ciocalteu reagent was added. The mix was incubated for 3 min in darkness at room temperature. Then, 192 µL of a solution containing Na₂CO₃ (0.4 %) and NaOH (2 %) was added. After 1 h of incubation at room temperature in darkness the absorbance was measured at 750 nm. The TPC was expressed as mg gallic acid equivalents (GAE) kg⁻¹ fw.

2.4.3. Total antioxidant capacity

The TAC was determined by three different methods: free radical scavenging capacity with 2,2-diphenyl-1-picrylhydrazil (DPPH) (Brand-Williams et al.1995), ferric reducing antioxidant power (FRAP) (Benzie and Strain 1999) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (Cano et al. 1998). Briefly for DPPH, a solution of 0.7 mM DPPH in methanol was prepared 2 h before the assay and adjusted to 1.1 (nm) immediately before use. A 21 µL aliquot of the TAC extract was added to 194 µL of this solution. The mixture was incubated for 1 h at room temperature in darkness. The TAC by DPPH was measured by the decrease in absorbance at 515 nm. Briefly for ABTS, 14 mM ABTS and 4.9 mM de K₂S₂O₈ stock solutions were mixed (1:1) and incubated for 16 h at room temperature in darkness. The absorbance of this
solution was adjusted to 0.7 nm. Then, 285 μL of the latter solution was added to 15 μL of TAC extract in a well plate and incubated for 6 min at room temperature in darkness. The TAC by ABTS was measured by the decrease in absorbance at 734 nm. Briefly for FRAP, a daily reaction solution containing sodium acetate buffer (pH 3.6), 10 mM TPTZ solution (in 40 mM HCl) and 20 mM FeCl₃ was prepared in a v:v:v proportion of 10:1:1 and incubated at 37 ºC for 2 h in darkness. Then, 6 μL of TAC extract was allowed to react with 198 μL of the FRAP solution for 40 min at room temperature in darkness. The TAC by FRAP was measured by the decrease in absorbance at 593 nm. All TAC data were expressed as mg of ascorbic acid equivalents (AAE) equivalent kg⁻¹ fw.

2.5. Statistical Analysis

The experiment was a two-factor (treatment×storage time) design subjected to analysis of variance (ANOVA) using Statgraphics Plus software (vs. 5.1, Statpoint Technologies Inc, Warrenton, USA). Statistical significance was assessed at the level $P=0.05$, and Tukey’s multiple range test was used to separate means. Pearson correlation analysis was performed to corroborate relationships between TPC and TAC.

3. RESULTS AND DISCUSSION

3.1. Enzymatic activity

3.1.1. Polyphenoloxidase

The initial PPO activity of CTRL-R1 and CTRL-R2 smoothies were 58±17 and 83±13 U kg⁻¹ fw, respectively, which was assigned as 100 % residual PPO activities (Figure 1). Refrigerated storage of CTRL samples affected their PPO enzyme activity ($P\leq0.5$). Accordingly, PPO activity of CTRL-R1 smoothie showed a progressive inactivation.
reaching 3% residual activity after 28 days at 5°C (Figure 1 A). Similarly, CTRL-R2 smoothie showed a progressive PPO inactivation reaching 3-18% residual activity after 24-28 days at 5°C (Figure 1 B). However, CTRL-R2 smoothie showed an initial great PPO inactivation, which reached minimum values of 15% after 10 days at 5°C 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°C (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°C) 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°C) 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°C. 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.
3.1.2. Peroxidase

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

3.1.3. Pectinmethylsterase

PME is a target enzyme in fruit and vegetable juices and smoothies to avoid phase separation of the colloidal suspension which is formed by two phases: serum and cloud (Chakraborty et al. 2014). The initial PME activities of CTRL-R1 and CTRL-R2 were $1.50 \pm 0.08$ and $0.38 \pm 0.05$ U kg$^{-1}$fw, respectively, which were assigned as the 100 %
residual PME activities (Figure 3). PME activity of tomatoes have been reported to be 4 and 9-fold higher compared to that of broccoli and carrots while PME activity in red peppers was not detectable (Espachs-Barroso et al. 2006; Castro et al. 2008; Houben et al. 2014). Accordingly, the lower tomato proportion in the R2 formulation may explain the lower initial PME activity of CTRL-R2 compared to CTRL-R1. After the thermal treatment, PME residual activities of R1 and R2 smoothies were 6 and 34 %, respectively. The thermal inactivation kinetics of PME in tomato juice was described reasonably well by first order inactivation kinetics with an inactivation D value of 4 min at 75 ºC (Terefe et al. 2009). Similarly, thermal treatments of 70-75 ºC for 5 min were able to completely inactivate PME activities in tomato, carrot and broccoli purées (Houben et al. 2013, 2014). Espachs-Barroso et al. (2006) found that activation energy of PME thermal inactivation from tomatoes was approximately 10 % higher than that for carrot. However, the PME activity and related thermal stability can greatly differ among produce varieties and ripening stage (Barrett and González 1994; De Sio et al. 1995). Accordingly, the higher PME thermal stability of R2 may be attributed to the presence of carrot with a high PME thermal stability in R2 smoothie. Houben et al. (2014) attributed the higher PME stability of carrots compared to broccoli to a protective effect of the carrot purée matrix as similarly observed Balogh et al. (2004). During refrigerated storage, PME residual activity of CTRL-R1 and CTRL-R2 samples decreased by 65 and 8 %, respectively, after 3 days at 5 ºC (Figure 3). The greater PME decrease in CTRL-R1 smoothie can be owed to its greater tomato concentration and presence of carrot which PME activities can be more sensible to the abiotic stress provoked by the cold storage. Accordingly, after this initial PME decrease due to the chilled shock, the PME was reactivated registering greater increases (up to 257 % relative activity after 21 days) in CTRL-R2 smoothie due to the commented greater
stability during chilled storage. In general, PME activities of heat treated R1 and R2 samples did not significantly changed during storage at 5 and 20 °C. However, the PME activity of treated R2 samples stored at 5 °C showed two maximum relative activities of 72 and 41 % after 7 and 35 days, although the activity at the end of shelf life was less than 14 %. Last behaviour was similar to that of CTRL-R2 samples but was not observed at 20 °C since those PME activity peaks could be owed to the chilled shock as explained before. In conclusion, thermal treatment was able to inactivate PME and maintain low activity levels of this enzyme during storage at 5 and 20 °C.

3.1.4. Phenylalanine ammonia-lyase

The initial PAL activities of CTRL-R1 and CTRL-R2 smoothies were 7.3±1.1 and 11.5±2.9 µmol cinnamic acid formed kg⁻¹ h⁻¹, respectively, although no significant (P≤0.05) differences were found between both smoothies (Figure 4 A). Bojórquez-Gálvez et al. (2010) reported approximately 5 µmol cinnamic acid formed kg⁻¹ h⁻¹ of PAL activity in tomato. The slightly higher PAL activity of our smoothies may be owed to the red pepper content which has reported high PAL activities ranging from 9.4 to 23.4 µmol cinnamic acid formed kg⁻¹ h⁻¹ depending of the variety (Perucka and Materska 2001). PAL activities were reduced in a 65-70 % after thermal treatment without significant differences among both smoothies. Rees and Jones (1996) reported a half-live of 1.4 min at 70 °C for partially purified PAL preparations in water (pH 8.0). The lower thermal stability of PAL found by those authors compared to our smoothies may be attributed to a better heat distribution in that aqueous solution and protective effects of the fibre particles of our smoothies.

The accumulation of phenolic compounds is a stress response caused by a change in 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 $404 \pm 25$ and $462 \pm 41$ mg GAE kg$^{-1}$ fw, respectively, without significant differences among them (Figure 5).
Similarly, Odriozola-Serrano et al. (2008) reported 311.0 mg GAE kg\(^{-1}\) fw in tomato juice. In general, phenolic degradation is induced during food processing, by chemical or enzymatic oxidation which can also lead to changes in bioavailability or biological activity (Tomás-Barberán and Espín 2001). However, the thermal treatment hereby applied did not induce significant changes in the TPC of the smoothies. In accordance with our results, Patras et al. (2009a) and Odriozola-Serrano et al. (2008) did not find significant changes of the TPC after thermal treatment of tomato juice (90 °C for 60 s) and tomato and carrot purées (70 °C for 2 min).

The initial TPC of CTRL samples increased throughout storage up to 18 % after 10 days, keeping these levels without significant changes until the end of storage (Figure 5). PAL increments like those observed in CTRL-R1 samples after 10 days is correspondent with the hereby found TPC increments of CTRL samples. On the other side, TPC of T-R1 and T-R2 samples decreased during storage registering levels 19 and 13-18 % lower after 58 and 40 days at 5 and 20 °C, respectively. Similarly, Odriozola-Serrano et al. (2009) found 16-24 % TPC decreases in thermally treated (90 °C for 60 s) tomato juice after 56 days at 4 °C. Since other enzymes apart from PAL are involved in the phenylpropanoid metabolism, the thermal treatment could inactivate some of them affecting the TPC biosynthesis observed in CTRL samples. Conclusively, thermal treatments did not induce TPC changes although they were enough to inactivate PPO and POD activities, leading to good TPC stability (<20 % degradation) during storage either at 5 or 20 °C.

3.3. Total antioxidant capacity

The initial total antioxidant capacities of CTRL-R1/R2 smoothies obtained by FRAP, ABTS and DPPH were 301±35/373±12, 467±29/591±42 and 91±22/182±34 mg AAE
kg\(^{-1}\) fw, respectively. The initial total antioxidant capacities of T-R1/R2 smoothies obtained by FRAP, ABTS and DPPH were 488±5/659±46, 527±42/637±40 and 233±24/287±24 mg AAE kg\(^{-1}\) fw, respectively. The low TAC by DPPH method may be explained since one of the main maxim absorbance wavelengths for lycopene (Naviglio et al. 2008), the main carotenoid of tomatoes, is close to that use for the DPPH method which may lead to the observed reduced TAC by DPPH. Similarly to our results, Keenan et al. (2010) found 3-fold higher TAC using FRAP method compared to DPPH in fruit smoothies.

Phenolic compounds are the major contributors to the antioxidant properties of fresh produce (Cisneros-Zevallos 2003). Antioxidant capacity of a food product may greatly differ depending of the analytical method used (Prior et al. 2005). Accordingly, a Pearson correlation using TPC and TAC data during storage was used to ascertain which TAC method was better correlated to TPC (Table 2). In general, TAC was highly correlated to TPC with \(r^2\) ranging from 0.57 to 0.88. In general, FRAP method achieved the best correlations \((r^2=0.69-0.88)\) closely followed by ABTS \((r^2=0.57-0.79)\). Accordingly, FRAP data during storage is presented (Figure 6).

Contrary to the unchanged TPC data after thermal treatment, TAC increased after thermal treatment registering FRAP method increments of 62 and 77 % in R1 and R2, respectively, comparing to CTRL smoothies. Similar TAC increments have been observed in tomato and carrot purées after thermal treatments (Patras et al. 2009b). It has been hypothesized that latter behaviour is related to an increase in the extractability of antioxidant components following thermal processing rather than an absolute increase.

According to TPC data, TAC of CTRL samples increased during storage registering a great increase of 51-55 % after 7 days compared to initial values being maintained (no
significant changes) those increased levels until the end of shelf life. However, and as
previously commented for TPC data, the TAC of treated samples progressively
decreased during storage at 5 and 20 ºC registering TAC levels 19-23 and 8-11 % lower
after 58 and 40 days, respectively. Similarly to our data on days 28 and 35, Keenan et
al. (2010) reported TAC (by FRAP) decreases of 19 % in heat-treated (70 ºC for 10
min) fruit smoothies after 30 days at 4 ºC.

4. Conclusions

A thermal treatment of red fresh vegetables smoothies of 3 min at 80 ºC inactivated
PPO, POD and PME which activities were minimal during subsequent storage either at
5 or 20 ºC. Moreover, the thermal treatment did not initially induce changes in the total
phenolics content of the samples, which was well correlated to PAL activity and total
antioxidant capacity, being these levels well preserved during shelf life even at 20 ºC.
Accordingly, this article presents two red fresh vegetables smoothies with stabilized
activities of degradative-quality enzymes and good levels of health-promoting
compounds during 58 and 40 days of storage at 5 and 20 ºC, respectively.

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**FIGURE AND TABLE CAPTIONS**

**Figure 1.** Polyphenoloxidase activity of untreated (CTRL) and heat-treated (T) red smoothies R1 (A) and R2 (B) stored at 5 and 20 °C (n=5±SD). Different letters denote significant differences ($P \leq 0.05$) among sampling days for the same treatment.
Figure 2. Peroxidase activity of untreated (CTRL) and heat-treated (T) red smoothies R1 (A) and R2 (B) stored at 5 and 20 °C (n=5±SD). Different letters denote significant differences (P≤0.05) among sampling days for the same treatment.

Figure 3. Pectinmethylesterase activity of untreated (CTRL) and heat-treated (T) red smoothies R1 (A) and R2 (B) stored at 5 and 20 °C (n=5±SD). Different capital letters denote significant differences (P≤0.05) among treatments for the same sampling day. Different lowercase letters denote significant differences (P≤0.05) among sampling days for the same treatment.

Figure 4. Phenylalanine ammonia-lyase activity of untreated (stored at 5 °C, A) and heat-treated (stored at 5 °C, B; stored at 20 °C, C) red smoothies R1 and R2 (n=5±SD). Different capital letters denote significant differences (P≤0.05) among treatments for the same sampling day. Different lowercase letters denote significant differences (P≤0.05) among sampling days for the same treatment.

Figure 5. Total phenolics content of untreated (stored at 5 °C, A) and heat-treated (stored at 5 °C, B; stored at 20 °C, C) red smoothies R1 and R2 (n=5±SD). Different capital letters denote significant differences (P≤0.05) among treatments for the same sampling day. Different lowercase letters denote significant differences (P≤0.05) among sampling days for the same treatment.

Figure 6. Total antioxidant capacity of untreated (stored at 5 °C, A) and heat-treated (stored at 5 °C, B; stored at 20 °C, C) red smoothies R1 and R2 (n=5±SD). Different capital letters denote significant differences (P≤0.05) among treatments for the same
sampling day. Different lowercase letters denote significant differences ($P \leq 0.05$) among sampling days for the same treatment.

Table 1. Composition and main chemical quality parameters of red vegetable smoothies (R1 and R2).

Table 2. Pearson correlation coefficients ($r$) between total phenolics content and different total antioxidant capacity methods (FRAP, ABTS and DPPH) of red vegetable smoothies, untreated (CTRL) and heat-treated, and stored at 5 or 20 °C.