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RED FRESH VEGETABLES SMOOTHIES WITH EXTENDED SHELF LIFE AS AN INNOVATIVE SOURCE OF HEALTH-PROMOTING COMPOUNDS

Short title: Health-promoting properties of red fresh vegetables smoothies

Noelia Castillejo[a], Ginés Benito Martínez-Hernández[a,b], Perla A. Gómez[b], Francisco Artés[a,b] and Francisco Artés-Hernández[a,b*]

[a] Postharvest and Refrigeration Group, Department of Food Engineering, Universidad Politécnica de Cartagena, Paseo Alfonso XIII, 48, 30203, Cartagena, Murcia, Spain.

[b] Institute of Plant Biotechnology, Universidad Politécnica de Cartagena, Campus Muralla del Mar s/n, 30202, Cartagena, Murcia, Spain.

* To whom correspondence should be addressed:

Tel: +34-968-325509; Fax: +34-968-325433.

E-mail: fr.artes-hdez@upct.es Web site: www.upct.es/gpostref

Abstract

Two fresh red vegetables smoothies rich in health-promoting compounds were developed. The smoothies showed a viscoelastic behaviour. According to sensory analyses, a shelf life of 28 days at 5 ºC was established for fresh blended smoothies while thermally-treated ones reached up to 40 days at 20 ºC and 58 days at 5 ºC. Total vitamin C degradation was 2-fold reduced during storage at 5 ºC regarding at 20ºC while the initial total carotenoids, lycopene and total chlorophylls contents were not greatly affected. A 250-g portion of such smoothies covers in a great extend the

established recommended daily nutrient intakes for dietary fibre, minerals and vitamin C of different population groups. In conclusion, a mild thermal treatment and low temperature storage greatly increased the shelf life of red fresh vegetables smoothies and reduced total vitamin C degradation.

Keywords: vitamin C; lycopene; chlorophylls; bioactive compounds; fibre; 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 avoid the low-density lipoprotein oxidation (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 (Yannakoulia, Kontogianni, & Scarmeas, 2014). Tomato, red pepper, carrot and broccoli have high contents of those health-promoting phytochemicals such as carotenoids, phenolic compounds, vitamins C and E, folates, glucosinolates and minerals, among others (Serrano et al., 2010; Dosz & Jeffery, 2013; Fernández-León et al., 2013; Sánchez-Rangel, Jacobo-Velázquez, Cisneros-Zevallos, & Benavides, 2014). Dietary fibre activates intestinal peristalsis, binds bile acids and water, and reduces blood cholesterol level and the risk of incidence of ischemic heart disease and postprandial glycaemia (Chen, Ma, Liang, Peng, & Zuo, 2011).

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

51 to preserve as much as possible the quality parameters (Artés-Hernández, Escalona,
52 Robles, Martínez-Hernández, & Artés, 2009). Smoothies are no alcoholic beverages
53 prepared from fresh or frozen fruit and/or vegetables, which are blended and usually
54 mixed with crushed ice to be immediately consumed. Often, some smoothies may
55 include other components like yogurt, milk, ice-cream, lemon water or tea. They have a
56 milk shake-like consistency that is thicker than slush drinks. Accordingly, smoothies
57 represent an excellent and convenient alternative to promote the daily consumption of
58 fruit and vegetables. The smoothie preparation involves a breakdown of plant
59 parenchyma, which leads to a dispersed solution consisting in a liquid phase (pectin and
60 other soluble solids) and a solid phase composed of insoluble solids (cell wall). The
61 main issue of the smoothie processing is the limited shelf-life of these products since
62 they are susceptible to spoilage (Buzrul, Alpas, Largeteau, & Demazeau, 2008) and
63 quality degradation. For that reason, in order to increase the shelf-life while keeping
64 quality, mild thermal treatments must be used during processing (Di Cagno, Minervini,
65 Rizzello, De Angelis, & Gobbetti, 2011; Rodríguez-Roque et al., 2015) and lowering
66 the storage temperature up to 5ºC recommended. However, the treatment should not be
67 much aggressive to preserve its nutritional and sensory quality. Thermal treatment
68 (generally in the range of 80 °C to 95 °C) is commercially applied for the inactivation of
69 spoilage enzymes in fruit purées and juices (Barba, Esteve, & Frigola, 2012;
70 Ludikhuyze & Hendrickx, 2002). However, thermal treatments may reduce
71 phytochemical contents of smoothies in detriment of related antioxidant properties. To
72 the best of our knowledge, there is no information about the effects of thermal
73 processing and subsequent storage on quality changes of fresh vegetable smoothies. For
74 that reason, the aim of this work was to study the effect of a mild conventional
75 pasteurization or avoiding the use of a thermal treatment on sensory, microbial and

physicochemical quality changes, as well as on selected bioactive compounds of two red fresh vegetable smoothies throughout the storage at 5 and 20 ºC.

2 Materials and methods

2.1 Plant material and smoothie preparation

Fresh vegetables (tomato, red pepper, broccoli and carrot) were purchased 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). According to the composition, two different red smoothies (R1 and R2) were prepared based in previous formulations, which were well accepted by a trained sensory panel. Table 1 presents the smoothies composition.

2.2. Thermal treatment and storage conditions

Smoothies were immediately placed in 15 mL falcon tubes 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, 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 ºC in iced water and then stored in darkness at 5 and 20 ºC. Fresh blended unheated samples were used as control (CTRL) which was just stored at 5 ºC. Five replicates per treatment and sampling day, for each storage temperature, were prepared. Samples of each treatment were taken on each sampling day and stored at -80 ºC until further analysis.

2.3. Rheological properties of smoothies

Rheological measurements were executed using ARG2 stress-controlled rheometer (TA Instruments, New Castle, DE, USA) equipped with serrated (to prevent wall depletion phenomena) plate-plate geometry (20 mm, gap 2 mm). A solvent trap saturated with water was used to prevent evaporation. For every measurement the smoothie sample was transferred to the rheometer geometry and the sample was allowed to equilibrate between the plates at 25°C for 1 min. Oscillatory tests were performed within the linear viscoelastic region. Storage modulus (G') and loss modulus (G") were determined in a frequency range of 100 to 0.2 Hz. The strain value was obtained by preliminary strain sweep oscillatory trials to determine the linear viscoelastic region. The strain sweep oscillatory tests were carried out at a frequency of 1 Hz and in a range of shear strain of 0.01 to 10 %. Flow tests were also used to cover shear rate range between 10^{-2} /s and 10^{2} /s. All experiments were carried out at 25 °C. Rheological data is presented as supplementary material. Three repetitions of the dynamic-mechanical experiments were performed for each smoothie sample.

2.4. Total dietary fibre and mineral content

The contents of pectin, hemicellulose, cellulose, lignin and ash in the smoothies were studied by thermogravimetric analysis (TGA), conducted on a TGA/DSC HT thermogravimetric analyser (Mettler-Toledo GmbH, Schwerzenbach, Switzerland) with the method described by Boluda-Aguilar and López-Gómez (2010) lightly modified. Fine powder from dried samples (105 ºC for 24 h) was obtained by mincer (IKA, A 11basic, Berlin, Germany). Approximately 10 mg of sample powder was used. Derivative thermogravimetric (DTG) curves were analysed by derivative weight loss (see supplementary material 2). The TG-DTG curves are presented as supplementary data. The temperature for the maximal weight loss (T_{max}) at 90 ºC is attributed to the

126 free water loss. The decomposition peaks at the T_{max} of 190, 270 and 321ºC are assigned
127 to pectin, hemicelluloses and cellulose, respectively (Boluda-Aguilar & López-Gómez,
128 2010; Zhou, Long, Meng, Li, & Zhang, 2013). The weight percentage of each
129 component in analysed samples is obtained as the mass loss produced during
130 volatilization.
131 The mineral content of the samples was analysed by X-ray fluorescence (XRF)
132 according to Martínez-Hernández, Gómez, Artés, and Artés-Hernández (2015a). For the
133 XRF analyses a spectrometerS4 Pioneer (Bruker Corporation, Billerica, MA, USA)was
134 used, equipped with a Rh anticathode X-ray tube (20-60 kV, 5-150mA and 4 kW
135 maximum), five analyser crystals (LiF200, LiF220, Ge, PET, and XS-55), sealed
136 proportional counter for light elements detection and a scintillation counter for heavy
137 elements with slight modifications. The recorded spectrum was evaluated by the
138 fundamental parameters method using the Spectra plus software EVA 1.7. Mineral
139 content was expressed as g kg^{-1}dry weight (dw) and mg kg^{-1}dw for major minerals and
140 trace elements, respectively. All samples were analysed in triplicate.
141
142 **2.5. Sensory evaluation**
143 Sensory analyses were performed according to international standards (ASTM STP 913
144 1986). Tests were conducted in a standard room (ISO 8589:2007) equipped with ten
145 individual taste booths. Samples (about 30 mL) were served at room temperature in
146 transparent plastic glasses coded with three random digit numbers. Still mineral water
147 was used as palate cleanser. The panel consisted of twelve assessors (six women/six
148 men, aged 22–68 years) screened for sensory ability (colour, flavour, visual appearance
149 and texture). A 5-point scale of damage incidence and severity was scored for off-
150 colour, off-odours, lumpiness, turbidity and precipitation/phase separation (5: none; 4:

151 slight; 3: moderate, limit of usability; 2: severe; 1: extreme). Visual appearance, flavour,
152 texture and overall quality (5: excellent, 4: good, 3: fair, limit of usability, 2: poor; 1:
153 extremely bad).
154
155 **2.6. Colour**
156 Colour was determined using a colorimeter (Minolta CM-2600d, Japan) calibrated with
157 a white reference plate (light source C), 2º observer and 8-mm viewing aperture.
158 Samples were introduced in a special glass tube mounted on a device connected to the
159 colorimeter. Measurements were recorded using the standard tristimulus parameters
160 (L*, a*, b*) of the CIE Lab system on three equidistant points of each replicate. Three
161 colour readings were taken turning the tube every caption and all three measurements
162 were automatically averaged by the device and recorded. Total colour differences (ΔE)
163 throughout storage compared to their respective initial values were calculated according
164 to equations previously described (Walkling-Ribeiro, Noci, Cronin, Lyng, & Morgan,
165 2010).
166
167 **2.7. Microbial analysis**
168 To determine the mesophilic, psychrophilic, *Enterobacteria*, and yeast and mould
169 growth, standard enumeration methods were used. Samples of 5 g were homogenised in
170 45 mL of sterile peptone saline solution (pH 7; Scharlau Chemie SA, Barcelona, Spain)
171 for 10 sin a sterile stomacher bag (model 400 Bags 6141, London, UK) using a
172 masticator (Colwort Stomacher 400 Lab, Seward Medical, London, UK). For the
173 enumeration of each microbial group, 10-fold dilution series were prepared in 9 mL of
174 sterile peptone saline solution. Mesophilic, *Enterobacteria* and psychrotrophic were
175 pour plated, and yeast and mould were spread plated. The following media and

incubation conditions were used: plate count modified agar (PCA) (Scharlau Chemie, Barcelona, Spain) for mesophilic and psychrotrophic aerobic bacteria, incubated at 30 °C for 48 h and at 5 °C for 7 days, respectively; violet red bile dextrose agar (Scharlau Chemie, Barcelona, Spain) for *Enterobacteria*, incubated at 37 °C for 48 h; and rose Bengal agar (Scharlau Chemie, Barcelona, Spain) for yeasts and moulds, incubated for 3–5 days at 22 °C. All microbial counts were reported as log colony forming units per gram of product (log CFU g^{-1}). Each of the three replicates was analysed by duplicate. The presence of Salmonella spp., *Listeria monocytogenes* and generic *Escherichia coli* was monitored according to the European legislation (Regulation EC 1441/2007 2007).

2.8. Physiochemical analyses

The pH, titratable acidity (TA) and total soluble solids content (SSC) of red vegetables smoothies was studied. A pH-meter was used to analyse the pH. The SSC of the smoothies was determined by a digital hand-held refractometer (Atago N1, Tokyo, Japan) at 25 ºC and expressed as ºBrix. TA was determined by the titration of 5 mL of juice plus 45 mL of distilled water with 0.1 mol L^{-1} NaOH to pH 8.1 (T50, Metter Toledo, Milan, Italy) and expressed as % (g citric acid 100 mL^{-1}). Three replicates per treatment were analysed.

2.9. Bioactive compounds

2.9.1. Vitamin C

The ascorbic (AA) and dehydroascorbic (DHA) acids were measured according to the method of Zapata and Dufour (1992) with modifications from Martínez-Hernández, Artés-Hernández, Gómez, and Artés (2013). Derivatised samples (20 μL) were injected on a Gemini NX (250 mm×4.6 mm, 5 μm) C18 column (Phenomenex, Torrance CA,

USA), using an HPLC (Series 1100 Agilent Technologies, Waldbronn, Germany) equipped with a G1322A degasser, G1311A quaternary pump, G1313A autosampler, G1316A column heater and G1315B photodiode array detector. The HPLC system was controlled by the software ChemStation Agilent, v. 08.03. AA and DHA were quantified using commercial standards (Sigma, St Louis, MO, USA). Calibration curves were made with at least six data points for each standard. Total vitamin C was calculated as the sum of AA and DHA and expressed as mg kg^{-1} fw. Each of the three replicates was analysed by triplicate.

2.9.2. Total carotenoids and chlorophylls content

Sample preparation for total carotenoids and chlorophylls determinations was conducted according to Martínez-Hernández, Gómez, Pradas, Artés, and Artés-Hernández (2011). An UV-visible spectrophotometer (8453, Hewlet Packard, Columbia, USA) was used to registered absorbances at 662, 644 and 470 nm. The equations developed by Wellburn (1994) were used to determine the individual levels of chlorophyll a (Cha = 10.05×A662 – 0.766×A644), chlorophyll b (Ch b = 16.37×A644 – 3.14×A662), total chlorophyll amount (Ca + Cb) and total carotenoids [TC = (1000×A470– 1.28×Ca– 56.7×Cb)/205]. Total chlorophyll and TC contents were expressed as mg kg^{-1} fw. Each of the three replicates was analysed by triplicate.

2.9.3. Lycopene

Lycopene content was determined according to Davis, Fish, & Perkins-Veazie (2003). Briefly, 1 g ground frozen sample was mixed with 5 mL of acetone containing 0.05% (w/v) butylhydroxytoluene, 5 mL 95% ethanol and 10 mL hexane. The extraction was carried out for 15 min in darkness inside a polystyrene box with ice and shaken

continuously at 200×g with the orbital shaker. After extraction, 3 mL distilled water was added, samples were shaken again for 5 min in the orbital shaker and the upper of the three layers formed was used as lycopene extract. Absorbances of the extracts were measured at 503 nm in the UV-visible spectrophotometer. The lycopene content was calculated according to Fish et al. (2002) as: lycopene= (A503×MW×DF)/(Ɛ); where MW is the lycopene molecular weight, DF the dilution factor and ε is the lycopene molar extinction coefficient (172,000 L mol cm^{-1} in hexane). Lycopene contents were expressed as mg kg^{-1} fw. Each of the three replicates was analysed by triplicate.

2.10. Statistical Analysis

The experiment was a one-factor (treatment) 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.

3. Results and discussion

3.1. Rheological properties of smoothies

The texture of a smoothie has to provide a balance between desired mechanical stability (for storage and handling) and desired instability (to elicit a specific texture attribute during mastication). Rheological properties are useful in determining the most ingredients proportions in the product development, quality control, and correlation of food texture to sensory attributes. Smoothies are viscoelastic food materials that exhibit both solid-like and fluid-like behaviour. The rheological characteristics of red smoothies are presented as supplementary data. The storage modulus (G') of smoothies was greater than the loss modulus (G") at any given point in the frequency sweep tests (see

supplementary material 1). This fact indicates a dominant contribution of the elastic component to the viscoelasticity of the investigated smoothies, behaviour typical for a viscoelastic solid. This means that the attractive forces become dominant due to the strong hydrogen bond and hydrophobic association (Basu, Shivhare, Singh, & Beniwal, 2011). Apparent viscosity of CTRL-R1 was higher than CTRL-R2 probably owed to the higher pectin content of R1 smoothie (see Supplementary material 1 and Table 2). The tanδ value (ratio between loss and storage modulus, also known as loss tangent) is a direct measure of the relative importance of viscous and elastic effects in the sample. For all the considered samples, tanδ was lower than 1 thus indicating a gel-like behaviour. While apparent of R1smoothie was reduced after thermal treatment, R2 smoothie showed the opposite behaviour (see Supplementary material 1) which may be explained by the different composition of smoothies. The effective shear rate range in the mouth is 40-50 s^{-1}, which would have implied actual sensory consistency (Wood &d Goff, 1973). The viscosity of CTRL-R1 samples was higher than CTRL-R2 within the shear rate range 40-50 s^{-1}. Accordingly, panellists scored better texture of R1 smoothies than R2 (as described latter), which is related to a greater smoothie viscosity of R1 smoothie.

3.2. Total dietary fibre and mineral content

The total dietary fibre content **(**DF), as well as their main components as pectin, hemicellulose and cellulose are depicted in Table 2. The total DF content of R1 and R2 smoothies were 4.7 and 4.8 % wet basis (wb), respectively. The higher total DF of R2 smoothie compared to R1 may be explained by the presence of carrots and higher pepper and broccoli contents in the smoothie formulation, having all those vegetables higher fibre contents. Pectin and hemicellulose content of smoothies accounted 1.4-1.5

276 and 1.2 % wb, respectively. Cellulose accounted 2.1 % wb for both smoothies.
277 According to The Code of Federal Regulations (FDA, 2014), food products which
278 contain 20 % or more of the recommended daily nutrient intakes (RNIs) for fibre (25 g
279 day^{-1}) are considered as an 'excellent source of fibre'. Accordingly, these fresh red
280 smoothies can be considered as an 'excellent source of fibre' since a portion of 250 g
281 provides 50 % of the RNIs for fibre.
282 The minerals content of both red smoothies are presented in Table 3. R1 smoothie
283 presented 1.1-1.5-fold higher P, Na, Al and Mn content than R2 smoothie. On the other
284 side, R2 smoothie presented 1.1-1.4-fold higher Fe, K, Ca, Zn and Sr content than R1.
285 A smoothie portion of 250 g provides 8-11, 2-3, 2-4 and 3-4 % of the RNIs for Mg, Ca,
286 Fe and Zn, respectively, covering population groups with special nutritional
287 requirements such as elders, pregnant women or adolescents (WHO, 2004).
288
289 **3.3. Sensory analysis**
290 Visual appearance, flavour, texture, off-colours, off-odours, lumpiness, turbidity,
291 precipitation/phase separation and overall quality of CTRL smoothies were reported to
292 be over the limit of acceptability up to 28 days at 5 ºC. Thermally-treated smoothies
293 maintained their sensory acceptation up to 40 days at 20 ºC and 58 days at 5 ºC (data
294 not shown). Accordingly, the shelf-life of the smoothies was established based in the
295 sensory analyses.
296
297 **3.4. Soluble solids content, pH and titratable acidity**
298 The initial SSC of CTRL-R1 and CTRL-R2 smoothies were 8.37 and 7.07 ºBrix,
299 respectively (Table 4). The higher SSC of R1 smoothie regarding R2 may be explained
300 by the higher tomato content of R1 (75 %) compared to R2 (56 %). Di Cagno et al.

301 (2011) reported a SSC of 13.1 ºBrix in red fruit smoothies. The higher tomato content
302 (56 and 75 %) compared with the low tomato (8 %) and high fruit contents (31 %
303 prunes and 26 % cherries) of fruit smoothies may explain the lower SSC of our
304 smoothies. The thermal treatment did not induce significant SSC changes in R1
305 smoothie but SSC of R2 lightly increased in 1.4 ºBrix after treatment. Accordingly, the
306 SSC increase of R2 smoothie may be explained by its carrot content. The hard texture
307 of carrot tissue may lead to carrot particles after blending. Accordingly, the soluble
308 solids extraction can be enhanced after thermal treatment as observed in R2 samples.
309 SSC of both untreated and thermally-treated smoothies did not significantly change
310 during storage either at 5 or 20 ºC.
311 The initial pH of untreated R1 and R2 smoothies were 4.36 and 4.31, respectively
312 (Table 4). Di Cagno et al. (2011) reported lower pH levels (3.5) in a red fruits smoothie
313 due to its high content of fruits, which have lower pH than vegetables. The pH of both
314 smoothies did not significantly change after the thermal treatment. The pH of treated
315 and untreated smoothies did not greatly change (<0.2 pH units) during storage either at
316 5 or 20 ºC.
317 The initial TA of untreated R1 and R2 smoothies was 0.25 and 0.22 mg citric acid 100^{-1}
318 g fw, respectively (Table 4). Keenan et al. (2010) reported higher TA values of 0.56 mg
319 citric acid 100^{-1} g fw in a fruit smoothie owed to the higher TA of fruits compared to
320 vegetables. Throughout conservation, TA of CTRL smoothies registered increases up to
321 34 and 54 % after 21 and 28 days at 5 ºC, respectively. Thermal treatment and storage at
322 5 ºC may reduce metabolic reactions since no great TA changes (<0.07 mg citric acid
323 100^{-1} g fw) were observed in those smoothies. Similarly, Di Cagno et al. (2011) did not
324 observe significant TA differences in heat-treated (80 ºC for 10 min) fruit/vegetable
325 smoothies throughout storage at 4 ºC. However, storage at 20 ºC of thermally-treated

smoothies induced a gradual TA reduction with values approximately 30 % lower at the end of storage regarding their respective initial levels. The latter behaviour is owed to the higher storage temperature, which enhances metabolic reactions that produce acidic compounds. In general, the TA behaviour of samples during storage was inversely correlated to pH behaviour.

3.4. Colour

The L*, a* and b* values of R1/R2 smoothies were 92.1/91.5, 16.1/13.4 and 37.4/38.7, respectively (data not shown). Thermal treatment induced light colour changes with ΔE values for R1 and R2 smoothies of 5.6 and 9.6, respectively. Walkling-Ribeiro et al. (2010) reported lower ΔE value (1.2) after a short thermal treatment (72 ºC for 15 s) of fruit smoothie. A great ΔE, of approximately 20 units, was observed after 3 days of storage of untreated smoothies, while treated smoothies only achieved ΔE of approximately 2-11 units after 7 days of storage at both temperatures. As observed, colour changes of smoothies during storage were greatly reduced in those treated samples, which are mostly due to the thermal inactivation of colour degradative enzymes such as polyphenoloxidase (PPO) and peroxidase (POD). Accordingly, great to nearly complete PPO and POD inactivations have been reported in broccoli and spinach puree after similar thermal treatments (Morales-Blancas, Chandia, & Cisneros-Zevallos, 2002; Wang et al., 2012, 2013). As expected, ΔE levels gradually increased throughout storage. However, storage at low temperature reduced the colour changes since ΔE of 20-21 and 24-26 were registered after 40 days at 5 and 20 ºC, respectively.

3.5. Microbial analysis

350 The initial microbial counts of CTRL-R1/R2 smoothies were 4.3/4.6, 4.0/4.6, 3.9/4.3
351 and 4.6/5.9 log CFU g^{-1} for mesophiles, psychrophiles, *Enterobacteria* and yeast and
352 moulds, respectively (Figure 1). Thermal treatment of R1/R2 smoothies achieved
353 mesophilic, psychrophilic, *Enterobacteria* and yeast and moulds reductions of
354 approximately 2/2.4, 1.7/2.2, 1.8/2.3 and 2.3/2.8 log units, respectively. Walkling et al.
355 (2010) reported mesophilic and yeast and moulds reductions of 3.5 and 3.7 log CFU g^{-1},
356 respectively, in a fruit smoothie after a thermal treatment of 70 ºC for 15 s. The dynamic
357 system used by Walkling et al. (2010) during heat treatment compared to our static
358 system may explain the better microbial reductions achieved by those authors.
359 During the first 10 days of storage, mesophilic counts of CTRL-R1 and CTRL-R2
360 smoothies increased by 0.5 and 0.3 log CFU g^{-1}, respectively. However, thermally-
361 treated R1/R2 smoothies stored at 5 and 20 ºC showed mesophilic increases of 0.6/1.0
362 and 1.7/1.9 log units, respectively, after 10 days. As expected, the microbial growth
363 rates were higher at high storage temperatures. Similarly, Walkling et al. (2010)
364 reported a mesophilic increment of 0.1-0.7 log CFU g^{-1} in a fruit smoothie after 7-14
365 days at 4 ºC. The observed higher mesophilic growth in treated samples could be owed
366 to the following hypotheses: 1) the vegetative or spore cells which resisted to the
367 thermal treatment, due to their higher thermal resistance and/or the protecting effects of
368 the smoothie matrix, could grow better due to the lower microbial competence for the
369 nutrients. 2) The used heat treatment completely inactivated the initial myrosinase
370 activity (163.0 nmoles sinigrin transformed per g fw of sample; data not shown), which
371 is responsible for the glucosinolates conversion to isothiocyanates. Isothiocyanates from
372 broccoli have shown high antimicrobial activities contrary to glucosinolates (Vig,
373 Rampal, Thind, & Arora, 2009). Accordingly, the glucosinolate-isothiocyanate
374 conversion was possible in untreated unheated samples, contrary to heat-treated

375 samples, with the observed preserving benefits from the isothiocyanates throughout
376 storage of smoothies. Therefore, our previous preliminary non-published data showed
377 that mesophilic increase of 2 log units in untreated R1 smoothie after 28 days at 5 ºC
378 was doubled when that untreated R1 smoothie was prepared without broccoli (data not
379 shown).
380 Attending to mesophilic counts of treated smoothies stored at 20 ºC, a typical microbial
381 growth curve was observed. Accordingly, lag (0-3rd day), exponential (3rd-14th day;
382 increases of 2-3 log units regarding initial levels), stationary (14-28th day) and decline
383 phases were observed. The absence of lag phase in R1 smoothie could be an artefact
384 since this phase can be shorter than 3 days at this high storage temperature but could be
385 extended due to the initial antimicrobial effect achieved with the oregano used in the
386 formulation of R2 smoothie. As expected, the reduction of storage temperature to 5 ºC
387 extended the exponential phase until approximately day 21th, with lower counts
388 increments (approximately 1 log unit) compared to those treated samples stored at 20
389 ºC.
390 Psychrotrophes showed a similar behaviour to mesophiles. However, increments of
391 psychrophiles were higher regarding mesophiles increases with approximately 3-4 log
392 unit psychrophiles increases for CTRL and treated samples stored either at 5 or 20 ºC
393 for 28 days. Psychotropic count changes of treated smoothies from day 28 to the end of
394 their shelf-life were below 1 log unit.
395 *Enterobacteria* counts of treated and CTRL samples increased progressively during
396 storage achieving approximately 1 log unit increases after 28 days at 5 ºC. However,
397 treated smoothies stored at 20 ºC registered *Enterobacteria* increments 2-fold higher
398 than those samples stored at 5 ºC after 28-35 days. After that maximum *Enterobacteria*

399 counts, those levels started to decrease until the end of their shelf-life reaching, in
400 general, similar levels to their respective initial counts.
401 Conclusively, thermal treatments of smoothies reduced 2-3 log units their initial
402 microbial loads being microbial growth rates of such treated samples better controlled
403 during storage at 5 ºC up to 58 days regarding samples stored at 20 ºC. Microbial loads
404 of treated smoothies were below 7 log CFU g^{-1} at the end of their shelf-life.
405
406 **3.7. Vitamin C**
407 Total vitamin C content, expressed as the sum of AA and DHA, of CTRL-R1 and
408 CTRL-R2 smoothies was 216 and 229 mg kg^{-1} fw, respectively (Figure 2). A smoothie
409 portion of 250 g provides approximately 130 % of the RNIs for vitamin C for adults and
410 80 % for lactating women which is the population group with the highest vitamin C
411 RNIs (WHO, 2004). Vitamin C content of red pepper is approximately 11-fold higher
412 than tomato (Vanderslice, Higgs, Hayes, & Block, 1990). Accordingly, the higher red
413 pepper content (21 %) of R2 smoothie compared to R1 (12 %) was more relevant than
414 the tomato concentrations of 56 and 75 %, respectively. DHA content of untreated R1
415 and R2 smoothies accounted the 14 and 20 % of the total vitamin C content,
416 respectively. Similarly, it has been reported that DHA of fresh tomatoes and red peppers
417 accounted the 3 and 22 % of total vitamin C, respectively, although these proportions
418 may differ depending of the variety (Lee & Kader, 2000). Thermal treatment
419 significantly degraded vitamin C of R1/R2 smoothies by 27/50 %. However, a 250 g
420 portion of thermally-treated R1/R2 smoothie still provides approximately 100/71 % of
421 the RNIs for vitamin C for adults and 56/41 % for lactating women (WHO, 2004).
422 Similarly, Benlloch-Tinoco, Igual, Salvador, Rodrigo and Martínez-Navarrete (2014)
423 reported 27 % vitamin C degradation in kiwifruit purée after thermal processing at 84

ºC for 5 min. AA content is easily oxidized during thermal treatments to DHA (Lee & Kader, 2000). Accordingly, AA contents of R1/R2 smoothies decreased by 51/72 % after thermal treatment with DHA increments of 70/40 %.

Storage of fresh fruits and vegetables implies AA oxidation to DHA being considered ascorbic acid oxidase (AAO) as the major enzyme responsible of this oxidation process (Lee & Kader, 2000). AAO of crushed broccoli florets was almost inactivated after thermal treatment at 65 ºC for 8 min (Munyaka, Oey, Van Loey, & Hendrickx, 2010). Accordingly, a great AA decrease/DHA increment of approximately 67/275 and 71/180 % was observed in CTRL-R1 and CTRL-R2 smoothies, respectively, after 3 days at 5 ºC. That behaviour was not observed in treated smoothies. Total vitamin C degradation rates were greatly reduced after 14-21 days. As expected, AA and DHA degradations were better controlled at lower storage temperature. Accordingly, while AA/DHA degradation of 75/42 % were observed in treated samples stored at 5 ºC after 21 days, treated samples stored at 20 ºC showed similar reductions earlier (14 days). At the end of shelf-life, total vitamin C contents of R1/R2 smoothies accounted approximately 14/17 % of their respective initial levels.

3.8. Total carotenoids and lycopene contents

The initial total carotenoids content of CTRL-R1 and CTRL-R2 smoothies was 52.5 and 65.2 mg kg^{-1} fw, respectively (Table 5). Lycopene accounted 53 and 74 % of the total carotenoids contents of R1 and R2 smoothies, respectively (Table 5). Since lycopene is the main carotenoid of tomatoes (Martínez-Hernández et al., 2015b), the high tomato content of smoothies may explain the high lycopene proportion. Carotenes are sensitive to heat, among other factors such as light, oxygen, and pH, and might be lost during thermal processing due to isomerization and oxidative degradation. However, lycopene

449 is likely to remain in a crystalline form during thermal processing of tomato and it is
450 therefore relatively stable (Martínez-Hernández et al., 2015b). Accordingly, thermal
451 treatment of smoothies did not significantly affect their total carotenoids or lycopene
452 contents. Similarly, lycopene content of tomato flesh was not changed after blanching at
453 85 ºC for 4 min (Urbonaviciene, Viskelis, Viskelis, Jankauskiene , & Bobinas, 2012).
454 The total carotenoids content of CTRL smoothies was quite stable during storage
455 registering maximum reductions of up to 13-16 % after 21 days keeping these levels
456 until the end of its shelf-life. A great total carotenoids decrease of 30-40 % was
457 registered in treated smoothies after 14-21 days at both storage temperatures. However,
458 total carotenoids content of treated smoothies was well maintained from days 14-21
459 registering even a slight and progressive total carotenoids increment until the end of
460 storage. Hence, treated smoothies registered 10-20 % lower total carotenoids content
461 after 58 days at 5 ºC and 40 days at 20 ºC, respectively. Since lycopene mainly
462 contributed to total carotenoids content, the lycopene behaviour during storage of
463 smoothies was similar to that of total carotenoids. Consequently, a heat treatment of the
464 smoothies just after blended greatly extended their shelf-life registering final total
465 carotenoids levels similar to those of CTRL samples independently (p<0.05) of the
466 storage temperature.
467
468 **3.9. Total chlorophylls**
469 The initial total chlorophylls content of CTRL-R1 and CTRL-R2 smoothies was 26.8
470 and 27.4 mg kg^{-1} fw, respectively (Table 6). Since smoothies contained approximately
471 12 % of broccoli, chlorophylls content are in accordance to those previously reported by
472 Fernández-León et al. (2013) in fresh-cut broccoli (Cv. Parthenon). Chlorophyll a and b

equally (50 %) accounted to the total chlorophylls content. The thermal treatment did not significantly affect the chlorophylls content of the smoothies.

No great chlorophylls changes were observed throughout the storage. Chlorophylls are highly susceptible to much enzymatic or non-enzymatic degradation during processing and storage. Pheideaoxygenase (PaO) pathway is the chlorophyll degradation pathway, which involves the following enzymes: chlorophyllase, Mg-dechelatase and peroxidase. According to data from Holden (1961), the low pH of our smoothies (4.35-4.40) inactivated chlorophyllase, which is responsible of the first step in PaO pathway. However, spinach purée with pH of 5.89 registered chlorophyll degradation up to approximately 25 % after 43 days at 4 ºC (Wang et al., 2013).

4. Conclusions

Two red fresh vegetables smoothies rich in health-promoting compounds were developed. The shelf-life, according to sensory and microbiological quality, of fresh blended (CTRL) smoothies was established in 28 days at 5 ºC. A mild thermal treatment of 3 min at 80 ºC after blended extended their shelf-life to 40 days at 20º C maintaining their health-promoting properties related to lycopene, total carotenoids and chlorophylls with no great changes in other quality parameters (total soluble solids content and pH). However, when the storage temperature of thermally-treated smoothies was at 5 ºC an extended shelf-life up to 58 days with better colour and vitamin C content retention. A 250-g portion of these smoothies can highly cover the established recommended daily nutrient intakes for dietary fibre, minerals and vitamin C of different population groups.

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655

FIGURE AND TABLE CAPTIONS

Table 1. Composition of red fresh vegetables smoothies (R1 and R2).

Table 2. Total dietary fibre and moisture content of red fresh vegetables smoothies (R1 and R2).

Table 3. Mineral content of red fresh vegetables smoothies (R1 and R2) (n=5±SD).

Table 4. pH, soluble solids content, titratable acidity, and total colour differences of untreated (CTRL) and heat-treated (HT) red fresh vegetables smoothies R1 (A) and R2 (B) stored at 5 and 20 ºC (n=5±SD). Different capital letters denote significant differences ($P \leq 0.05$) 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 5. Total carotenoids and lycopene content of untreated (CTRL) and heat-treated (HT) red fresh vegetables smoothies R1 (A) and R2 (B) stored at 5 and 20 ºC (n=5±SD). Different capital letters denote significant differences ($P \leq 0.05$) 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 6. Total chlorophylls content of untreated (CTRL) and heat-treated (HT) red fresh vegetables smoothies R1 (A) and R2 (B) stored at 5 and 20 ºC (n=5±SD). Different capital letters denote significant differences ($P \leq 0.05$) among treatments for

680 the same sampling day. Different lowercase letters denote significant differences
681 ($P \leq 0.05$) among sampling days for the same treatment.
682
683 **Figure 1.** Mesophilic (A), psychrophilic (B), *Enterobacteria* (C) and yeast and moulds
684 (D) counts (log CFU g^{-1}) of untreated (CTRL; first column) and heat-treated (HT;
685 second and third columns) red fresh vegetables smoothies R1 (first file) and R2 (second
686 file) stored at 5 and 20 ºC (n=5±SD). Different capital letters denote significant
687 differences ($P \leq 0.05$) among treatments for the same sampling day. Different lowercase
688 letters denote significant differences ($P \leq 0.05$) among sampling days for the same
689 treatment.
690
691 **Figure 2.** Total vitamin C (ascorbic acid and dehydroascorbic acid) of untreated
692 (CTRL; first column) and heat-treated (HT; second and third columns) red fresh
693 vegetables smoothies R1 (first file) and R2 (second file) stored at 5 and 20 ºC
694 (n=5±SD). Different capital letters denote significant differences ($P \leq 0.05$) among
695 treatments for the same sampling day. Different lowercase letters denote significant
696 differences ($P \leq 0.05$) among sampling days for the same treatment.
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SUPPLEMENTARY MATERIAL

Supplementary material 1. Evolution of the storage and loss moduli with frequency (A) and viscous flow curves at 25 ºC of R1 and R2 smoothies.

Supplementary material 2. TG and DTG curves of R1 (A) and R2 (B) smoothies.