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Orange juice processing using a continuous flow ultrasound-assisted supercritical CO_2 system: microbiota inactivation and product quality

Paniagua-Martínez, I.[1,3], Mulet, A.[1], García-Alvarado, M.A.[2] Benedito, J.*[1]

1: Food Technology Department. Universitat Politècnica de València. Camino de Vera s/n, Valencia, Spain.

2: Chemical and Biochemical Engineering Department. Instituto Tecnológico de Veracruz. Av. Miguel A. de Quevedo 2779. Veracruz, Mexico.

3: Universidad Veracruzana, Facultad de Bioanálisis, Carmen Serdán e Iturbide S/N, col. Flores Magón 91700, Veracruz, Veracruz, México.

*Corresponding author: Grupo de Análisis y Simulación de Procesos Agroalimentarios, Departamento Tecnología de Alimentos, Universitat Politècnica de València, Camí de Vera s/n, E46022, Valencia, Spain. Tel.: +34-96-3879147 Fax:+34-96-3879839.

E-mail address: jjbenedi@tal.upv.es (J. Benedito)

Abstract

The feasibility of using supercritical CO_2 assisted by ultrasound (SC-CO_2-HPU) in continuous mode (3.06 min residence time) for the non-thermal pasteurization of orange juice was evaluated. The proposed technology was effective for microbial inactivation; complete inactivation was obtained for *E. coli* and total aerobic mesophilic bacteria while 99.7% reduction for *S.cerevisiae*. Results showed that the SC-CO_2-HPU treatment brought about small changes in the pH, ºBrix and titratable acidity of the juice. Furthermore, although SC-CO_2-HPU technology produced a higher browning index (211%) and greater changes in color, it was possible to improve the cloud of juice by 173%; what is more, a smaller percentage of phenolic compounds (6.5%) and ascorbic acid (5.5%) was lost compared to the thermally pasteurized juice (10 % decrease in both parameters). Moreover, the antioxidant capacity could be increased (12%) with respect to the natural juice. Therefore, SC-CO_2-HPU technology appears to be effective for microbial pasteurization and the mild process conditions used could lead to an increase in the juice quality.

Industrial relevance

The demand for high quality processed foods which preserve their natural and fresh-like characteristics has awakened a growing interest in non-thermal technologies. The ultrasound-assisted SC-CO_2 continuous system is an innovative non-thermal technology that could represent a development in the area of emerging technologies. This technology allows high quality products to be obtained by preserving their natural bioactive compound content while maintaining their fresh-like organoleptic characteristics. In fact, food experts working in academia, industry or governmental agencies worldwide foresee that non-thermal emerging technologies will be among the most impactful novel food processing technologies for the next decade in terms of product commercialization.

Keywords: Non-thermal process, supercritical CO_2, ultrasound, continuous regime, orange juice

1. Introduction

In recent years, while developed countries have witnessed a rise in the consumption of processed fruit juices, that of fresh citrus fruit has been on the wane (Tiwari, O'Donnell, Muthukumarappan, & Cullen, 2009a). Worldwide, orange juice is a very popular product due to its high nutritional value, its bioactive components, such as phenolic compounds, vitamin C and carotenoids, and its sensory characteristics (Ortuño, Balaban, & Benedito, 2014).

Despite its low pH, this juice needs to be processed because it is of limited stability due to microbial growth and enzyme activity, which can cause unpleasant organoleptic changes or the degradation of compounds during storage (Fabroni, Amenta, Timpanaro, & Rapisarda, 2010; Ferrentino, Plaza, Ramirez-Rodrigues, Ferrari, & Balaban, 2009; Khandpur, & Gogate, 2016 ; Liu et al, 2010; Zinoviadou et al, 2015).

Although thermal pasteurization remains the most commonly-used method for the preservation of juices, there is growing interest in developing alternative techniques. The new techniques are expected to minimize changes in the nutritional and organoleptic characteristics of food, obtaining fresher and richer juices than traditional thermal technology. Two such techniques are high hydrostatic pressure (HHP) and pulsed electric fields (PEF), which result in better quality retention and adequate shelf life; however, they cannot inactivate enzymes, such as PME, well enough to produce a shelf-stable juice, unless they are combined with elevated temperatures. In addition, these new technologies involve high investment and operational costs, which is an important obstacle to their industrial application (Niu et al, 2010; Ozuna, Paniagua-Martínez, Castaño-Tostado, Ozimek, & Amaya-Llano, 2015; Tiwari, Muthukumarappan, O'donnell, & Cullen, 2009b; Vervoort et al., 2011). Moreover, at present, HHP processing consists of batch processes, which limits its use because of its low processing capacity (Damar & Balaban, 2006).

For the purposes of processing large volumes of liquid food, such as orange juice, a continuous preservation process is more desirable. This objective can be attained by applying supercritical fluids, a non-thermal preservation technique in which both CO_2 and the product are pumped through the system by high-pressure pumps, mixed and maintained in contact for a period of time (Fabroni et al., 2010; Paniagua-Martínez, Mulet, García-Alvarado, & Benedito, 2016).

Supercritical CO_2 (SC-CO_2) has a density close to that of liquids, as well as gas properties like high diffusivity and low viscosity; therefore, it has excellent transport properties. Furthermore, these properties can be controlled by temperature and pressure

changes (Calix, Ferrentino, & Balaban, 2008; Niu et al, 2010.; Wimmer & Zarevúcka, 2010). Supercritical CO_2 is considered an excellent alternative to solvents because of its non-toxic and non-flammable nature and its relatively low critical pressure and temperature (73.6 bar, 31.0 °C). Moreover, the SC-CO_2 has a lethal effect on bacteria (Garcia González et al., 2007). This effect is directly proportional to the applied pressure, time and temperature. SC-CO_2 acts on bacteria as follows: first, solubilization occurs in the external liquid phase, causing carbonic acid formation (which dissociates into bicarbonate and hydrogen ions); therefore, it increases cell membrane fluidity and permeability, increasing the diffusion of CO_2 into the cell and causing a decrease in intracellular pH. Thus, the inactivation/inhibition of key cellular metabolic enzymes for microorganisms occurs. As a result, a disorder in the electrolyte balance of intracellular constituents is produced and vital constituents of cells and cell membranes are extracted (Fabroni et al., 2010; Garcia González et al., 2007; Kincal et al., 2005; Ortuño, Martínez-Pastor, Mulet, & Benedito, 2013; Paniagua et al., 2016).

Despite all the aforementioned advantages of SC-CO_2 inactivation, even the continuous systems require long treatment times and high pressures and temperatures (Fabroni et al., 2010; Kincal et al., 2005) to ensure the safety and stability of food, limiting the efficiency of the inactivation process, compromising the food quality and increasing processing costs. In this sense, there is growing interest in process intensification, with the simultaneous application of different non-thermal technologies, in the search for synergistic effects. One of the techniques that synergistically improves the inactivation mechanisms of SC-CO_2 is high power ultrasound (HPU), which accelerates and improves heat and mass transfer processes (Ortuño et al., 2013, 2014; Paniagua et al., 2016).

When high power ultrasound propagates in a liquid, cavitation bubbles are generated by pressure changes. These microbubbles collapse violently in the succeeding compression cycles of a propagated sonic wave. This results in localized high temperatures, pressures and significant shearing effects. Consequently, the intense local energy and high pressure bring about a localized pasteurization effect (without causing significant temperature increases, while shortening processing time and cutting energy consumption) (Abid et al., 2013; Tiwari, Mu hukuma appan, O'Donnell, & Cullen, 2008a; Tiwari et al., 2009). Therefore, with the combination of SC-CO_2 and HPU (SC-CO_2-HPU), an increase is produced both in the solubilization rate of SC-CO_2 in the liquid and in the mass transfer due to the vigorous stirring produced by the ultrasonic field. Thereby, a quick saturation of CO_2 in the medium is achieved, as well as the intensification of the inactivation mechanisms. Furthermore, cavitation and agitation produced by the HPU cause cell wall damage, increasing the SC-CO_2 penetration, the

intracellular compound extraction and the death of microbial cells. In addition, thermal, chemical and mechanical effects induced by HPU cavitation contribute to enzyme inactivation (Tiwari et al., 2008a). The combined use of use of SC-CO_2 and HPU can be considered as a green processing technique since it can contribute to the reduction of energy and waste, the increase of the product quality and safety and the decrease of the carbon and water footprint (Chemat et al., 2017).

Ortuño, Martínez-Pastor, Mulet, and Benedito (2012) reported that by using a batch-mode SC-CO_2 at 350 bar and 36 °C for 25 min, a reduction of 1 log-cycle in *Escherichia coli* DH1 (*E. coli*) was obtained in orange juice. However, Kincal et al. (2005) reported that a continuous SC-CO_2 treatment (210 bars, 34.5 °C, 10 min residence time) caused at least a 5 log-cycle reduction in pathogens (*E. coli* O157: H7, *Salmonella* Typhimurium and *Listeria monocytogenes*). Consequently, it can be expected that batch-mode equipment requires a much longer inactivation time compared to continuous SC-CO_2 systems. There are a few studies of batch-mode SC-CO_2 intensified using ultrasound (SC-CO_2-HPU); two of them prove the complete inactivation of the *E.coli* and *S.cerevisiae* population in orange juice after 1.5 min (225 bar, 36 °C) and 5 min (350 bar, 36 °C) of treatment, respectively (Ortuño et al., 2012, 2013). In order to improve the efficiency of batch SC-CO_2 treatments, a continuous system was developed by Paniagua et al. (2016) who studied the inactivation of *S. cerevisiae* in apple juice, using the continuous flow SC-CO_2-HPU at different juice residence times (3.06-9.2 min), temperatures (31-41 °C) and pressures (100-300 bars). The results demonstrated that the maximum inactivation achieved by the system was 7.8 log-cycles. However, there are no studies covering either the use of this continuous technique (SC-CO_2-HPU) for other types of juices or the effect of the process on the product quality. Therefore, the aim of this study was to determine the effect of SC-CO_2-HPU treatment in a continuous regime on both the inactivation of the microbiota and the quality attributes of orange juice.

2. Materials and methods

2.1. Orange juice

Valencia Navel oranges (*Citrus sinensis*) were purchased from a local market and kept at 4 °C for 2 days until juice extraction. Orange juice was obtained by washing, peeling and extracting the fruit juice (Ultra Juicer, Robot Coupe J80, USA). Juice extraction took place just prior to the treatment application; consequently, an extraction was required for each experiment. Each experiment required about 1.5 L of juice, 1 L was used for processing (SC-CO_2-HPU and thermal pasteurization), and 0.5 L served

as control. Juices were not inoculated and only the inactivation of the microbiota was considered.

2.2. SC-CO_2-HPU processing

Laboratory continuous regime equipment was designed and built for supercritical CO_2 assisted by high power ultrasound (SC-CO_2-HPU) (Figure 1) (Paniagua et al., 2016).

The SC-CO_2-HPU process applied to the juice was as follows: first, liquid carbon dioxide was supplied from the tank to the chiller reservoir in which it was compressed to 200 bar by means of the injection of pressurized gaseous N_2. The liquid CO_2 was supplied from the bottom of the chiller reservoir (which stores it at −18 °C) to the pump where it was compressed at the target pressure. The equipment was stabilized at the treatment pressure (P) and temperature (T) by flowing SC-CO_2 at a constant flow rate of 5 mL/min. Thereafter, the ultrasound equipment was connected, and once the process conditions (P, T) were attained, the sample to be treated was pumped to the mixing point (7, Fig.1) where it mixed with the SC-CO_2. The mixture went into the sonication vessel (8, Fig. 1), where the HPU was applied. For the experiments with HPU, the power applied during the whole experiment was 40 W±5W (I=250 ±10mA; U=220 ±5 V, measured with a Digital Power Meter, Yokogawa, Model WT210). Pressure and temperature were kept constant during the experiment. The mixture of juice/SC-CO_2 exiting the sonication vessel went into the holding tube (14, Fig. 1) and, finally, into the separation vessel (15, Fig. 1), where it was depressurized and the CO_2 separated from the juice and recirculated to the reservoir (3, Fig. 1). Prior to each experiment, the different sections of the equipment through which the product flows were cleaned and sanitized with disinfectant solution (Delladet VS2, Diversey, Spain) and distilled and autoclaved water. To determine the effect of temperature on both the quality parameters and on the inactivation of the microbiota of orange juice, samples (0.5 L) were treated by SC-CO_2-HPU in a continuous system at 100 bar and different temperatures (31, 36 and 41 °C). The pressure and temperature conditions were selected according to Paniagua et al. (2016), taking into account that low pressures reduce the operating costs while maintaining an acceptable microbial inactivation. The flow rate of juice was 25 mL/min and the residence time 3.06 min. The process conditions were selected from previous experiments in order to attain adequate inactivation levels. All the experiments were run in triplicate.

2.3. Heat treatment

To evaluate the effect of conventional thermal treatment on the quality parameters and microbiota inactivation of orange juice, the juice was pasteurized (PASC Computer Controlled Laboratory pasteurizer, EDIBON, Spain) at 90 °C for 1 minute. For this purpose, the juice was placed in a feed tank, driven by a pump to a plate heat exchanger, rapidly heated to the desired temperature and taken to the holding tube where it remained throughout the processing time. After the treatment, the juice was cooled rapidly in a water bath (4 °C). Experiments were run in triplicate. Thus, it was possible to compare the SC-CO_2-HPU processing results (quality and microbiology) with those of the conventional heat treatment.

2.4. Microbiota analysis

The viability of *E. coli*, total aerobic mesophilic and *S. cerevisiae* in the orange juice samples was determined by the plate count method to evaluate the effect of both treatments (SC-CO_2-HPU in continuous system and thermal pasteurization) on the microbiota of orange juice. Each sample was serially diluted with sterilized distilled water. 100 µL of the appropriate dilution (10^{-1} and 10^{-2}) were plated in triplicate on LB Agar, PCA Agar or YPD Agar plates and incubated for 24 h at 37 °C, 35 °C or 30 °C, for *E. coli*, total aerobic mesophilic or *S. cerevisiae,* respectively, before counting. Results were expressed as $-\log(N/N_0)$, where N_0 is the initial number of cells in the control sample and N is the number of cells in the sample after the different treatments. When the total microbial inactivation was achieved, results were expressed as $\log(N_0)$.

2.5. Physico-chemical analysis of orange juice

All the physico-chemical measurements were taken in triplicate.

2.5.1. pH and °BRIX

The pH of treated and untreated orange juice samples was measured using a digital pH-meter (pH Crison 25, Spain). Samples were measured in triplicate at room temperature.

Soluble solids were measured using a refractometer (Pocket Digital Refractometer Hand-held, Atago, Japan). Measurements were taken in triplicate at room temperature.

2.5.2. Titratable acidity

Titratable acidity was measured using the method described by Kincal et al. (2006), using NaOH 0.1 N. Results were obtained in triplicate and expressed as grams of citric acid per 100 mL of juice.

252 2.5.3. Phenolic compounds

253 Total phenolic compounds were determined by the method described by Gao,
254 Ohlander, Jeppsson, Björk, and Trajkovski (2000) applying 1:3 dilution factor of the
255 samples. The quantification of the phenolic compounds with respect to a standard curve
256 of gallic acid with concentrations between 110.4 and 552 ppm was performed. Results
257 were expressed as ppm equivalent of gallic acid.
258
259 2.5.4. Antioxidant capacity (FRAP)

260 Antioxidant capacity was assessed by the method described by Pulido, Bravo, and
261 Saura-Calixto (2000) using the FRAP reagent and applying 1:20 dilution factor of the
262 samples. To obtain the results, a calibration curve of Trolox with concentrations between
263 50 and 750 μM was built, plotting the concentration of Trolox versus absorbance at 30
264 minutes. The antioxidant capacity of samples at 30 minutes with the FRAP reagent was
265 expressed as the equivalent Trolox concentration at 30 minutes.
266
267 2.5.5. Browning index

268 Browning index was used to discover the effect of treatments on juice browning. For
269 this purpose, a spectrophotometric method was used after centrifuging and filtering the
270 samples. This method is described by Xu et al. (2011). In the present study, however,
271 the centrifugation time was 10 minutes and the angular velocity 12600 rpm.
272
273 2.5.6. Color

274 Color was measured using a colorimeter (Spectrophotometer CM-2500d, Konica
275 Minolta, Japan) based on the L*, a*, b* color coordinates (Kincal et al., 2006; Ferrentino
276 et al, 2009). Color measurements were taken in triplicate.

277 The total color difference (ΔE) was determined from Equation 1, which indicates the
278 magnitude of the color change after treatment.
279

280 $$\Delta E=[\ (L-L_0)^2+(a-a_0)^2+(b-b_0)^2\ ]^{½} \quad (1)$$

281

282 where: L_0, a_0 and b_0 are the color values of untreated juice and L, a and b those of the
283 treated samples. Differences in perceivable color can be classified as very different (ΔE
284 > 3), different (1,5 < ΔE < 3) and slightly different (ΔE < 1,5) (Tiwari, Muthukumarappan,
285 O'donnell, & Cullen, 2008b).
286
287 2.5.7. Cloud

To evaluate the loss of cloud or juice clarification after treatment, a spectrophotometric method was used after sample centrifugation, as described by Ferrentino et al. (2009).

Absorbance was recorded as the cloud value with distilled water used as blank. The percentage of cloud change was calculated by Equation 2.

$$\text{Percentage cloud change} = \frac{\text{final cloud value} - \text{initial cloud value}}{\text{initial cloud value}} \cdot 100 \quad (2)$$

2.5.8. Ascorbic acid

The ascorbic acid content was measured using the 2, 6 dichloroindophenol titrimetric method (AOAC 967.21). The ascorbic acid reduced the indicator dye, 2, 6 dichloroindophenol, to a colorless solution through oxidation–reduction reactions.

2.6. Statistical Analysis

Using the statistical package, Statgraphics Centurion XVI, a multifactorial ANOVA was carried out, and LSD (Least Significant Differences) were identified in order to evaluate the influence of the treatments considered (Ortuño et al., 2012).

3. Results and discussions

3.1. *Microbiota inactivation after the* SC-CO_2-HPU treatment

The inactivation of the microbiota of orange juice is shown in Table 1. After the SC-CO_2-HPU treatment was applied, the total inactivation of the initial microbial load of *E.coli* and total aerobic mesophilic bacteria was measured at the different temperatures employed. However, the initial population of *S.cerevisiae* could not be completely inactivated, obtaining levels of inactivation of 2.60, 2.24 and 2.19 log cycles at 31, 36 and 41 ºC, respectively, which corresponds to average reductions of 99.7, 99.4 and 99.3%, respectively. The use of different temperatures produced no significant differences ($p>0.05$) in the level of *S.cerevisiae* inactivation. The difficulty of achieving the complete inactivation of *S. cerevisiae* could be related with its thicker cell wall, which measures 124.8 nm, in comparison with *E.coli*, which is 17.7 nm but also mainly to the different structure of bacteria and yeast (Ortuño et al., 2014). In a similar way to the results of the present study, Ortuño et al. (2014) obtained a reduction of 7 and 4 log units in *E.coli* and *S.cerevisiae*, respectively, starting from the same initial cell concentration,

for a treatment of orange juice with SC-CO_2-HPU (225 bar; 31ºC; 6 min). In the same way, but using SC-CO_2-HPU in continuous system (200 bar, 36°C), Paniagua-Martínez et al. (2016) obtained reductions of 6.8 log cycles in *S. cerevisiae* in 3.1 min of residence time using apple juice as model medium. Fabroni et al. (2010), studied the effect of continuous SC-CO_2 (130-230 bar, 5.08 L/h juice flow rate, 1.96-3.91 L/h of CO_2 flow rate and 15 min of residence time) on the inactivation of total aerobic mesophilic bacteria and yeast population in blood orange juice, obtaining reductions of 3 log cycles for each type of microorganism.

On the other hand, the thermal pasteurization treatment attained the complete inactivation of the assessed microbiota.

3.2. Effect of the SC-CO_2-HPU treatment on the physico-chemical properties of orange juice

3.2.1. pH, °Brix, Titratable acidity (TA)

The results of pH, °Brix and TA are shown in Table 2. The continuous treatment of orange juice using SC-CO_2-HPU had a non-significant ($p>0.05$) effect on the pH of the juice, similarly to what happens in the case of the thermal treatment (Table 2). No significant ($p<0.05$) differences were observed between the pH of the juice after the SC-CO_2-HPU or the pasteurization treatments. This could be due to the short treatment time and to the low initial pH value of the juice. In this regard, for pH values of 3.7-3.8, the dissociation of the carbonic acid formed by the dissolution of the CO_2 into the juice is difficult, due to the high dissociation constants of carbonic acid and the bicarbonate (pKa=6.57 and pKa=10.62, respectively) (Zhou, Wang, Hu, Wu, & Liao, 2009). Kincal et al. (2006) observed a change of between 0.14% and 0.54% in the pH of orange juice treated with a continuous SC-CO_2 process (380, 720 and 1070 bar; 0.40-1.18 ratio CO_2/juice; 40ºC; 10 min). Fabroni et al. (2010) observed an increase in the pH of orange juice of around 1.47% after a treatment with a continuous SC-CO_2 process (230 bar; 5.08 L/h juice; 3.91 L/h CO_2; 36ºC; 15 min), as well as a percentage of 1.18% after thermal pasteurization. As can be observed from Table 2, the range of pH values of the control samples used in the different experiments comprises the range of the pH of the treated ones; this points to the scarce impact of the treatment on this quality attribute, the natural variability being more noticeable than the possible effect of the treatment.

In the case of the °Brix, the results obtained showed a slight decrease at 31 ºC (-0.81%), 36 ºC (-1.74%) and 41 ºC (-1.41%), although non-significant ($p<0.05$) differences between the control and processed juice samples were observed for any

treatment. Gasperi et al. (2009) studied the use of a batch SC-CO_2 treatment (100 bar; 36 ºC; 10 min) on apple juice, and found a reduction percentage of 0.85%. Kincal et al. (2006) obtained reductions of approximately 1.80% after a continuous SC-CO_2 treatment (380 bar; 0.40 ratio CO_2/juice; 40ºC; 10 min) of orange juice. As happened for the pH, the natural variability of the juice is more important than the possible influence of the treatment on the ºBrix.

Finally, the acidity results showed that, although an average reduction of 4.94% was found for the SC-CO_2-HPU treated samples, the differences were not significant ($p>0.05$) due to the high degree of variability of the natural orange juice and the resulting treatments.

In a similar way, both Kincal et al. (2006) and Tiwari et al. (2008a) found non-significant changes in acidity after a continuous SC-CO_2 treatment (720 bar; 0.64 ratio CO_2/juice; 40ºC; 10 min) and an ultrasonic process (8.61-22.79 W/cm^2; 2-10 min), respectively.

3.2.2. Phenolic compounds and antioxidant capacity

The content of phenolic compounds significantly ($p<0.05$) decreased after the continuous SC-CO_2-HPU treatment compared to that of the untreated juice, for all the temperatures studied (-3.54, -3.68 and -4.15% at 31, 36 and 41ºC, respectively; Figure 2). The differences among the treatments for this parameter were only significant ($p>0.05$) between 31 and 41ºC. Moreover, a significant difference ($p<0.05$) in phenolic compounds was found between the SC-CO_2-HPU and pasteurization treatments; thus, while the average decrease in phenolic compounds for SC-CO_2-HPU treatments was of -3.79±0.9%, the thermal pasteurization brought about a decrease of -10%. This greater loss of phenolic compounds could be due to the high degree of degradation of carbohydrates and organic acids during the thermal processing, which could give rise to furfurals and other carbonyl compounds which may form condensation products with polyphenols (Fabroni et al., 2010). The results found in this study coincide with those reported by Fabroni et al. (2010), who found reductions of 5.27% after a continuous SC-CO_2 treatment (130 bar; 5.08 L/h juice; 1.96 L/h CO_2; 36ºC; 15 min) of orange juice and 9.99% for a conventional thermal pasteurization treatment (90ºC, 30 s). Therefore, it seems that the use of HPU, which intensifies the microbial inactivation, do not negatively affect the amount of phenolic compounds in the processed orange juice. Similarly, Rawson et al. (2011a) observed no reduction in the content of phenolic compounds after a HPU treatment (24.1–60 µm; 25-45 ºC; 2-10 min) of watermelon juice.

The antioxidant capacity results showed a significant ($p<0.05$) decrease in the samples processed with SC-CO_2-HPU compared to the control samples (Figure 3), except for the treatment at 31 ºC in which a significant ($p<0.05$) increase (12.13%) was obtained. However, between 36 and 41 ºC, there were no significant differences, leading the treatments to reductions of 3.68 and 3.96%, respectively. Therefore, the use of temperatures of over 31ºC in the SC-CO_2-HPU treatment leads to a greater reduction in the juice antioxidant capacity. On the other hand, thermal pasteurization presented a significantly ($p<0.05$) greater reduction (-9.07%) in the antioxidant capacity compared to the continuous SC-CO_2-HPU treatments. Fabroni et al. (2010) reported similar results after a continuous treatment with SC-CO_2 (130-230 bar; 5.08 L/h juice; 3.91 L/h CO_2; 36 ºC; 15 min) of orange juice: the percentages of antioxidant capacity decreased by between 1.39 and 2.53% versus 5.50 and 10.89% for pasteurized juice. However, in the present study, an increase in the antioxidant capacity was observed at 31ºC. It has been widely reported that the use of HPU can lead to an increase in the antioxidant capacity of vegetable samples, due to the increased extraction of active compounds. Therefore, two effects could be superimposed in the present study: the increase in the antioxidant capacity due to HPU and the decrease due to the SC-CO_2 treatment and the temperature. In the case of 31ºC, the result of these two effects brought about an increase in the antioxidant capacity, since the greater quantity of compounds extracted from juice pulp would compensate for the decrease produced by the SC-CO_2 treatment.

3.2.3. Browning index and color

Tables 3 and 4 show the results obtained for the browning index and color, respectively. The SC-CO_2-HPU treatment of orange juice produced a significant ($p<0.05$) increase in the browning index when compared with the control sample at every treatment temperature; the higher the temperature, the greater the browning index increase (Table 3). However, the only significant differences found were those between the treatment at 31 ºC and the other two temperatures considered. The average change in the browning index for the SC-CO_2-HPU treated samples was of 226%. An even greater difference between the variation of the browning index in treated and untreated samples was observed by Tiwari et al. (2008b) when working on sonicated orange juice (40-100% amplitude), where it increased by 636.8%. Those authors attributed the browning of the samples to the destruction of the pigments, mainly carotenoids, produced by the HPU. One of the main factors contributing to the browning of orange juice is ascorbic acid oxidation, leading to the appearance of reactive carbonyl groups, such as furfural and 5-hydroxymethylfurfural, which can be precursors of non-enzymatic browning (Bharate, & Bharate, 2012; Bull et al., 2004; Yeom, Streaker, Zhang, & Min,

2000). In addition, the browning effect could be linked to the decomposition of sugars or caramelization (Vervoort et al., 2011) as well as to the Maillard reactions between reducing sugars and free amino groups, leading to the formation of melanoidins, which are compounds that cause dark browning (Ibarz-Martínez, Pagán, Garza, & Ibarz, 2010). Vervoort et al. (2011) reported that the non-enzymatic browning is accelerated by the temperature and processing time, as observed for the SC-CO_2-HPU treatment (Table 3).

However, the changes in the browning index are much larger for the SC-CO_2-HPU treatment than for that of the thermal pasteurization. This shows that, although temperature is an influential factor as regards juice browning, the mixing of the juice with SC-CO_2 and/or the application of ultrasound are much more determinant.

On the other hand, the non-enzymatic browning produced by the treatment with SC-CO_2-HPU was also observed from the results of the color analysis (Table 4). For every treatment, there was a decrease in the L*, a*, b* values. Thus, the decrease in the L* value showed a loss in brightness or increase in darkness which is directly related with juice browning (Tiwari et al., 2008b; Yeom et al., 2000), although it could also be related with the juice cloud, because the reflected light is affected by the cloud (Liu, Hu, Zhao, & Song, 2012). The decrease in a* and b* values showed the color change to tonalities less red and yellow. Considering the ΔE parameters, the greatest color difference was obtained in samples treated with SC-CO_2-HPU at 36 °C, followed by 41 and 31 °C, while a smaller color difference was observed for the pasteurized juice. Moreover, in this case, the ΔE values do not point to a relationship with temperature. Therefore, with ΔE values above 3, the color changes were noticeable for every treatment considered. A similar finding was observed by Fabroni et al, (2010) in orange juice samples after continuous SC-CO_2 treatment (130 bar; 5.08 L/h juice; 1.96-3.91 L/h CO2; 36 ºC; 15 min) and thermal pasteurization (88-91 °C, 30s). These authors also observed a decrease in L*, a*, b* values for both types of treatments, obtaining ΔE values of 7.87-11.89 and 2.88-6.23 for the continuous treatment of SC-CO_2 and thermally pasteurized juice, respectively. The color changes that take place after SC-CO_2-HPU treatment could also be related to the cavitation effect of HPU, which regulates various physical, chemical and biological reactions, between them, carotenoid degradation due to the free radicals formed during the treatment (Abid et al., 2013; Tiwari et al., 2008a).

3.2.4. Cloud

The cloud is related with the particle suspension which is composed of a complex mixture of proteins, pectins, lipids, hemicellulose, cellulose, and other minor components

(Niu et al., 2010; Tiwari et al., 2009b). This is an important attribute that positively affects the turbidity, taste, aroma and characteristic color of orange juice. Its loss is mainly attributed to the enzymatic activity of the PME, which causes phase separation in the juice and the resulting loss of cloud (Bull et al., 2004; Polydera, Galanou, Stoforos, & Taoukis, 2004).

The SC-CO_2-HPU treatment of orange juice significantly ($p<0.05$) increased the cloud values when compared with the control at every temperature considered (Figure 4), showing that there is a significant ($p<0.05$) difference between the treatments at 31 and 36 ºC when compared to that at 41 ºC. The average increases in the cloud value were of 195.0, 198.4 and 270.6% at 31, 36 and 41ºC, respectively; therefore, the cloud value increased when the treatment temperature rose. In a similar way, after the SC-CO_2 treatment (400 bar; 55ºC; 10-60 min) of orange juice, Niu et al. (2010) obtained percentages of cloud increase of 91.33-115.48%. Also, Tiwari et al. (2008a), after the ultrasonic treatment of orange juice (8.61-22.79 W/cm2; 2-10 min) obtained increases of 63-222%. As can be observed, after the SC-CO_2-HPU treatment of juice, the cloud was preserved and improved. This phenomenon is mainly due to the reduction in the enzymatic activity of the PME, as well as the system depressurization, which homogenizes juice, causing the breakdown or reduction of the colloidal particles in the juice (Kincal et al., 2006; Liu et al., 2012). Another factor that contributed to the increase in the cloud is the HPU effect, which produced the rupture of the linear molecule pectin, reducing its molecular weight (Tiwari et al., 2009b). The cloud values obtained after pasteurization were significantly ($p<0.05$) lower than those obtained after SC-CO_2-HPU treatment which indicates that the use of this novel SC-CO_2-HPU-based technology could improve some quality attributes of orange juice while reducing the processing time compared to when only SC-CO_2 or HPU is used.

3.2.5. Ascorbic acid

The continuous SC-CO_2-HPU orange juice treatment at the different temperatures considered produced a statistically significant reduction ($p<0.05$) in ascorbic acid when compared with the control (Figure 5). However, a considerable percentage of ascorbic acid was preserved after treatments, observing reductions of only 4.55, 5.85, and 6.50%, at 31, 36, and 41 °C, respectively. As can be observed, the ascorbic acid loss increased as the temperature rose, although, in the range considered, the only significant ($p<0.05$) differences that exist are those between the treatment at 31 and that carried out at 41ºC. This slight degradation may be due to the formation of free radicals produced by the effect of cavitation generated by the HPU, leading to the oxidation of polar organic

compounds, such as ascorbic acid and total phenols; and it may also be due to the thermolysis produced inside bubbles and the subsequent activation of the Maillard reaction (Tiwari et al, 2009a; Rawson et al, 2011b). A significant difference ($p < 0.05$) between the ascorbic acid content of the juice after SC-CO_2-HPU treatment and thermal pasteurization was also observed, with a greater loss of ascorbic acid in the case of the thermal pasteurization (-10.05%). The greater reduction in ascorbic acid in the latter treatment can be explained by the application of a higher processing temperature, since ascorbic acid is a thermolabile nutrient (Sanchez-Moreno et al., 2005) and seems to be more affected by high temperatures than by the use of the combined treatment (SC-CO_2-HPU). Similar behavior was observed by Fabroni et al. (2010) after the continuous SC-CO_2 processing of orange juice, finding a lower reduction of ascorbic acid in SC-CO_2-HPU treated samples (6.37%, 130 bar; 5.08 L / h juice; 1.96 L / h CO_2; 36 ° C; 15 min) compared to the thermally pasteurized ones (88-91 °C, 30s). Also, Tiwari et al. (2009a) obtained reductions of 1.46-5.17% of ascorbic acid after the ultrasonic treatment of orange juice (from 0.33 to 0.88 W / mL; 10.2 min) and a reduction of 7.14% after thermal pasteurization. Despite the decrease in ascorbic acid when using SC-CO_2-HPU, at the lowest temperature, less than half is reduced if compared to the case of thermal pasteurization.

In order to apply new processing techniques at industrial level it is important to consider the best process conditions (Boukroufa, Boutekedjiret, Petigny, Rakotomanomana, & Chemat, 2015). In this regard, according to the results of the present study, the use of a low pressure (100 bar), close to the critical pressure of CO_2 (72.9 bar), and a low temperature (31ºC), which provides the best juice quality, would facilitate the scaling of the process to the juice industry. Nevertheless, since the use of ultrasound in liquid food could lead to degradation of some chemical compounds (Jacotet-Navarro et al., 2016), further research should be conducted to evaluate the influence of ultrasound intensification on possible degradation of bioactive compounds in juices.

4. Conclusions

The SC-CO_2-HPU continuous treatment was effective for microbial inactivation in orange juice, the effectiveness being dependent on the microbial cell wall thickness. The SC-CO_2-HPU continuous treatment did not affect the pH, ºBrix or Titratable acidity of the juice. Moreover, compared with thermal pasteurization, the loss of phenolic compounds was small and the antioxidant capacity could even be increased with respect to the untreated juice. Although the treatment affected the color of the juice, causing an overall

546 darkening, the cloud and, therefore, the stability of the treated juices were greatly
547 improved. The obtained results demonstrated the potential of the continuous SC-CO_2-
548 HPU inactivation technique, the use of mild process conditions leading to an increase in
549 the quality of the product processed using this technique. Moreover, the fact that the
550 proposed technique works in a continuous mode greatly facilitates its industrial
551 implementation.
552
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560
561 **6. References**
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697

Highlights

- Ultrasound-assisted continuous SC-CO_2 reduced the orange juice microbiota
- The treatment produced greater changes in color (darkening) than thermal pasteurization.
- The cloud of the treated juice and the phenolic and vitamin contents were improved by 173%, 6.5% and 5.5%, respectively.
- The antioxidant capacity of the treated juice increased with respect to the fresh (untreated) one.
- Ultrasound intensification may improve the acceptance of SC-CO_2 processing by the food industry.

Figure Captions

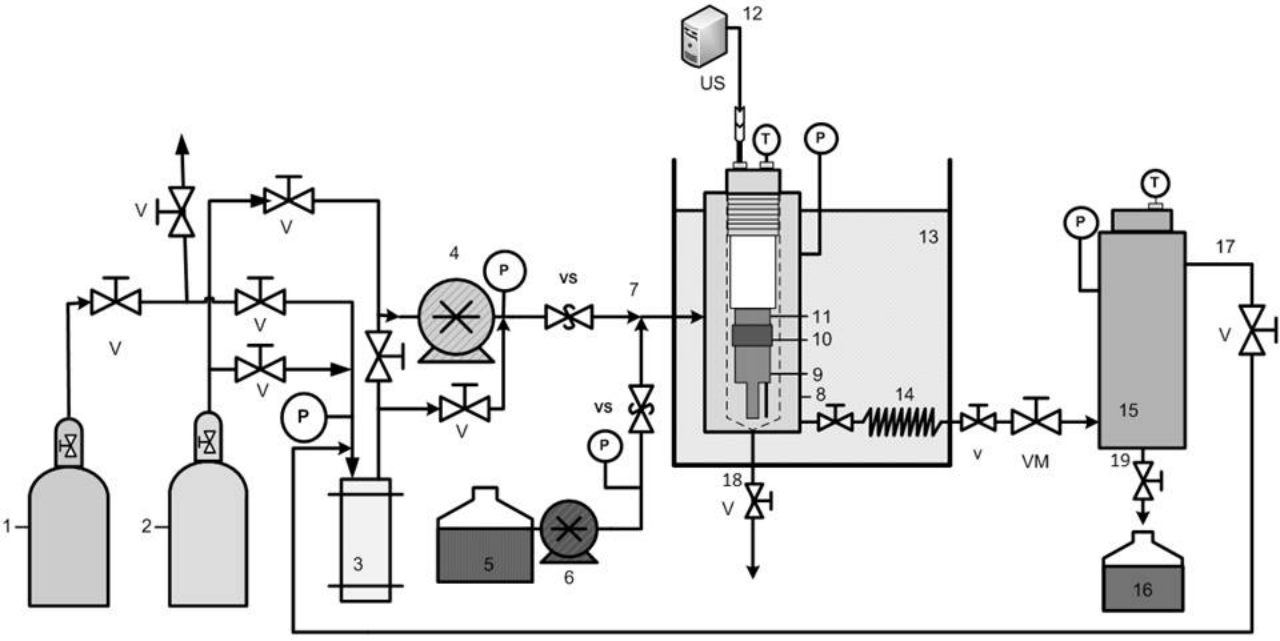
Fig. 1. Supercritical CO_2 continuous treatment system. 1. CO_2 tank; 2. N_2 tank; 3. Chiller reservoir; 4. CO_2 Pump; 5. Liquid reservoir; 6. Liquid Pump; 7. Mixing point; 8.Sonication vessel; 9. Sonotrode; 10. Insulation joint; 11. Ceramics; 12. Power generation unit; 13. Thermostatic bath; 14. Continuous contact tube; 15. Separation vessel; 16. Treated sample; 17. CO_2 Recirculation; 18. Sonication vessel output, 19. Separation vessel output, V. valve; VS. non-return valve; VM. micrometric valve; P. Manometer; T. temperature sensor.

Fig. 2. Loss of phenolic compound content in orange juice after different treatment conditions.

Fig. 3. Percentage variation of antioxidant capacity in orange juice under different treatment conditions.

Fig. 4. Percentage variation of cloud in orange juice under different treatment conditions.

Fig. 5. Percentage variation of ascorbic acid in orange juice under different treatment conditions.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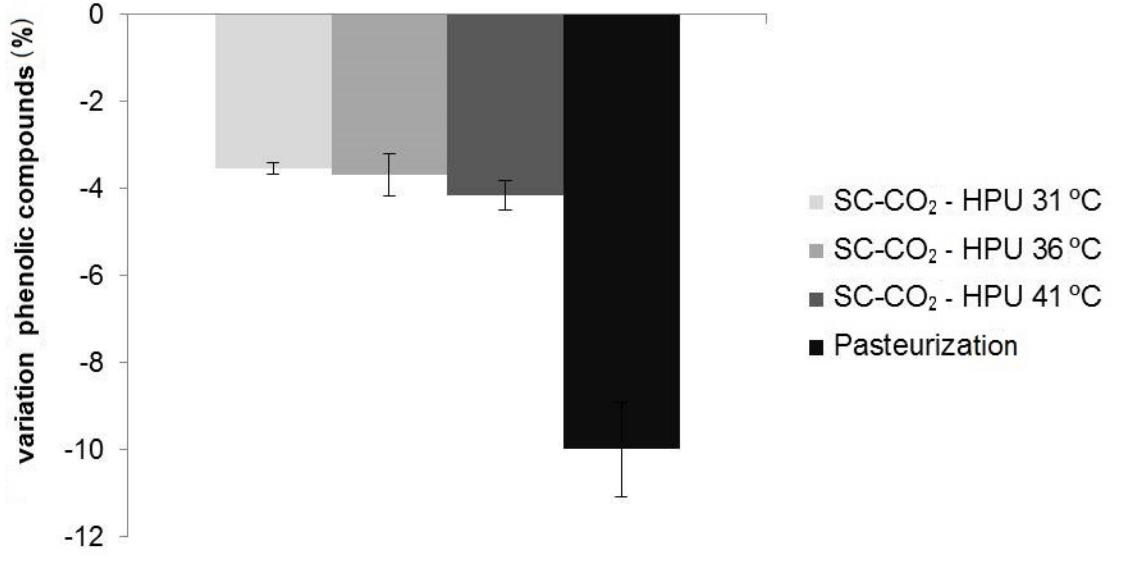
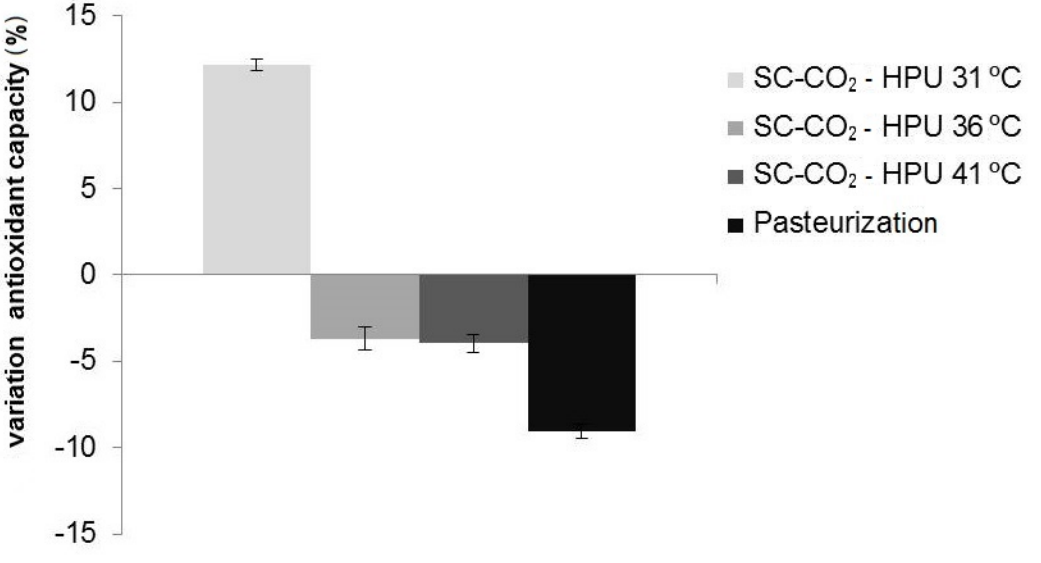

variation phenolic compounds (%)
0
-2
-4
-6
-8
-10
-12
SC-CO2 - HPU 31 ºC
SC-CO2 - HPU 36 ºC
SC-CO2 - HPU 41 ºC
Pasteurization


variation antioxidant capacity (%)
15
10
5
0
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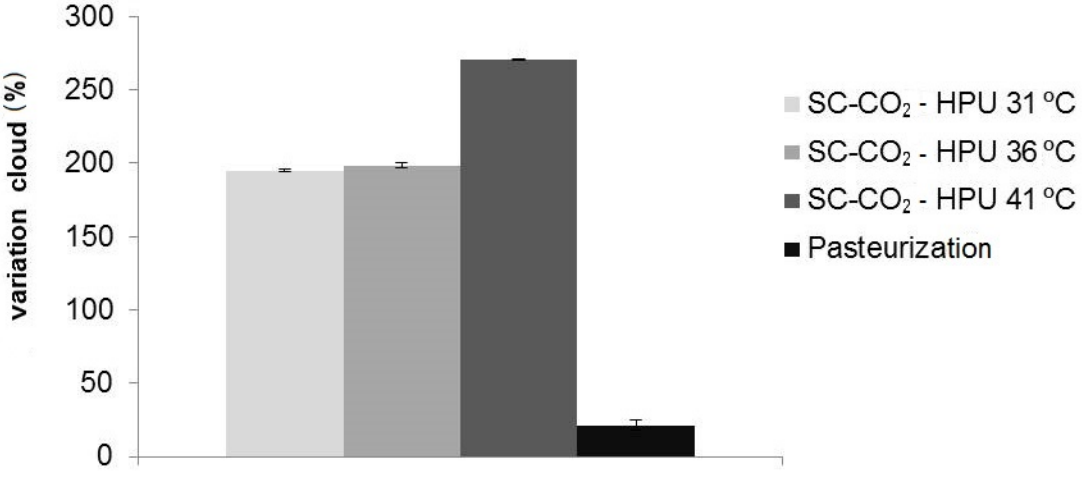
SC-CO2 - HPU 31 ºC
SC-CO2 - HPU 36 ºC
SC-CO2 - HPU 41 ºC
Pasteurization


variation cloud (%)
300
250
200
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100
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0
SC-CO2 - HPU 31 ºC
SC-CO2 - HPU 36 ºC
SC-CO2 - HPU 41 ºC
Pasteurization

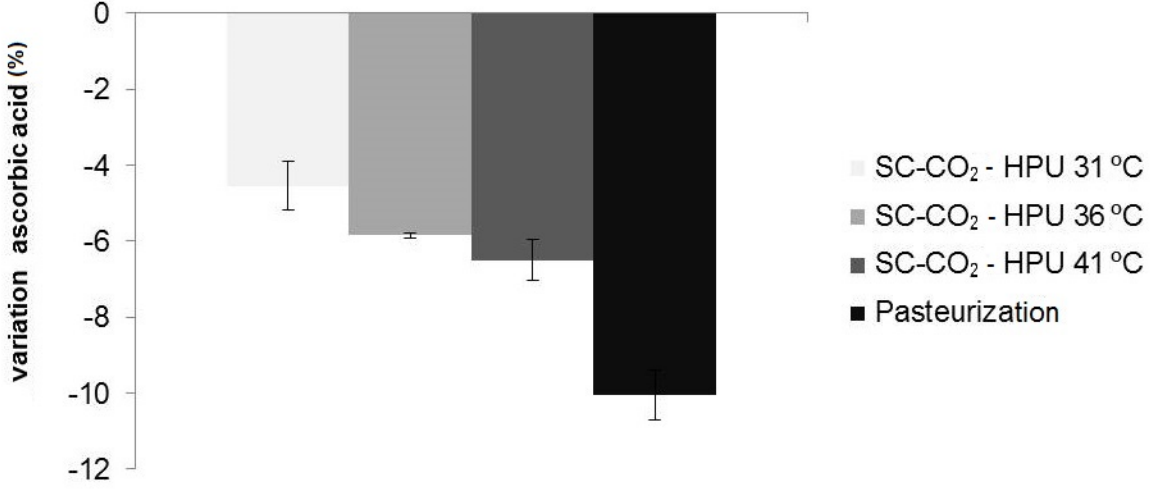
variation ascorbic acid (%)
0
-2
-4
-6
-8
-10
-12
SC-CO2 - HPU 31 ºC
SC-CO2 - HPU 36 ºC
SC-CO2 - HPU 41 ºC
Pasteurization

TABLE 1. Inactivation of microbiota in orange juice after SC-CO_2+HPU and thermal pasteurization treatments

Treatment / conditions	*E.coli**			*S.cerevisiae**			Total aerobic mesophilic*		
	N_0	N	Log N_0	N_0	N	-Log N/N_0	N_0	N	Log N_0
SC-CO_2-HPU/ 100 bar, 31 ºC	5.75E+03	0.00E+00	3.47±0.61	1.20E+05	2.00E+02	2.61±0.40	1.22E+03	0.00E+00	2.95±0.43
SC-CO_2-HPU/ 100 bar, 36 ºC	1.24E+04	0.00E+00	3.80±0.63	4.53E+04	2.55E+02	2.24±0.21	3.66E+03	0.00E+00	3.50±0.31
SC-CO_2-HPU/ 100 bar, 41 ºC	1.35E+04	0.00E+00	3.84±0.79	1.58E+05	1.04E+03	2.19±0.02	9.65E+03	0.00E+00	3.95±0.23
Thermal pasteurization/ 90 ºC, 1 min	9.11E+02	0.00E+00	2.82±0.43	6.16E+03	0.00E+00	3.54±0.54	1.03E+03	0.00E+00	2.88±0.48

TABLE 2. pH, ºBrix and titratable acidity values of orange juice after SC-CO2+HPU and thermal pasteurization treatments

	pH			ºBrix			Titratable acidity (g citric acid/100ml)*		
	Control	Treated	Variation	Control	Treated	Variation	Control	Treated	Variation
Treatment / conditions									
SC-CO_2-HPU/ 100 bar, 31 ºC	3.58±0.04^{a}	3.60±0.05^{a}	0.56±0.01%	12.23±0.23^{a}	12.13±0.23^{a}	-0.81±0.3%	0.874±0.02^{a}	0.835±0.021^{ab}	-4.46±0.15%
SC-CO_2-HPU/ 100 bar, 36 ºC	3.63±0.03^{a}	3.67±0.02^{a}	1.10±0.08%	11.46±0.06^{bc}	11.26±0.05^{c}	-1.74±0.05%	0.757±0.05^{abc}	0.718±0.05^{bc}	-5.15±0.26%
SC-CO_2-HPU/ 100 bar, 41 ºC	3.68±0.005^{a}	3.69±0.005^{a}	0.27±0.05%	12.20±0.17^{a}	12.03±0.11^{a}	-1.39±0.12%	0.747±0.04^{bc}	0.708±0.04^{c}	-5.22±0.52%
Thermal pasteurization/ 90 ºC, 1 min	3.61±0.08^{a}	3.62±0.09^{a}	0.28±0.09%	12.03±0.12^{a}	11.86 ±0.05^{ab}	-1.41±0.23%	0.836±0.04^{ab}	0.829±0.04^{ab}	-0.83±0.11%

Different letters for the same quality parameter within a row and column indicate significant differences ($p < 0.05$)

TABLE 3. Browning index of orange juice after SC-CO_2+HPU and thermal pasteurization treatments

	Browning Index (A420 nm)		
Treatment / conditions	Control	Treated	Variation
SC-CO_2-HPU/100 bar, 31 ºC	0.21±0.00^{a}	0.66±0.02^{b}	216.40±7.85%
SC-CO_2-HPU/100 bar, 36 ºC	0.23±0.01^{a}	0.75±0.01^{c}	228.13±4.49%
SC-CO_2-HPU/100 bar, 41 ºC	0.22±0.00^{a}	0.72±0.01^{c}	233.49±4.03%
Pasteurization/90 ºC, 1 min	0.21±0.01^{a}	0.22±0.01^{a}	5.38±0.19%

Different letters within a row and column indicate significant differences ($p < 0.05$)

TABLE 4. Color values of orange juice after SC-CO_2+HPU and thermal pasteurization treatments

	Color*						
	Control			Treated			
Treatment / conditions	L*	a*	b*	L*	a*	b*	ΔE
SC-CO_2-HPU/ 100 bar, 31 ºC	33.86±2.04	4.61±1.11	56.75±3.57	29.76±2.88	0.805±1.59	49.73±4.84	8.85±0.60
SC-CO_2-HPU/ 100 bar, 36 ºC	40.52±0.96	5.54±0.35	64.30±2.45	33.86±1.73	0.98±0.73	55.79±2.47	11.74±1.25
SC-CO_2-HPU/ 100 bar, 41 ºC	33.96±1.39	6.37±0.93	6.37±0.93	30.21±0.67	1.44±0.23	50.54±1.01	8.70±1.86
Pasteurization/ 90 ºC, 1 min	35.00±0.25	8.25±1.20	58.74±0.26	32.42±0.13	4.52±0.28	54.47±0.18	6.27±0.87