



# Preservation of bioactive compounds of a green vegetable smoothie using short time–high temperature mild thermal treatment

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## Abstract

Smoothies represent an excellent and convenient alternative to promote the daily consumption of fruit and vegetables in order to obtain their health-promoting benefits. Accordingly, a green fresh vegetables smoothie (77.2% cucumber, 12% broccoli and 6% spinach) rich in health-promoting compounds was developed. Soluble solids content, pH and titratable acidity of the smoothie were  $4.3 \pm 0.4$  Bx,  $4.49 \pm 0.01$  and  $0.22 \pm 0.02$  mg citric acid  $100^{-1}$  g fw, respectively. Two thermal treatments to reduce microbial loads and preserve quality were assayed: T1 (3 min at 80 °C) and T2 (45 s at 90 °C). Fresh blended unheated samples were used as control (CTRL). The smoothie presented a viscoelastic behaviour. T1 and T2 treatments reduced initial microbial loads by 1.3–2.4 and 1.4–3.1 log units, respectively. Samples were stored in darkness at 5 and 15 °C. Colour and physicochemical changes were reduced in thermal-treated samples throughout storage, which were better preserved at 5 °C rather than at 15 °C. Vitamin C changes during storage were fitted with a Weibullian distribution. Total vitamin C losses of T1 and T2 samples during storage at 15 °C were greatly reduced when they were stored at 5 °C. Initial total phenolic content ( $151.1 \pm 4.04$  mg  $kg^{-1}$  fw) was 44 and 36% increased after T1 and T2 treatments, respectively. The 3-*p*-coumaroyl quinic and chlorogenic acids accounted the 84.7 and 7.1% relative abundance, respectively. Total antioxidant capacity ( $234.2 \pm 20.3$  mg Trolox equivalent  $kg^{-1}$  fw) remained constant after the thermal treatments and was better maintained during storage in thermal-treated samples. Glucobrassicin accounted the 81% of the initial total glucosinolates content ( $117.8 \pm 22.2$  mg  $kg^{-1}$  fw) of the smoothie. No glucosinolates losses were observed after T2 treatment being better preserved in thermal-treated samples. Conclusively, a short time–high temperature mild thermal treatment (T2) showed better quality and bioactive compounds retention in a green fresh vegetable smoothie during low temperature storage.

## Keywords

Phenolic compounds, glucosinolates, vitamin C, antioxidants, quality, beverages

Date received: 29 March 2016; accepted: 31 March 2016

## INTRODUCTION

Clinical and epidemiological research indicates that at least 80% of current chronic diseases and premature deaths are preventable with changes in diet and consumer lifestyle (Anand et al., 2008). Fruit and

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vegetables have a high content of phytochemicals responsible of preventative effects on cardiovascular diseases, cancers, hypertension and other chronic conditions such as diabetes and obesity derived from their phytochemicals (Boeing et al., 2012). However, fruits and vegetables consumption consistently is below the 400 g of fruits and vegetables daily intake which has been worldwide promoted by several programmes such as '5 A Day' (WHO/FAO, 2003). Smoothies represent an excellent and convenient alternative to promote the daily consumption of fruit and vegetables. Smoothies are non-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, lemonade or tea. They have a milk shake-like consistency that is thicker than slush drinks (Rodríguez-Verástegui et al., 2015). Recent research has shown that daily consumption of green smoothies may enhance health quality of consumers (Maeda, 2013). The main issue of the smoothie processing is the limited shelf-life of these products since they are susceptible to spoilage (Buzrul et al., 2008) and quality degradation. For that reason, mild thermal treatments must be used during processing in order to increase the shelf-life while keeping quality (Di Cagno et al., 2011). Furthermore, storage at low temperature up to 5 °C is recommended. However, the treatment should not be much aggressive to preserve its nutritional and sensory quality. Thermal treatment (generally in the range of 80–95 °C) is applied for the inactivation of spoilage enzymes in smoothies, fruit purées and juices (Barba et al., 2012). However, thermal treatments may reduce the phytochemical content of smoothies, such as antioxidants among others, in detriment of related health-promoting properties. Studies about the effects of thermal processing and subsequent storage on bioactive compounds and quality changes of fresh vegetable smoothies are very scarce and no previous data for green smoothies are so far reported. For that reason, the aim of this work was to study the effect of two different mild conventional heat treatments on quality changes, as well as on selected bioactive compounds of a green fresh vegetable smoothie throughout storage at 5 and 15 °C.

## MATERIALS AND METHODS

### Plant material and smoothie preparation

Fresh vegetables, purchased at a local supermarket, were sanitised with 75 mg l<sup>-1</sup> NaClO during 2 min and then rinsed with cold tap water for 1 min. Cucumbers were peeled and all vegetables were then cut and blended (MX2050 blender, Braun, Germany). The green smoothie composition was 77.2% cucumber,

12% broccoli and 6% spinach. The nutritional composition of the smoothie was determined with the software DIAL 1.0 (Ortega-Anta et al., 2008) and it is presented as Supplementary Material 1. Citric acid (4.8%) was added in order to decrease the pH below 4.5 and reduce microbial growth of the smoothie during subsequent storage.

### Thermal treatments and storage conditions

Thermal treatments were applied by using a Mastia thermoresistometer described by Conesa et al. (2009). Immediately after blending, the sterilised vessel of the thermoresistometer was filled with 400 ml of the smoothie. For treatment T1, the thermoresistometer was programmed to increase the initial smoothie temperature ( $8 \pm 2$  °C) with a heating rate of 30 °C/min to 80 °C, then maintained for 3 min and cooled down to a final temperature of 40 °C (heating rate of 30 °C/min). For treatment T2, the thermoresistometer was programmed to increase the initial smoothie temperature with a heating rate of 30 °C/min to 90 °C, then maintained for 45 s and cooled down to a final temperature of 40 °C (heating rate of 30 °C/min). After thermal treatments, the smoothie temperature was cooled down to 4 °C submerging the vessel in an ice water bath while continuously agitation was programmed in the thermoresistometer. Samples were stored in darkness at 5 and 15 °C up to 49 days depending on storage temperature. Fresh blended unheated samples were used as control (CTRL). Five replicates per treatment and sampling day, for each storage temperature, were prepared.

### Rheological properties of smoothies

Rheological measurements were executed using an 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) according to Castillejo et al. (2016). Briefly, oscillatory tests were performed within the linear viscoelastic region for the samples previously equilibrated (25 °C for 1 min) between the plates. Storage modulus ( $G'$ ) and loss modulus ( $G''$ ) were determined in a frequency range of 100–0.2 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 0.01–10%. Flow tests were also used to cover shear rate range between 10<sup>-2</sup> /s and 10<sup>2</sup> /s. All experiments were carried out at 25 °C. Rheological data are presented as Supplementary Material 2. Three repetitions of the dynamic-mechanical experiments were performed for each smoothie sample.

### Total dietary fibre (DF) 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) as previously described (Castillejo et al., 2016). Briefly, derivative thermogravimetric curves of approximately 10 mg of sample powder (dried at 105 °C for 24 h) were analysed by derivative weight loss (see Supplementary Material 3). The temperature for the maximal weight loss ( $T_{\max}$ ) at 90 °C is attributed to the free water loss. The decomposition peaks at the  $T_{\max}$  of 190, 270 and 321 °C are assigned to pectin, hemicelluloses and cellulose, respectively (Castillejo et al., 2016). The weight percentage of each component in the analysed samples was obtained as the mass loss produced during volatilisation.

The mineral content of the samples was analysed by X-ray fluorescence (XRF) according to Martínez-Hernández et al. (2015). For the XRF analyses a spectrometer S4 Pioneer (Bruker Corporation, Billerica, MA, USA) was used, equipped with a Rh anticathode X-ray tube (20–60 kV, 5–150 mA and 4 kW maximum), five analyser crystals (LiF200, LiF220, Ge, PET and XS-55), sealed proportional counter for light elements detection and a scintillation counter for heavy elements with slight modifications. The recorded spectrum was evaluated by the fundamental parameters method using the Spectra plus software EVA 1.7. Mineral content was expressed as g kg<sup>-1</sup> dry weight (dw) and mg kg<sup>-1</sup> dw for major minerals and trace elements, respectively. Each of the five replicates was analysed by duplicate.

### Informal sensory evaluation

An informal sensory evaluation was conducted by an untrained panel of eight assessors to address if differences in visual appearance, flavour, texture, off-colours, off-odours, lumpiness, turbidity, precipitation/phase separation and overall quality were noticeable among the three sample types. Accordingly, shelf-life of the samples was established based on overall quality scores.

### Microbial analysis

Mesophilic, psychrophilic, *Enterobacteriaceae*, and yeast and mould growth were determined using standard enumeration methods according to Castillejo et al. (2016). All microbial counts were reported as log colony forming units per gram of product (log CFU g<sup>-1</sup>). Each of the five 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).

### Physiochemical analyses

The pH, titratable acidity (TA) and total soluble solids content (SSC) of smoothies were determined according to Castillejo et al. (2016). Briefly, 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 °C and expressed as °Bx. TA was determined by the titration of 5 ml of juice plus 45 ml of distilled water with 0.1 mol l<sup>-1</sup> NaOH to pH 8.1 (T50, Mettler Toledo, Milan, Italy) and expressed as g citric acid 100 ml<sup>-1</sup>. Three replicates per treatment were analysed.

Colour was determined using a colorimeter (Minolta CR-300 Series, Japan) calibrated with a white reference plate (light source C), 2° observer and 8 mm viewing aperture. Samples were introduced in a special glass tube mounted on a device connected to the colorimeter. Measurements were recorded using the standard tristimulus parameters ( $L^*$ ,  $a^*$ ,  $b^*$ ) of the CIE Lab system on three equidistant points of each replicate. Three colour readings were taken turning the tube at every read and all three measurements were automatically averaged by the device and recorded. Total colour differences ( $\Delta E$ ) throughout storage compared to their respective initial values were calculated according to equations previously described (Walkling-Ribeiro et al., 2010).

### Chlorophylls content

Chlorophyll content was determined according to Martínez-Hernández et al. (2011). Briefly, 0.5 g ground frozen (-80 °C) sample was mixed with 9 ml of hexane and 15 ml of a mixture of methanol/acetone (1:2, v/v). The extraction was carried out for 4 h (darkness in ice bed shaken continuously at 200 × G). After extraction, 25 ml of a solution 1 M NaCl was added, samples were shaken again in a vortex (Heidolph, Reax Control, Kelheim, Germany) and the upper of the three layers formed was used as chlorophyll extract. An aliquot of 1 ml was placed into a quartz cuvette (Hellma GmbH & Co., Müllheim, Germany) and the absorbance (A) at 662 and 644 nm was measured using a UV-visible spectrophotometer (Hewlett Packard, model 8453, Columbia, USA). The equation  $Ch = [10.05 \times A_{662} - 0.766 \times A_{644}] + [16.37 \times A_{644} - 3.14 \times A_{662}]$  developed by Wellburn (1994) was used to determine the total chlorophyll content (Ch) which was expressed as mg kg<sup>-1</sup> fw. Each of the three replicates was analysed by triplicate.

### Bioactive compounds

**Vitamin C.** The ascorbic (AA) and dehydroascorbic (DHA) acids were measured based on the method of Zapata and Dufour (1992) with instruments and

methodology described by Martínez-Hernández et al. (2013). Briefly, 5 g ground frozen ( $-80^{\circ}\text{C}$ ) sample was placed into a 25 ml Falcon tube and 10 ml of cold ( $4^{\circ}\text{C}$ ) buffer (0.1 M citric acid, 0.05% EDTA, 4 mM sodium fluoride and 5% MeOH) were added. The mixture was homogenised (Ultraturrax T25 basic, IKA, Berlin, Germany) for 10 s, filtered (four-layer cheesecloth) and pH adjusted (6 N NaOH) to 2.35–2.4. Subsequently, 750  $\mu\text{l}$  filtered (0.45  $\mu\text{m}$  polyether sulphone filter) purified extract (Sep-Pak cartridges C18, Waters, Dublin, Ireland) was derivatised with 250  $\mu\text{l}$  of 7.7 M 1,2-phenylenediamine for 37 min in darkness at room temperature. Immediately after derivatisation, 20  $\mu\text{l}$  were injected on a Gemini NX (250 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ ) 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. 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  $\text{mg kg}^{-1}$  fw. Each of the five replicates was analysed by triplicate.

**Simultaneous analysis of phenolic compounds and intact glucosinolates.** A smoothie sample of 9 g was homogenised (Ultra-turrax T-25, Ika-Labortechnik, Staufen, Germany) in 7 ml 70% MeOH under an ice water bath to avoid enzymatic activations. Immediately, samples were heated at  $70^{\circ}\text{C}$  for 15 min in a water bath under continuous agitation to inactivate myrosinase. Then, the samples were centrifuged (13,000  $\times$  g, 10 min,  $4^{\circ}\text{C}$ ). The supernatants were collected, filtered through 0.20  $\mu\text{m}$  syringe PTFE filters and used as intact glucosinolates/phenolic extracts.

Analysis and identification of phenolic and intact glucosinolates were conducted according to Fernández-León et al. (2013). An ultra high-performance liquid chromatography instrument (Shimadzu, Kyoto, Japan) equipped with a DGU-20A degasser, LC-30AD quaternary pump, SIL-30AC autosampler, CTO-10AS column heater and SPDM-20A photodiode array detector was used. Chromatographic analyses were carried out onto a Kinetex C18 column (100 mm  $\times$  4.6 mm, 2.6  $\mu\text{m}$  particle size; Phenomenex, Macclesfield, UK). Phenolic acids were quantified as equivalents of chlorogenic acid (5-caffeoylquinic acid; Sigma, St Louis, MO, USA) and sinapic acid (Sigma, St Louis, MO, USA). Glucosinolates were quantified as sinigrin equivalents. The results were expressed as  $\text{mg kg}^{-1}$  fw. Each of the five replicates was analysed by duplicate.

LC/UV-PAD/ESI-MSn analyses were carried out in an Agilent HPLC 1100 series equipped with a photodiode array detector and mass detector in series (Agilent Technologies, Waldbronn, Germany).

**Total antioxidant capacity (TAC).** TAC extraction and analysis were conducted using the same instruments and methodology described by Rodríguez-Verástegui et al. (2015) using 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). All TAC data were expressed as  $\text{mg}$  of Trolox equivalents  $\text{kg}^{-1}$  fw. Each of the five replicates was analysed by duplicate.

### Statistical analysis

The experiment was a two-factor (treatment  $\times$  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.

## RESULTS AND DISCUSSION

### Rheological properties

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). The rheological properties of the smoothie are presented as Supplementary Material 2.  $G'$  of smoothies was greater than  $G''$  at any given point in the frequency sweep tests (see Supplementary Material 2). This fact indicates a dominant contribution of the elastic component to the viscoelasticity of the smoothie, behaviour typical for a viscoelastic solid. This means that the attractive forces become dominant due to the strong hydrogen bond and hydrophobic association (Basu et al., 2011). The  $\tan\delta$  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. The  $\tan\delta$  of samples was lower than 1 thus indicating a gel-like behaviour. Apparent viscosity of the green smoothie was not greatly changed after thermal treatment as observed in Supplementary Material 2. The effective shear rate range in the mouth is 40–50  $\text{s}^{-1}$ , which would have implied actual sensory consistency (Wood and Goff, 1973). The viscosity of T1 samples was slightly higher than T2 samples within the shear rate range 40–50  $\text{s}^{-1}$ .

### Total DF and mineral content

The total DF content of the smoothie was  $4.4 \pm 0.2\%$  wet basis (wb) (data not shown). Pectin and hemicellulose contents of the smoothie were  $0.8 \pm 0.1$  and  $1.9 \pm 0.3\%$  wb, respectively. The smoothie accounted  $1.8 \pm 0.3\%$  wb of cellulose (data not shown). According to The Code of Federal Regulations (FDA, 2015), food products which contain 20% or more of the recommended daily nutrient intakes (RNIs) for fibre ( $25 \text{ g day}^{-1}$ ) are considered as an 'excellent source of fibre'. Accordingly, this green smoothie can be considered as an 'excellent source of fibre' since a portion of 250 g provides approximately 50% of the RNIs for fibre.

The P, S, Na, K, Ca, Mg, Cl, Al and Si contents of the smoothie were  $0.75 \pm 0.01$ ,  $0.48 \pm 0.00$ ,  $0.47 \pm 0.01$ ,  $4.24 \pm 0.00$ ,  $0.41 \pm 0.00$ ,  $0.30 \pm 0.00$ ,  $0.48 \pm 0.00$ ,  $0.01 \pm 0.00$  and  $0.04 \pm 0.00 \text{ g kg}^{-1}$  fw, respectively (data not shown). The Fe, Mn and Zn contents of the smoothie were  $8.72 \pm 0.27$ ,  $3.11 \pm 0.24$  and  $5.93 \pm 0.13 \text{ mg kg}^{-1}$  fw, respectively (data not shown). A 250 g portion of this smoothie provides 29–34, 9–15, 7–12 and 15–21% of the RNIs for Mg, Ca, Fe and Zn, respectively, covering population groups with special nutritional requirements such as elders, pregnant women or adolescents (FAO/WHO, 2004).

### Informal sensory evaluation

Visual appearance, flavour, texture, off-colours, off-odours, lumpiness, turbidity, precipitation/phase separation and overall quality of CTRL smoothie were reported to be over the limit of acceptability up to 21 days at  $5^\circ\text{C}$  by an informal sensory panel test of eight assessors (data not shown). Thermally treated smoothies maintained their sensory acceptance up to 49 days at  $5^\circ\text{C}$ . Inappropriate storage at  $15^\circ\text{C}$  reduced sensory acceptance to seven days. Accordingly, the shelf-lives of the smoothies were established based on those sensory analyses.

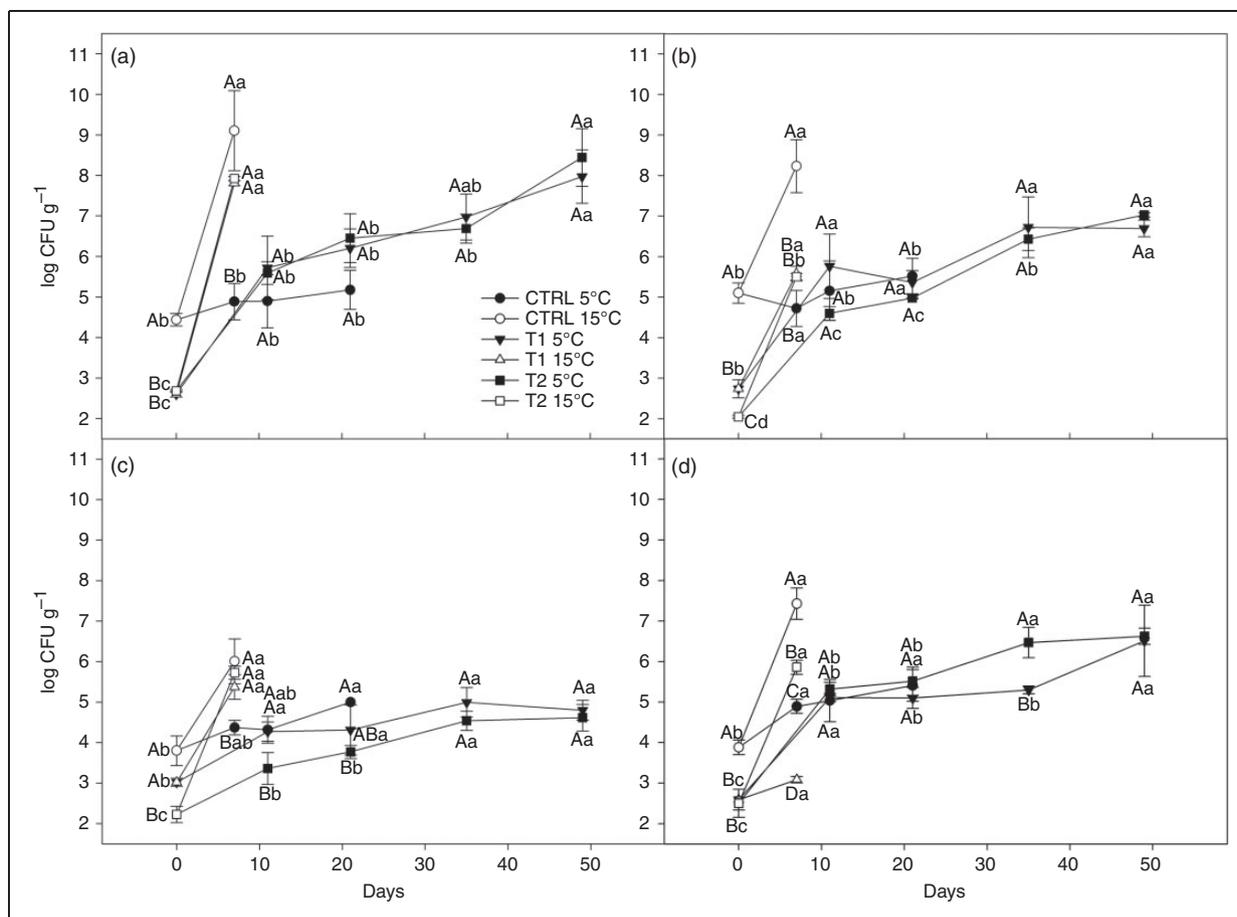
### Microbial analysis

The mesophilic load of CTRL smoothie on processing day was  $4.4 \pm 0.2 \text{ log CFU g}^{-1}$  (Figure 1(a)). Thermal treatments reduced mesophilic load by 1.7–1.8 log units without differences among them. Zhao et al. (2014) reported 2 log units mesophilic reductions in cucumber juice heat treated at  $85^\circ\text{C}$  for 15 s. The similar microbial reduction in the juice with shorter treatment time may be owed to the fibres and other particles contained in the smoothie which may difficult the heat transmission contrary to the juice. Great mesophilic increases of 4.7 and 5.2 log units were observed in CTRL and thermal-treated smoothies after seven days at  $15^\circ\text{C}$ , respectively.

However, storage at  $5^\circ\text{C}$  greatly reduced mesophilic growth of CTRL and heat-treated samples compared to those stored at  $15^\circ\text{C}$  since increments of 3.6–3.8 and 0.7 log units were registered after 21 days at  $5^\circ\text{C}$ . Heat-treated samples stored at  $5^\circ\text{C}$  showed a great mesophilic growth (2.9–3.1 log units) in the first 11 days remaining those loads almost unchanged ( $<0.4$  log units changes) until day 35. However, those microbial loads of T1 and T2 samples were increased by 1.4 and 1.9 log units, respectively, from day 39 to day 49. The higher mesophilic growth of heat-treated samples could be explained by different hypotheses: (1) the vegetative or spore cells which resisted to the thermal treatment, due to their higher thermal resistance and/or the protecting effects of the smoothie matrix, could grow better due to the lower microbial competence for the nutrients. (2) The used heat treatment completely inactivated the initial myrosinase activity ( $163.0 \text{ nmole sinigrin transformed per g fw of sample}$ ; data not shown), which is responsible for the glucosinolates conversion to isothiocyanates. Isothiocyanates from broccoli have shown high antimicrobial activities contrary to glucosinolates (Vig et al., 2009). Accordingly, the glucosinolate–isothiocyanate conversion was possible in untreated unheated samples, contrary to heat-treated samples, with the observed preserving benefits from the isothiocyanates throughout storage of smoothies. Therefore, our previous preliminary non-published data showed that mesophilic increase of 2 log units in untreated smoothie after 28 days at  $5^\circ\text{C}$  was doubled when that untreated smoothie was prepared without broccoli (data not shown).

Initial psychrophilic count of CTRL smoothie was  $5.1 \pm 0.3 \text{ log CFU g}^{-1}$  (Figure 1(b)). Psychrophilic counts were reduced by 2.4 and 3.1 log units after T1 and T2 treatments, respectively. During storage at  $15^\circ\text{C}$ , psychrophilic counts of CTRL, T1 and T2 samples augmented by 3.1, 2.8 and 3.5 log units after seven days, respectively. However, and similarly to mesophilic data, although heat-treated samples showed great increases of 2.6–3.0 log units after 11 days at  $5^\circ\text{C}$  loads changes were lower than 2.4 log units in the last 38 days of storage. In contrast, CTRL samples did not significantly change after 21 days at  $5^\circ\text{C}$ . CTRL and heat-treated samples registered psychrophilic counts of  $5.5 \pm 0.4$  and  $6.7 \pm 0.8$ – $7.0 \pm 0.1 \text{ log CFU g}^{-1}$  after 21 and 49 days at  $5^\circ\text{C}$ , respectively.

Initial *Enterobacteriaceae* counts of  $3.8 \pm 0.4 \text{ log CFU g}^{-1}$  were only significantly reduced after T2 treatment by 1.6 log units (Figure 1(c)). The *Enterobacteriaceae* levels of CTRL and T1 samples stored at  $15^\circ\text{C}$  increased by 2.2–2.3 after seven days while T2 samples increased in a greater extent with 3.5 log units increments after seven days. CTRL and thermal-treated samples registered *Enterobacteriaceae*



**Figure 1.** Mesophilic (a), psychrophilic (b), *Enterobacteriaceae* (c) and yeast and moulds (d) counts ( $\log \text{CFU g}^{-1}$ ) of untreated (CTRL) and heat-treated (T1 and T2) green vegetables smoothies stored at 5 and 15°C ( $n=5 \pm \text{SD}$ ).

counts of  $5.0 \pm 0.4$  and  $4.6 \pm 0.3$ – $4.8 \pm 0.3$   $\log \text{CFU g}^{-1}$  after 21 and 49 days of at 5°C, respectively.

Yeasts and moulds counts of CTRL smoothie of  $3.9 \pm 0.2$   $\log \text{CFU g}^{-1}$  were reduced by 1.3–1.4 log units after heat treatments without significant differences among them (Figure 1(d)). Similar to mesophiles, dynamic heating system during a milder heat treatment (70°C for 15s) induced greater yeasts and moulds reductions (3.7 log units) in a fruit smoothie (Walkling-Ribero et al., 2010). Furthermore, data from Zhao et al. (2014) showed better heat transmission in cucumber juice compared to our smoothie since initial yeasts and moulds (4  $\log \text{CFU g}^{-1}$ ) were greatly reduced below the detection limit (1  $\log \text{CFU g}^{-1}$ ). Yeasts and moulds counts of CTRL and T2 samples were incremented by 3.6 and 3.4 log units, respectively, after seven days at 15°C while T1 samples only increased in 0.5 log units. Similar to mesophilic and psychrophilic, yeasts and moulds counts greatly augmented by 2.5 and 2.8 log units after 11 days followed by a 1.3–1.4 log units increment in the last 38 days of storage at 5°C. CTRL and thermal-treated samples

showed final yeasts and moulds counts of  $5.4 \pm 0.4$  and  $6.5 \pm 0.9$ – $6.6 \pm 0.2$   $\log \text{CFU g}^{-1}$  after 21 and 49 days at 5°C, respectively.

Conclusively, thermal treatments, with better initial microbial reductions achieved by T2, combined with low temperature storage, kept microbial loads below 6 log units after 39 days at 5°C. Although CTRL samples showed a similar microbial behaviour to heat-treated samples during low temperature storage, thermal treatment is needed to inactivate quality-degradation enzymes, as recently reported in vegetable smoothies (Rodríguez-Verástegui et al., 2015), in order to reduce colour changes of smoothies during storage as shown later.

### SSC, pH and TA

CTRL smoothie showed an initial SSC of  $4.3 \pm 0.4^\circ \text{Bx}$  (Table 1). Di Cagno et al. (2011) reported a higher SSC of  $10.8^\circ \text{Bx}$  in a green smoothie due to the high fruit content (40% kiwifruits, 7% fennels, 8% spinach and 15% papaya). Thermal treatment did not induce SSC

**Table 1.** Soluble solids content (SSC), pH, titratable acidity (TA), total colour differences ( $\Delta E$ ) and total chlorophylls content of untreated (CTRL) and heat-treated (T1 and T2) green vegetables smoothies stored at 5 and 15 °C ( $n=5 \pm SD$ )

Treatment	T <sup>a</sup> (°C)	Day	SSC (°Bx)	pH	TA (g citric acid 100 ml <sup>-1</sup> )	$\Delta E$	Total chlorophylls (mg kg <sup>-1</sup> fw)	
CTRL	–	0	4.30 ± 0.35Aa	4.49 ± 0.01Ab	0.22 ± 0.02Ab	–	58.9 ± 1.7Aa	
T1	–	0	4.17 ± 0.15Aa	4.36 ± 0.03Cb	0.24 ± 0.01Ac	5.02 ± 0.02Ab	19.0 ± 0.5Bb	
T2	–	0	4.17 ± 0.12Aa	4.42 ± 0.03Ba	0.26 ± 0.00Ab	4.97 ± 0.09Ab	21.2 ± 3.4Ba	
CTRL	15	7	4.23 ± 0.13Ba	4.33 ± 0.09BCb	0.35 ± 0.01Ba	10.16 ± 0.38 – –	43.7 ± 0.8Ab	
		5	7	4.97 ± 0.12Aa	4.59 ± 0.06Aab	0.30 ± 0.01Ba	7.07 ± 1.29 – b	45.4 ± 1.1Ab
		5	11	4.40 ± 0.36Aa	4.48 ± 0.05Ab	0.27 ± 0.01Aab	8.63 ± 0.22 – ab	45.4 ± 1.9Ab
		5	21	4.57 ± 0.15Aa	4.62 ± 0.03Aa	0.29 ± 0.03Aa	10.73 ± 0.69 – a	27.8 ± 4.9Ac
T1	15	7	4.17 ± 0.15Ba	4.23 ± 0.04Cb	0.66 ± 0.07Aa	6.47 ± 0.35 – a	17.2 ± 0.7Bc	
		5	11	4.17 ± 0.12Aa	4.49 ± 0.01Aab	0.23 ± 0.02Ac	6.00 ± 0.19 – b	20.8 ± 1.3Bab
		5	21	4.30 ± 0.00Ba	4.59 ± 0.02ABa	0.26 ± 0.02Ac	7.50 ± 0.37 – a	26.7 ± 3.8Aa
		5	35	4.27 ± 0.12Aa	4.47 ± 0.09Aab	0.33 ± 0.03Ab	7.55 ± 0.93 – a	11.5 ± 3.2Bc
T2	15	5	49	3.27 ± 0.31Ab	4.45 ± 0.09Aab	0.62 ± 0.03Aa	7.66 ± 0.22 – a	15.5 ± 2.7Abc
		7	4.23 ± 0.15Ba	4.49 ± 0.10ABa	0.33 ± 0.03Bb	6.20 ± 0.17 – a	19.7 ± 1.5Ba	
		5	11	4.17 ± 0.42Aa	4.47 ± 0.02Aa	0.25 ± 0.02Ab	7.11 ± 0.57 – a	24.8 ± 1.8Ba
		5	21	4.30 ± 0.00Ba	4.49 ± 0.07Ba	0.29 ± 0.04Ab	..	16.2 ± 2.5Ba
T2	15	5	35	4.27 ± 0.25Aa	4.44 ± 0.05Aa	0.36 ± 0.13Aab	7.90 ± 0.70 – a	19.1 ± 2.4Aa
		5	49	3.50 ± 0.20Ab	4.47 ± 0.15Aa	0.47 ± 0.05Ba	7.46 ± 0.84 – a	22.3 ± 7.8Aa

Different capital letters denote significant differences ( $P \leq 0.05$ ) among treatments stored at the same temperature for the same sampling day. Different lowercase letters denote significant differences ( $P \leq 0.05$ ) among sampling days for the same treatment stored at the same temperature.

changes. SSC of all smoothies remained quite constant after seven days of storage at 15 °C. Contrary, CTRL smoothie showed a SSC increase of 0.7°Bx after seven days at 5 °C followed by a decrease. Accordingly, CTRL smoothie registered 4.6 ± 0.2°Bx after 21 days at 5 °C with no differences regarding its respective initial level. SSC levels of T1 and T2 smoothies remained stable up to 35 days of storage at 5 °C followed by a decrease of 0.9 and 0.7°Bx, respectively, from day 35 to day 49. The observed SSC decreases during storage may be owed to the sugars and other soluble solids used by microorganisms and enzymatic systems as substrates in several metabolic reactions.

The initial pH and TA of CTRL smoothie were 4.49 ± 0.01 and 0.22 ± 0.02 mg citric acid 100<sup>-1</sup> g fw, respectively (Table 1). Data from Di Cagno et al. (2011) showed a higher TA of 0.6 mg citric acid 100<sup>-1</sup> g fw in a green smoothie owed to the high kiwifruit content (40%) showing this fruit a high TA. Similar to SSC, pH and TA did not register significant differences after thermal treatments. The pH of CTRL and T1 samples was slightly reduced by 0.2 and 0.4 pH units, respectively, after seven days at 15 °C although pH of T2 samples remained stable. Correspondingly, TA of CTRL and T1 samples increased 0.1 and 0.4 mg citric acid 100<sup>-1</sup> g fw, respectively, after seven days at 15 °C while T2 did not register differences

during this period. No great pH and TA changes in CTRL samples were observed after 21 days at 5 °C. The pH of thermal-treated samples was constant throughout storage at both storage temperatures. Similarly, no pH changes were observed in untreated and heat treated (100 °C for 60 s) spinach puree after 43 days at 4 °C (Wang et al., 2013). Correspondingly, TA values of T1 and T2 did not change for 35 days at 5 °C followed by an increase from day 35 to day 49 registering values 0.38 and 0.20 mg citric acid 100<sup>-1</sup> g fw higher, respectively, compared to their respective initial levels. As previously observed, microbial growth may be greatly reduced by thermal treatment and subsequent low temperature storage. Microorganisms consume sugars and other soluble solids during growth producing metabolic acidic products. Accordingly, SSC and pH decreased, and TA increased during storage of the smoothie being these changes greatly reduced in thermal-treated samples and during cold storage.

#### Colour differences and total chlorophylls and carotenoids contents

Colour is one of the most important quality parameters of smoothies to evaluate its storage quality. Green colour of vegetables is mainly due to chlorophylls which may be degraded due to certain degrading

enzymes (chlorophyllase, Mg-dechelataase and peroxidase). The initial  $L^*$ ,  $a^*$  and  $b^*$  values of CTRL smoothie were  $41.9 \pm 1.9$ ,  $-14.0 \pm 0.7$  and  $22.7 \pm 0.1$ , respectively (data not shown). The initial total chlorophylls content of CTRL smoothie was  $58.9 \pm 1.7 \text{ mg kg}^{-1} \text{ fw}$  (Table 1) accounting chlorophyll a 82% of total content (data not shown). Meng et al. (2014) found approximately  $75 \text{ mg kg}^{-1} \text{ fw}$  total chlorophylls content in fresh-cut cucumber. The higher total chlorophylls content of our smoothie may be owed to the spinach contribution which has high chlorophylls content. Total chlorophyll content decreased by 64–68% after heat treatments without differences among them (Table 1). Accordingly, an initial  $\Delta E$  of  $4.97 \pm 0.09$ – $5.02 \pm 0.02$  was observed after thermal treatments without significant differences among them (Table 1). However, milder thermal treatment ( $72^\circ\text{C}$  for 15 s) reported lower  $\Delta E$  value (1.2) in fruit smoothie (Walkling-Ribeiro et al., 2010). Accordingly,  $a^*$  value, the most important index evaluating instrumental colour in green vegetables, was increased from  $-11.2$  to  $-6.5$  in spinach puree after heat treatment at  $100^\circ\text{C}$  for 60 s (Wang et al., 2013).

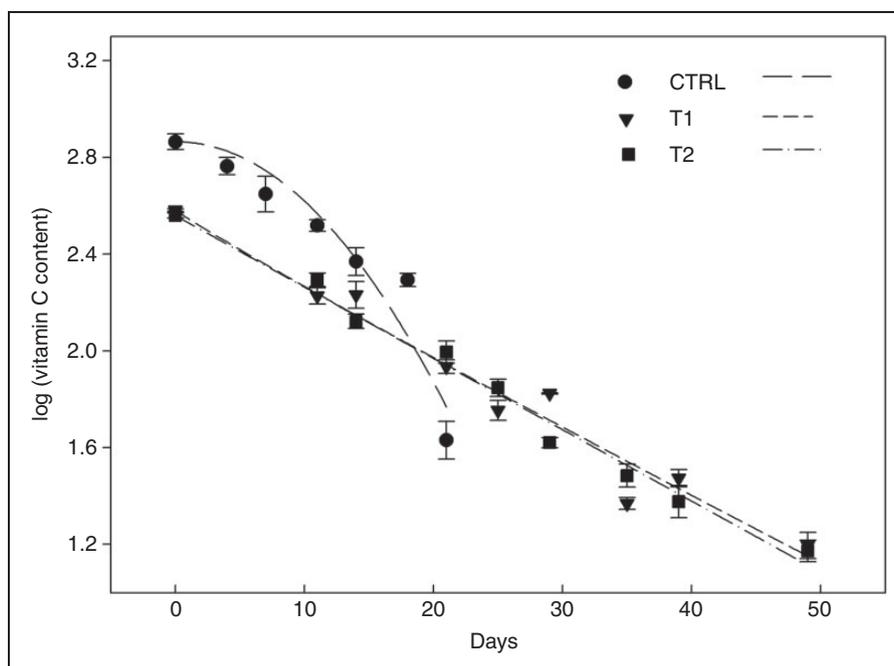
Storage of CTRL samples either at 15 or  $5^\circ\text{C}$  induced total chlorophyll losses of approximately 26 and 53% after seven and 21 days at  $5^\circ\text{C}$ , respectively (Table 1). Although T1 and T2 samples stored at  $5^\circ\text{C}$  showed higher total chlorophylls degradation trends

(67–71%), these differences were not significant after seven days at  $5^\circ\text{C}$  regarding CTRL samples. In general, thermally treated samples did not show total chlorophylls changes during storage at  $5^\circ\text{C}$  registering similar levels after 49 days compared to their respective initial levels. Accordingly, thermal treatments reduced colour changes during storage since CTRL smoothie registered  $\Delta E$  of  $10.16 \pm 0.38$  and  $10.73 \pm 0.69$  after seven days at  $15^\circ\text{C}$  and 21 days at  $5^\circ\text{C}$ , respectively, while T1/T2 registered  $\Delta E$  values of  $6.47 \pm 0.35$ – $6.20 \pm 0.17$ / $7.66 \pm 0.22$ – $7.46 \pm 0.84$  after 49 days at  $5^\circ\text{C}$ . Similarly, spinach puree heat treated at  $100^\circ\text{C}$  for 60 s only showed  $a^*$  changes of 2.3 while untreated spinach puree reported  $a^*$  changes of 6.5 after 43 days at  $4^\circ\text{C}$  (Wang et al., 2013).

As observed, these heat treatments can reduce colour changes related to chlorophylls levels in the green smoothie due to inactivation of colour-degrading enzymes. Accordingly, great to nearly complete inactivations have been reported in broccoli and spinach puree after similar thermal treatments (Wang et al., 2012, 2013).

### Total vitamin C

The initial total vitamin C (ascorbic acid + DHA) of CTRL smoothie was  $731.5 \pm 53.3 \text{ mg kg}^{-1} \text{ fw}$  (Figure 2). Ascorbic acid is easily oxidised by the enzymes ascorbate oxidase and ascorbic acid peroxidase



**Figure 2.** Total vitamin C (logarithmic scale) in untreated (CTRL) and heat-treated (T1 and T2) green vegetables smoothies stored at  $5^\circ\text{C}$  ( $n = 5 \pm \text{SD}$ ). Experimental (points) and fitted values are derived from the Weibullian model (lines).

to DHA which exhibits antioxidant properties in addition to antiscorbutic activity equivalent to that of ascorbic acid (Munyaka et al., 2010). During smoothie preparation blending disrupts plant cells allowing enzymes to access their substrates located in different plant cell locations. Accordingly, no ascorbic acid was detected in CTRL or thermally treated smoothies on processing day due to the rapid ascorbic acid to DHA enzymatic conversion (data not shown). The ascorbic acid to DHA conversion was possible since the applied thermal treatments did not completely inactivate the vitamin C oxidative enzymes as previously reported (Munyaka et al., 2010). It is well known that vitamin C is a very thermolabile vitamin (Lee and Kader, 2000). Accordingly, heat treatments reduced by approximately 50% the initial total vitamin C level of the smoothie without significant differences among treatments. Lower vitamin C content ( $430 \text{ mg l}^{-1}$ ) has been reported in a green smoothie (40% kiwifruits, 7% fenels, 8% spinach and 15% papaya) which was treated at  $80^\circ\text{C}$  for 10 min (Di Cagno et al., 2011).

Total vitamin C losses of 76–87% were observed after seven days at  $15^\circ\text{C}$  without significant differences among samples (data not shown). However, latter great total vitamin C loss was reduced by 50% when CTRL samples were stored at  $5^\circ\text{C}$ . Similarly, storage of T1 and T2 samples at  $5^\circ\text{C}$  reduced by 1.5- and 2-fold, respectively, the total vitamin C losses (85–91%) observed after 11 days at  $15^\circ\text{C}$ . CTRL and heat-treated samples showed total vitamin C contents of  $42.7 \pm 7.3$  and  $14.9 \pm 1.5/15.7 \pm 1.9 \text{ mg kg}^{-1} \text{ fw}$  after 21 and 49 days at  $5^\circ\text{C}$ , respectively, without differences among thermal treatments.

According to FAO/WHO, vitamin C intake is required to promote optimal health (FAO/WHO, 2004). A 250 g portion of this smoothie provides approximately 400% of the RNIs for vitamin C for adults and 260% for lactating women which is the population group with the highest RNIs for vitamin C (FAO/WHO, 2004). However, vitamin C of fruit and vegetables beverages may greatly decrease during storage due to oxidative and enzymatic degradative processes, among others (Lee and Kader, 2000). Accordingly, it is important to predict the vitamin C degradation during the smoothie storage to know the maximum storage time that ensures the minimum vitamin C RNIs. Experimental data related to total vitamin C changes during storage at  $5^\circ\text{C}$  were well fitted ( $R^2_{\text{ADJ}} > 95\%$ ; Table 2) with the cumulative form of the Weibull distribution (equation (1)). Calculations were estimated with the GInaFit application (version 1.6) for Microsoft Excel (Geeraerd et al., 2005). However

$$\log_{10} X = \log_{10} X_0 - \left(\frac{t}{\delta}\right)^p \quad (1)$$

**Table 2.** Estimates of Weibullian distribution parameters  $\delta$  and  $p$  and adjusted  $R^2$  for vitamin C content changes in untreated (CTRL) and heat-treated (T1 and T2) green vegetables smoothies during storage at  $5^\circ\text{C}$

	$\delta$	$p$	$R^2_{\text{ADJ}}$
CTRL	20.07	2.02	0.92
T1	33.62	0.95	0.96
T2	33.88	0.98	0.99

where  $X$  is the vitamin C content,  $X_0$  is the initial vitamin C content,  $t$  is the storage time (days),  $\delta$  represents the time needed for the first decimal reduction (days) and  $p$  is the shape parameter. Table 2 shows the calculated parameters  $\delta$  and  $p$  for the vitamin C curves determined with the Weibull model. While vitamin C curves of CTRL smoothie stored at  $5^\circ\text{C}$  showed downward concavity ( $p > 1$ ), T1 and T2 samples showed upward concavity ( $p < 1$ ). Since total vitamin C content did not significantly change during storage at  $15^\circ\text{C}$  and no intermediate data were analysed between processing and seventh day of storage these data were not modelled. The maximum storage time at  $5^\circ\text{C}$  of a smoothie portion of 250 g that ensured the minimum vitamin C RNI ( $45 \text{ mg day}^{-1}$ ) for CTRL T1 and T2 samples was 15.2, 10.7 and 10.8 days, respectively. At the end of CTRL and T1–T2 smoothies shelf-lives the vitamin C contents still represented 30 and 10% of the RNIs, respectively. As observed, total vitamin C degradation of thermal-treated smoothies was higher than CTRL samples. DHA can be rapidly and irreversibly hydrolysed to 2,3-diketogulonic acid (2,3-DKG) hence losing its antiscorbutic activity (Deutsch, 2000). The applied thermal treatments may increase the extraction of those compounds involved in the vitamin C degradation to 2,3-DKG increasing its reaction rates according to the observed reduced vitamin C levels of thermal-treated samples during storage.

### Phenolic compounds

The phenolic compounds of the smoothie were identified by their chromatographic behaviour, UV spectra and HPLC/MS (Supplementary Materials 3 and 4). Initial total phenolic content (calculated as the sum of identified phenolics) of CTRL smoothie was  $151.1 \pm 4.0 \text{ mg kg}^{-1} \text{ fw}$  (Table 3). The initial total phenolic content of CTRL was increased by 44 and 36% after T1 and T2 treatments, respectively. The apparent increases of these phenolic compounds could be primarily due to the cell membrane and wall ruptures of plant material, releasing phytochemicals from the insoluble portion of the smoothie. The lower phenolic

**Table 3.** Phenolic contents of untreated (CTRL) and heat-treated (T1 and T2) green vegetables smoothies stored at 5 and 15°C (n = 5 ± SD)

		Phenolic compounds (mg kg <sup>-1</sup> fw)									
		3- <i>p</i> -coumaroyl quinic acid					1,2-disinpoyl Total				
T <sup>a</sup>	Chlorogenic acid	Sinapic acid	(1)	(2)	gentiobioside	1-sinapoyl-2-feru	1,2'-trisinapoyl	-2-feruloylgen	tiobioside	individual phenols	
Treatment (°C)	Day acid	acid	(1)	(2)	gentiobioside	loylgentiobioside	gentiobioside	gentiobioside	gentiobioside	phenols	
CTRL	0	10.76 ± 1.39Ab	4.63 ± 0.27Ba	42.78 ± 1.41Ca	85.20 ± 2.65Ba	0.52 ± 0.05Ab	1.46 ± 0.36Cb	4.38 ± 0.25Ba	1.35 ± 0.22Ba	151.09 ± 4.04Ca	
T1	0	12.88 ± 0.16Aa	5.58 ± 0.08Aa	77.89 ± 1.19Ba	107.09 ± 4.01Aa	0.45 ± 0.02Aab	3.69 ± 0.15Aa	8.53 ± 0.40Aa	2.46 ± 0.03Aa	218.56 ± 3.19Aa	
T2	0	12.01 ± 0.34Aa	4.54 ± 0.19Ba	82.78 ± 1.83Aa	91.55 ± 1.74Bb	0.46 ± 0.01Aa	2.89 ± 0.19Ba	9.21 ± 0.24Aa	2.47 ± 0.01Aa	205.91 ± 2.97Ba	
CTRL	15	11.98 ± 2.28Ab	3.38 ± 0.29Bb	35.20 ± 1.35Bb	36.35 ± 3.03Cb	0.68 ± 0.05Aa	0.51 ± 0.14Ca	0.72 ± 0.14Cb	0.58 ± 0.10ABb	89.40 ± 3.13Cb	
	5	10.13 ± 1.13Ab	4.39 ± 0.40Aa	11.86 ± 1.53Cb	71.64 ± 3.99Db	0.34 ± 0.00Bc	1.06 ± 0.04ABbc	0.55 ± 0.04Cb	0.41 ± 0.01Bb	100.39 ± 6.87Cb	
	5	11.03 ± 0.88Ab	4.32 ± 0.21Aa	8.90 ± 2.11Bb	68.33 ± 2.92Bb	0.34 ± 0.00Bc	0.87 ± 0.23ABc	0.70 ± 0.24Bb	0.41 ± 0.01Bb	94.21 ± 3.15Cb	
	5	14.82 ± 1.33Aa	4.58 ± 0.18Aa	4.18 ± 0.46Cc	64.54 ± 5.02Bb	0.34 ± 0.01Ac	0.57 ± 0.34Ac	0.54 ± 0.03Ab	0.76 ± 0.26Ab	90.34 ± 7.05Cb	
T1	15	10.79 ± 0.16Ab	5.11 ± 0.40Aa	71.95 ± 2.74Ab	108.63 ± 3.06Aa	0.43 ± 0.10Ba	1.30 ± 0.39Ab	3.91 ± 0.25Ab	1.10 ± 0.41Ab	203.22 ± 4.48Ab	
	5	11.76 ± 1.03Ab	4.39 ± 0.47Ab	35.47 ± 1.11Ab	53.78 ± 0.39Cc	0.47 ± 0.05Aa	0.73 ± 0.37Ac	2.41 ± 0.49Ac	0.85 ± 0.21Abc	105.71 ± 1.09Bd	
	5	12.95 ± 2.39Bab	4.64 ± 0.57Aab	16.12 ± 0.92Bd	91.15 ± 4.81Ab	0.36 ± 0.03Ab	0.73 ± 0.16Ac	1.01 ± 0.97Ac	0.57 ± 0.20Ac	124.14 ± 5.44Bc	
	5	13.72 ± 2.54Ab	4.85 ± 0.09Aab	24.37 ± 2.26Bc	88.44 ± 3.98Ab	0.36 ± 0.01Ab	0.76 ± 0.12Ac	1.76 ± 0.52Ac	0.84 ± 0.28Abc	128.40 ± 4.33Bc	
	5	14.95 ± 2.02Aab	5.16 ± 0.34Aab	22.73 ± 3.10Ac	96.09 ± 2.60Ab	0.39 ± 0.01Aab	1.38 ± 0.15Ab	4.08 ± 0.50Ab	1.31 ± 0.06Ab	140.70 ± 0.91Ab	
T2	15	8.86 ± 0.46Ab	4.75 ± 0.18Aa	36.11 ± 2.03Bb	95.14 ± 1.32Ba	0.36 ± 0.02Bb	0.57 ± 0.18Bb	1.80 ± 0.52Bb	0.73 ± 0.22ABb	148.64 ± 3.62Bb	
	5	11.94 ± 2.01Aa	4.69 ± 0.43Aa	41.34 ± 3.54Abc	82.37 ± 2.56Ab	0.36 ± 0.02Ba	0.66 ± 0.22Ab	0.52 ± 0.08Bc	0.35 ± 0.00Bd	140.05 ± 4.35Ac	
	5	12.10 ± 1.94ABa	4.44 ± 0.50Aa	40.31 ± 3.12Ac	83.26 ± 6.28Ab	0.37 ± 0.03Aa	0.72 ± 0.15Ab	0.97 ± 1.03Ac	0.66 ± 0.13Abc	140.98 ± 7.60Ac	
	5	13.97 ± 0.54Aa	4.83 ± 0.29Aa	51.14 ± 3.65Ab	91.90 ± 8.22Ab	0.39 ± 0.05Aa	0.63 ± 0.13Ab	2.54 ± 0.14Ab	0.81 ± 0.16Ab	162.10 ± 11.87Ab	
	5	14.86 ± 1.98Aa	4.69 ± 0.07Aa	32.12 ± 5.77Ac	82.67 ± 1.95Bb	0.41 ± 0.06Aa	0.87 ± 0.29Ab	1.35 ± 0.71Bbc	0.53 ± 0.11Bcd	131.30 ± 6.48Ac	

Different capital letters denote significant differences ( $P \leq 0.05$ ) among treatments stored at the same temperature for the same sampling day. Different lowercase letters denote significant differences ( $P \leq 0.05$ ) among sampling days for the same treatment stored at the same temperature.

increment of T2 samples may be owed to the lower treatment time which did not produce great cell disruption as observed in T1 samples. Accordingly, the content of the main phenolic acids (3-*p*-coumaroyl quinic, chlorogenic and sinapic acids) remained unchanged after T2 treatment. The greatest phenolic acids increments after T1/T2 treatments were those corresponding to 1-sinapoyl-2-feruloylgentiobioside, 1,2,2'-trisinapoylgentiobioside, 3-*p*-coumaroyl quinic acid (1) and 1,2'-disinapoyl-2-feruloylgentiobioside with 153/98, 95/110, 82/94 and 82/83% compared to the respective initial contents of CTRL samples.

Attending to phenolic acids changes during storage, the levels of 3-*p*-coumaroyl quinic acid (1), 1-sinapoyl-2-feruloylgentiobioside, 1,2,2'-trisinapoylgentiobioside and 1,2'-disinapoyl-2-feruloylgentiobioside smoothies decreased throughout storage for both treatments and storage temperatures registering the greatest losses in the first seven days of storage. Storage at 5 °C of CTRL smoothies greatly reduced the 3-*p*-coumaroyl quinic acid (2) and 1-sinapoyl-2-feruloylgentiobioside losses of 57 and 65% at 15 °C to 16 and 27%, respectively. However, 3-*p*-coumaroyl quinic acid (1) and 1,2'-disinapoyl-2-feruloylgentiobioside showed the opposite behaviour with losses of 18–57 and 70–72% in those CTRL smoothies stored for seven days at 15 and 5 °C, respectively. On the other side, 1,2,2'-trisinapoylgentiobioside and 1,2-disinapoylgentiobioside levels of CTRL samples decreased by approximately 85 and 33%, respectively, after seven days independently of the storage temperature. Among thermally treated samples sinapic acid contents did not change after 49 or seven days at 5 or 15 °C, respectively. In general, T1 samples registered sevenfold lower 3-*p*-coumaroyl quinic acid (1) losses and 1.1–1.6-fold lower losses for 1-sinapoyl-2-feruloylgentiobioside, 1,2,2'-trisinapoylgentiobioside and 1,2'-disinapoyl-2-feruloylgentiobioside regarding T2 samples after seven days either at 5 or 15 °C. Accordingly, among thermal treatment conditions, the lower temperature treatment better retained latter four phenolic acids during storage compared to higher temperature treatment time. 3-*p*-coumaroyl quinic (2) and sinapic acids did not register great changes throughout storage in all samples at 5 °C or thermally treated samples stored at 15 °C. However, 3-*p*-coumaroyl quinic (2) and sinapic acids decreased by 57 and 27%, respectively, in CTRL samples stored for seven days at 15 °C. Attending to chlorogenic acid, no significant changes in CTRL samples were observed for 7–11 days at 15 or 5 °C. Chlorogenic acid of CTRL samples stored at 5 °C increased from day 11 to day 21 by 38%. However, T1 and T2 samples stored at 15 °C registered 16 and 26% chlorogenic acid losses after seven days, respectively. PAL is the key enzyme in the phenols biosynthesis pathway which is activated under abiotic

stresses (Cisneros-Zevallos, 2003) such as the wounding produced during smoothie blending. Accordingly, PAL was activated in untreated red smoothies after 10 days of storage at 5 °C being this enzyme activation, retarded to 20–30 days either at 5 or 20 °C in thermally treated samples (similar conditions as T1) (Rodríguez-Verástegui et al., 2015). Furthermore, PAL activation of CTRL samples was double of that from heat-treated samples (Rodríguez-Verástegui et al., 2015). Accordingly, the observed chlorogenic acid increment in CTRL samples may be owed to PAL activation. However, a lower PAL activation of heat-treated samples may lead to the observed unchanged levels in T1 and T2 samples stored at 5 °C and reduced levels in those samples stored at 15 °C as a negative counterbalance between chlorogenic acid biosynthesis and its degradation at this high storage temperature. As it has been previously reviewed the changes of the phenolic profile of fruit blends during storage greatly depend on the phenolic compound and storage conditions as also observed in our smoothie data (Chen et al., 2013).

Conclusively, phenolic contents increased after thermal treatment, in a greater extend in T1 samples, being this phenolic increment associated with subsequent enhanced bioaccessibility in the gastrointestinal tract (Bugianesi et al., 2004). In general, phenolic levels decreased during storage, except chlorogenic and sinapic acids, registering the greatest losses in the first 7–11 days showing T1 samples lower degradation rates.

## TAC

The initial TAC of CTRL smoothie obtained by FRAP, ABTS and DPPH were  $234.2 \pm 20.3$ ,  $395.7 \pm 22.4$  and  $54.4 \pm 16.6$  mg Trolox equivalents  $\text{kg}^{-1}$  fw, respectively (Table 4). 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 on the analytical method used (Prior et al., 2005). Accordingly, a Pearson correlation using total phenolic content and TAC data during storage was used to determine which TAC method was better correlated to total phenolic content. FRAP method achieved the best correlations ( $r^2 = 0.67$ ) closely followed by ABTS ( $r^2 = 0.53$ ). Consequently, only FRAP data are discussed.

Contrary to the apparent increase of total phenolic content after thermal treatment, TAC did not register significant differences after heating. Similarly, Keenan et al. (2010) did not find significant TAC changes after heat treatment (70 °C for 10 min) of a fruit smoothie while total phenolic content increased. Vitamin C also plays an important contribution to the TAC of the

**Table 4.** Total antioxidant capacity (FRAP, ABTS and DPPH methods) of untreated (CTRL) and heat-treated (T1 and T2) green vegetables smoothies stored at 5 and 15 °C (n = 5 ± SD)

Treatment	T <sup>a</sup> (°C)	Day	Total antioxidant capacity (mg Trolox equivalents kg <sup>-1</sup> fw)		
			FRAP	ABTS	DPPH
CTRL	–	0	234.2 ± 20.3Aa	395.7 ± 22.4Ab	54.4 ± 16.6Ab
T1	–	0	247.1 ± 12.4Aa	411.4 ± 34.8Aa	85.1 ± 23.8Aa
T2	–	0	231.0 ± 5.9Aab	390.0 ± 39.2Aa	69.1 ± 6.6Aa
CTRL	15	7	237.1 ± 18.7ABa	458.1 ± 25.0Aa	97.7 ± 18.3ABa
		5	218.1 ± 14.2Bab	383.2 ± 7.1Bb	35.1 ± 15.1Cbc
	5	11	127.9 ± 18.2Bc	251.9 ± 33.2Ac	17.5 ± 7.5Bc
		21	174.4 ± 28.1Bbc	372.6 ± 38.0Ab	13.8 ± 11.9Bc
T1	15	7	260.4 ± 14.9Aa	352.9 ± 18.2Ba	119.5 ± 13.5Aa
		5	226.6 ± 0.7Aa	287.8 ± 21.8Ab	43.5 ± 11.9Ab
	5	21	243.4 ± 20.9Aa	415.0 ± 44.6Aa	45.2 ± 4.0Ab
		35	255.5 ± 19.8Aa	302.6 ± 18.1Ab	56.9 ± 8.5Aab
		49	249.8 ± 19.0Aa	326.9 ± 13.4Ab	70.3 ± 9.4Aab
T2	15	7	230.5 ± 15.7ABa	379.4 ± 38.5Ba	77.6 ± 6.8Ba
		5	152.3 ± 8.3Bc	260.1 ± 8.2Ac	37.7 ± 2.9ABb
	5	21	204.5 ± 21.0ABb	388.0 ± 8.5Aa	49.1 ± 3.9Aab
		35	272.2 ± 19.7Aa	289.3 ± 9.8Abc	45.0 ± 17.6Aab
		49	248.7 ± 22.6Aab	337.3 ± 30.4Aab	54.0 ± 13.8Aab

FRAP: ferric reducing antioxidant power; ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); DPPH: 2,2-diphenyl-1-picrylhydrazil.

Different capital letters denote significant differences ( $P \leq 0.05$ ) among treatments stored at the same temperature for the same sampling day. Different lowercase letters denote significant differences ( $P \leq 0.05$ ) among sampling days for the same treatment stored at the same temperature.

smoothie. Accordingly, the unchanged TAC may be a result of the above described vitamin C reduction after thermal treatment.

The TAC levels of all samples remained quite constant throughout storage at both temperatures, except CTRL samples stored at 5 °C which showed a TAC decrease of 45% after 11 days followed by an increase registering final levels of  $174.4 \pm 28.1$  mg Trolox equivalents kg<sup>-1</sup> fw. Similarly, Keenan et al. (2010) reported TAC decreases in heat-treated (70 °C for 10 min) fruit smoothies after 10 days at 4 °C. Accordingly, thermal treatments avoided TAC losses of the smoothie during storage at both temperatures probably due to the heat inactivation of enzymes involved in the degradation of antioxidant compounds.

### Intact glucosinolates

The glucosinolates of the smoothie were identified by their chromatographic behaviour, UV spectra and HPLC/MS (Supplementary Materials 3 and 5). The initial total glucosinolate content of CTRL samples was  $128.77 \pm 16.26$  mg kg<sup>-1</sup> (Table 5). Total glucosinolate content was not affected by any of the thermal treatments. However, different patterns were observed

among individual glucosinolates. Isothiocyanates are the biologically active breakdown products from glucosinolates which lack of those chemopreventive properties. However, the presence of the epithiospecifier protein (ESP), among other factors, may lead to other breakdown products different from isothiocyanates. The thermal treatments applied may ensure the thermal degradation of ESP since this protein was completely inactivated in broccoli florets after 60 °C for 5 min (Matusheski et al., 2004), being the ESP inactivation probably even enhanced in our smoothie due to better heat transmission. Regarding to structure–activity relationships, it was generally observed that glucosinolates with a hydroxyl function in the side chain are more labile compared to their corresponding non-hydroxylated relatives (Hanschen et al., 2014). Accordingly, while glucobrassicin content did not change for any of the thermal treatments, 4-hydroxyglucobrassicin was reduced by 29% after T1 treatment. Furthermore, T1 treatment induced 4-methoxyglucobrassicin decrease of 49%. T2 treatment did not induce significant changes among glucosinolates contrary to T1. Glucoraphanin content of the smoothie increased by 36% after T1 treatment. It has been widely reported that aliphatic glucosinolates (such as

**Table 5.** Intact glucosinolates contents of untreated (CTRL) and heat-treated (T1 and T2) green vegetables smoothies stored at 5 and 15 °C (n = 5 ± SD)

Treatment	T <sup>a</sup> (°C)	Day	Glucosinolates (mg kg <sup>-1</sup> fw)					
			Glucoraphanin	Glucobrassicin	4-Hydroxygluco brassicin	4-Methoxy glucobrassicin	Total glucosinolates	
CTRL	–	0	4.48 ± 0.31Ba	95.25 ± 22.33Aa	16.96 ± 0.38Aa	1.12 ± 0.09Aa	117.81 ± 16.26Aa	
T1	–	0	6.08 ± 0.57Aa	108.30 ± 5.51Aa	12.03 ± 0.51Ba	0.57 ± 0.11Ba	126.98 ± 5.53Aa	
T2	–	0	4.18 ± 0.38Bab	123.14 ± 1.50Aa	17.39 ± 0.63Aa	1.33 ± 0.28Aa	144.04 ± 1.23Aa	
CTRL	15	7	1.57 ± 1.51ABb	101.02 ± 2.61Aa	7.18 ± 0.70Cb	0.18 ± 0.10Ab	109.94 ± 3.10ABa	
		5	7	3.15 ± 0.39Aab	108.52 ± 11.14Aa	10.03 ± 0.54ABb	0.85 ± 0.23Aa	122.55 ± 10.49Aa
		5	11	1.84 ± 1.21Abc	107.08 ± 4.41Ba	8.38 ± 0.71Bc	1.28 ± 1.54Aa	118.57 ± 6.71Ba
		5	21	0.83 ± 0.58Ac	99.87 ± 3.53Aa	7.36 ± 0.49Ac	1.70 ± 0.36Aa	109.76 ± 3.85Aa
T1	15	7	0.93 ± 0.11Bb	95.79 ± 4.84Aa	8.71 ± 0.84BCb	0.03 ± 0.04Ab	105.46 ± 5.62Bb	
		5	11	0.07 ± 0.07Ad	80.06 ± 0.07Cb	12.65 ± 0.07Aa	0.83 ± 0.16Aa	93.61 ± 1.46Cc
		5	21	0.65 ± 0.17Ad	102.30 ± 3.16Aa	7.12 ± 0.94Ac	0.40 ± 0.47Ba	110.47 ± 3.50Ab
		5	35	1.97 ± 0.15Ac	71.26 ± 6.36Ab	8.14 ± 0.21Bc	0.17 ± 0.06Aa	81.55 ± 6.23Ad
		5	49	2.89 ± 0.38Ab	58.81 ± 1.87Bc	9.59 ± 0.50Ab	0.29 ± 0.21Aa	71.58 ± 1.76Bd
T2	15	7	1.70 ± 0.08ABa	104.98 ± 6.85Ab	11.30 ± 1.19Ab	1.95 ± 1.94Aa	119.94 ± 4.28ABb	
		5	11	0.70 ± 0.30Ac	125.31 ± 6.32Aa	9.83 ± 1.40Bb	1.16 ± 0.14Aa	137.01 ± 5.65Aa
		5	21	0.54 ± 0.06Ac	105.36 ± 2.36Ab	9.01 ± 1.56Ab	1.14 ± 0.19ABa	116.05 ± 1.28Ab
		5	35	1.39 ± 0.13Bbc	83.50 ± 9.48Ac	10.50 ± 0.77Ab	1.13 ± 1.25Aa	96.52 ± 10.74Ac
		5	49	2.56 ± 0.52Aa	73.83 ± 7.62Ac	9.22 ± 0.64Ab	1.57 ± 1.51Aa	87.19 ± 9.21Ac

Different capital letters denote significant differences (P ≤ 0.05) among treatments stored at the same temperature for the same sampling day. Different lowercase letters denote significant differences (P ≤ 0.05) among sampling days for the same treatment stored at the same temperature.

glucoraphanin) are more heat stable than indole glucosinolates (such as glucobrassicin 4-hydroxyglucobrassicin and 4-methoxyglucobrassicin) for temperature treatments below 110 °C (Hanschen et al., 2014). Therefore, the apparent glucoraphanin increment may be owed to a better heat stability of this glucosinolate together with an enhanced extractability of this compound during T1, the longest treatment.

Glucosinolates contents of CTRL samples did not change after seven days at 5 °C, except 4-hydroxyglucobrassicin which decreased by 41%. However, storage of CTRL samples at 15 °C for seven days induced 58, 65 and 84% decreases of 4-hydroxyglucobrassicin, glucoraphanin and 4-methoxyglucobrassicin, respectively, although glucoraphanin was preserved. Similarly, Rangkadilok et al. (2002) reported a 50% decrease in glucoraphanin in ‘Marathon’ heads after seven days at 15 °C, but no decrease after seven days at 4 °C. The breakdown of glucosinolates by myrosinase is usually a very rapid event which is greatly enhanced after mechanical homogenisation such as smoothie preparation (Verkerk et al., 1997). In agreement to our data, myrosinase activity of ‘Marathon’ heads was probably greatly reduced at 4 °C while it was enhanced at 15 °C. Glucosinolates levels from day 7 to day 21 did not change at 5 °C except glucoraphanin that greatly

decreased by 81%. Similarly, glucoraphanin showed the greatest losses among glucosinolates in broccoli florets stored at 4 °C (Verkerk et al., 2001).

Regarding to thermal-treated samples, no great glucosinolates changes were observed after 11 days at 5 °C except 4-hydroxyglucobrassicin of T2 samples that, similarly to CTRL samples, decreased by 43%. The observed 4-hydroxyglucobrassicin reduction may be owed to the commented higher degradation of these glucosinolates with a hydroxyl function. From day 11 to the end of storage at 5 °C, low glucosinolates losses were observed in those thermal-treated samples (<26%). In the same way, no great glucosinolates losses (<30%) were observed in those T1 and T2 samples stored at 15 °C for seven days. Van Eylen et al. (2007) reported a residual myrosinase activity of 23% in broccoli juice treated at 60 °C for 3 min. Accordingly, the low glucosinolates losses of thermal-treated samples during storage at low temperature may be owed to a complete myrosinase inactivation.

Conclusively, short-time/high temperature treatment (T2) did not induce individual glucosinolates losses regarding T1 samples (<49% losses). The glucosinolates degradation observed in CTRL samples during storage was greatly reduced in both thermal-treated samples.

## CONCLUSIONS

This study presents a green fresh vegetables smoothie with excellent nutritional, microbial and physicochemical quality during a shelf-life of 49 days at 5 °C. Mild thermal treatments were necessary during processing to preserve its quality achieving T2 (45 s at 90 °C) better microbial reductions and health-promoting compounds preservation (related to phenolics and glucosinolates contents). Furthermore, low temperature storage at 5 °C is recommended to preserve quality and safety. A 250 g portion of this green smoothie can highly cover the established RNIs for DF, minerals and vitamin C of different population groups.

## ACKNOWLEDGEMENTS

We are grateful to M. Otón for his skilful technical assistance.

## DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The authors are grateful to Spanish Ministry of Economy and Competitiveness (MINECO) Project AGL2013-48830-C2-1-R and FEDER for financial support.

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