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Daniel A. Jacobo-Velázquez, Carmen Hernández-Brenes

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Stability of avocado paste carotenoids as affected by high hydrostatic pressure processing and storage

Daniel A. Jacobo-Velázquez[a] *and Carmen Hernández-Brenes*[a,*]

[a] Department of Biotechnology and Food Engineering, School of Biotechnology and Food. Tecnológico de Monterrey-Campus Monterrey. E. Garza Sada 2501 Sur, C.P. 64849, Monterrey, N.L., México

Running head: Stability of carotenoids in HHP treated avocado.

*To whom correspondence should be addressed: Dr. Carmen Hernández-Brenes, Tecnológico de Monterrey-Campus Monterrey. E. Garza Sada 2501 Sur. Centro de Biotecnología-FEMSA 305-A., C.P. 64849, Monterrey, N.L., Mexico. Tel.: +52-818-358-14-00 Ext. 4817. Fax: +52-818-328-4136. E-mail: chbrenes@itesm.mx

ABSTRACT

When compared to thermal and chemical alternatives, high hydrostatic pressure (HHP) processing is the most effective non-thermal technology to process avocados. Herein we report the effects of HHP-processing (600 MPa, 3 min) and storage (40 days, 4°C) on the stability of avocado paste (*Persea americana*, cv.Hass) carotenoids. Likewise, the effects of HHP-processing and storage on hydrophilic and lipophilic oxygen radical absorbance capacities (ORAC) of the product were studied. Pressurization induced a significant increase (aprox. 56%) in concentrations of total extractable carotenoids. Highest increases for individual carotenoids were observed for neoxanthin-b (513%), followed by α-cryptoxanthin (312%), α-carotene (284%), β-cryptoxanthin (220%), β-carotene (107%), and lutein (40%). Carotenoid levels declined during storage, but at the end of the product's sensory shelf-life were higher than those initially present in unprocessed avocado paste. Interestingly, ORAC-values followed a different trend than carotenoids; they decreased immediately after HHP-processing and increased during storage, therefore indicating that carotenoids appear to be minor contributors to the total antioxidant capacity of the fruit.

Highlights

- A chemical characterization and quantification of carotenoid profiles for the widely consumed avocado cultivar *Persea americana* cv. Hass was performed, determining new carotenoids not previously reported for the same.
- High hydrostatic pressure (HHP) processing (600 MPa for 3 min) of avocado paste, under commercial conditions, caused a significant increase (~56%) in the concentration of total carotenoids extracted from avocado paste immediately after processing.
- Stability of carotenoid profiles of the HHP processed avocado paste matrix during refrigerated storage were also studied for 40 days; indicating that avocado lipoxygenase type II isozymes

appear to be responsible for the observed degradations in avocado total carotenoid contents during storage (aprox. 50% lower than freshly processed samples).

At the end of the sensory shelf-life of the product (20 days) the contents of total carotenoids in the processed avocado paste were similar to the values of samples immediately after processing; indicating that the nutritional and nutraceutical value, based on carotenoids and antioxidant capacity, of HHP-processed avocado paste processed under commercial conditions paste was retained.

Keywords: Hass avocado; High hydrostatic pressure (HHP) processing; Carotenoids; Oxygen Radical Absorbance Capacity (ORAC) value.

1. Introduction

Avocado (*Persea americana*) is an important source of bioactive molecules that protect human cells against the detrimental effect of free radicals. The main antioxidants present in avocado pulp are the oxygenated carotenoids (xanthophylls, mainly lutein) (Heinonen, Ollilainen, Linkola, Varo, &

Koivistoinen, 1989; Lu, Arteaga, Zhang, Huerta & Heber, 2005), as well as vitamins C, and E (Bergh, 1992). In addition, avocado contains persenone A and B which are bioactive molecules that protect against inflammation and carcinogenesis (Kim et al., 2000). According to the American Diet Association (ADA), avocado can be classified as a functional food due to its high nutritional value and proven beneficial effects for human health (ADA, 1999). Previous reports have shown that avocado pulp can be used to reduce LDL cholesterol (Colquhoun, Moores, Somerset, & Humphries, 1992), as well as to improve cardiovascular health (Bergh, 1992), prevent cancer (Lu et al., 2005), and to control blood glycemic levels (Lerman-Garber, Ichazo-Cerro, Zamora-Gonzalez, Cardoso-Saldana, & Posadas-Romero, 1994). Lu et al. (2005) demonstrated that an acetone extract from Hass avocado inhibited the growth of androgen-dependent (LNCaP) and non-androgen-dependent (PC-3) cancer cells. The authors attributed the observed bioactivity to the high contents of lutein and vitamin E (α-tocopherol and γ-tocopherol) present in the extract. In addition, avocado contains high levels of monounsaturated fatty acids. Therefore, carotenoids present in the avocado food matrix are better absorbed in the gastrointestinal tract (Unlu, Bohn, Clinton, & Schwartz, 2005).

High hydrostatic pressure (HHP) has been pointed as the most effective technology to stabilize and extend the shelf-life of avocado pulp (López-Malo, Palou, Barbosa-Cánovas, Welti-Chanes, & Swanson, 1998; Jacobo-Velázquez & Hernández-Brenes, 2010a; Jacobo-Velázquez, Ramos-Parra, & Hernández-Brenes, 2010b; Jacobo-Velázquez & Hernández-Brenes, 2011). Daily approximately 80 tons of avocados are HHP processed in Mexico alone. Therefore at the present, commercially processed avocado paste is being exported for human consumption around the world with acceptable microbiological and sensory stabilities during its refrigerated storage. However, there are no reports in literature on the effects of HHP processing under commercial conditions and its subsequent refrigerated storage time on the stability of nutrients and bioactive compounds present in avocados. It is well known that HHP processing has little effect on low molecular weight molecules when evaluated immediately after pressurization, contributing to the conservation of vitamins and pigments in fruits and vegetables

when compared with thermal processing (Polydera, Stoforos, & Taoukis, 2004; Pandrangi & Balasubramaniam, 2005). Prior work on the stability of fruit nutrients after HHP processing, showed in some instances higher or lower concentrations of bioactive molecules immediately after HHP processing, indicating a potential effect on the bioavailability of the compounds (De Ancos, González, & Cano, 2000; Kim, Park, Cho, & Park, 2001; Fernández-García, Butz, & Tauscher, 2001; Sánchez-Moreno, Plaza, De Ancos, & Cano, 2003).

The main deterioration mechanisms of HHP processed avocado paste (Hass variety) have been previously reported by Jacobo-Velázquez et al. (2010a). The authors observed a pH decline during the refrigerated storage of HHP processed avocado paste, as well as the activation of lipoxygenase (LOX) enzyme during storage. Biochemical changes that were correlated by the authors with increases in the sensory perception of sour and rancid flavors during the refrigerated storage of HHP treated avocado pastes (Jacobo-Velázquez et al., 2011). In addition it has been previously observed that avocado contains LOX type II isoenzymes (Jacobo-Velázquez, Hernández-Brenes, Cisneros-Zevallos, & Benavides, 2010c), which are capable of catalyzing the degradation of carotenoids through a co-oxidation mechanism (Robinson, Wu, Domoney, & Casey, 1995). Based on this information, for this study we hypothesized that carotenoids present in HHP processed avocado paste although are not destroyed immediately after processing could be degraded by LOX II isoenzymes during the subsequent product storage period.

The objectives of the present project therefore were to evaluate the stability of avocado paste carotenoid profiles during HHP processing and subsequent refrigerated storage. In addition, the changes in the hydrophilic and lipophilic oxygen radical absorbance capacity (ORAC) of avocado paste were also determined after processing and during storage in order to increase the knowledge on the processing and storage stability of other avocado bioactive molecules such as the phenolic compounds, vitamins C, and E.

2. Materials and methods

2.1. Fruit samples and chemicals

Avocados (*Persea americana* Mill, cv. Hass) used in the study were obtained from the Mexican region of Michoacán. Butylated hydroxytoluene (BHT) was obtained from Spectrum Quality Products (New Brunswick, NJ., USA). All-*trans*-β-carotene, β-apo-8´carotenal, ascorbic acid, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), 2,2'-azobis (2-amidinopropane)-dihydrochloride (AAPH), and fluoresceinsodium salt (FL) were obtained from Sigma-Aldrich Co. (St. Louis, MO., USA). Lutein was purchased from Indofine Chemical Co. (Hillsborough, NJ., USA). Randomly methylated β-cyclodextrin (RMCD) was obtained from Fisher Scientific Int. (Winnipeg, MB., Canada). Potassium hydroxide (KOH), sodium chloride (NaCl), acetone and hexane were purchased from Desarrollo de Especialidades Químicas (San Nicolás de los Garza, NL., México). Methyl tert-butyl ether (MTBE, HPLC grade), and water (HPLC grade) were purchased from Fisher Scientific Int. (Winnipeg, MB., Canada). Methanol (HPLC grade) was obtained from Fermont-Productos Químicos Monterrey (Monterrey, NL., México) and isopropyl alcohol (HPLC grade) was obtained from Omnisolv (Gibbstown, NJ., Darmstad, Germany).

2.2. Samples preparation, high hydrostatic pressure (HHP) processing and storage conditions

Avocados were washed, sanitized, peeled and manually macerated into avocado paste as previously described (Jacobo-Velázquez et al., 2010a; 2010b; 2011). Prior to packaging, the paste was vacuum de-aerated (-88.2 kPa) with an Ultravac UV2100A pump (Koch Equipment LCC, Kansas, City, MO, USA). Individual avocado paste samples (200 g) were packaged into oxygen impermeable plastic bags (14 cm length x 12.6 cm width x 3 cm height) of a thickness of 0.018 cm. Vacuum packaging (-67.9 kPa for 2 s) was performed with a Multivac R230 model 542 packaging machine (Multivac, Wolfertschwenden, Germany), and placed in cold water (1-3°C for 20 min), prior to processing, to reach an internal temperature of ~5°C.

Avocado paste packages were commercially processed at Avomex International S.A de C.V. (Sabinas, Coahuila, México). Samples were pressurized at 600 MPa for 3 min, using a 215L ULTRA HHP processing unit (Avure Technologies, Kent, WA, USA). The time required to reach 600 MPa was 3.5 min. The decompression time was 2.75 min. Purified water was used as pressurization media. Samples were pressurized at a vessel temperature of 23°C. Processing conditions were closely monitored and recorded to assure experimental reproducibility. After processing, the samples were placed in a cold water bath (1 to 3°C) and transported under refrigeration (4°C) to the Biotechnology Center (Centro de Biotecnología-FEMSA) at Tecnológico de Monterrey (Monterrey, NL, México). Avocado paste samples (27 plastic bags, 200 g each) were stored under refrigeration conditions (4±1°C) for 40 days and sampled before and after HHP processing, and every 5 days during the storage period.

2.3. Lipoxygenase (LOX) activity assay

LOX activity was determined by the method previously described by Anthon & Barret (2003), and adapted for avocado as described by Jacobo-Velázquez et al., (2010), which consisted in monitoring the formation of conjugated dienes from linoleic acid, using linoleic acid as the substrate for the enzyme extract. The formation of conjunated dienes from linoleic acid was monitored spectrophotometrically as 234 nm. In order to quantify the residual activity of LOX, the activity was determined in the fresh avocado paste (non-processed) and in the processed product (time 0 days).

2.4. Dissolved oxygen concentration

Dissolved oxygen concentration inside of the packed HHP treated avocado paste was determined with an YSI electrode model 55112FF (YSI Inc., Yellow Spring, Ohio, USA.). The electrode was placed in the center of the HHP processed product through a small incision in the corner of the plastic bags, avoiding the transfer of oxygen from the environment to the product. The measurements were conducted by triplicate and the concentration of O_2 was expressed as μg of O_2 per g of avocado paste in fresh weight (FW).

2.5. Identification and quantification of avocado carotenoids by HPLC-DAD

2.5.1. Extraction of carotenoids and saponification

Avocado carotenoids were extracted following the procedure described by Lu et al. (2005) with slight modifications. Extractions were performed at room temperature (≈20°C) and under dark conditions. BHT was added to the solvents (0.1% w/v) used for the extractions. Avocado paste (2 g) was added with β-apo-8´carotenal (8 ppm) as an internal standard. The avocado paste samples with the internal standard added were homogenized with acetone (10 mL) using an Ultra-Turrax homogenizer (IKA Works, Inc., Wilmington, NC) and placed in an ultrasonic bath (Branson 1510, Branson Ultrasonic Corp., Danbury, CT, USA) for 2 min. The homogenates were then filtered under vacuum through Whatman No. 1 filter paper (New Jersey, USA) to obtain the acetone extract. This procedure was repeated three times to ensure the complete extraction of carotenoids present in the samples. The acetone extracts were pooled and concentrated in a centrifugal evaporator SpeedVac® SC210A (Thermo Electron Corporation, Milford, MA., USA), operating at 37°C for approximately 1 h until acetone was completely evaporated from the extract.

The concentrated acetone extracts were saponified at room temperature for 10 h with a 9M KOH solution (2 mL) prepared in an ethanol-water solution (50%). Saponification was performed in closed vials with nitrogen in the headspace. The saponified extracts were then diluted with bidistillated water (10 ml) and transferred to separatory funnels where 3 consecutive extractions with hexane (12 mL) were carried out. Each extraction was performed by manually shaking the separatory funnel for 1 min. The aqueous fractions were discarded and the hexane fraction were successively washed (approximately 10 times) with bidistillated water (10 mL) until neutrality was achieved in the hexane fraction (phenolphthalein was used as indicator). In those cases where an emulsion between the hexane and aqueous fraction was observed, a 30% (p/v) NaCl solution (0.5 mL) was added to destabilize the emulsion. The procedure was repeated until no turbidity was observed at the interface. The neutralized hexane fraction was recovered and the solvent was concentrated in a centrifugal evaporator operating at 37°C for approximately 1 h. The remaining solvent was evaporated to dryness with a continuous flow of

nitrogen. The dried samples were re-suspended with isopropyl alcohol (1 mL) and passed through polytetrafluoroethylene (PTFE) membranes (0.45 µm) prior to injection to the chromatographic system.

2.5.2. Analysis of avocado carotenoids by HPLC-PDA

The qualitative and quantitative analysis of carotenoids was performed by high performance liquid chromatography with photodiode array detector (HPLC-PDA) according to the procedure described by Pott, Marx, Neidhart, Mühlbauer, & Carle (2003) with slight modifications. The HPLC system used was composed of two 515 binary pumps, a 717-plus autosampler, and a 996-photodiode array detector (Waters Corp., Mildford, MA). Carotenoids were separated on a 4.5 mm x 250 mm, 5 µm, C-30 reverse-phase column (YMC carotenoid, Wilmington, USA). The mobile phases consisted of methanol/MTBE/water (81:15:4, v:v, phase A) and MTBE/methanol/water (90:6:4, v:v, phase B). The gradient solvent system was 0/100, 90/25, 100/100, 140/100 (min/% phase A) at a flow rate of 1 mL/min. Chromatographic data was processed with the Millennium software v3.1 (Waters Corp., Mildford, MA).

2.5.3. Preparation of standard curves

For the quantification of carotenoids, standard curves of all-*trans*-β-carotene, β-apo-8´carotenal, and lutein were prepared. To prepare the standard curves, stock solutions of all-*trans*-β-carotene and β-apo-8´carotenal were prepared in hexane whereas a stock solution of lutein was prepared in ethanol. The concentrations of each carotenoid in the stock solutions were determined spectrophotometrically using the extinction coefficient $A_{1cm}^{1\%}$ for each compound ($A_{1cm}^{1\%}$ = 2560 at 450 nm for all-*trans*-β-carotene; $A_{1cm}^{1\%}$ = 2470 at 447 nm for β-apo-8´carotenal; $A_{1cm}^{1\%}$ = 2550 at 445 nm for lutein). Once the concentration of each standard in the stock solutions was determined they were mixed to obtain a solution containing the three standards at a concentration of 15 ppm each. The solvents of the solution with 15 ppm of each compound were evaporated to dryness with a centrifugal evaporator and re-suspended with isopropyl alcohol (HPLC grade). The carotenoid mixtures re-suspended in isopropyl alcohol were diluted to

obtain final concentrations of 12, 9, 6, 4, 3, 2, 1 and 0.5 ppm. The dilutions were passed through polytetrafluoroethylene (PTFE) membranes (0.45 µm) prior to injection to the HPLC.

2.5.4. Interpretation of UV-Visible spectra

The identification of avocado carotenoids was performed by three different procedures depending on the information available for each chromatographic peak. The methods of identification were: (a) identification by interpretation of UV-Visible spectra characteristics and comparison with previous reports (De Ancos et al., 2000; Chen, Peng, & Chen, 1996; Mercadante, Rodríguez-Amaya, & Britton, 1997; Lee, 2001); (b) identification by comparison with retention time and UV-Visible absorption spectra characteristics of commercial standards; (c) identification by order of elution reported in the literature for the same chromatographic conditions (Lee, 2001; Meléndez-Martínez, Britton, Vicario, & Heredia, 2005; Waters, 2005).

2.6. Determination of hydrophilic and lipophilic oxygen radical absorbance capacity (ORAC)

The hydrophilic (H-ORAC) and lipophilic (L-ORAC) antioxidant capacity of avocado paste were determined with the procedure reported by Prior et al. (2003). The samples were lyophilized in a VirTis Free Mobile Serie freeze dryer (SP Industry, Inc. Gardiner, NY, USA). The lyophilized samples (1 g) were homogenized with hexane (5 mL) using a vortex (2 min at room temperature). The homogenates were then placed in an ultrasonic bath for 2 min and filtered under vacuum with Whatman filter paper No. 1 to recover the hexane extract. This procedure was repeated three times to assure the complete extraction of the lipophilic fraction. The hexane extracts were pooled and placed in a water bath at 30°C and the solvent was evaporated with a continuous flow of nitrogen. The extracts were dissolved in acetone (10 mL) and used to determine the L-ORAC.

The solid residues recovered in the filter paper were re-extracted with an acetone/water/acetic acid (70:29.5:0.5, AWA) solution, following similar procedures described for the lipophilic extractions. Briefly, residues were homogenized with AWA (5 mL) using a vortex (2 min at room temperature). The homogenates were then placed in an ultrasonic bath for 2 min and filtered under vacuum with a

Whatman filter paper No. 1 to recover the AWA extract. This procedure was repeated three times to assure the complete extraction of the hydrophilic fraction. The extracts were pooled, diluted with AWA solution to a final volume of 25 mL and used to determine the H-ORAC.

The determinations of the H-ORAC and L-ORAC were performed in a microplate reader Synergy HT (Bio-Tek® Instruments, Winooski, Vermont, EUA.) equipped with temperature control and two injector pumps. The equipment was programmed to maintain the temperature of the assay constant at 37°C. Calculations for the ORAC values were obtained following the procedure described by Prior et al. (2003).

2.7. Statistical analysis

Analyses were performed using three replicates. Data represents the mean value of samples and bars indicate their standard error. Analyses of variance (ANOVA) were conducted using JMP software version 5.0 (SAS Institute Inc. Cary, NC, USA) and mean separations performed using LSD test ($p < 0.05$).

3. Results and discussion

3.1. Avocado paste lipoxygenase (LOX) activity as affected by high hydrostatic pressure (HHP) processing

Pressurization of avocado paste at 600 MPa for 3 min resulted in residual LOX activity levels of ~44%, in reference to the unprocessed samples. Castellanos-Dohnal (2005) reported similar results (~43% of residual LOX activity) in avocado pastes processed under the same HHP conditions. In addition, Jacobo-Velázquez et al., (2010a) reported a LOX residual activity of ~55% after pressurization of avocado paste, also under the same HHP commercial conditions (600 MPa for 3 min). Interestingly, the authors observed reactivation of the enzyme during the refrigerated storage of samples processed under the same conditions. LOX type II isoenzymes, which are present in avocados, are capable of catalyzing the degradation of carotenoids through a co-oxidation mechanism (Robinson et al., 1995;

Jacobo-Velázquez et al., 2010c). However, LOX is capable of carotenoid co-oxidation only when dissolved O_2 is present in the food matrix. Therefore, in the present study we deemed relevant to determine the concentration of dissolved O_2 in the avocado paste packaged under vacuum and treated with HHP. Results indicated that avocado paste samples contained ~0.15 ppm of dissolved oxygen, demonstrating that O_2 was present in the HHP treated avocado paste product and thus available for LOX catalyzed oxidative reactions.

3.2. Carotenoid analyses

The identification and quantification of individual carotenoids in the acetone extracts of avocado paste were performed for unprocessed (Fig. 1A) and HHP processed samples (Fig. 1B). Similar individual carotenoid chromatographic profiles were obtained for both samples. The tentative identifications of the individual carotenoids present in the avocado Hass cultivar are reported in Table 1. The identity was assigned based on the absorption maxima of each chromatographic peak by comparison with the absorption maxima of commercial standards and prior literature data. Although the retention times are variable in HPLC methodologies, the order of elution of the individual carotenoids was considered as an additional criterion for identity when prior publications used the same chromatographic conditions (Lee, 2001; Meléndez-Martínez et al., 2005; Waters, 2005).

The carotenoids identified in the unprocessed avocado paste included lutein, β- and α- carotene, β- and α- cryptoxanthin, luteoxanthin isomers, neoxanthin, zeaxanthin, and two other possible xanthophylls (Fig. 1). The carotenoid that showed highest concentration in the avocado pulp was the lutein (~56% of total). These results are consistent with previous reports on the identification of the main carotenoids present in Hass avocados (USDA-NCC, 1998) (Table 1). However, in the present study, we identified and quantified additional carotenoids not previously reported for the Hass cultivar that included neoxanthin a and b, luteoxanthin isomers a and b, and α- cryptoxanthin (Fig. 1, peaks 1, 2, 3, 4, and 8, respectively). At same, the presence of the newly observed carotenoids has also been reported for the Nabal avocado cultivar (Gross, Gabai, & Lifshitz, 1972; 1973; 1974). The additional

carotenoids detected in the present study can be attributed to the stationary phase used. Herein a C-30 reverse phase column was used to separate and identify carotenoids by HPLC-PDA, whereas in previous reports a C-18 reverse phase was used. The use of the C-30 column increased the selectivity and thus showed a higher capacity to separate possible stereoisomers of the carotenoids present in avocado matrix (Su, Rowley, & Balazs, 2002). In addition, it has been reported that the maturity index and the harvest season can also affect the carotenoid profiles and their relative concentrations in the fruit as it also affects other metabolites present (Gross et al., 1974; Slater, Shankman, Shepherd, & Alfin-Slater, 1975; Lu et al., 2005).

3.3. Concentration of total and individual carotenoids as affected by high hydrostatic pressure (HHP) processing

The concentration of total carotenoids in the HHP processed avocado samples was ~56% higher when compared to the unprocessed sample (Fig. 2). Similar results have been published for orange juice and caqui fruit, in which the authors observed increases of 9-43% in total carotenoids extracted after HHP processing (De Ancos et al., 2000; De Ancos, Sgroppo, Plaza, & Cano, 2002). It also has been observed that the extractability of carotenoids increases as the pressure treatments intensify (De Ancos et al., 2000; 2002; Sánchez-Moreno et al., 2003). High pressure treatments are known to induce morphological changes in plant cells, which result in the rupture of cell membranes and subsequent leaching of intracellular constituents into the food matrix, facilitating the extraction of compounds such as the carotenoids (Shimada et al., 1993; Cheftel, 1995; Torres & Velázquez, 2005). In addition, it is well known that pressurization affects the structure of macromolecules, including proteins (Cheftel, Hendrickx, Ludikhuyze, Van den Broeck, & Weemaes, 1998; Butz & Tauscher, 2002; Butz et al., 2003). In plants, carotenoids are associated to proteins located in the cell membrane surface. Therefore, it is likely that the HHP induced modifications of proteins structure may result in the destruction of protein-carotenoid complexes, facilitating the extractions of carotenoids from the food matrixes (De Ancos et al., 2002; Sánchez-Moreno et al., 2003).

The concentrations of individual carotenoids before and after HHP processing are shown in Table 2. The individual carotenoid that showed the highest percent increase in concentration was neoxanthin b (513%), followed by α- cryptoxanthin (312%), α-carotene (284%), β-cryptoxanthin (220%), β-carotene (107%) and lutein (40%). Zeaxanthin was the carotenoid found in the lowest levels in unprocessed avocado paste and was not detected in the HHP processed samples (t_0, Table 2). HHP processing also decreased by 16% the concentration of an unidentified carotenoid peak labeled as a possible xanthophyll b (Fig. 1A, peak 9). The differences in pressure-induced changes, for individual compounds, can be attributed to the degree of association of each individual carotenoid to their cell membrane binding proteins (Sánchez-Moreno et al., 2003). The observed increases in the extractability of carotenoids caused by HHP processing may have a positive impact on the absorption of the compounds during human digestion, enhancing their bioavailability (De Ancos et al., 2000; 2002; Cheftel 1995). However, to test these hypothesis further pharmacokinetic studies on digestion models with HHP processed samples are needed.

3.4. Concentration of total and individual carotenoids as affected by storage time

The concentration of total carotenoids in the product remained constant during the first 10 days of refrigerated storage (Fig. 2). A similar behavior was observed for most individual carotenoids (Table 2). However, some carotenoids such as zeaxanthin, β– and α- cryptoxanthin, β– and α- carotene showed a significant increase in concentration, reaching their maximum percent rises (125%, 418%, 526%, 269%, and 186%, respectively) at 10 days of storage (t10) and in reference to un-processed samples. The increased concentrations observed for certain carotenoids during the first days of storage of HHP processed samples supports the hypothesis formulated by Castellanos-Dohnal (2005) on the gradual release of compounds from the interior of the cell to the matrix of pressurized foods during their storage. It is likely that HHP processing affects the structure of chloroplast (organelles that contains the carotenoids) and the permeability of the cell membrane, facilitating the release of the molecules during the subsequent product storage period (Préstamo & Arroyo, 1998; Gonzalez, Anthon, & Barret, 2010).

At 15 days of storage, the concentration of total carotenoids in the HHP treated avocado paste showed a significant decline (≈33%, $p < 0.05$) (Fig. 2). A previous publication on HHP avocado paste, identified the residual activity of LOX and its reactivation after 15 days of storage as one of the principal deterioration mechanism of the product during its shelf-life (Jacobo-Velázquez et al., 2010a). Avocado LOX is a type II isozyme, which is capable to catalyze the degradation of carotenoids through a co-oxidation mechanism (Jacobo-Velázquez et al., 2010c). Therefore, the decreased concentration of carotenoids observed at 15 days of storage in the present study, appears to be related with the reactivation of LOX enzyme previously reported. Interestingly, the concentration of total carotenoids increased at 20 days suggesting further release of compounds from the interior of the cells to the food matrix during storage. However, in the subsequent days of storage, the concentration of carotenoids in the product decreased reaching a minimum value that was ~50% lower at 40 days of storage, when compared to the time 0 storage day samples.

Regarding the concentration of individual carotenoids during the complete storage period, results demonstrated that certain compounds such as zeaxanthin, neoxanthin a, α-carotene, and luteoxanthin isomers a and b, were almost completely degraded (Table 2). However, other carotenoids such as lutein and β-carotene showed small degradation percentages (20% and 24%, respectively) after 40 days of storage when compared to the freshly processed samples of day 0, indicating that these compounds were less susceptible to oxidation during storage. Pérez-Gálvez & Mínguez-Mosquera (2001) reported that the stability of carotenoids present in pepper (*Capsicum annuum L.)* was related to the chemical structure of the carotenoids and was also affected by the nature and concentration of other oxidizing molecules in the samples. The authors determined the rate of degradation of individual carotenoids using mono and poli-unsaturated fatty acids as the oxidizing agents and concluded that the rate of degradation of each carotenoid due to mechanism of auto-oxidation (non-enzymatic) was directly related to the stability of the peroxi-carotenoids intermediates produced. The higher the stability of those intermediates, the lower the rate of degradation of carotenoids. Pérez-Gálvez & Mínguez-Mosquera

(2001) found that capsanthin and capsorubin, two of the major carotenoids in pepper, showed lower degradation than other carotenoids present in the pepper tissue such as β–carotene and certain xanthophylls. The observed differences were attributed to the functional groups present in each compound. In the particular case of capsanthin and capsorubin, the compounds contain ketone groups which give major stability to the peroxi-carotenoids intermediates retarding the process of auto-oxidation.

In the present study, the carotenoids identified in Hass avocados showed different degrees of stability during the refrigerated storage period of the HHP processed samples (Table 2), which also can be attributed to differences in their chemical structures. Pérez-Gálvez & Mínguez-Mosquera (2001) found that carotenoids are less susceptible to degradation when the substrates of oxidation are monounsaturated fatty acids. Avocados are known as rich sources of monounsaturated fatty acids, particularly oleic acid (Hu, 2003; Chong, Sinclair, & Guymer, 2006). Therefore, it is possible that the avocado matrix itself is beneficial for carotenoid stability protecting them against non-enzymatic oxidative processes due to the nature of its lipids. Consequently, the observed degradation of carotenoids of avocado paste during storage is likely due to the enzymatic action of LOX rather than processes of auto-oxidation. Therefore, the differences in the decrease in concentration of individual carotenoids observed during storage are possibly related to the susceptibility of each compound of being co-oxidized by LOX enzyme.

If we consider the previously calculated sensory shelf-life of 20 days for avocado paste processed and stored under the same conditions as the present study (Jacobo-Velázquez et al., 2010b; Jacobo-Velázquez et al., 2011), the concentration of total carotenoids remains similar to the levels contained in the freshly processed samples (day 0 storage time). At that particular point in the product's shelf-life (20 days of storage), HHP processing offered a potential nutritional benefit since the carotenoid levels were higher (56%) than those found in the unprocessed avocado paste. In order to decrease LOX reactivation levels during storage and retain even higher concentrations of carotenoids during storage; it would be

interesting to perform further work on the optimization of the processing conditions to completely inactivate LOX enzymatic activity after processing as previously reported by Palou et al., (2000) for guacamole (avocado paste salted and acidulated).

3.5. Hydrophilic and lipophilic antioxidant activity of avocado paste as affected by high hydrostatic pressure (HHP) processing

The total antioxidant activity of the matrix, estimated as the sum of the ORAC values for the hydrophilic (H-ORAC) and lipophilic (L-ORAC) fractions of the unprocessed avocado paste was 124 μmol of trolox equivalments (TE) per g of product dry weight (Fig. 3 A & B). The L-ORAC value for unprocessed avocado paste (L-ORAC = 111 μMol of TE per g dry weight) as shown in Fig. 3A was higher than the H-ORAC value (Fig. 3B). Avocado fruit contains important concentrations of vitamin E (Bergh, 1992), persenone A and B (Kim et al., 2000), and carotenoids (Heinonen et al., 1989; Lu et al., 2005), which are non-polar compounds that are possibly contributing to the observed lipophilic antioxidant activity. The H-ORAC value of the unprocessed avocado fruit was ~13 μMol of TE per g dry weight. The hydrophilic antioxidant activity of avocado can be attributed mainly to phenolic compounds (Vinson, Su, Zubik, & Bose, 2001; Wu et al., 2004; Jacobo-Velázquez & Cisneros-Zevallos, 2009) and vitamin C (Bergh, 1992) previously known to be present in the pulp. H-ORAC and L-ORAC values obtained in the present study were different than those reported by Wu et al. (2004) for Hass avocados. The H-ORAC value reported by the authors was higher than the one found in the present study, whereas the L-ORAC value was much lower (19.71 μMol of trolox equivalments per g dry weight) (Wu et al., 2004). These differences can possibly be attributed to changes in the maturity index of the fruit and the season of harvest, factors that induce variability in the composition of the fruit (Slater et al., 1975).

As it can be observed in Fig. 3 A&B, HHP decreased total ORAC values by ~36.5% in reference to unprocessed samples. In particular, H-ORAC values decreased by ~10.4% in comparison with those of

unprocessed samples (Fig. 3A). This decrease in H-ORAC values may be attributed to the degradation of phenolic compounds or vitamin C during HHP processing. It has been reported that HHP treatments combined with temperature when applied to orange juice (100 MPa/60°C/5 min and 400 MPa/40°C/1 min) decreased the concentrations of vitamin C by ~10% (Sánchez-Moreno et al., 2003). Likewise, other studies have reported activation of vitamin C degrading enzymes during pressurization such as peroxidase and ascorbic acid oxidase (Talcott, Percival, Pittet-Moore, & Celoria, 2003). Therefore the possible degradation of vitamin C during HHP processing appears to be a feasible explanation for the observed decreases in H-ORAC values. L-ORAC values decreased by ~40% after pressurization, contributing even more to the lower total ORAC values observed immediately after the HHP treatment of avocado pulp (Fig. 3B). Interestingly, the decrease in the L-ORAC and total ORAC values were not correlated with the increases in the concentration of total carotenoids extracted after pressurization (Fig. 2). De Ancos et al. (2000) observed a similar behavior in persimmon fruit pure. Authors observed increases in the amounts of some individual carotenoids, which resulted in increases of vitamin A values (75-87 retinol equivalents/100 g). However, those increases were not correlated with the total antioxidant activity of the fruit. Although avocado is a rich source of other lipophilic antioxidants such as tocopherols, previous reports indicate that they are stable to HHP treatments in other food systems (Moltó-Puigmartí, Permanyer, Castellote, & López-Sabater, 2011; Barba, Esteve, & Frigola, 2012). Therefore, the observed decreases in L-ORAC values of HHP treated avocados are possibly related to the degradation of some carotenoids after processing (for instance zeaxanthin, Table 2) rather than total carotenoid content. However, further research would be required to better understand the contribution of each individual carotenoid as well as other lipophilic compounds to the L-ORAC value of avocado paste.

3.6. Hydrophilic and lipophilic antioxidant activity of avocado paste as affected by storage time

Fig. 3 A&B also contains the changes in L- and H-ORAC values, respectively, for HHP treated avocado paste during 25 days of refrigerated storage. The activity was monitored during the selected period of time based on the previously calculated sensory shelf-life of the product reported by *(*Jacobo-Velázquez et al., 2010a; 2010b*)*. Both H-ORAC (Fig. 3A) and L-ORAC (Fig. 3B) values of avocado paste increased during storage. Although levels decline after processing, at day 5, avocado paste recovered the hydrophilic and lipophilic ORAC values observed in the unprocessed avocado paste. H-ORAC values (Fig. 3A) then remained constant during the subsequent storage days. L-ORAC values (Fig. 3B), however, increased until day 10, reaching a maximum mean value of 133.62±5 μMol of TE per g of product dry weight. After day 15, L-ORAC values started to decline and reached the value of the unprocessed avocado sample.

In general, increases in L-ORAC and H-ORAC values observed during storage can be attributed to the migration of antioxidant compounds from the cell to the avocado paste matrix, enhancing the extraction of both hydrophilic and lipophilic compounds. Most of the antioxidants in avocado pulp are stored within plant cell organelles. It is known that the application of HHP induces morphological changes (compression of the vacuoles, cellular enlargement, formation of filaments, modification of the nuclei and organelles, aggregation of citoplasmatic proteins, etc) that result in the rupture of the membrane and the subsequent liberation of intracellular constituents (Shimada et al., 1993; Cheftel, 1995). The hypothesis of HHP liberation of intracellular constituents into the product matrix during avocado paste storage can be supported by observations published by Castellanos-Dohnal (2005) and Jacobo-Velázquez et al., (2010a; 2011). Results which appear to be consistent with the observed increments in the ORAC values throughout the shelf-life of the HHP processed avocado pulp (Fig. 3). H-ORAC values increased and then remained constant during storage, suggesting that all hydrophilic antioxidant compounds were liberated into the food matrix. On the other hand, L-ORAC values increased during the first days, and then decreased, indicating that lipophilic antioxidant compounds released during storage were possibly degraded by enzymatic (LOX) and/or non-enzymatic reactions.

4. Conclusion

In this study it was learned that HHP processing (600 MPa for 3 min) of avocado paste caused a significant increase (~56%) in the concentration of total carotenoids extracted from avocado paste. It was also observed that those bioactive compounds were gradually degraded during refrigerated storage of the product, resulting in total carotenoid contents that were ~50% lower than freshly processed samples, after 40 days. However, at the end of the sensory shelf-life of the product (20 days) the contents of total carotenoids in the processed avocado paste were similar to the values of samples immediately after processing (day 0). Therefore, the carotenoid based nutraceutical value of the product was retained at the end of its shelf-life. Results also suggested that avocado LOX type II isozymes participate in the oxidation of carotenoids during the refrigerated storage of the HHP processed avocado pulp. Total ORAC values for the product decreased immediately after HHP processing, possibly due to degradation of antioxidant compounds (such as the phenolic compounds, and/or vitamin C and E) not measured in the present study. However, ORAC values increased again during storage reaching values similar to those observed in the unprocessed samples. Results from this study also indicated that carotenoids have little contribution to the elevated L-ORAC values of Hass avocado, thus it would be interesting to explore the antioxidant activity of additional lipophilic antioxidant compounds present in the fruit.

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Figure captions

Fig. 1. Typical HPLC-PDA avocado paste carotenoid chromatograms (shown at 450 nm) obtained from unprocessed (A) and high hydrostatic pressure (HHP) processed (600 MPa for 3 min) samples (B). Tentative identification of the chromatographic peaks was performed as indicated in Table 1. Peak assignments included: (1) Neoxanthin a; (2) Neoxanthin b; (3) Luteoxanthin isomer a; (4) Luteoxanthin isomer b; (5) Lutein; (6) Zeaxanthin; (7) Possible xanthophyll a; (8) α-cryptoxanthin; (9) Possible xanthophyll b; (10) β-cryptoxanthin; (11) α-carotene; (12) β-carotene. IS = Internal standard (apo-β-8´-carotenal).

Fig. 2. Effects of high hydrostatic pressure (HHP) processing (600 MPa for 3 min) and storage (4°C for 40 d) on the concentration of total carotenoids in avocado paste. Values represents the mean of 3 replications with their standard error bars. Data points with different letters indicates statistical difference by the LSD test ($p < 0.05$). *Unprocessed avocado paste.

Fig. 3. Effects of high hydrostatic pressure (HHP) processing (600 MPa for 3 min) and storage (4°C for 25 d) on the antioxidant activity (ORAC values) of avocado paste. (A) Hydrophilic ORAC value. (B) Lipophilic ORAC value. Values represent the mean of 3 replications with their standard error bars. DW = Dry weight. Data points with different letters indicate statistical differences of the means by the LSD test ($p < 0.05$). *Unprocessed avocado paste.

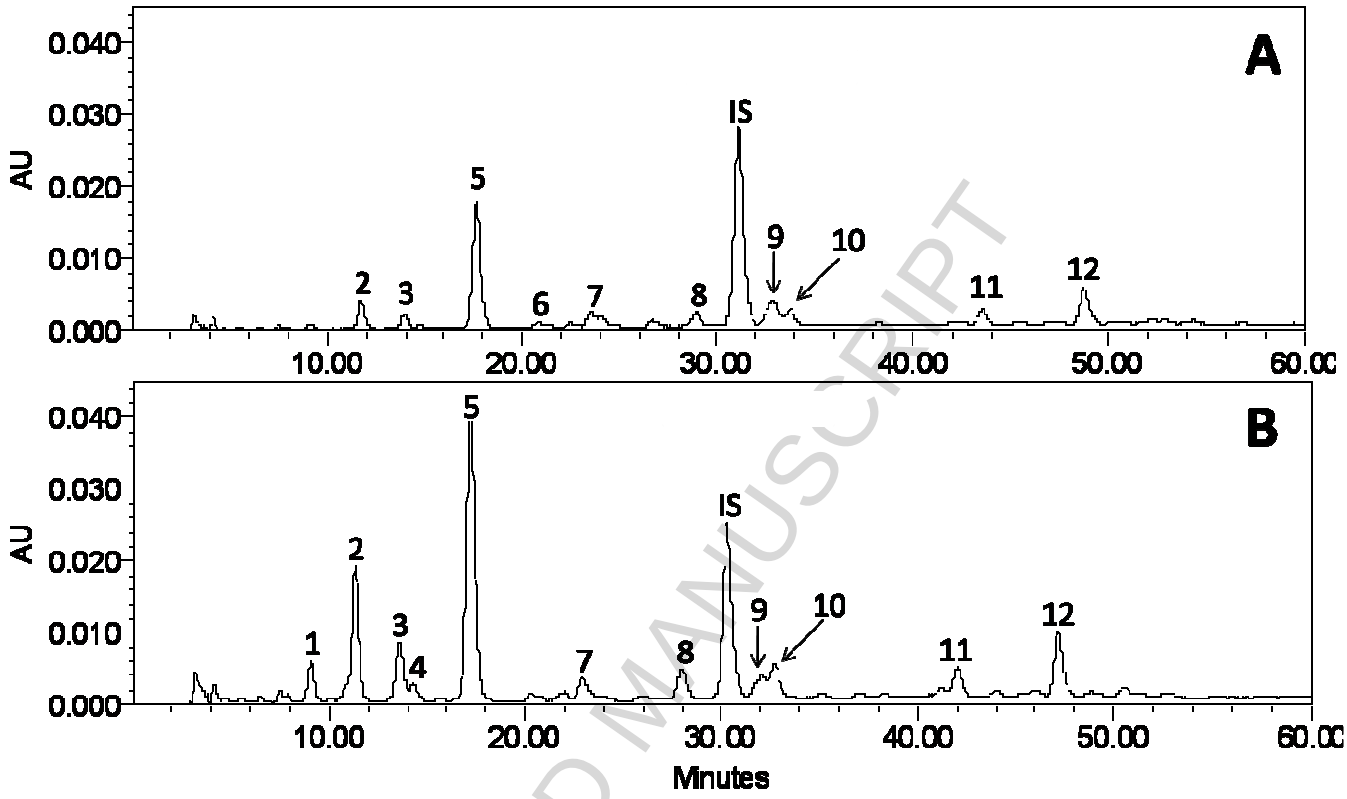

Fig. 1.

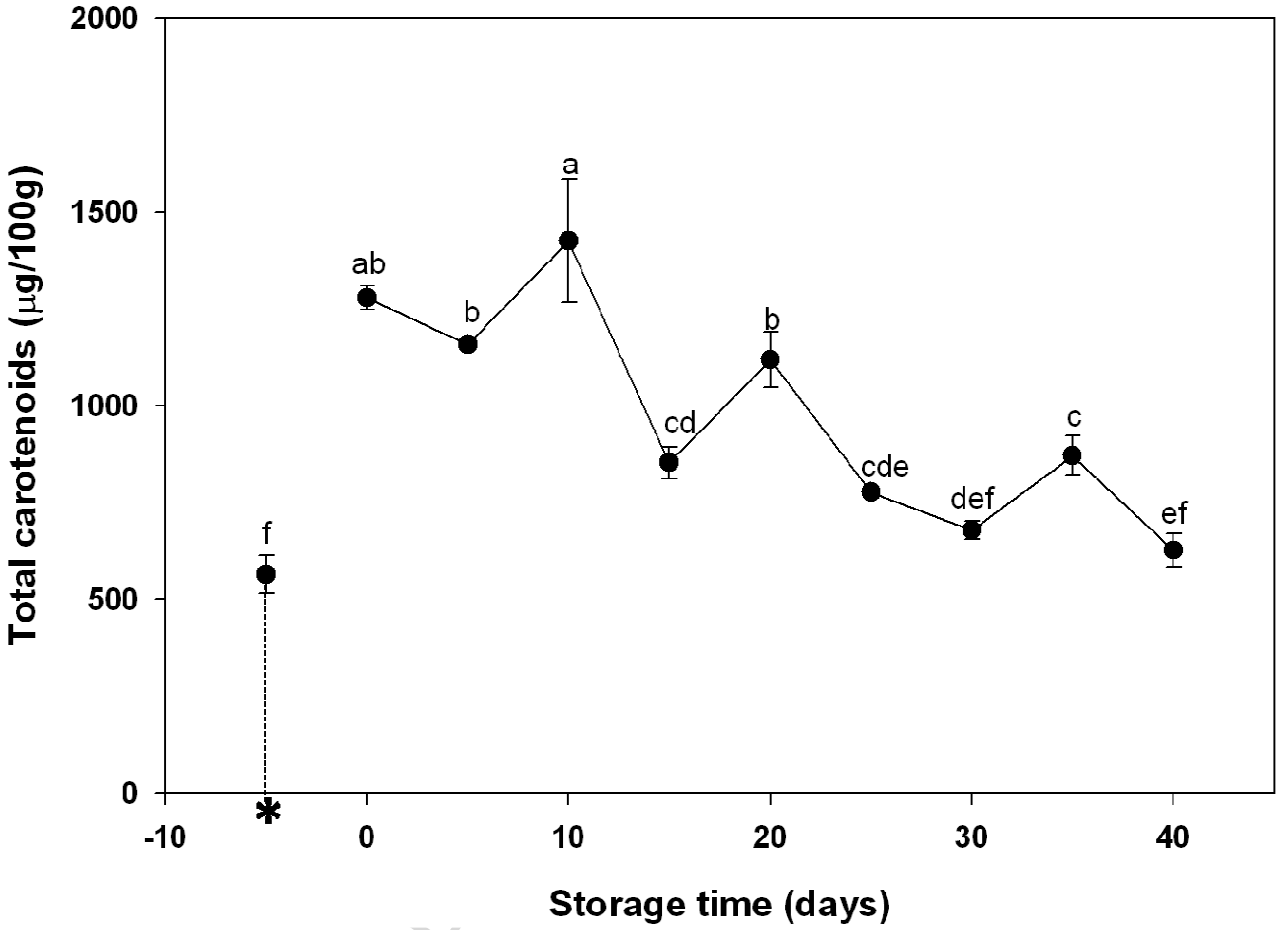

Fig. 2.

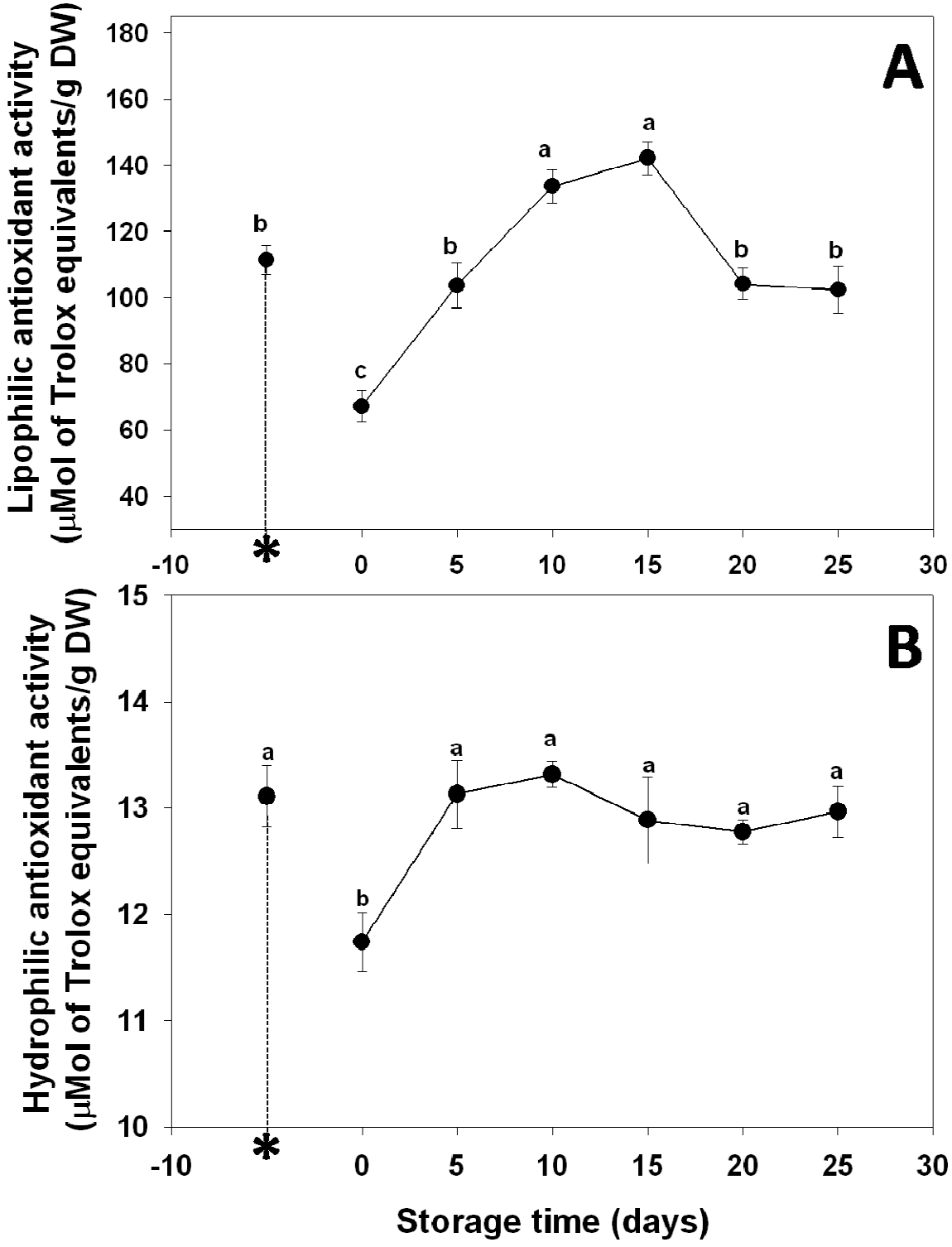

Fig. 3.

Table 1. Tentative identification of avocado paste (*Persea americana* var. Hass) carotenoid chromatographic profiles obtained by HPLC-PDA.

Peak number[1] (retention time)	λmax[2] (nm)	Tentative identification	Previously reported in avocado[3]	Method of identification[4]
1 (9.10)	414.1, 438.2, 469.7	Neoxanthin a		A, C
2 (11.36)	416.8, 438.2, 469.7	Neoxanthin b		A, C
3 (13.58)	397.2, 421.3, 447.9	Luteoxanthin isomer a		A, C
4 (14.32)	397.2, 423.7, 445.5	Luteoxanthin isomer b		A, C
5 (17.24)	(421.9), 445.5, 474.5	Lutein	i, iii	A, B, C
6 (20.30)	(430), 450.3, 481.8	Zeaxanthin	i, iii	A, C
7 (22.94)	(431.84), 462.4, 486.85	Possible xanthophyll a		A
8 (28.03)	(424), 447.9, 474.5	α-cryptoxanthin		A, C
9 (32.07)	(433.28), 472.3, 486.7	Possible xanthophyll b		A
10 (32.71)	(429), 450.3, 481.8	β- cryptoxanthin	i, ii, iii	A, C
11 (42.04)	(419), 445.5, 472.1	α-carotene	i, ii, iii	A, C
12 (47.18)	(429), 452.7, 479.4	β-carotene	i, ii, iii	A, B, C

[1]Number of peak assigned according to the order of elution from the C_{30} stationary phase (Fig. 1). [2]Wavelengths of maximum absorption in the UV/Vis spectra of each chromatographic peak, values in parenthesis indicates a shoulder in the peak. [3]Previously reported by, (i) Heinone et al. (*1*), (ii) USDA-NCC (*30*), (iii) Lu et al. (*2*). [4]Method applied for the identification of the peak, (A) Identification by spectral interpretation of the wavelengths of maximum absorption in the UV/Vis spectra and comparison with wavelengths of maximum absorption reported in the literature (De Ancos et al., 2000; Chen et al., 1996; Mercadante et al., 1997; Lee, 2001); (B) Identification by comparison with the retention time and wavelengths of maximum absorption in the UV/Vis spectra of commercial standards; (C) Identification by the order of chromatographic elution reported in the literature (Lee, 2001; Meléndez-Martínez et al., 2005; Waters, 200).

Table 2. Effects of high hydrostatic pressure (HHP) processing (600 MPa for 3 min) and storage (4°C for 40 days) on the concentrations of individual carotenoids of avocado paste (*Persea americana* var. Hass).

Peak	Carotenoid	Concentration (μg/100 g)[1, 2]				
		Unprocessed	Storage time (t_{days})			
			t_0	t_5	t_{10}	t_{15}
1	Neoxanthin a	ND	44.30±1.12 a	19.53±0.76 cd	28.08±5.52 b	16.08±2.88 de

2	Neoxanthin b	32.78±7.84	de	201.10±8.33	a	151.20±1.52	b	165.68±27.90	b	78.53±7.08	cd
3	Luteoxanthin isomer a	14.09±5.36	e	118.43±33.32	a	81.92±0.84	bc	54.79±11.08	d	33.97±4.03	de
4	Luteoxanthin isomer b	ND		15.47±1.34	a	12.11±2.33	ab	6.89±0.77	b c	2.27±0.31	c
5	Lutein	313.95±6.93	f	440.34±2.48	a b	432.95±1.09	ab	457.74±15.69	a	397.23±9.80	cd
6	Zeaxanthin	6.00±2.33	b	ND		5.20±1.18	b	13.49±1.81	a	ND	
7	Possible xanthophyll a	1.73±0.18	d	22.03±1.44	c	67.67±2.33	a	44.90±15.37	b	17.52±2.60	cd
8	α-cryptoxanthin	15.88±4.29	e	65.45±0.29	b	60.03±0.06	bc	99.42±16.47	a	48.46±4.04	bc d
9	Possible xanthophyll b	49.00±5.17	b	40.98±2.43	b	65.81±1.16	a	45.73±6.34	b	24.30±0.24	cd
10	β-cryptoxanthin	26.86±4.87	d	85.88±2.09	b	71.55±1.74	bc	139.17±27.34	a	64.18±6.90	bc d
11	α-carotene	16.49±2.83	bc d	63.46±4.34	a	28.18±2.92	b	47.18±15.75	a	30.38±0.92	b
12	β-carotene	87.11±12.22	e	180.19±3.82	b c	162.08±3.82	bc d	321.22±26.39	a	139.99±9.32	cd e

[1]Concentrations are reported as lutein equivalents for the xanthophylls (peaks 1, 2, 3, 4, 5, 6, 7, 8 y 10) and as β-carotene equivalents for the carotenoids with provitamin A activity (peaks 9, 11, 12 y 13). [2] Values represents the mean of 3 replications ± standar error of the mean. [3]Different letters in the same row indicates statistical difference by the LSD test ($p < 0.05$). ND = Not detected.

Table 2. (continued) Effects of high hydrostatic pressure (HHP) processing (600 MPa for 3 min) and storage (4°C for 40 days) on the concentrations of individual carotenoids of avocado paste (*Persea americana* var. Hass).

Peak	Carotenoid	Concentration (μg/100 g)[1, 2]				
		Storage time (t_{days})				
		t_{20}	t_{25}	t_{30}	t_{35}	t_{40}
1	Neoxanthin a	24.80±1.62 b c[3]	11.02±0.62 e	ND	ND	ND

		87										
2	Neoxanthin b	188.22±17.47	a b	73.98±6.20	c d	23.43±10.72	e	86.70±34.14	b c d	21.41±8.74	e
3	Luteoxanthin isomer a	91.03±10.17	a b	37.04±3.40	d e	17.56±2.23	e	67.33±6.00	b c d	4.79±2.24	e
4	Luteoxanthin isomer b	17.70±4.34	a	3.90±0.26	c	ND		ND		ND	
5	Lutein	420.60±5.86	b c	387.10±21.21	d	386.50±4.74	d	410.68±13.19	b c d	354.03±11.33	e
6	Zeaxanthin	ND		ND		ND		ND		ND	
7	Possible xanthophyll a	16.14±1.00	c d	12.80±0.30	c d	12.13±6.08	c	7.44±3.64	c d	18.02±4.15	c d
8	α-cryptoxanthin	49.56±13.39	b c d	50.21±5.88	b c d	29.83±4.17	d e	36.31±10.69	c d e	23.35±0.56	e
9	Possible xanthophyll b	20.26±0.96	c d e	13.49±1.87	e	15.53±2.28	d e	26.80±1.80	c	24.01±1.077	c d
10	β-cryptoxanthin	60.54±24.22	b c d	45.38±7.20	c d	34.53±3.49	c d	49.60±13.61	b c d	43.07±14.27	c d
11	α-carotene	28.61±6.36	b	21.57±0.10	b c	14.80±0.31	b c d	9.07±0.38	c d	0.30±0.29	d
12	β-carotene	202.23±14.65	b	119.23±10.92	d e	135.73±19.57	c d e	177.75±38.69	b c	137.60±11.94	c d e

[1]Concentrations are reported as lutein equivalents for the xanthophylls (peaks 1, 2, 3, 4, 5, 6, 7, 8 y 10) and as β-carotene equivalents for the carotenoids with provitamin A activity (peaks 9, 11, 12 y 13). [2] Values represents the mean of 3 replications ± standar error of the mean. [3]Different letters in the same row indicates statistical difference by the LSD test ($p < 0.05$). ND = Not detected.