ENRICHED FUNCTIONAL CARROT SMOOTHIE WITH BIOSYNTHESIZED PHENOLIC COMPOUNDS

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Abstract

Carrots are worldwide highly consumed due to their sensory characteristics and health-promoting properties. However, their low phenolic/antioxidant levels may be greatly increased with abiotic stresses. Accordingly, phenolic/antioxidant enhancements and related phenylalanine ammonia-lyase (PAL) activity of carrot shreds under different treatments (UVc radiation and hyperoxia conditions) were studied after a pre-enrichment incubation of 72 h at 15ºC. Subsequently, a carrot smoothie prepared from enriched carrot shreds was heat-treated (90ºC for 30 s) and stored at 5ºC up to 14 days. Heat-treated smoothies showed a good physicochemical and microbiological quality (<6 log CFU g^{-1}) after 14 days at 5ºC, although those non-irradiated samples registered lower psychrophilic and yeasts and molds levels. Heat-treated smoothie from non-

irradiated shreds stored under hyperoxia conditions, showed the highest total phenolic content of 13,82 mg ChAE kg^{-1} fw (87 % chlorogenic acid) after 14 days at 5ºC. Total phenolics content was in accordance with PAL and total antioxidant capacity. Conclusively, a pre-enrichment incubation of carrot shreds under hyperoxia conditions allowed to obtain a functional smoothie with great phenolic levels and good microbiological and physicochemical quality up to 14 days at 5ºC.

Keywords: wounding; hyperoxia; UVc radiation; antioxidant.

1. INTRODUCTION

The crescent consumer's knowledge on functional foods has led to an increasing interest in foods not only intended to feed, but also to prevent chronic and nutritional-related diseases as well as to improve overall human well-being. High intake of fruit and vegetables has been proved to prevent a grand array of diseases, such as degenerative disorders, cancer and cardiovascular among others (Slavin and Lloyd 2012). However, the current lifestyle turns difficult the preparation of these plant products. Thus, their consumption should be promoted through the development of attractive ready-to-eat products that should be processed with minimal and non-aggressive treatments to preserve as much as possible the quality parameters of the raw materials (Artés et al. 2009). Accordingly, smoothies represent an excellent and convenient alternative to promote the daily consumption of fruits and vegetables (Rodríguez-Verástegui et al. 2015). Smoothies are nonalcoholic beverages prepared from fresh or frozen fruit and/or vegetables, which are blended without filtering 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 (Castillejo et al. 2015). Fruits and vegetables are rich in phenolic compounds among other bioactive compounds. Phenolic compounds are great antioxidants related to several health-promoting properties such as anti-inflammatory, antitumoral, as well as preventing neurodegenerative and chronic disorders (El Gharras 2009). Phenylalanine ammonia-lyase (PAL) is the key enzyme of primary (shikimate) and secondary (phenylpropanoid) pathways and is, therefore, involved in the biosynthesis of polyphenolic compounds (Dixon and Paiva 1995). It is well reported that PAL activity may be enhanced by an array of biotic and abiotic stress-induced mechanisms, such as wounding, radiation exposure, hyperoxia storage, water stress, ultrasounds, chilling injury, low minerals, hormones and pathogen attack, among others (Cisneros-Zevallos 2003; Cuéllar-Villarreal et al. 2016). Previous studies have shown that wounding, low UVc doses and hyperoxia storage, singly, enhance phenolic content on carrots and other foodstuff (Alegria et al. 2012; Avena-Bustillos et al. 2012; Becerra-Moreno et al. 2012; Jacobo-Velázquez et al. 2011). Carrots occupy the sixth place among the list of most consumed vegetables in the American diet, although its total phenolics content (TPC) is almost the lowest among them (Chun et al. 2005). Accordingly, the health benefits derived from carrots could be increased by enhancing their phenolics levels during a controlled pre-enrichment incubation by using postharvest abiotic stresses. Furthermore, synergistic effects on phenolics increments after combined application of different stresses may occur. Nevertheless, the effects on phenolic/antioxidant levels after combined application of wounding, intermediate UVc dose and hyperoxia atmosphere on carrots has not been studied yet. Therefore, a functional phenolic-enriched carrot smoothie may be developed previously applying abiotic stresses on carrot material, singled or combined, during a pre-enrichment incubation prior to smoothie preparation. Moreover, a mild heat treatment of the

smoothie may guarantee the food safety criteria and physicochemical quality of this functional carrot smoothie during refrigerated storage.

Accordingly, the aim of this study was to optimize a pre-enrichment treatment of carrots to maximize the phenolic/antioxidant levels in order to obtain a functional carrot smoothie with enhanced phenolic/antioxidants contents. Furthermore, the effects of a mild heat treatment and subsequent refrigerated storage on the enriched phenolic/antioxidant levels of the functional carrot smoothie were also studied.

2. MATERIALS AND METHODS

2.1. Plant material

Fresh carrots (*Daucus carota* L. cv. Nantes) were purchased at a local market in Cartagena (Southeast of Spain) in April. Carrots were carefully inspected, selecting those with similar visual appearance and size (14-15 cm long and 2-3 cm diameter). Subsequently, carrots were sanitized in a cold room (8°C) with chlorine (100 ppm NaClO; 5°C; pH 6.5±0.1) for 2 min, rinsed with tap water at 5ºC for 1 min and drained in a perforated basket for 1 min. A ratio of 300 g plant material: 5 L chlorine was used. Carrots were wounded to shreds (2mm×3mm×40-60 mm) with a food processor (FreshExpress+, Moulinex, Lyon, France). Pre-enrichment treatments were conducted immediately after wounding.

2.2. Pre-treatment treatments and incubation of plant material

The UVc treatment chamber used was detailed by Artés-Hernández et al. (2009). Carrot shreds were placed between the two lines of UVc lamps at 17.5 cm above and below over a 35 mm thick bi-oriented PP film mounted on polystyrene net (130×68 cm) that minimized blockage of the UVc radiation. The applied UVc intensity of 67.6 Wm^2 was

101 calculated as the mean of 18 UVc readings on each side of the net using a VLX 254
102 radiometer (Vilber Lourmat, Marne la Vallee, France). Thus, both sides received the
103 same UVc intensity. The UVc light intensity was kept constant and the applied dose
104 was varied by altering the exposure time at the fixed distance. A UVc radiation
105 treatment of 4 kJ UVc m^{-2} (exposure time of 139 s) was applied. Non-irradiated samples
106 were used as control (hereinafter 'CTRL').
107 Samples to be stored under hyperoxia conditions (hereinafter 'HO') were placed in
108 plastic containers (30 cm diameter, 60 cm height) and connected to an air-flow-through
109 system supplied with humidified flows of either air or a gas mixture containing 80 % O_2
110 (balanced with N_2). In order to ensure a good air flow through carrot shreds, these
111 samples were distributed in opened plastic petri dishes (8.5 cm diameter, 1 cm height).
112 CO_2 partial pressures were kept <0.15 kPa to avoid any physiological effect exerted by
113 CO_2 such as anaerobic metabolism. Samples stored under air conditions were used as
114 control (hereinafter 'Air'). Gas treatments were applied at 15ºC for 72 h in darkness.
115 Pre-enrichment incubation of carrots, as well as smoothie preparation and subsequent
116 storage, is summarized in Figure 1.
117

118 **2.3. Carrot smoothie preparation, heat treatment and storage conditions**

119 Carrot smoothie was prepared in a food processor (Robot Cook®, Robot Coupe,
120 Vincennes Cedex, France) using sterilized water in a relation of 1:1 (carrot weight:
121 water volume). Heat treatment of carrot smoothie was applied using a Mastia
122 thermoresistometer described by Conesa et al. (2009). Immediately after smoothie
123 blending, the sterilized vessel of the thermoresistometer was filled with 400 mL of the
124 smoothie. The thermoresistometer was programmed to increase the initial smoothie
125 temperature (8±2ºC) with a heating rate of 30ºC/min to 90ºC, then maintained for 30 s

and cooled down to a final temperature of 40ºC (cooling rate of 30ºC/min). After heat treatment, the smoothie temperature was cooled down to 5ºC submerging the vessel in an ice-water bath while continuously agitation was programmed in the thermoresistometer. Subsequently, 15-mL aliquots of heat-treated samples were taken in aseptic conditions in sterile Falcon tubes through the thermoresistometer sampling port. Samples were stored in darkness at 5ºC. Non heat-treated carrot smoothie was used as control. Visual appearance, flavor, texture, off-colors, off-odors, lumpiness, turbidity, precipitation/phase separation and overall quality of heat-treated smoothie conducted by an informal sensory panel test of 8 persons were reported to be over the limit of acceptability up to 14 days at 5 ºC. Sampling was conducted on processing day (0) and after 7 and 14 days at 5ºC. Five replicates per pre-treatment and sampling day were prepared.

2.4. Analyses

2.4.1. Physiochemical analyses

The pH, titratable acidity (TA), total soluble solids content (SSC) and color of samples were determined according to Castillejo et al. (2015). TA and SSC were expressed as g citric acid 100 mL^{-1} and ºBrix, respectively. Total color differences (ΔE) and browning index differences (ΔBI) were calculated according to equations previously described (Palou et al. 1999).

2.4.2. Microbial analysis

Mesophilic, psychrophilic and yeast and mold growth was determined using standard enumeration methods according to Castillejo et al. (2015). All microbial counts were reported as log colony forming units per gram of product (log CFU g^{-1}). Each of the

five replicates was analyzed by duplicate. The presence of Salmonella spp., *Listeria monocytogenes* and generic *Escherichia coli* was monitored throughout storage of smoothies according to the European legislation (EC_1441/2007 2007).

2.4.3. Phenylalanine ammonia-lyase

PAL activity was analyzed according to Ke and Saltveit (1986) with modifications (Jacobo-Velázquez et al. 2011). Concisely, 2 g of sample was mixed with polyvinylpolypyrrolidone (0.2 g) and homogenized in cold 50 mM borate buffer (pH 8.5) containing 400 μL L^{-1} β-mercaptoethanol. Homogenates were filtered through four layers of cheesecloth and then centrifuged at 10,000×G for 20 min at 4°C. Supernatants were used as enzyme extract. Two sets of UV-Star well plates (Greiner Bio-One, Frickenhausen, Germany) containing 69 μL of PAL extract plus 200 μL ultrapure water were prepared for every sample and pre-incubated at 40 ºC for 5 min. Afterwards, 30 μL of either water (blank) or 100 mM L-phenylalanine substrate solution (freshly prepared before assay) were added to each of the well for every sample set. The absorbances of sample sets were measured at 290 nm using a Multiscan plate reader (Tecan Infininte M200, Männedorf, Switzerland) at time 0 and after 1 h of incubation at 40 ºC. The PAL activity was expressed as μmol of *t*-cinnamic acid synthesized kg^{-1} fw h^{-1} using a *t*-cinnamic acid standard curve (0-6.75 mM). Each of the three replicates was analyzed by duplicate.

2.4.4. Phenolic compounds

Extraction to determine phenolic compounds and total antioxidant capacity (TAC) extract was conducted by homogenization (Ultra Turrax® model 18T, IKA-Werke GmbH & Co. KG, Germany) of 2 g of sample in 8 mL methanol for 20 s under ice-

water bath. Subsequently, extracts were centrifuged at 13500×G for 20 min at 4 ºC and supernatants were collected and analyzed. Extracts for individual phenolic compounds were further filtered through a 0.22 µm polyethersulphone filter and stored at −80 ºC in amber vials until UPLC analysis.

Total phenolic content (TPC) was analyzed by Folin–Ciocalteu reagent method as previously described (Martínez-Hernández et al. 2011). Briefly, a 19 µL aliquot of TPC extract was placed on a 96 PS flat bottom well plate (Greiner Bio-One, Frickenhausen, Germany) and 29 µL of Folin–Ciocalteu reagent 2 N (Sigma, St Louis, MO, USA) were added. Samples were incubated for 3 min in darkness at room temperature. After incubation, 192 µL of a solution containing Na_2CO_3 (4 g L^{-1}) and NaOH (20 g L^{-1}) were added and the reaction was carried out for 1 h at room temperature in darkness. Subsequently, absorbance was read at 750 nm using the same microplate reader as described before. TPC was expressed as chlorogenic acid (Sigma, St Louis, MO, USA) equivalents (ChAE) in mg kg^{-1} fresh weight (fw). Each of the three replicates was analyze by duplicate.

Analyses of individual phenolic compounds were conducted as previously described (Alegria 2015) with some modifications. Briefly, samples of 20 µL were analyzed using an Ultra High-Performance liquid chromatography (UPLC) 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. The UPLC system was controlled by the software LabSolutions (Shimadzu, v. 5.42 SP5). Chromatographic analyses were carried out onto a Kinetex C18 column (100 mm×4.6 mm, 2.6 µm particle size; Phenomenex, Macclesfield, UK) with a KrudKatcher Ultra HPLC guard column (Phenomenex, Macclesfield, UK). The column temperature was maintained at 25 ºC. The mobile phase was acidified water (A; formic acid to final

pH 2.3) and acidified methanol (B; formic acid to final pH 2.3). The flow rate was 1.5 mL min^{-1}. Gradient program used was 0/88, 1.2/88, 2.4/85, 8.3/70, 9.4/50, 11.8/50, 20.8/55, 22.0/60 (min/% phase A). Then, column equilibration was conducted at 0 % A for 2.2 min. Chromatograms were recorded at 320 nm. Phenolic acids were quantified as standards of chlorogenic acid (3-CQA), ferulic acid (Sigma, St Louis, MO, USA), isochlorogenic acid A (3,5-CQA) and C (4,5-CQA) (ChromaDex, Irvine, CA, USA). The calibration curves were made with at least six data points. The results were expressed as mg kg^{-1} fw. Each of the three replicates was analyzed by duplicate.

2.4.5. Total antioxidant capacity

The extracts were analyzed for TAC according to Brand-Williams et al. (1995) with modifications (Martínez-Hernández et al. 2013). Briefly, a solution of 0.7 mM 2,2-diphenyl-1-picrylhydrazil (DPPH) in methanol was prepared 2 h before the assay and adjusted to 1.10±0.02 nm immediately before use. A 21 µL aliquot of the previously described extract was placed on a 96 PS flat-bottom well plate and 194 µL of DPPH was added. The reaction was carried out for 30 min at room temperature in darkness and the absorbance at 515 nm was measured using the Multiscan plate reader. Results were expressed as mg Trolox equivalent antioxidant capacity kg^{-1} fw. Each of the three replicates was analyze by duplicate.

2.5. Statistical Analyses

A complete randomized design in triplicate, with two-way ANOVA (treatment×storage), by Post Hoc Tuckey HSD tests, were used with SPSS software (v. 21, IBM, USA).

3. RESULTS

3.1. Physicochemical quality

Carrot shreds showed an initial pH and TA of 6.32-6.37 and 0.32-0.48 % (Table 1), respectively, similar to previous data (Pushkala et al. 2012). The pH of the shreds decreased and TA increased during pre-enrichment incubation as a combined effect of microbial growth and phenolic acids enrichment as shown later. Accordingly, pH/TA of non-irradiated and irradiated shreds decreased to 3.20-4.05/4.75-5.22 and 4.87-5.02/3.42-4.02, respectively, after pre-enrichment incubation. The higher acidification observed in non-irradiated samples may be explained by the higher phenolic acids content of these samples as it will be discussed later. No clear influence of the atmosphere storage conditions on pH and TA of carrot shreds after pre-enrichment incubation was observed. Similarly, no significant differences on pH and TA values were observed among air or hyperoxia-stored (80 % O_2) blueberry fruit during storage up to 35 days at 4 ºC (Zheng et al. 2003). Carrot smoothies from CTRL-Air, CTRL-HO, UVc-Air and UVc-HO carrot shreds showed initial pH/TA values of 3.35/2.05, 4.13/1.07, 5.03/0.68 and 5.15/1.00, respectively. Heat treatment did not change (p<0.05) initial pH and TA of carrot smoothies. Quality of carrot beverages is difficult to maintain during storage due to its low acidity. The pH of carrot beverages is usually acidified with citric acid, or other acidulants, to approximately 3.8 as a general commercial practice by the food industries in order to reduce microbial growth and degradative enzymatic and non-enzymatic reactions during storage (Quitão-Teixeira et al. 2009; Talcott and Howard 1999). Alternatively, acidification of carrot juice through fermentation has been proposed as a preservation method combined with pasteurization (Tamminen et al. 2013). Accordingly, the spontaneous fermentation occurred during pre-enrichment incubation of carrot shreds allowed to naturally reduce the pH extending

the shelf-life with a desirable mild acidic taste. In general, heat-treated carrot smoothies did not show great pH/TA changes throughout storage at 5ºC with final pH/TA values of 3.8-4.5/0.87-1.14. Similarly, acidified blanched carrot juice showed more stable pH and TA values than non-acidified juices up to 21 days of storage at 4ºC (Yu and Rupasinghe 2012).

Carrot shreds showed initial SSC of 7.85-7.92 (Table 1) similar to previous data (Martínez-Hernández et al. 2016). Carrot shreds stored under hyperoxia conditions presented higher SSC compared to air-stored samples after pre-enrichment incubation. Similarly, SSC of blueberry fruit increased during hyperoxia (80 % O_2) storage at 5ºC (Zheng et al. 2003). The latter behavior may be explained by the reduced microbial growth under hyperoxia conditions, as shown later, and consequently lower microbial sugars consumption. SSC of carrot smoothies was not greatly changed after heat treatment. In general, SSC of non-heat-treated carrot smoothies decreased through storage as consequence of microbial growth. Contrary, SSC of heat-treated smoothies generally did not register great changes due to the lower microbial loads. Kaur and Sharma (2013) also reported unchanged SSC in pasteurized carrot juice after 15 days at 5 ºC.

Color is an important parameter for conformity determination of carrot beverages quality. ΔE is a colorimetric parameter extensively used to characterize the variation of colors during processing and storage of food products. BI represents the purity of brown color and is reported as an important parameter in processes where enzymatic or non-enzymatic browning take place (Palou et al. 1999). For that reason, ΔE and BI have been satisfactorily used to assess color quality of carrot beverages after processing treatments and subsequent storage (Kaur and Sharma 2013). UVc pre-treatment of carrot shreds induced initial mild browning (ΔE=5.6, ΔBI= 89.4; data not shown).

Browning observed in some fruits and vegetables after UVc radiation has been attributed to the increased peroxidase (POD) activity (Tomás-Barberán and Espín 2001). However, such browning of carrots shreds after the low UVc dose used was not visually observed. Irradiated shreds showed higher BI after pre-enrichment incubation which may be owed to the pre-activated POD during UVc pre-treatment (Table 1). Furthermore, pre-enrichment incubation under hyperoxia conditions induced slightly higher ΔE and ΔBI compared to carrot shreds incubated under air conditions. *β*-carotene, the main pigment responsible of the bright orange color of carrots, is very susceptible to isomerization and oxidation (Knockaert et al. 2012). Furthermore, POD activity may increase under hyperoxia storage as previously reported (Yang et al. 2009). Accordingly, the observed greater color degradation under hyperoxia compared to air conditions may be explained by a *β*-carotene degradation and incremented POD activity. Heat treatment of carrot smoothies induced low color changes (ΔE<25, ΔBI<96) which correspond to undetected visual color changes by a trained panel test (Kaur and Sharma 2013). Accordingly, *β*-carotene degradation in carrot puree and juice was very low, or even enhanced, due to higher extractability after such heat treatment as previously modeled (Lemmens et al. 2010; Marx et al. 2003; Quitão-Teixeira et al. 2009). Color changes of heat-treated carrot smoothies during storage were lower compared to untreated smoothies which may be owed to heat inactivation of color-degradative enzymes and reduced *β*-carotene degradation under such low storage temperature. Accordingly, only 5.5 % residual POD activity was reported in carrot juice after a similar heat treatment and it was even reduced to 2 % after 14 days at 4ºC (Quitão-Teixeira et al. 2009). Attending to pre-enrichment treatments, all smoothies from irradiated carrots shreds showed slightly higher color changes after 14 days of storage at 5ºC. Nevertheless, all heat-treated smoothies from stressed carrot shreds

(CTRL-HO, UVc-Air and UVc-HO) presented a good physicochemical quality after 14 days of storage at 5ºC.

3.2. Microbiological quality

Carrot shreds showed initial mesophilic, psychrophilic and Y+M loads of 5.4, 5.1 and 4.8 log CFU g^{-1}, respectively (Table 2). UVc pre-treatment reduced initial microbial loads of carrots shreds by 1.1-1.3 log units. Similar microbial reductions have been reported in Bimi® broccoli after a UVc dose of 4.5 kJ m^{-2} (Martínez-Hernández et al. 2011). The observed sanitation effect of UVc is due to the capacity of this non-ionizing radiation to induction the formation of pyrimidine dimers which distort the DNA helix and block microbial cell replication. Consequently, the cells become unable to repair their radiation-damaged DNA and die (Bintsis et al. 2000). Pre-enrichment incubation of shreds under air conditions led to mesophilic/psychrophilic and Y+M growth of 3.2/2.8 and 4.7 log units, respectively, after 72 h. However, pre-enrichment incubation of non-irradiated shreds under HO conditions greatly limited mesophilic/psychrophilic and Y+M growth to only 1.1/1.0 and 2.7 log units, respectively, after 72 h. Likewise, total viable counts were better controlled under hyperoxia (90 %) compared to air storage (Amanatidou et al. 2000). The observed microbicidal effects during hyperoxia storage may be explained by several factors such as the unfavorable effects on the oxidation–reduction potential of the system, the oxidation of enzymes having sulfhydryl groups or disulfide bridges, and the accumulation of injurious reactive O_2 species (Kader and Ben-Yehoshua 2000). The sanitizing effects of UVc radiation and hyperoxia storage in other fresh-cut fruit and vegetables have been previously reviewed (Artés et al. 2009). UVc irradiated shreds showed greater microbial growth compared to non-irradiated samples during pre-enrichment incubation. Latter detrimental effect of UVc

pre-treatment during storage, contrary to benefit from initial sanitation, may be explained by several hypothesis: 1) repair systems such as UV-induced enzymatic photorepair and expression of excision-repair genes that may restore DNA integrity in exposed microbial cells (Bintsis et al. 2000). Accordingly, those microorganisms with restored genetic material may show greater growth rates. 2) Pant cell disruption caused by UVc radiation leads to leakage of electrolytes (Martínez-Hernández et al. 2013) such as sugars which favors microbial growth.

In general, heat treatment reduced initial microbial loads of carrot smoothies (7-8 log units) below detection limits (1 log CFU mL^{-1} for mesophilic/psychrophilic and 2 log CFU mL^{-1} for Y+M). Accordingly, the applied heat treatment was enough to achieve pasteurization levels. Microbial loads of untreated smoothies were over 10 log CFU mL^{-1} after 7 days at 5 ºC (data not shown). Mesophilic counts of heat-treated carrot smoothies increased during storage registering final loads of 3.5-4.1 log CFU mL^{-1}, without significant differences among pre-treatments, after 14 days at 5ºC. Smoothies from UVc-HO and UVc-Air pre-treated shreds showed the highest psychrophilic growths with 4.7 and 3.5 log units increments, respectively, after 14 days at 5 ºC. Meanwhile, smoothies from CTRL-HO and CTRL-Air shreds registered psychrophiles increments of 2.9 and 1.4 log units, respectively, after 14 days at 5 ºC. As observed, psychrophilic growth in pasteurized smoothies was higher as the stress level from pre-enrichment incubation augmented following this order: HO>UVc>UVc+HO. Latter behavior may be explained since as the stress level increased surviving microorganisms after heat treatment acquired greater adaptation to grow under unfavorable conditions such as low temperature storage. Similarly, heat-treated smoothies from UVc-HO and UVc-Air shreds registered 0.8 and 1.3 log CFU mL^{-1} increments, respectively, while the other two pre-treatments did not register significant ($p<0.05$) changes after 14 days at 5

351 ºC. Salmonella spp., *Listeria monocytogenes* and generic *Escherichia coli* were
352 monitored throughout storage of smoothies meeting European legislation limits
353 (Comission Regulation (EC) No 1441/2007 2007). Phenolic acids are known to exhibit
354 antimicrobial activity against a variety of microorganisms (Wen et al. 2003). In the
355 same line, carrot juice have shown great antilisterial properties (Beuchat and Brackett
356 1990). Application of combined preservative factors (called hurdles) is used by food
357 industries according to the hurdle technology to achieve effective preservation of foods
358 (Leistner 2000). Consequently, the good microbiological quality (microbial loads<6 log
359 units) of all heat-treated carrot smoothies after 14 days of storage may be owed to the
360 combination of achieved acidic pH, enhanced antimicrobial compounds (phenolic acids)
361 and low storage temperature.
362
363 **3.3. Phenylalanine ammonia-lyase activity**
364 PAL is the key enzyme between primary (shikimate pathway) and secondary
365 (phenylpropanoid) metabolism pathways involved in the biosynthesis of polyphenolic
366 compounds (Dixon and Paiva 1995). Carrot shreds showed initial PAL activity of 12.5-
367 16.2 µmol cinnamic acid formed kg^{-1} h^{-1} fw (Table 3) similar to previous data
368 (Martínez-Hernández et al. 2016). UVc pre-treatment did not induce significant
369 ($p<0.05$) changes in the PAL activity of carrot shreds at day 0. PAL activity of shredded
370 carrots greatly increased after pre-enrichment period. CTRL-HO carrot shreds showed
371 the highest increments with a PAL activity of 224.9 µmol cinnamic acid formed kg^{-1} h^{-1}
372 fw after 72 h of pre-enrichment period. The rest of pre-treatments showed PAL
373 activities ranging from 86.2 to 102.7 after pre-enrichment period without significant
374 differences among them. PAL activation after wounding and hyperoxia storage has been
375 reported as an abiotic stress response being proposed ATP and reactive oxygen species

as signaling molecules (Jacobo-Velázquez et al. 2011). Fresh carrot smoothie from CTRL-HO shreds showed an initial PAL activity of 112.43 µmol cinnamic acid formed kg^{-1} h^{-1} fw while the activity of this enzyme ranged from 40.6 to 52.5 µmol cinnamic acid formed kg^{-1} h^{-1} fw or the rest of smoothies. Pasteurization of carrot smoothie greatly reduced PAL activity by 81-95 % without significant differences ($p<0.05$) among pre-treatments. Likewise, heat treatment (70ºC for 3 min) of vegetables red smoothies (pH 4.4) led to reductions of PAL activities of 65-70 % (Rodríguez-Verástegui et al. 2015). In general, PAL activity of smoothies decreased throughout storage registering final activities of 22.4/11.7 µmol cinnamic acid formed kg^{-1} h^{-1} fw for smoothies from CTRL-HO shreds while the rest of samples ranged among 1.0-5.7 µmol cinnamic acid formed kg^{-1} h^{-1} fw. PAL activation due to wounding stress occurred during smoothie preparation may be greatly reduced at low storage temperatures. Accordingly, PAL activity of red vegetables smoothies greatly incremented after 20 days at 5ºC (Rodríguez-Verástegui et al. 2015). Accordingly, no PAL activation was observed in the carrot smoothies in this storage period of 14 days at 5ºC. Thus, the decrease of PAL activity observed in the carrot smoothies throughout storage may be owed to the low storage temperature and acidic pH conditions as previously reported in PAL preparations (Gareth Rees and Hugh Jones 1996).

3.4. Phenolic compounds

Carrot shreds reported initial TPC of 187.3 mg CHA kg^{-1} fw (Table 3). Similar TPC have been reported for the same carrot cultivar (Alegria et al. 2010). The major individual phenolic compounds identified were 3-CQA, 3,5-CQA, 4,5-CQA and ferulic acid (Table 4). The phenolic contents of carrots were unchanged ($p<0.05$) immediately after UVc pre-treatment. The TPC of carrot shreds increased by approximately 2060,

1510, 1170 and 760 % in CTRL-HO, CTR-Air, UVc-Air and UVc-HO samples, respectively, after 72 h of pre-enrichment incubation. Postharvest abiotic stresses such as wounding, UVc radiation and hyperoxia storage have been reported to greatly increment the contents of phenolic compounds in carrots during subsequent storage (Alegria et al. 2012; Martínez-Hernández et al. 2011). This phenolic biosynthesis has been reported to be a consequence of PAL activation after these abiotic stresses being proposed ATP and reactive oxygen species as signaling molecules (Jacobo-Velázquez et al. 2011). UVc-HO showed the lowest phenolic accumulation during pre-enrichment incubation among the rest of treatments probably owed to a partial PAL denaturation by such UVc treatment delaying the stress-enhanced activity of this enzyme. The pre-enrichment incubation of carrot shreds allowed to obtain carrot smoothies with TPC of 710.4-1925.7 mg CHA kg^{-1} fw, representing 3-CQA the 87.3 % of the sum of phenolic compounds. 3-CQA, an ester of caffeic acid with quinic acid with great antioxidant capacity compared to other phenolic compounds, has been reported as the main phenolic compound in carrots (Castelluccio et al. 1995). The identified minor phenolic compounds 3,5-CQA, 4,5-CQA and ferulic acid accounted 7.8, 2.4 and 2.5 % of the sum of phenolic compounds, respectively. Heat treatment of carrot smoothies did not induce significant ($p<0.05$) changes of TPC or individual phenolic compounds. Consistently, no TPC changes were reported between untreated and heat-treated carrot purees and juices (Patras et al. 2009; Quitão-Teixeira et al. 2009).

The TPC of untreated smoothies registered a mild TPC increment of 10-25 % at day 7 showing the smoothie from CTRL-HO shreds the highest increment. This TPC increment at day 7 is in accordance to the ferulic acid and 3,5-CQA increments (Table 4) and to the greater PAL activity observed of these samples regarding the rest of smoothies (Table 3). However, heat-treated smoothies did not show the same behavior

426 at day 7. Similar phenolic increments have been reported in red vegetables smoothies
427 during low temperature storage (Rodríguez-Verástegui et al. 2015). Interestingly, great
428 TPC increments of approximately 610-850 % were registered in heat-treated smoothies
429 at day 14 comparing to their respective initial levels. Heat-treated smoothies from non-
430 irradiated air/HO shreds showed the highest TPC at day 14 with approximately
431 10960/13824 mg CHA kg^{-1} fw. This great TPC enhancement of non-irradiated samples
432 were due to 3-CQA and ferulic acids enhancements. The greater phenolic biosynthesis
433 observed in smoothies from air-incubated carrot shreds is in accordance to the still
434 higher PAL activities of these smoothies at day 14. However, PAL activity of those
435 samples at day 14 may not explain such great increments of phenolic compounds
436 observed in heat-treated smoothies. Accordingly, this enhanced biosynthesis of phenolic
437 compounds in heat-treated smoothies at day 14 may be owed to other enzymes different
438 to PAL involved in the phenylpropanoid pathway. Heat treatment (100 ºC for 45 s) of
439 carrots has been reported to induce TPC enhancements during subsequent storage of
440 carrot shreds at 5 ºC comparing to untreated samples (Alegria et al. 2012). Accordingly,
441 the heat treatment applied to the carrot smoothies could trigger signals related to other
442 enzymes different from PAL involved in the phenylpropanoid pathway although the
443 activation of these enzymes could be retarded until day 14 due to the low storage
444 temperature.
445
446 **3.5. Total antioxidant capacity**
447 The initial TAC of carrot shreds was 1102.3±97.3 mg Trolox equivalent kg^{-1} fw (Table
448 3). TAC increased during pre-enrichment incubation being highly correlated (R^2=0.90)
449 to TPC. Among pre-enrichment treatments, non-irradiated carrot shreds stored under
450 hyperoxia conditions registered the highest TAC enhancements as observed for TPC.

Similar high TAC-TPC correlations have been previously reported after wounding and hyperoxia storage of carrots (Jacobo-Velázquez et al. 2011). The specific antioxidant capacity (ratio of total antioxidant capacity over total soluble phenolics) has been proposed as a useful index to provide information of the effectiveness of phenolic compounds to neutralize free radicals (Cisneros-Zevallos 2003; Heredia and Cisneros-Zevallos 2009). A higher specific antioxidant capacity means phenolic compounds have a higher capacity to stabilize free radicals (Reyes et al. 2007). Irradiated samples showed higher specific antioxidant capacities compared to non-irradiated samples reporting UVc-HO shreds the highest value with 1010.7 µmol Trolox mg^{-1} ChAE. Similar specific antioxidant activity was reported by induced carrot phenolics after postharvest abiotic stresses (Cisneros-Zevallos 2003).

Smoothies from carrots without UVc pre-treatment showed the highest TAC levels with 1462.2 (hyperoxia) and 1343.8 (air) µmol Trolox mg^{-1} ChAE. On the other side, smoothies from irradiated carrots showed lower TAC levels with 829.1 (hyperoxia) and 1056.9 (air) µmol Trolox mg^{-1} ChAE. Similar to TPC, heat treatment of carrot smoothies did not induce significant ($p<0.05$) changes of TAC. TAC of smoothies increased by 64-227 % after 14 days of storage at 5 ºC. The greatest TAC increments after 14 days in un-heated smoothies was observed in those samples from non-irradiated carrots. However, the highest TAC increments in heat-treated smoothies were registered by UVc pretreated samples. Latter behavior may be explained since UVc pre-treatment was able to compensate subsequent reduction of activities of enzymes involved in the phenylpropanoid pathway after heat treatment.

5. Conclusions

Carrot is a vegetable highly consumed which low phenolic levels could be naturally increased leading to a phenolic/antioxidant enriched plant material to produce a functional carrot smoothie. The phenolic levels of shredded carrots used for the smoothie preparation were greatly enhanced after pre-enrichment incubation for 72 h at 15 ºC up to 2060 % in those non-irradiated shreds stored under hyperoxia conditions. The total antioxidant capacity was highly correlated to total phenolic content. The high temperature-short time heat treatment reduced microbial loads below the detection limits with low growth during subsequent refrigerated storage. The physicochemical quality was good for all smoothies at the end of storage. UV-C pretreatment of carrot shreds resulted in carrot smoothies at the end of storage with higher psychrophilic and yeasts and molds loads and lower phenolic levels. Accordingly, pre-enrichment incubation of carrot shreds under hyperoxia conditions allowed to obtain a functional smoothie with great phenolic levels and good microbiological and physicochemical quality up 14 days at 5 ºC.

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693 **Figure and table captions**

694 **Figure 1.** Flow diagram of pre-enrichment incubation of carrots, smoothie preparation
695 and storage conditions.

696

697 **Table 1.** pH, titratable acidity (TA; %), soluble solids content (SSC; º Brix), total color
698 differences (ΔE) and browning index differences (ΔBI) changes of carrot shreds after
699 different treatments (hyperoxia, UVc radiation and controls) during pre-enrichment
700 incubation (72 h at 15 ºC) and subsequent storage at 5 ºC of prepared non-treated or
701 heat-treated (90 ºC for 30 s) smoothie (n=5±SD). Different capital letter denotes
702 significant differences ($p < 0.05$) among different treatments for the same sampling day.
703 Different lowercase letter denotes significant differences ($p < 0.05$) among different
704 sampling days for the same treatment.

705

706 **Table 2.** Mesophilic, psychrophilic and yeasts and molds counts (log CFU g^{-1}) of carrot
707 shreds after different treatments (hyperoxia, UVc radiation and controls) during pre-
708 enrichment incubation (72 h at 15 ºC) and subsequent storage at 5 ºC of prepared non-
709 treated or heat-treated (90 ºC for 30 s) smoothie (n=5±SD). (n=5±SD). Different capital
710 letter denotes significant differences ($p < 0.05$) among different treatments for the same
711 sampling day. Different lowercase letter denotes significant differences ($p < 0.05$)
712 among different sampling days for the same treatment.
713
714 **Table 3.** Phenylalanine ammonia-lyase activity (PAL; µmol *t*-cinnamic acid synthesized
715 kg^{-1} fw h^{-1}), total phenolic content (TPC; Chlorogenic acid equivalent kg^{-1} fw) and total
716 antioxidant capacity (TAC; mg Trolox equivalents kg^{-1} fw) of carrot shreds after
717 different treatments (hyperoxia, UVc radiation and controls) during pre-enrichment
718 incubation (72 h at 15 ºC) and subsequent storage at 5 ºC of prepared non-treated or
719 heat-treated (90 ºC for 30 s) smoothie (n=5±SD). (n=5±SD). Different capital letter
720 denotes significant differences ($p < 0.05$) among different treatments for the same
721 sampling day. Different lowercase letter denotes significant differences ($p < 0.05$)
722 among different sampling days for the same treatment.
723
724 **Table 4.** Individual phenolic contents (mg kg^{-1} fw) of carrot shreds after different
725 treatments (hyperoxia, UVc radiation and controls) during pre-enrichment incubation
726 (72 h at 15 ºC) and subsequent storage at 5 ºC of prepared non-treated or heat-treated
727 (90 ºC for 30 s) smoothie (n=5±SD). (n=5±SD). Different capital letter denotes
728 significant differences ($p < 0.05$) among different treatments for the same sampling day.
729 Different lowercase letter denotes significant differences ($p < 0.05$) among different
730 sampling days for the same treatment.

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