LWT - Food Science and Technology 58 (2014) 519–526

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

High hydrostatic pressure effect on chemical composition, color, phenolic acids and antioxidant capacity of Cape gooseberry pulp (*Physalis peruviana* L.)

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A R T I C L E I N F O

Article history:
Received 28 August 2013
Received in revised form
3 April 2014
Accepted 6 April 2014
Available online 13 April 2014

Keywords:
High hydrostatic pressure
Antioxidant capacity
Phenolic compounds
Functional foods
Cape gooseberry

A B S T R A C T

The aim of this study was to evaluate the effects of high hydrostatic pressure (HHP) at 300, 400 and 500 MPa/1, 3 and 5 min on nutritional and antioxidant properties of Cape gooseberry pulp after immediate application and after 60 days of storage. Proximal analysis, color, phenolic acids content and antioxidant capacity were determined. When analyzing the immediate effect of different treatments, a clear influence of HHP was observed in all the components of the proximal analysis. Regarding color, none of the three chromatic parameters showed significant differences with control leading to a minimum ΔE at 300 MPa/3 min. Changes in bound and free phenolic acids were evidenced after treatments. The maximum levels of TPC as well as antioxidant capacity were observed at 500 MPa/5 min. By the end of storage, all treated samples discolored leading to $\Delta E = 14.9$ at 500 MPa/5 min. The profile of free and bound phenolic acids presented differences compared to Day 0. The antioxidant capacity by means of ORAC increased for treatments above 300 MPa/5 min indicating the effectiveness of these treatments for the production of functional products based on gooseberry pulp. For treatments above 400 MPa/3 min, molds and yeasts were not detected.

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1. Introduction

Many chronic diseases, such as cardiovascular diseases and cancer, are the leading causes of death in many developed and developing countries. Oxidative stress is believed to be an important contributing factor in the development of these diseases, and the potent antioxidant properties of phytochemicals, including the phenolic compounds found in fruits and vegetables especially, may help to prevent the effects of oxidative stress on the body (Ramadan, 2011; Su et al., 2014; Zhang et al., 2013).

Cape gooseberry (*Physalis peruviana* L.) is a potential candidate for the processing of new functional foods because of its nutritional properties as well as its biologically active components (Hassanien, 2011). In particular, the pulp of the fruit contains high levels of phenolic compounds, carotenoids, vitamin E and vitamin C (Ramadan & Moersel, 2007; Repo de Carrasco & Encina, 2008; Rop, MLcek, Jurikova, & Valsikova, 2012; Valdenegro, Henríquez, Lutz, Almonacid, & Simpson, 2010). Therefore, the challenge to preserve the nutraceutical properties suggests the application of non-thermal innovative technologies for the stabilization of this pulp. Among these technologies, the application of high hydrostatic pressure (HHP) has the potential to produce high-quality foods that display characteristics of fresh products, are microbiologically safe and have an extended shelf life (Ferrari, Maresca, & Ciccarone, 2010; Huang et al., 2013). During HHP the food is subjected to pressure

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http://dx.doi.org/10.1016/j.lwt.2014.04.010
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generally in the range of 100–1000 MPa at or around room temperature (Cao et al., 2012). Over the last 20 years, HHP has been investigated and several commercial products, including fruit juices, i.e. mandarin, grapefruit, apple, orange, carrot juices and broccoli–apple juice mixture treated by HPP are currently available on market (Ahmed, Ramaswamy, & Hiremath, 2005; Barba, Esteve, & Frigola, 2011; Bull et al., 2004; McInerney, Seccafien, Stewart, & Bird, 2007). HHP processing preserves nutritional value with only a minimal effect on the product quality and sensory properties of fruits and vegetables, since low molecular weight food compounds, such as flavoring agents, pigments, and some vitamins are not altered, because covalent bonding is not affected by pressure (Oey, Lille, Van Loey, & Hendrickx, 2008). The effect of HHP can vary depending on processing conditions (pressure, hold time, pH and temperature) and food form (whole, pieces, puree or juice). The food matrix can be altered by these variables, consequently impacting on the effectiveness of HHP (Vega-Gálvez et al., 2011) Several studies have been conducted in which HHP has been applied to various food matrices, especially fruits and other vegetable beverages (Barba et al., 2011; Ferrari et al., 2010; McInerney et al., 2007; Sánchez-Moreno, Plaza, de Ancos, & Cano Plaza, 2006; Wolbang, Fitos, & Treeby, 2008).

The aim of this research was to evaluate the effect of HHP treatment on physico-chemical properties, color (L^*, a^*, b^* and ΔE), total phenolic contents, free and bound phenolic acids as well as antioxidant activity of Cape gooseberry pulp immediately after application of HHP and after 60 days of storage at 4 °C.

2. Material and methods

2.1. Sample preparation

Cape gooseberry was cultivated and purchased in the region of La Serena city, Chile. The samples were selected to provide a homogenous group, based on date of harvest, color, size, and freshness according to visual analysis. Before pressurization, the fruits were pressed and homogenized in a blender (Philips, HR1720, Amsterdam). The gooseberry pulp was packed in polyethylene flexible pouches, and kept under chilling conditions in a refrigerated room (4 °C) for until further HHP processing.

2.2. High hydrostatic pressure treatment and storage conditions

Pressure treatments were performed in an isostatic pressing system (Avure Inc., Kent, WA, USA), with a cylindrical pressure chamber (length 700 mm, diameter 60 mm). Water was used as the pressurizing medium. A pressure level of 300, 400 and 500 MPa with holding times of 1, 3 and 5 min at room temperature were used. Samples were packed in polyethylene bag then heat-sealed and exposed to high hydrostatic pressure treatment. For each experiment trial, a control sample was prepared. The analyses of the proximal composition as well as quality parameters were performed immediately after processing (Day 0) and after 60 days of storage (Day 60). All experiments were done in triplicate. The HHP-treated pulp was stored at 4 ± 2 °C.

2.3. Microbial analysis

To count viable natural microorganisms in gooseberry pulp, the total plate count method was used. Twenty five mL or grams of each sample was obtained aseptically and homogenized with a 225 mL peptone saline solution 0.1% (Difco, Detroit, USA) in a filter stomacher bag using a Stomacher® (Biocheck, S.A., Barcelona, Spain) at 240 rpm for 60 s. Further decimal dilutions were made with the same diluent, and duplicates of at least three appropriate dilutions were plated on appropriate media. The samples were analyzed for mold and yeast (M&Y). One mL of the initial dilution was spread on three plates (0.3, 0.3 and 0.4 mL) of Dicloran Rose Bengal Chloramphenicol (DRBC, Difco, Detroit, USA) agar, and 0.1 mL of each subsequent was spread on one DRBC plate. Plates were then incubated at 25 °C for 3–5 days, and plates with 15–300 colonies were counted. Microbial data were transformed into logarithms of the number of colony-forming units (log CFU/g).

2.4. Proximal analysis

Crude protein content was determined using the Kjeldahl method with a conversion factor of 6.25 (AOAC no. 960.52). Lipid content was analyzed gravimetrically following Soxhlet extraction (AOAC no. 960.39). Crude fiber was estimated by acid/alkaline hydrolysis of insoluble residues (AOAC no. 962.09). Crude ash content was estimated by incineration in a muffle furnace at 550 °C (AOAC no. 923.03). The available carbohydrate was estimated by difference. The moisture content was determined gravimetrically (AOAC no. 934.06). All measurements were carried out in triplicate.

2.5. Color analysis

The color of the samples was measured using a HunterLab colorimeter HunterLab, Miniscan XE Plus 45/0-L (Hunter Associates Laboratory Inc., Virginia, USA) fitted with a 2.5 cm diameter aperture. Color was expressed in HunterLab units L^* (lightness/darkness), a^* (redness/greenness) and b^* (yellowness/blueness), standard illuminant D65 and observer 10° (Nuñez-Mancilla, Pérez-Won, Uribe, Vega-Gálvez, & Di Scala, 2013). Five replicate measurements were performed and the results were averaged. In addition, total color difference (ΔE) was calculated using the following Eq. (1), where L_0, a_0 and b_0 were the control values for fresh pulp.

$$\Delta E = \left[\left(a^* - a_0\right)^2 + \left(b^* - b_0\right)^2 + \left(c^* - c_0\right)^2\right]^{0.5} \quad (1)$$

2.6. Total phenolic content

Total phenolic content (TPC) was determined colorimetrically by the Folin–Ciocalteau method according to Chuah et al. (2008) with modifications. 0.5 mL Aliquot of the Cape gooseberry pulp extract solution was transferred to a glass tube, 0.5 mL of Folin–Ciocalteau reagent and 2 mL of 20% Na_2CO_3 solution were added and mixed well in a vortex. After 15 min of incubation at ambient temperature, 10 mL of ultra pure water were added and the precipitate formed was removed by centrifugation during 5 min at 4000 × g. Finally, the absorbance was measured in a spectrophotometer (Spectronic 20® Genesys™, Illinois, USA) at 725 nm and compared to a gallic acid equivalent (GAE) calibration curve. Results were expressed as mg GAE/100 g d.m. All reagents were purchased from Merck (Merck KGaA, Darmstadt, Germany). All measurements were done in triplicate.

2.7. Extraction of phenolic acids

2.7.1. Free phenolic acids

Extraction was performed by a shaker holding 4.0 g Cape gooseberry pulp in 30 mL 69% acetone for 63 min at 250 rpm, according to the method of Lopez-Martinez et al. (2009) with some modifications. After centrifugation at 5000 rpm for 3 min, the supernatant was removed and extraction was repeated once more time in a similar way for 30 min by a shaker. The combined extracts

were evaporate to 37 °C and redissolved in 10 mL MeOH–formic acid (99:1). Aliquots of 10 μL were injected into the HPLC column. All measurements were done in triplicate.

2.7.2. Bound phenolic acids

After extraction of free phenolic acids (PAs), 30 mL of 3 N NaOH was added directly to the residue and was put into the shaker for 88 min according to the methodology of Lopez-Martinez et al. (2009). The hydrolyzate was acidified to pH 3 with concentrated HCl. The liberated PAs in the clear solution were extracted seven times with 10 mL ethyl acetate. The pooled ethyl acetate extracts were evaporated under a rotary evaporator in vacuum to 37 °C. The dry residue was dissolved in 10 mL MeOH–formic acid (99:1). Aliquots of 10 μL were injected into the HPLC column. All measurements were done in triplicate.

2.7.3. Chromatographic conditions: high performance liquid chromatography analysis

An HPLC system, Agilent 1200, equipped with a high pressure pump; automatic injector; a UV–visible diode array detector; controlled by ChemStation software, was used for the analysis. The analytical column was a Kromasil 100-5C18 (250 × 4.6 mm) (Eka chemical, Sweden). The flow rate was 0.7 mL/min and the eluates were monitored at 280 and 310 nm at 25 °C. The mobile phase was composed of solvent A (formic acid 0.1%, pH 3) and B (100% acetonitrile). The elution was as follows: initial conditions 87% A and 13% B; a linear gradient of solvent B was used from 13 to 55% from 0 to 18 min, from 55 to 60% from 18 to 23 min, from 60 to 13% from 23 to 25 min, and then returns to initial conditions by 2 min. The phenolic extracts and standard compounds were analyzed under the same analysis conditions. Identification of some of the main phenolic acids (gallic, protocatechuic, chlorogenic, caffeic, syringic, vanillic, *p*-coumaric, trans-sinapic, ellagic, salicylic, trans-ferulic and trans-cinnamic acid) in MeOH–formic acid (99:1), it was performed by comparisons to the retention times and then their spectra, the peak area of maximum absorption wavelength. The results of the main phenolic compounds were expressed as mg/100 g d.m. All measurements were done in triplicate.

2.8. Antioxidant capacity

The antioxidant capacity was determined based on three analysis (DPPH radical scavenging assay), Ferric reducing/antioxidant power (FRAP) and Oxygen radical absorbance capacity (ORAC).

2.8.1. DPPH assay

The antioxidant capacity determined by DPPH assay followed the procedure described by Turkmen, Sari, and Velioglu (2005) with some modifications. Different dilutions of the extracts were prepared in triplicate. An aliquot of 2 mL of 0.15 mM DPPH radical in methanol was added to a test tube with 1 mL of the sample extract. The reaction mixture was vortex-mixed for 30 s and left to stand at room temperature in the dark for 20 min. The absorbance was measured at 517 nm, using a spectrophotometer (Spectronic® 20 GenesysTM, Illinois, USA). 80% (v/v) methanol was used to calibrate the spectrophotometer. Calibration curves were made for each assay using Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid). The results were expressed as μmoles of Trolox equivalents (TE)/100 g d.m. All measurements were done in triplicate.

2.8.2. FRAP assay

The antioxidant activity of the extracts was determined by a modified method of the ferric reducing antioxidant power (FRAP) assay according to Benzie and Strain (1999). To prepare the FRAP reagent, a mixture of 0.1 mol/L acetate buffer (pH 3.6), 10 mmol/L TPTZ, and 20 mmol/L ferric chloride (10:1:1 v:v:v) was made. Then, 1.8 mL reagent, 0.18 mL water and 0.06 mL sample were added. The reaction mixture was incubated at 37 °C for 120 min. The increase in absorbance was monitored at 593 nm vs. a blank. FRAP reagent was freshly made up each measuring day and the blank consisted in 1.8 mL reagent 0.18 mL water and 0.06 mL methanol (80%v/v). The final absorbance of each sample was compared with those obtained from the standard curve made from Trolox and the results were expressed as μmoles TE/100 g d.m. All measurements were done in triplicate.

2.8.3. ORAC assay

The ORAC assay followed the procedure described by Zhang et al. (2010) with some modifications. A fluorescein stock solution (100 mol/L) in phosphate buffer (75 mmol/L, pH 7.4) was prepared and kept at 4 °C in the dark. Fresh working fluorescein solution (100 mmol/L) was prepared daily by diluting the stock solution in phosphate buffer. Next, 200 μL of the working fluorescein solution were added to each 40 μL of pulp sample or Trolox standard prepared in phosphate buffer in a black 96-well plate and incubated for 20 min at 37 °C. The assay was initiated by adding the peroxyl radical generator prepared in phosphate buffer. Specifically, 35 μL of 2,2′-azobis-2-amidinopropane (AAPH, 0.36 mol/L) was added and the fluorescence was measured (ex = 485 nm and em = 535 nm) every minute using a Victor3 multilabel plate reader (Perkin–Elmer, Turku, Finland) maintained at 37 °C until the lecture had declined to less than 5% of the initial reading. Standards and samples were run in triplicate. Results for ORAC were determined using a regression equation relating Trolox concentrations and the net area under the kinetic fluorescein decay curve. The ORAC value of each *Physalis* extract was expressed in μmoles TE/100 g d.m.

2.9. Statistical analysis

The effect of pressure on each quality parameter was analyzed using Statgraphics Plus 5 (Statistical Graphics Corp., Herndon, VA, USA) applying an analysis of variance (ANOVA). Differences among the mean values were analyzed using the least significant difference (LSD) test with a significance level of $\alpha = 0.05$ and a confidence interval of 95% ($P < 0.05$). In addition, the multiple range test (MRT) included in the statistical program was used to demonstrate the existence of homogenous groups within each of the parameters.

3. Results and discussion

3.1. Effect of HHP on molds and yeasts

The number of surviving cells in gooseberry pulp after HHP treatment was determined by monitoring M&Y. The initial count of M&Y in untreated pulp was 3.123 ± 0.063 log CFU/g. M&Y in the pulp was not detected after HHP treatment (Day 0). After 60 days of storage, the inactivation achieved with the different treatments depends on the pressure intensity. It was observed that the highest the pressure and time exposure, the highest the inactivation (Huang et al., 2013). In particular for treatments above 400 MPa/3 min, M&Y were not detected. This study focused on the changes of the quality aspects of the gooseberry pulp after HHP treatments, and the analyses concerning the changes were presented as following.

3.2. Effect of HHP on proximal analysis

Table 1 shows the effects of HHP on the chemical composition of fresh (control) and treated samples (Day 0 and Day 60). Proximate

Table 1
Proximate analysis of fresh and processed Cape gooseberry pulp immediately after HHP (Day 0) and after storage (Day 60) (g 100 g^{-1} sample).

Treatments	Moisture[a]	Ash	Fat	Protein crude (N*6.25)	Crude fiber	Carbohydrate
Day 0						
Control	78.61 ± 0.24^{a}	6.58 ± 0.95^{a}	5.70 ± 0.17^{a}	5.64 ± 0.11^{ab}	6.33 ± 0.17^{ac}	60.80 ± 0.62^{a}
300 MPa/1 min	82.00 ± 0.75^{bcd}	6.12 ± 0.17^{ab}	3.39 ± 0.18^{b}	6.23 ± 0.77^{bd}	6.77 ± 0.16^{b}	86.40 ± 1.19^{b}
300 MPa/3 min	81.04 ± 2.89^{abcd}	11.66 ± 1.78^{c}	1.18 ± 0.11^{c}	4.94 ± 1.03^{ab}	6.57 ± 0.12^{bc}	74.94 ± 6.00^{c}
300 MPa/5 min	78.56 ± 1.89^{ab}	8.97 ± 0.03^{d}	0.78 ± 0.08^{ac}	4.57 ± 1.13^{a}	8.37 ± 0.87^{d}	84.46 ± 7.33^{b}
400 MPa/1 min	83.19 ± 0.62^{cd}	6.72 ± 0.64^{a}	1.16 ± 0.16^{c}	8.56 ± 1.06^{c}	5.18 ± 0.68^{a}	84.24 ± 1.85^{b}
400 MPa/3 min	83.63 ± 0.82^{d}	5.77 ± 0.32^{ab}	0.87 ± 0.02^{c}	6.31 ± 1.10^{bd}	2.87 ± 0.38^{e}	89.53 ± 1.52^{b}
400 MPa/5 min	83.76 ± 1.74^{d}	6.23 ± 0.46^{ab}	5.13 ± 0.55^{d}	1.14 ± 0.14^{e}	3.48 ± 0.14^{e}	84.63 ± 3.66^{b}
500 MPa/1 min	83.60 ± 2.02^{d}	6.12 ± 0.20^{ab}	1.00 ± 0.05^{c}	10.3 ± 0.66^{f}	5.17 ± 0.60^{a}	75.39 ± 2.60^{c}
500 MPa/3 min	80.44 ± 1.19^{abc}	5.24 ± 0.32^{b}	0.86 ± 0.10^{ac}	5.65 ± 0.55^{ab}	6.62 ± 0.45^{bc}	91.68 ± 0.13^{b}
500 MPa/5 min	88.23 ± 3.34^{e}	8.11 ± 0.35^{d}	6.26 ± 0.53^{e}	7.67 ± 0.47^{cd}	1.47 ± 0.21^{f}	62.11 ± 0.45^{a}
Day 60						
Control	83.41 ± 0.22^{a}	8.28 ± 0.94^{ac}	1.20 ± 0.43^{a}	8.07 ± 0.16^{ab}	5.01 ± 0.21^{a}	81.46 ± 0.41^{a}
300 MPa/1 min	80.17 ± 0.14^{b}	6.82 ± 1.42^{ab}	0.94 ± 0.06^{abc}	6.77 ± 0.83^{bcd}	7.16 ± 2.73^{ab}	85.17 ± 1.79^{b}
300 MPa/3 min	80.99 ± 0.13^{b}	7.99 ± 1.27^{a}	1.04 ± 0.30^{abc}	7.20 ± 1.21^{bcd}	9.93 ± 2.78^{b}	87.50 ± 0.14^{bcd}
300 MPa/5 min	82.42 ± 0.75^{a}	5.59 ± 0.03^{b}	1.11 ± 0.10^{ab}	9.33 ± 1.61^{a}	5.74 ± 0.03^{a}	86.33 ± 0.26^{bc}
400 MPa/1 min	82.25 ± 0.99^{c}	5.45 ± 0.09^{b}	0.44 ± 0.03^{de}	6.77 ± 0.59^{bcd}	4.46 ± 0.73^{a}	88.66 ± 3.80^{cd}
400 MPa/3 min	82.01 ± 0.56^{c}	5.55 ± 0.29^{b}	0.27 ± 0.03^{e}	6.07 ± 0.89^{d}	5.44 ± 0.34^{a}	89.86 ± 1.18^{d}
400 MPa/5 min	81.96 ± 0.07^{c}	6.24 ± 0.04^{b}	0.09 ± 0.01^{e}	7.21 ± 0.04^{bcd}	5.40 ± 0.09^{a}	85.61 ± 0.30^{bc}
500 MPa/1 min	79.91 ± 1.04^{b}	9.73 ± 0.05^{cd}	0.64 ± 0.16^{cd}	8.24 ± 0.29^{abc}	5.61 ± 0.31^{a}	90.20 ± 1.61^{d}
500 MPa/3 min	80.71 ± 0.19^{b}	11.19 ± 0.04^{de}	0.88 ± 0.06^{abc}	6.67 ± 0.31^{cd}	5.38 ± 0.32^{a}	80.69 ± 1.25^{a}
500 MPa/5 min	80.25 ± 1.04^{b}	11.26 ± 0.85^{e}	0.84 ± 0.03^{bc}	6.99 ± 1.12^{bcd}	5.24 ± 0.43^{a}	77.38 ± 0.85^{e}

Values are mean ± s.d. ($n = 3$).
a–e different letters in the same column indicate significant differences ($P < 0.05$).
[a] g/100 g Sample.

analysis of fresh Cape gooseberry was close to those reported by Puente, Pinto-Muñoz, Castro, and Misael (2011) and Hassanien (2011). Analyzing the immediate effect of HHP on moisture, an increased in the sample moisture value was observed. The increase in moisture content may be due to increase water absorption by the protein, since it is known that high hydrostatic pressure can increase the hydration of proteins ($P < 0.05$). Similar results were found by Briones-Labarca, Venegas-Cubillos, Ortiz-Portilla, Chacana-Ojeda, and Maureira (2011) and Briones-Labarca, Muñoz, and Maureira (2011) working with apple at 500 MPa/10 min. The application of HHP did not show a clear trend regarding the content of ashes, however, working at 300 MPa/3 min resulted in a notable increased compared to control ($P < 0.05$) (Briones-Labarca, Venegas-Cubillos, et al., 2011). Except for 500 MPa/5 min, the other treatments decreased the fat content ($P < 0.05$). Regarding proteins, working at 400 MPa/1 min and 500 MPa/1 and 5 min increased the amount of crude protein. Regarding crude fiber some treatments decreased the initial content (400 MPa/1, 3 and 5 min and 500 MPa/5 min), and others increased it (300 MPa/3 and 5 min) given that the increase in moisture probably has a dilution effect on other constituents of the high pressure-treated pulp samples (Briones-Labarca, Venegas-Cubillos, et al., 2011; Briones-Labarca, Muñoz, et al., 2011). When analyzing the carbohydrate content immediately after HHP, except for the treatment at 500 MPa/5 min all the others presented a clear increase in the initial content. After storage for 60 days at 4 °C, pressurized samples presented lower moisture content than the control samples. Regarding ashes, the treatments at 500 MPa/3 and 5 min showed an increase in this component compare to control samples. The control samples showed a reduction of 78.9% of fat compared to fresh samples (control at Day 0). When analyzing the pressurized samples, all treatments led to a decrease in fat content, however a clear pattern was not observed ($P < 0.05$). Regarding proteins, significant differences were not evidence in the range of pressure studied. Regarding crude fiber and carbohydrates, similar trend was found for these components when analyzing samples at day 0 (Briones-Labarca, Venegas-Cubillos, et al., 2011; Briones-Labarca, Muñoz, et al., 2011).

3.3. *Effect of HHP on color*

Color attributes of fresh and HHP processed fruit pulp immediately after HHP as well as over storage are presented in Table 2. The chromatic parameters of fresh Cape gooseberry were $a_0 = 23.67 \pm 0.30$, $b_0 = 59.85 \pm 0.29$ and $L_0 = 54.44 \pm 0.34$. Control samples presented changes in all the chromatic parameters ($P < 0.05$). When analyzing the immediate effect of the different treatments on color, none of the three chromatic parameters showed significant differences with control samples ($P < 0.05$). The minimum ΔE value was achieved at 300 MPa/3 min. Regarding the effect of pressure on ΔE after the storage of 60 days, the three parameters (L^*, a^* and b^*) reduced their values resulting in a $\Delta E = 19.78$ for control samples which according to Barba et al. (2011) implies great changes in color compared to that of the original fresh sample. The different treatments affected the chromatic parameters. The observed changes in these parameters indicated browning of samples. However, significant differences were not detected among treatments ($P < 0.05$). The parameter a^* decreased its values at all levels of pressure applied indicating loss of red color. These changes indicated that the color of the pulp became discolored probably due to the residual activity of enzymes, which induced the enzymatic browning of phenolic compounds in the pulp (Huang et al., 2013; Oey et al., 2008). Similar results were found in previous work processing pomegranate juice between 350 and 550 MPa (Varela-Santos et al., 2012). Moreover, the behavior of certain physico-chemical parameters differ because of the HHP treatment intensity applied and also according to the food matrix to which it is applied (Ahmed et al., 2005; Huang et al., 2013). For example, Guerrero-Beltrán, Swanson, and Barbosa-Cánovas (2004) demonstrated the good color stability and increased shelf life of peach purée, containing ascorbic acid, treated at 500 MPa/5 min and stored refrigerated at a temperature of 3 °C. Dede, Alpas, and Bayindirli (2007), working on high hydrostatic pressure and thermal processing of carrot and tomato juices, observed that high pressure treatment of 250 MPa/35 °C/15 min produced lower color difference compared to that of the fresh sample. Perera et al. (2009) also reported the effect of HHP on color of fruit products and

Table 2
Changes in chromatic parameters (L^*, a^*, b^*) and ΔE of Cape gooseberry pulp immediately after HHP (Day 0) and after storage at 4 °C (Day 60).

Treatments	a^*	b^*	L^*	ΔE
Day 0				
Control	$23.67 \pm 0.30^{af,A}$	$59.85 \pm 0.29^{a,A}$	$54.44 \pm 0.34^{a,a}$	0.00 ± 0.00
300 MPa/1 min	$18.33 \pm 0.42^{b,A}$	$54.40 \pm 0.77^{b,A}$	$46.23 \pm 0.19^{b,A}$	$11.23 \pm 0.56^{a,A}$
300 MPa/3 min	$24.31 \pm 0.31^{c,A}$	$61.14 \pm 0.54^{c,A}$	$48.87 \pm 0.13^{c,A}$	$5.78 \pm 0.10^{b,A}$
300 MPa/5 min	$21.52 \pm 0.69^{d,A}$	$57.87 \pm 0.95^{de,A}$	$46.87 \pm 0.55^{d,a}$	$8.46 \pm 0.93^{ce,A}$
400 MPa/1 min	$21.25 \pm 0.42^{d,A}$	$57.22 \pm 0.57^{de,A}$	$46.70 \pm 0.17^{d,a}$	$8.54 \pm 0.42^{c,A}$
400 MPa/3 min	$18.77 \pm 0.17^{b,A}$	$56.34 \pm 0.21^{d,A}$	$45.97 \pm 0.13^{b,A}$	$10.40 \pm 0.09^{d,A}$
400 MPa/5 min	$21.29 \pm 0.60^{d,A}$	$57.70 \pm 0.60^{ef,A}$	$47.33 \pm 0.41^{e,A}$	$7.83 \pm 0.71^{ce,A}$
500 MPa/1 min	$23.22 \pm 0.31^{ae,A}$	$59.29 \pm 0.43^{ag,A}$	$48.10 \pm 0.05^{f,A}$	$6.40 \pm 0.06^{bf,A}$
500 MPa/3 min	$22.75 \pm 0.83^{e,A}$	$57.17 \pm 0.97^{ag,A}$	$47.82 \pm 0.44^{fg,A}$	$6.81 \pm 0.64^{f,A}$
500 MPa/5 min	$20.89 \pm 0.51^{f,A}$	$58.55 \pm 0.93^{fg,A}$	$47.54 \pm 0.24^{eg,A}$	$7.76 \pm 0.59^{e,A}$
Day 60				
Control	$16.30 \pm 0.22^{ac,B}$	$46.50 \pm 0.50^{a,B}$	$41.40 \pm 0.22^{a,B}$	$19.78 \pm 0.41^{a,B}$
300 MPa/1 min	$17.70 \pm 0.17^{b,B}$	$51.68 \pm 0.65^{b,B}$	$42.85 \pm 0.18^{bd,B}$	$15.40 \pm 0.53^{bd,B}$
300 MPa/3 min	$16.80 \pm 0.24^{cd,B}$	$46.10 \pm 0.40^{a,B}$	$40.30 \pm 0.30^{c,B}$	$20.91 \pm 0.48^{a,B}$
300 MPa/5 min	$16.70 \pm 0.10^{ac,B}$	$50.00 \pm 0.30^{c,B}$	$41.10 \pm 0.40^{ac,B}$	$18.48 \pm 0.46^{c,B}$
400 MPa/1 min	$17.60 \pm 1.0^{b,B}$	$49.90 \pm 0.44^{d,B}$	$42.60 \pm 1.50^{bd,B}$	$16.64 \pm 1.68^{de,B}$
400 MPa/3 min	$17.50 \pm 0.32^{b,B}$	$51.30 \pm 0.70^{bd,B}$	$43.40 \pm 0.40^{de,B}$	$15.24 \pm 0.52^{b,B}$
400 MPa/5 min	$15.80 \pm 0.70^{a,B}$	$49.50 \pm 0.72^{cd,B}$	$43.80 \pm 0.64^{e,b}$	$16.77 \pm 1.13^{e,B}$
500 MPa/1 min	$17.40 \pm 0.31^{b,B}$	$51.60 \pm 0.60^{b,B}$	$43.80 \pm 0.22^{e,B}$	$14.9 \pm 0.60^{b,B}$
500 MPa/3 min	$15.90 \pm 0.51^{a,B}$	$49.10 \pm 1.00^{cd,B}$	$42.40 \pm 0.31^{b,B}$	$17.94 \pm 0.99^{ce,B}$
500 MPa/5 min	$15.80 \pm 0.20^{a,B}$	$48.70 \pm 0.60^{c,B}$	$42.10 \pm 0.23^{bf,B}$	$18.38 \pm 0.32^{c,B}$

Values are mean ± s.d. ($n = 3$).
a–e different letters in the same file indicate significant differences ($P < 0.05$).
A–D different letters in the same column indicate significant differences ($P < 0.05$).

concluded that minimal color changes were caused by HHP treatments.

3.4. *Effect of HHP on total phenolic content*

The influence of different treatments on total phenolic content (TPC) of Cape gooseberry pulp is shown in Table 3. The initial content of TPC was 212.84 ± 5.49 mg GA/100 g d.m. When analyzing the immediate effect of HHP on fresh samples, it can be observed that some treatments increased whilst others reduced the initial content of TPC ($P < 0.05$). The maximum TPC was observed at 500 MPa/5 min. Based on these results, it can be concluded that HHP treatment, due to changes in fruit pulp microstructure, produces changes in the distribution and aggregation of phenolics compounds. High pressure treatment can increase the rate of mass transfer resulting in an enhancement of solvent penetration into the cells by disrupting the cellular walls and hydrophobic bonds in the cell membrane, which may lead to a high permeability (Prasad, Yang, Yi, Zhao, & Jiang, 2009). Thus, this increase in total phenols may be related to an increased extractability of some antioxidant components such as anthocyanins, amino acids and protein with phenolic hydroxyl group after HHP treatment (Cao et al., 2011).

Table 3
Changes in total phenolic content of Cape gooseberry for control (Day 0) and after storage at 4 °C (Day 60).

Treatments	TPC (mg GAE/100 g d.m.)	
	Day 0	Day 60
Control	$212.84 \pm 5.49^{af,A}$	$233.84 \pm 6.83^{a,B}$
300 MPa/1 min	$164.68 \pm 2.44^{b,A}$	$169.25 \pm 7.92^{b,A}$
300 MPa/3 min	$205.47 \pm 9.72^{ac,A}$	$182.00 \pm 10.94^{cd,A}$
300 MPa/5 min	$120.41 \pm 1.31^{d,A}$	$193.70 \pm 4.11^{d,B}$
400 MPa/1 min	$153.74 \pm 6.03^{be,A}$	$216.82 \pm 1.80^{e,B}$
400 MPa/3 min	$201.86 \pm 10.78^{ac,A}$	$171.16 \pm 8.71^{bc,A}$
400 MPa/5 min	$220.34 \pm 1.95^{f,A}$	$190.91 \pm 7.35^{d,B}$
500 MPa/1 min	$193.08 \pm 2.28^{c,A}$	$208.07 \pm 4.81^{e,B}$
500 MPa/3 min	$142.14 \pm 5.80^{e,A}$	$188.84 \pm 9.31^{d,B}$
500 MPa/5 min	$268.72 \pm 7.16^{g,A}$	$190.42 \pm 7.12^{d,B}$

Values are mean ± s.d. ($n = 3$).
a–e different letters in the same file indicate significant differences ($P < 0.05$).
A–D different letters in the same column indicate significant differences ($P < 0.05$).

Similar results were obtained for apricot nectars, carrot and tomato puree, and cashew apple (Huang et al., 2013; Patras, Brunton, Da Pieve, & Butler, 2009; Queiroz et al., 2010).

After 60 days of storage the TPC of the control samples increased to 233.84 mg GA/100 g d.m. ($P < 0.05$). However, some treatments showed reduction in the TPC relate to control samples. This may be due to higher residual activity of enzymes such as polyphenoloxidase and peroxidase in HHP-treated fruit pulp, since these enzymes substrates are the phenols endogenous in pulp (Cao et al., 2011).

3.5. *Effect of HHP on phenolic acids*

It has been found that phenolic compounds exist in significant quantities in cell walls of plants. These compounds, called bound phenolics, are covalently conjugated to cell wall components (Su et al., 2014). Furthermore, it is hypothesized that bound phenolics were able to provide a slow and continuous release of phenolics in the lower gastrointestinal tract via the simultaneous action of β-glucosidases and esterases of gut microflora. Therefore, the profile of free and bound phenolics in fruits and vegetables could provide us some insight into their potentials of improving human health (Zhang et al., 2013).

Five phenolic compounds were detected in free and bound fractions of Cape gooseberry pulp, which were syringic, vanillic, p-coumaric, trans-ferulic and cinnamic. The free and bound phenolic contents in pulp of Cape gooseberry and their percentage contributions to the total phenolic contents are presented in Table 4. The free and bound phenolic contents ranged from 2.36 to 4.44 mg/100 g d.m. and from 0.56 to 3.0 mg/100 g d.m., respectively. In control samples, syringic acid only existed in free form and p-coumaric vanillic acid and trans-ferulic only in bound form. When analyzing the effect of pressure on the content of each fraction (Day 0) it can be observed that pressure influence the quantity of

Table 4
Free and bound phenolics acids of Cape gooseberry pulp as affected by HHP at Day 0.

Acids[a]		Control	300 MPa/1 min	300 MPa/3 min	300 MPa/5 min	400 MPa/1 min	400 MPa/3 min	400 MPa/5 min	500 MPa/1 min	500 MPa/3 min	500 MPa/3 min
Syringic	F	2.31 ± 0.11[A]	1.18 ± 0.03[B]	1.10 ± 0.05[B]	2.29 ± 0.13[A]	2.53 ± 0.18[A]	2.38 ± 0.03[A]	3.23 ± 0.22[C]	3.16 ± 0.26[C]	nq	nq
	B	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Vanillic	F	nq	nq	nq	nd	nd	nd	nd	nd	nd	nd
	B	1.87 ± 0.99[ABC]	1.82 ± 0.28[AB]	nd	nd	2.21 ± 0.32[C]	2.09 ± 0.08[BC]	1.43 ± 0.21[DE]	1.31 ± 0.08[D]	1.16 ± 0.09[D]	1.68 ± 0.05[AE]
p-Coumaric	F	nd		nd	nd	nd	nd	nd	nd	nd	nd
	B	1.70 ± 0.19[AD]	2.21 ± 0.09[B]	1.16 ± 0.08[C]	1.05 ± 0.04[C]	1.69 ± 0.05[A]	2.12 ± 0.14[B]	1.23 ± 0.22[C]	1.87 ± 0.02[ADE]	2.03 ± 0.04[BE]	1.90 ± 0.15[DE]
Trans-ferulic	F	nq	1.50 ± 0.09[A]	0.84 ± 0.05[B]	0.77 ± 0.28[B]	nq	nq	nq	nq	nq	nq
	B	0.93 ± 0.12[A]	1.28 ± 0.18[ABD]	0.46 ± 0.06[C]	0.33 ± 0.03[C]	1.23 ± 0.08[AB]	1.34 ± 0.06[ABD]	1.17 ± 0.62[AB]	1.09 ± 0.11[A]	1.66 ± 0.23[D]	1.56 ± 0.11[BD]
Cinnamic	F	0.55 ± 0.04[A]	0.57 ± 0.05[A]	nd	nd	0.63 ± 0.07[AB]	0.71 ± 0.05[AB]	0.76 ± 0.18[B]	0.61 ± 0.11[AB]	0.76 ± 0.09[B]	0.99 ± 0.07[C]
	B	0.24 ± 0.05[A]	0.34 ± 0.03[AB]	nd	nd	0.41 ± 0.03[AB]	0.38 ± 0.04[AB]	0.47 ± 0.22[B]	0.44 ± 0.08[AB]	0.73 ± 0.06[C]	1.01 ± 0.14[D]

Values are mean ± standard deviation ($n = 3$).
nd: No detected.
nq: No quantifiable; its retention time matches only (not its spectrum).
Similar letters in the exponential in the same row show there are no significant differences ($P > 0.05$) according to the classification obtained by the multiple range test.
[a] mg/100 g d.m.; F: free; B: bound.

each fraction. For example, at 300 MPa/3 and 5 min the acids vanillic acid and cinnamic were not detected. In addition, at 300 MPa/1, 3 and 5 min the trans-ferulic acid presented both free and bound fractions indicating conversion of free to bound phenolics or even the complete depletion of this compound. These results could be related to incomplete inactivation of oxidative enzymes (Keenan, Brunton, Gormley, & Butler, 2011). Moreover, the loss of total phenols could be due to the oxidation degradation of phenolic compounds and the polymerization of phenolic compounds with proteins (Wang et al., 2012).

After 60 days of storage, the phenolics acids presented the profiles shown in Table 5. The control phenolic acids have changed their original free to bound ratio profiles. The application of HHP reduced the content of both fractions ($P < 0.05$). In particular, the free form of the vanillic acid disappeared at all the treatments applied. Although the degradation of some phenolic acids yielding other phenolic acids during HHP requires more investigations, some authors attributes this to the effects of enzymes. Thus, the reduction of the content of the phenolic acids could be due to the presence of enzymes that were not inactivated by HHP (Keenan, Rößlea, Gormley, Butler, & Brunton, 2012; Queiroz, Mendes Lopes, Fialho, & Valente-Mesquita, 2008).

3.6. Effect on antioxidant capacity

Table 6 shows the antioxidant capacity of fresh and pressurized pulp samples determined by means of three analyses (DPPH, FRAP and ORAC). Initial antioxidant activity was 94.07, 2763.66 and 3243.09 μmol TE/100 g d.m. for DPPH, FRAP and ORAC, respectively. The different levels obtained from these assays may indicate a relative difference in the ability of antioxidant compounds in the extracts to quench aqueous peroxyl radicals (Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Byrne, 2006). As can be observed from Table 5, the immediate application of HHP showed significant increased in antioxidant capacity of Cape gooseberry pulp. Regarding DPPH, all treatments increased the initial value, for FRAP and ORAC some treatments showed increased in the antioxidant capacity ($P < 0.05$). The three methods presented the highest values of antioxidant capacity at 500 MPa/5 min. These results are in accord previous investigations working with of tomato, carrot and blackberry purees; and orange juice (Patras et al., 2009; Polydera, Stoforos, & Taoukis, 2005). Besides, comparable results were reported by previous work determining the effects of HHP (300–500 MPa/1–3 min) on antioxidant capacity of aloe vera gel by means of DPPH (Vega-Gálvez et al., 2011).

Typically, HHP treatments are thought to influence the extraction yield of some bioactive compounds. However, the effect of pressure on antioxidant activity is not the same among food products. Literature data varies considerably with the effects both time–temperature and food matrix dependent (Keenan et al., 2012; Oey et al., 2008).

When analyzing the samples after storage (Day 60), all treatments decreased the antioxidant capacity of samples measured by means of DPPH compared to control samples at Day 60. Regarding ORAC, a significant increased was observed at 300 MPa/5 min and 400 MPa/1 min. The treatment at 300 MPa/5 min presented the highest value of antioxidant capacity. Some authors have reported that high hydrostatic pressure processing either increases or maintains the antioxidant depending on different level pressure and holding time (Di Scala et al., 2013). Comparable results were reported for pomegranate juices treated with HHP after 35 days storage (Varela-Santos et al., 2012) and blueberry juices (Barba et al., 2012). Jacobo-Velazquez and Hernandez-Brenes (2012) reported that antioxidant capacity (ORAC) of avocado paste carotenoids decreased immediately after HHP treatment and then

Table 5
Free and bound phenolics acids of Cape gooseberry pulp as affected by HHP at Day 60.

Acids[a]		Treatments									
		Control	300 MPa/1 min	300 MPa/3 min	300 MPa/5 min	400 MPa/1 min	400 MPa/3 min	400 MPa/5 min	500 MPa/1 min	500 MPa/3 min	500 MPa/3 min
Syringic	F	4.44 ± 0.19^{a}	2.82 ± 0.19^{bc}	2.35 ± 1.07^{c}	3.18 ± 0.52^{b}	2.63 ± 0.04^{bc}	2.55 ± 0.17^{bc}	2.42 ± 0.10^{c}	2.15 ± 0.15^{c}	2.50 ± 0.15^{c}	2.26 ± 0.12^{c}
	B	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Vanillic	F	4.05 ± 0.42	nq	nq	nd	nd	nd	nd	nd	nd	nd
	B	3.00 ± 0.11^{a}	1.75 ± 0.45^{b}	1.60 ± 0.26^{b}	1.77 ± 0.30^{b}	2.38 ± 0.40^{c}	1.70 ± 0.05^{b}	1.67 ± 0.15^{b}	1.46 ± 0.26^{b}	1.87 ± 0.03^{b}	1.69 ± 0.14^{b}
p-Coumaric	F	nd	nd	nd	nd	nd	nd	nd	nd ±	nd	nd
	B	2.72 ± 0.37^{a}	1.78 ± 0.30^{bf}	1.21 ± 0.11^{c}	1.69 ± 0.19^{b}	2.43 ± 0.29^{ad}	1.31 ± 0.12^{ce}	1.86 ± 0.17^{bf}	1.23 ± 0.10^{cf}	2.08 ± 0.02^{d}	1.59 ± 0.12^{be}
Trans-ferulic	F	2.36 ± 0.07^{a}	nq	nq	nq						
	B	1.75 ± 0.18^{a}	1.10 ± 0.29^{bd}	0.07 ± 0.06^{c}	1.10 ± 0.12^{bd}	1.41 ± 0.27^{e}	0.88 ± 0.17^{bc}	$0.98\ 0.05^{bcd}$	$0.75\ 0.03^{cd}$	1.07 ± 0.06^{b}	$1.17\ 0.09^{de}$
Cinnamic	F	3.49 ± 0.08^{a}	nd	nq	0.11 ± 0.03^{b}	0.53 ± 0.04^{b}	1.67 ± 0.14^{c}	1.52 ± 0.07^{c}	1.53 ± 0.07^{c}	1.68 ± 0.05^{c}	1.59 ± 0.01^{c}
	B	0.56 ± 0.04^{ae}	0.51 ± 0.01^{abe}	0.40 ± 0.03^{bc}	0.73 ± 0.17^{d}	0.62 ± 0.07^{e}	0.51 ± 0.06^{abe}	0.45 ± 0.03^{abc}	0.37 ± 0.03^{c}	0.49 ± 0.05^{ab}	0.42 ± 0.01^{bc}

Values are mean ± standard deviation ($n = 3$).
nd: No detected.
nq: No quantifiable; its retention time matches only (not its spectrum).
Similar letters in the exponential in the same row show there are no significant differences ($P > 0.05$) according to the classification obtained by the multiple range test.
[a] mg/100 g d.m. F: free; B: bound.

increased with storage. The effect of pressure on the antioxidant capacity is not the same among the food products. In addition, apparently exposure of plant foods to high hydrostatic pressures would change plant matrix structures influencing the extraction methods prior determining antioxidant capacity. More investigations are required in order to relate antioxidant capacity of processed fruits with microstructure and physico-chemical changes induced by application of high hydrostatic pressure.

4. Conclusion

Cape gooseberry pulp subjected to HHP at 300, 400 and 500 MPa during 1, 3 and 5 min were affected in terms of quality after processing and storage (60 days at 4 °C). The immediate analysis of quality indices of the treated pulp evidences clear influence of HHP on chemical composition (moisture, fat, protein, and carbohydrates), color, bound and free phenolic acids, total phenolic content as well as antioxidant capacity. The maximum TPC was observed at 500 MPa/5 min. Regarding antioxidant capacity, all treatments increased its value when DPPH method was used compared to control samples. The highest value observed by means of ORAC determination was at 500 MPa/5 min. After 60 days of storage at 4 °C all treated samples decreased their chromatic parameters leading to $\Delta E = 14.9$ at 500 MPa/5 min. The profile of free and bound phenolic acids presented differences compared to Day 0. TPC did not show significant differences compared to control samples and antioxidant capacity increased for treatments above 300 MPa/5 min.

These results showed that this technology has a potential use in the preservation of Cape gooseberry pulp quality, which would be highly beneficial for the development of new functional food products based on this pulp.

Table 6
Changes in antioxidant capacity (DPPH, FRAP and ORAC) of Cape gooseberry pulp immediately after HHP (Day 0) and after storage at 4 °C (Day 60).

Treatments	Antioxidant capacity (μmol TE/100 g d.m.)		
	DPPH	FRAP	ORAC
Day 0			
Control	$94.07 \pm 4.06^{a,A}$	$2763.66 \pm 53.25^{abf,A}$	$3243.09 \pm 176.25^{ac,A}$
300 MPa/1 min	$116.67 \pm 11.47^{b,A}$	$2899.69 \pm 109.14^{a,A}$	$3557.43 \pm 352.40^{ab,A}$
300 MPa/3 min	$110.74 \pm 10.89^{b,A}$	$2659.52 \pm 89.48^{bf,A}$	$2816.15 \pm 365.01^{ac,A}$
300 MPa/5 min	$121.05 \pm 0.50^{b,A}$	$2429.89 \pm 83.04^{c,A}$	$2174.24 \pm 264.86^{c,A}$
400 MPa/1 min	$153.67 \pm 6.31^{c,A}$	$3275.26 \pm 103.73^{d,A}$	$6432.72 \pm 7.85^{d,A}$
400 MPa/3 min	$172.39 \pm 3.44^{d,A}$	$3412.41 \pm 136.57^{e,A}$	$5486.52 \pm 111.21^{de,A}$
400 MPa/5 min	$138.00 \pm 1.18^{e,A}$	$2353.57 \pm 53.99^{c,A}$	$4059.54 \pm 269.43^{ab,A}$
500 MPa/1 min	$159.68 \pm 4.02^{c,A}$	$2784.24 \pm 86.24^{af,A}$	$4776.06 \pm 318.07^{be,A}$
500 MPa/3 min	$139.25 \pm 2.17^{e,A}$	$2602.17 \pm 16.57^{b,A}$	$4441.12 \pm 200.95^{de,A}$
500 MPa/5 min	$210.22 \pm 10.08^{f,A}$	$3583.20 \pm 18.36^{e,A}$	$7176.02 \pm 224.35^{d,A}$
Day 60			
Control	$171.01 \pm 2.44^{a,B}$	$2366.7 \pm 34.20^{a,B}$	$2984.06 \pm 150.20^{ac,A}$
300 MPa/1 min	$139.10 \pm 4.07^{bc,B}$	$2181.7 \pm 32.57^{bf,B}$	$3106.46 \pm 344.75^{ab,A}$
300 MPa/3 min	$145.15 \pm 4.24^{c,B}$	$2295.0 \pm 59.32^{ac,B}$	$3316.18 \pm 823.04^{ab,A}$
300 MPa/5 min	$133.16 \pm 9.88^{b,A}$	$2795.1 \pm 71.97^{d,B}$	$3544.88 \pm 578.09^{b,B}$
400 MPa/1 min	$135.05 \pm 5.50^{b,B}$	$2628.3 \pm 70.99^{e,B}$	$3097.97 \pm 381.62^{ab,B}$
400 MPa/3 min	$67.89 \pm 7.83^{d,B}$	$2251.3 \pm 48.15^{bc,B}$	$3016.09 \pm 504.96^{ab,B}$
400 MPa/5 min	$75.72 \pm 5.46^{d,B}$	$2267.5 \pm 92.57^{abc,A}$	$3298.27 \pm 429.73^{ab,B}$
500 MPa/1 min	$107.52 \pm 9.46^{e,B}$	$2063.7 \pm 87.09^{f,B}$	$3320.71 \pm 129.26^{b,B}$
500 MPa/3 min	$133.60 \pm 3.70^{b,A}$	$2105.2 \pm 59.39^{f,B}$	$2385.22 \pm 223.65^{cd,B}$
500 MPa/5 min	$107.28 \pm 6.29^{e,B}$	$1848.7 \pm 22.88^{g,B}$	$1892.25 \pm 218.73^{d,B}$

Values are mean ± s.d. ($n = 3$).
a–e different letters in the same file indicate significant differences ($P < 0.05$).
A–D different letters in the same column indicate significant differences ($P < 0.05$).

Acknowledgments

The authors gratefully acknowledge the financial support of project FONDECYT 1120102 and Research Department of Universidad de La Serena (DIULS), La Serena, Chile, as well as CONICYT for Jessica Lopez fellowship.

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