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OPTIMIZATION OF THE SUPERCRITICAL CO₂ PASTEURIZATION PROCESS FOR THE PRESERVATION OF HIGH NUTRITIONAL VALUE OF POMEGRANATE JUICE

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ABSTRACT

This work aims to develop a high-pressure pasteurization process for pomegranate juice by supercritical CO₂ (SC-CO₂). The process was optimized in a mutibatch-system through Design of Experiment (DoE) and Response Surface Methodology. The optimized SC- CO₂ process (12.7MPa, 45°C, 40min) was compared with conventional thermal pasteurization (90°C, 1 min) and High Hydrostatic Pressure process (600MPa, 3 min) for the microbiological inactivation and the change in quality parameters of total phenols content (TPC), antioxidant activity (AAt) and colour, up to 28 days of storage at 4°C. SC-CO₂ treated juice was microbiological stable during the entire shelf life as well as colour and TPC. The antioxidant activity showed a significant ($p<0.05$) decrease during the storage period, however this evidence was observed also for the thermal and high hydrostatic processed samples. Overall, SC-CO₂ process is a promising technology for the development of innovative pasteurization technologies for high nutritional juice.

1. INTRODUCTION

Food has a key role in disease prevention and treatment and in recent decade it has been increased awareness by population. Thus, the production and consumption of healthy foods has gained much importance. Consumers are paying more and more attention to what they eat, preferring foods that can bring healthy-outcome without the addition of artificial ingredients [1,2]. Among these foods, pomegranate (*Punica granatum*) is a high functional fruit and it can bring countless beneficial effects to the human health. Not by chance, it was used as a medicine by ancient populations [3,4].

Pomegranate Juice (PJ) is obtained from the endocarp of the fruit, which consists of numerous red arils. The PJ is rich in polyphenols, secondary metabolites characterised by complex chemical structures that carry out antioxidant activity [5], as well as in bioactive compounds typical of the plant kingdom. These are secondary metabolites of plants and their importance is recognized for their remarkable antioxidant power [5]. This characteristic is well known in tea, red grape and red fruits and PJ shows the highest activity [6]. The importance of polyphenols has been highlighted by several studies, which reported the beneficial effects associated with their consumption, i.e. as preventive and protective actions against various diseases like cardiovascular and neurodegenerative diseases and tumour [7-9]. The main phenolic families found in pomegranates are the punicalagins, the ellagitannins (in the monomeric form as ellagic acid) and the anthocyanins. Ellagic acid is the focus of many recent studies because it seems to be the main responsible of beneficial effects associated with the prevention of prostate cancer [10,11]. Whereas anthocyanins are of fundamental importance, not only for its antioxidant power, but also for its aromatic-sensory characteristics, in fact it is thanks to this compound that the pomegranate takes on its typical intense red colour [12].

The preservation over time of valuable PJ should guarantee microbiological safety and the preservation of its original bioactive molecules. To date, thermal pasteurization is still the most widely used technology for sanitizing the-PJ [13], which assures high microbiological safety and enzymatic inactivation [14,15]. However, thermal pasteurization involves chemical and nutritional changes, with serious, repercussions on colour, flavour and taste changes, thus negatively affecting the consumer's acceptance of the final product [16]. For this reason, additives and sugars are commonly added to fruit juices not only to improve the taste but also

to mask off-flavours produced by thermal processes [17]. Furthermore, the nutritional component may also be compromised by the involvement of high temperatures [18].

In the last two-decade non-thermal technologies (i.e. High Hydrostatic Pressure (HHP), High Pressure Homogenization (HPH), Pulsed Electric Field (PEF) and Ultrasounds (US)) have been investigated to avoid the use of high temperatures and maintain fresh-like qualities. However, some of them are still at research or pilot scale stage and more studies are needed to demonstrate their effects on the nutritional component [19]. Specifically, HPH process depends on processing parameters (membrane, pore size, transmembrane pressure), type of juice and microorganism target, while US require a combination effect with mid-temperature or pressure in order to obtain a consistent (≥ 5 -log) reduction of microorganisms [20]. PEF is a promising alternative technology that preserves the original sensory and nutritional characteristic of the juices, requires short processing time and low temperature and results to be energy saving with respect to the thermal process, however its association to bad consumer perception discourage their use [21]. Moreover PEF alone is not sufficient on the reduction of the bacteria spore populations [22]. Among non-conventional technologies, HHP is emerging on the juices market. This type of process involves pressures higher than 100MPa and it is commonly used in USA, Japan and Europe for the treatment of liquid and solid foods (e.g. juice, meat, fish, sauces). However, HHP process require high initial capital cost and is difficult to be afforded by SMEs [23].

A promising high-pressure alternative can be found when carbon dioxide (CO₂) is used at supercritical conditions (SC). SC-CO₂ exploits bactericidal properties and it can inactivate a wide range of microorganisms. SC-CO₂ minimally change the sensorial quality of the products and colour, aroma and flavour of the product are similar to the fresh one [24-26]. The efficacy of SC-CO₂ pasteurization depends on process parameters, therefore proper optimization studies should be carried out to determine the effect and interaction between process variables. Recent applications of the technology for the preservation of high functional juice involved pineapple juice [27], orange juice [28], strawberry juice [29], elderberry juice [30], whey-grape juice [31,32] and mulberry juice [33] but, to the best of our knowledge, no study on SC-CO₂ pasteurization of PJ is found in the literature. The objective of this work is to develop and optimize a new process for the pasteurization

of PJ at low temperature with the use of SC-CO₂ focusing on the microbial stability and the preservation of chemical components and principal sensory attributes. For this purpose, experiments were designed [34, 35] to vary the operating parameters of time, temperature and pressure in order to find the optimal conditions for microbiological inactivation of the microbiota. This study is a first comparative investigation on the effects of different treatment processes on PJ, considering not only to the effects linked to the technologies, but also the shelf life of the treated product. The variation of the polyphenolic content and its antioxidant activity as a result of the process were monitored with shelf life studies: microbiological and chemical components were analysed for 28 days of storage at refrigeration temperature (4°C). The results of the proposed technology (SC-CO₂) are compared to those obtained with thermally pasteurized juice and HHP.

2. Materials and methods

2.1. Sample preparation

Pomegranate juice was produced with fresh pomegranates (*Punica granatum* L. cv. *Wonderful*) harvested in Spain during September 2018. The juice was prepared in October 2018 by Ortoromi s.c.a in industrial conditions. The arils were manually separated, and the juice was squeezed with an industrial screw extractor, homogenized and finally divided into 250 mL bottles, which were immediately frozen at -20 °C. To avoid influence of native tissue enzymes on the stability of nutritional components in pomegranate juice (§3.1) 0.1% (w/w) of ascorbic acid was added to the juice before bottling. Prior to each treatment, the samples were thawed at 4 °C overnight.

2.2. Supercritical CO₂ plant

The treatment with supercritical CO₂ was carried out in a multibatch plant [36] consisting of equivalent 10 reactors in parallel with an internal volume of 15 mL. Before each use, the reactors were cleaned, rinsed with water and autoclaved. Each reactor was filled with 5 mL of juice under safety cabinet in sterile conditions. A magnetic micro-stirrer (Microstirrer, Velp Scientifica, Italy) maintained the juice mixed during the entire length of the process. Temperature was maintained constant using a thermostatic bath controlled by a temperature controller. Liquid CO₂ (purity 99.99 %, Rivoira, Italy) was cooled in a chiller and fed to the reactors through a membrane pump (Gilson 307, Gilson, France) at a constant rate of about 6 MPa/min. A pressure controller keeps constant the operating pressure during the process. At the end of the process the plant was depressurized in about 1 min acting on a micrometric valve. All the juices were collected in sterile 15ml falcon tubes in aseptic conditions under a safety cabinet. The juice was processed within 1-hour for microbiological, colour, pH and brix analysis, once it cooled down to ambient temperature.

2.2.1 Design of Experiment

For what concerns the study of the micro-organism's inactivation experiments it was designed using a central composite design [34]. Three factors were considered in the experimental design, each moving on three levels: temperature (moving on levels: 35, 40, 45 °C), pressure (8, 12 and 16 MPa) and time (0, 20, 40 min).

The treatment time of 0 min corresponds to the pressurization and depressurization step. Each point was replicated three times and the central one was replicated nine times. The order of the experiments was randomised. The effect of the three independent variables on inactivation rate was modelled using a polynomial response surface.

The optimal condition was further analysed for the chemical characterization (TPC, antioxidant activity, anthocyanins and Ellagic acid contents). The juice was frozen after the process at $-20\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ and stored until the analysis. Moreover, the juice was stored for 28 days at 4°C and the changes in the microbial content and the TPC, antioxidant activity, anthocyanins and Ellagic acid contents were determined during storage.

2.3. High Hydrostatic Process (HHP) and plant

HHP was carried out in an industrial plant (*Hiperbaric 420*, Spain). Bottles 250 mL (filled with 220 mL of juice) were placed in a cylindrical vessel at an initial temperature of $10\text{ }^{\circ}\text{C}$ and pressurized at 600 MPa for 3 minutes. Pressurization took place in 3 minutes at a constant rate of 200 MPa/min, while depressurization was instantaneous. Deionized water at $10\text{ }^{\circ}\text{C}$ was used as transmitting pressure medium.

The temperature of samples increased up to $24\text{ }^{\circ}\text{C}$ due to adiabatic compression. During depressurization, the temperature decreased to the initial temperature. The bottles were frozen at -20°C and stored until the analyses. The same microbiological and physico-chemical measurements as for the SC-CO₂ treated samples were carried out after the treatment and during refrigerated storage.

2.4. Heat treatment

200 mL of pomegranate juice was placed in a 500 mL flask and heated by a heating plate incorporating magnetic stirring (F60 Stirrer, Falc Instruments, Italy) for maintaining appropriate temperature in the whole sample. Temperature was measured with a digital thermometer (YC-717, YCT, Taiwan). Once the temperature reached 90°C , the hot plate was switched off and the sample was pasteurized at 90°C for one minute[37]. After pasteurization the flask was transferred to an ice and water bath to cool down the juice immediately. The thermally treated juice was collected in 15 mL falcon tubes, filled with 10 mL of treated juice in aseptic conditions. For chemical analysis the juice was frozen at -20°C and stored until the analyses.

The same microbiological and physico-chemical measurements as for the SC-CO₂ treated samples were carried out after the treatment and during refrigerated storage.

2.5. Polyphenol oxidases and peroxidases activity

The following reagents were used for enzymatic analysis: poly-vinylpyrrolidone (PVP) (~110 µm) (Sigma-Aldrich), catechol (>99%) (Sigma-Aldrich), hydrogen peroxide (30%), p-Phenylenediamine (Sigma-Aldrich) and Triton™ X-100 (Sigma-Aldrich), sodium phosphate dibasic hepta-hydrate and sodium dihydrogen phosphate monohydrate (Sigma-Aldrich). The activity of enzymes POD and PPO were analysed according to the protocol described by [38].

The following reagent solutions were made: 0.2 M and 0.05 M (pH 6.5) of phosphate buffer solutions, and an extraction mixture (prepared with the 0.2 M phosphate buffer, 4% polyvinylpyrrolidone PVPP 1% Triton X-100 and 1 M NaCl). At the beginning of the analysis, a quantity of extraction mixture was added to the samples (1:1, w/w), then the solutions were mixed and centrifuged at 24,000 x g (5417R, Eppendorf, Hamburg) for 30 minutes at 4 °C. The supernatant of each sample was used to determine the PPO and POD activities. For the PPO assay, 200 µL of the supernatant was introduced into 1 mL of 0.05 M phosphate buffer (pH 6.5) and 0.07 M catechol was added. The absorbance was measured spectrophotometrically (Perkin Elmer UV/Vis Spectrometer Lambda 11) at $\lambda = 420$ nm (25 °C) during 10 minutes of reaction.

For the POD activity assay, 1 mL of 0.05 M phosphate buffer (pH=6.5) was added to 100 µL of the sample supernatant, then was added 100 µL of 1% p-phenylenediamine (w/v) (diluted in 0.05 M phosphate buffer) and 100 µL of 1.5% (v/v) hydrogen peroxide (diluted in 0.05 M of phosphate buffer).

The changes of absorbance were measured at $\lambda=485$ nm (25 °C) during 10 minutes of reaction. The results of enzymatic assays were expressed as a $\Delta A/\text{min/g}$ of fresh weight (FW) of the analysed juice.

2.6. Microbial analysis

The juice was serially diluted (1:10 dilution) in Phosphate Buffer Solution (PBS); mesophilic bacteria, yeast and moulds were quantified before and after the treatments through the standard plate count techniques. Total mesophilic bacteria were included in Plate Count Agar medium (PCA, Sacco, Italy) by pouring the PCA

on 1 mL of the decimal dilutions. Plates were incubated at 30 °C for 72 h. Yeast and moulds were quantified by spreading 0.1 mL of the decimal dilution on Rosa Bengal Agar Istisan 96/35 (RBA, Sacco, Italy). Plates were incubated at ambient temperature for 96 h.

The limit of quantification (LoQ) and the limit of detection (LoD) for mesophilic bacteria was set at 250 CFU/mL and 10 CFU/mL, respectively, whereas for yeast and moulds was 2500 CFU/mL and 100 CFU/mL, respectively. To reduce the limit of detection up to to 1 CFU/mL, enrichment tests were performed: the treated juice was incubated for 24 h at 30 °C and then the sample was analysed as described previously.

2.7. Phenolic compounds analysis

For each treatment the juice was thawed and mixed vigorously with Vortex (Velp scientifica, 2x3 Advanced Vortex Mixer, Italy) then was divided into 1.5 mL Eppendorfs (1.5 mL each) and stored at 4 °C for 28 days.

Before the analysis, PJ was centrifuged ($14,000 \times g$ for 10 min at 4 °C) and then supernatant was filtered on PTFE microfilters with a pore size of 0.45 μm (Waters, USA). . For the SC-CO₂ process, only the juice treated at the optimum condition (12,7MPa, 45°C and 40 min) was considered.

2.7.1. Total phenolic content (TPC) and Antioxidant Activity (AAt)

The content of total polyphenols was analysed with spectrophotometer (Thermo Electron Corporation *Helios γ* , VISIONlite™ rate Spectrophotometer software, US) at 765 nm using *Folin Ciocalteu* coulometric assay as described by Gao, Ohlander, Jeppsson, Björk, & Trajkovski (2000) with some modifications. 0.2 mL of sample (diluted with distilled water in proportion 1:200, v/v) was added to one millilitre of *Folin-Ciocalteu* reagent (diluted in distilled water in proportion 1:10, v/v) (Sigma-Aldrich, Germany). Sample were mixed with vortex and allowed to stand for 3 minutes from the starting of reaction. Then 0.8 millilitres of 15% (w/v) sodium carbonate solution (diluted in distillate water) was added. The reaction mixture was incubated, in dark conditions, at room temperature for 60 minutes. The standard curve was prepared with Gallic acid monohydrate (0,2-8,0 $\mu\text{g/mL}$) (Sigma-Aldrich, Germany) and the content of total phenolic compounds were expressed as mg of gallic acid equivalent/ L (mg GAE/L).

The antioxidant activity (AAt) was analysed with spectrophotometer (Thermo Electron Corporation Helios y, VISIONlite™ rate Spectrophotometer software, US) at 515 nm using 2,2-diphenyl-1 picrylhydrazyl- DPPH coulometric assay[39]. The DPPH mother solution was prepared by dissolving 0.025 g of 2,2-Diphenil-1-picrylhydrazyl (Sigma-Aldrich, Germany) in 1 L of ethanol (0.06 mM) (PanReac ApplicChem, Spain), mixed for 4 hours at room temperature and stored in fridge at 4 °C until use. The working solution was prepared by diluting the DPPH mother solution with ethanol to obtain absorbance of 0.8 at 515 nm. 0.06 mL sample (diluted in distilled water in proportion 1:200, v/v) was mixed with 0.190 mL of ethanol and 1 mL of DPPH solution. The blank sample was prepared with ethanol, whereas control sample was prepared by mixing 0.250 mL of ethanol with 1 mL of DPPH solution with absorbance of 0.6. From the adding of DPPH solution, the samples were then incubated for 40 minutes, in dark condition, at room temperature. The standard curve was prepared with ascorbic acid diluted in ethanol (0-10 µg/mL). The antioxidant activity was calculated according to equation (1):

$$\text{antioxidant activity} = \left[\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \right] * 100 \quad (1)$$

2.7.2. Anthocyanins profile by HPLC

The anthocyanins profile was carried out using the method described by [40] with small modifications reported by [41]. HPLC analysis was carried out by Waters 2695 connected with a Photodiode Array Detector 2996 (Waters, USA) using a reversed phase Sunfire C18 column (250 × 4.6 mm, 5 µm, Waters, USA) with Sunfire C18 guard column (20 × 4.6 mm, 5 µm, Waters, USA) at a temperature of 25 °C, a flow rate of 1 mL/min and detection at 520 nm. The samples were eluted using a gradient of 80% solution of acetonitrile in 4.5% formic acid (solvent A) and 4.5% formic acid (solvent B), as follows (time, corresponding composition): 0 min, 0%A; 7min, 15%A; 15min, 15%A; 21min, 100%A; 26min, 0%A. Monomers of the anthocyanins were identified by comparing their retention times with those of the standards and with LCMS analysis. All results were expressed in mg of pelargonidin-3-glucosides/100 mL of juice.

2.7.3. Ellagic acid determination

HPLC Agilent technologies 1260 infinity (II), equipped with a DAD detector was used for the analysis. The injected sample volume was 10 μ L, the temperature was 30.0 °C and the mobile phase contained Formic acid solution (0.1%, v/v) (Solvent A) and Acetonitrile (solvent B). The following gradient was applied: 0 min, 0% Solvent B, 25 min, 70% Solvent B, 26 min, 0% Solvent B, 30 min, 0% Solvent B. The flow rate was 1 mL/min, the data acquisition was in the range 280-545 nm and compounds were detected at 254 nm. This method was applied specially to detect and quantify the ellagic acid presence.

2.8 Physico-chemical characterization

The colour changes were measured with a ColorQuest XE colorimeter (Hunter Lab, Germany), in glass cuvettes with an optical path of 5 mm. Results were expressed using the CIELab system, with illuminant D65. The colour parameters were expressed as L (whiteness or brightness/darkness), a (redness/greenness), and b (yellowness/blueness), whereas total colour differences (ΔE), were calculated according to Equation (2).

$$\Delta E = \sqrt{(L_0 - L)^2 + (a_0 - a)^2 + (b_0 - b)^2} \quad (2)$$

The pH of PJ samples was measured using a digital pH meter (VWR pHenomenal 1100L, IT), calibrated with commercial buffer solution at 7.0 and 4.0. A sample of 10 ml was placed in a 20 ml beaker and agitated with a magnetic stirrer. The pH measurement was taken after a stabilization at room temperature

An optical refractometer with automatic temperature compensation (triple scale refractometer MR200ATC) was used for the measurement of Brix (resolution 0.2%). A drop of PJ was taken with 1ml plastic Pasteur pipettes placed on the prism and after 1 minute the measurement was taken. For SC-CO₂ treated juice, the pH and brix were taken 1 hour after the treatment, once the PJ had cooled down to ambient temperature.

2.9 Statistical analysis of the data

All statistical analyses were carried out using Statistica 10 StatSoft® software. The significant differences were estimated based on the Analysis of the Variance (ANOVA) with Fisher's test (p -value ≤ 0.05). The results were expressed as a mean \pm standard deviation of a minimum of two replicates, performed on samples prepared according to the same procedure

3 RESULTS

3.1. Enzymatic analyses: Polyphenol oxidases and peroxidases activity

The endogenous oxidoreductive enzymes are one of the most important factors responsible for the loss of nutritive food components during processing and storage [41-43]. It is well known that enzymes inactivation is often more difficult than inactivation of most microorganisms in fruit juice [15]. The enzymes activity after SC-CO₂ processes depends on process parameters [44]. However, the enzymatic inactivation capacity of SC-CO₂ process can be very limited also at high pressure and temperature especially in vegetable products [45]. For this reason, a preliminary investigation on the inactivation of POD and PPO was performed at the highest process variable (16MP, 45°C, 40 min), before carrying out the optimization of the microbial inactivation. The POD and PPO activities were analysed in pomegranate juice in non-treated and after SC-CO₂ treatment samples considering (or not) the addition of ascorbic acid (Table 1), a common natural additive in the fresh juice industry. We observed that the addition of ascorbic acid (0.1 %) inhibits significantly both enzymes. Moreover, no PPO activity was observed in juice without addition of vitamin C, what indicates that this enzyme has no activity in pomegranate juice. For instance, POD activity in the fresh no-treated juice (0.033 ΔA/min/g) was 70, 50, 15 and 5 times lower in comparison to the POD activity observed in beetroot, carrot, celery and apple juices respectively [38,43,45,46] which indicates that oxidoreductive enzymes may not to be an important problem from the technological point of view. On the other hand our previous research proved that even slight activity of oxidoreductive enzymes may cause significant deterioration of nutritional value during long time storage of fruit juices[42]. After the treatment with SC-CO₂, a good POD-activity reduction (around 69% reduction) was observed in the raw/natural juice. However, even the harshest SC-CO₂ conditions were not enough for total POD inactivation when the ascorbic acid was not added. To avoid influence of native tissue enzymes for the stability of nutritional components in pomegranate juice, the ascorbic acid was added to the juice to all further experiments. The synergetic effects of SC-CO₂ and organic acid has been previously proved for stabilization of elderberry juice and inactivation of native enzymes [30].

3.2. Microbial Inactivation

As demonstrated in previous literature, SC-CO₂ has usually an effective bactericidal action [24]. When CO₂ is at supercritical conditions, it has peculiar characteristic of extreme solubility that allows its penetration into cells and membrane modification. In this way the intracellular electrolyte balance and pH is altered and, accordingly, metabolism processes that lead to cellular inactivation are triggered [27]. However, the inactivation process is complex, and the effectiveness of SC-CO₂ is matrix-dependent. Furthermore, other factors may affect microbial resistance (e.g. process and product parameters). To demonstrate that SC-CO₂ can be used in an effective pasteurization process for pomegranate juice, the effect of SC-CO₂ on microbial inactivation has been studied to verify if the process was able to ensure the expected microbiological safety of the product. The effect of three main process factors: temperature T , pressure P and treatment time t on the microbiological inactivation was studied, following the central composite design presented in Section 2.2.1. The outcomes of the experiments were analyzed through a response surface model, a multi-linear regression model of the second order (Equation 3), where y_1 (the logarithm of the inactivation of mesophilic bacteria) is the following:

$$y_1 = \beta_0 + \beta_1 T + \beta_2 P + \beta_3 t + \beta_4 TP + \beta_5 Tt + \beta_6 T^2 + \beta_7 P^2 + \beta_8 t^2 \quad (3)$$

In the response surface model the logarithm of the mesophilic bacteria inactivation is expressed as a function of factors through linear (i.e., T, P, t) terms, quadratic terms (i.e., T^2, P^2, t^2), and also interactions between temperature and pressure, and temperature and processing time (i.e., TP, Tt). Figure 1 shows the response surface as a function of the factors, compared to the experimental points (red dots). In particular, it can be seen that the surface accurately fits the experimental data (the surface is always very close to the red points), being the determination coefficient of $R^2 = 0.92$. Figure 1a shows that, once the pressure is fixed, the inactivation of mesophilic bacteria increases with an increase in temperature and treatment time and reaches a maximum at a temperature of 45 °C and a treatment time of 40 minutes, i.e. the maximum values considered in the analyses. Figure 1b and Figure 1c show the dependence of the inactivation of mesophilic bacteria on temperature and pressure and on time and pressure. In both cases it can be noticed that, within the range considered, the bacterial inactivation is much less sensitive to pressure, meaning that an increase in this parameter do not lead to an increase in bacterial inactivation. Figure 2 shows the contour plots related

to the response surface. The grey areas highlight the part of the experimental domain where total inactivation of mesophilic bacteria can be achieved. It is confirmed that bacterial inactivation is much more sensitive on temperature and treatment time than on pressure. The maximum value for the inactivation curve of mesophilic bacteria corresponds to a temperature of 45 °C, a treatment time of 40 minutes and a pressure of 12.7 MPa. These conditions represent the optimum value of temperature, pressure and treatment time for the inactivation of mesophilic bacteria in PJ. It is worth noticing that it is possible to reduce the operating pressure in order to obtain an optimal inactivation of mesophilic bacteria. To validate the modelling procedure, the experiment at the optimal inactivation condition ($T=45^{\circ}\text{C}$, $t=40$ minutes, $P=12.7$ MPa) was carried out. A triplicate test was conducted that demonstrated the effectiveness of the modelling strategy. In all these cases, the total inactivation of mesophilic bacteria was obtained through the optimal process parameters. Since, in addition to bacteria, juices are often spoiled with high level of moulds and yeast due to of the low pH that characterizes them (<4.5) [47], the effectiveness of the process on yeasts and moulds was also studied. A new response surface representing the inactivation of yeasts and moulds was fitted:

$$y_2 = \beta_0 + \beta_1 T + \beta_2 P + \beta_3 t + \beta_4 P^2 + \beta_5 t^2 \quad (4)$$

The logarithm of the inactivation of yeasts and moulds, y_2 , depends linearly on all three factors and also on the squared pressure and time. The corresponding response surface (which provides a good fitting with $R^2 = 0.90$) is shown in Figure 3 and shows a similar behaviour to the one of the mesophilic bacteria inactivation. The grey areas represent the part of the experimental domain where there is a full inactivation of yeasts and moulds. Since these areas are larger than those for total inactivation of mesophilic bacteria, this proves that in pomegranate juice it is easier to inactivate yeasts and moulds than mesophilic bacteria. Also, in this case a complete inactivation was found at the following conditions: $T=45^{\circ}\text{C}$, $t=40$ minutes and $P=12.7$ MPa. Yeast and moulds were also more sensitive to the SC-CO₂ compared with mesophilic bacteria as we recently demonstrated for coriander [48,49] and chicken breast [50].

Shelf life tests were carried out on juice treated under optimum conditions and microbiological counts were performed, using untreated, pasteurized (90°C, 1 min) and HHP (600 MPa, 3 minutes) treated sample juices

as a comparison for the inhibitory effects. The results of microbiological and shelf life analysis are shown in Table 2. The non-treated juice had 4.3 Log (CFU/ml) of mesophilic bacteria and 4.2 Log (CFU/ml) of yeast and moulds and remained almost stable during the preservation time. After the different treatments, the growth of the microorganisms was under the Limit of Detection (<LoD) and there was no growth during the shelf life test in all the treated juices. These results are probably due to the interaction of different factors, that strongly affects the resistance of the microorganisms: in addition to process parameters, environmental parameters such as the very low-pH of the pomegranate juice (<3.0), could make an environment unfavourable to the growth of bacteria thus driven to a microbiological stability over time. However, the comparison between different processes demonstrated that PJ treated with SC-CO₂ has a similar microbiological stability compared with HHP and the thermally stabilized ones.

3.3. Total Phenols Content (TPC) and Antioxidant Activity (AAt)

The analyses were performed at different times of storage. The samples were thawed and analysed together every 7 days of storage. All the samples were compared at single time point of storage. At time 0 the Total Phenols Content (TPC) content was between 605.5 and 873.1 µg/mL GAE and only HHP sample (713.9 µg/mL) didn't showed significant difference compared to the fresh non-treated juice (686.8 µg/mL). These values are similar-to those reported for orange juice [51].

After the SC-CO₂ treatment the juice increased 22% its TPC, instead, the thermally treated sample showed a decrease of 15%. Even if SC-CO₂ sample was the only one that showed an increase in the TPC content, its Antioxidant Activity (AAt) at time 0 was lower (32%) than the other samples (44.5-68% AAt). AAt is strictly correlated to phenols chemical structure, hence the total amount of AAt depends on the phenols-pool and specifically on the presence of the different functional group of specific phenols [52]. Therefore, at equal concentrations, polyphenols poor in functional groups might not have the same antioxidant capacity as those rich in these groups.

In literature there are many data about the chemical composition of PJ, the phenols content and antioxidant activity; however the behaviour over-time during storage is not reported elsewhere. Mirsaedghazi et al. [53] showed the change in TPC and antioxidant activity of fresh non-treated PJ after a storage period at -25°C

observing a decrease in TPC, antioxidant activity and anthocyanins content within 20 day. In our work, at the end of the 28 days of storage, the range of TPC concentration of the samples was between 335 and 1292.9 mg/L GAE. Non-treated sample lost 53% of TPC content, the SC-CO₂ sample lost 23%, while the HHP sample showed an almost double content of TPC with respect to time 0. Our finds are consistent with Patras et al.[54], who reported higher values of TPC and AAt after HHP applications in strawberry and blackberry purées. The higher content could be linked to a better extraction of the bioactive compounds due to the high pressure. Moreover, HHP-treated juice showed a preservation in antioxidant (22.5%) activity at the end of the storage while a decrease was found in all other samples, until reaching undetectable level in the samples treated with SC-CO₂ and thermal pasteurization. The strong reduction of AAt in these samples might be caused by the different ratio air/PJ during storage. While for the thermal and SC-CO₂ process, the juice was produced at lab scale and stored in Eppendorfs (ratio PJ:air 2:1), the HHP PJ was stored in the plastic bottles used for the treatment (ratio PJ:air 7:1). The different quantity of oxygen could have influenced the AAt for the thermal and SC-CO₂ PJ because oxygen has a strong effect on AAt [59]. Additional studies should be performed in the future to confirm this hypothesis.

3.4. Anthocyanins' profile and Ellagid acid content

Anthocyanins are the main responsible of the intense red colour of the juice. Anthocyanins were recorded at 520 nm, and the content was characterized by the comparison of their UV-Vis spectra, retention times with specific references. From the analyses, five well distinct compounds were found: Delphinidin 3,5-diglucoside, cyanidin 3,5-diglucoside, delphinidin 3-glucoside, cyanidin 3-glucoside and pelargonidin 3-diglucoside. Non significant ($p > 0.05$) differences in the anthocyanins' profile were found among the different treated juices at time 0 (Figure 4). During storage time, an overall variation of the single compound amounts from the beginning to the end of storage was observed in all the processed samples due, probably, to a natural oxidation process. The HHP showed the highest content for every single compound, followed by SC-CO₂ and finally by thermally-treated (Figure 4). The anthocyanins loss percentage after 28 days was on average 44% in HHP, 73% in SC-CO₂ and 84.4% in thermally-treated juices. This trend was also observed in the colour value (Figure 5) which confirms that this category of compounds is the main responsible of the redness of juice.

Following a hydrophilic stationary phase was applied for the determination of specific polyphenols, and the compounds were recorded between 280 and 545nm. The main known type is Ellagitannin, that belong to tannins, among this the most important in PJ are punicalagin, punicalin and ellagic acid (EA). EA is the main phenolic compound to which most of beneficial effects of PJ are associated [7]. In the nature it is normally bounded with sugars to higher phenolic compounds and only in PJ and strawberries is possible to find a great amount of free form of it. For this reason, EA was quantified at the beginning and at the end of storage (Table 4) to support this study and obtain preliminary results for further studies. The compound was recorded in all the treated juices, at 350nm which is the optimal wavelength for the determination of this compound. At time 0, no significant differences were observed between the treated and non-treated samples: the amounts of EA were between 40-60 µg/mL values which agree with those found in the literature [55]. After 28 days of storage, only non-treated and thermally treated juices preserved all the content of EA, instead a decrease was found in HHP (from 53.6 to 30.8 µg/mL) and a drastic decrease in SC-CO₂ (from 42.8 to 19.1 µg/mL). Probably the high pressure alone or in combination with CO₂ could have promoted the formation of an EA complex during the storage that is not detectable as free EA. Literature confirm the possibility that EA might be released in acidic conditions in strawberry [60, 61]. This hypothesis can be in accordance with the increased TPC content during storage for HHP PJ, however it is not very clear the effect for the SC-CO₂ treated juice and more studies should be performed. Daniel et al.[56], showed the stability of EA in unhydrolyzed strawberry even after 90 days of storage at -20°C. The results in our study were obtained in triplicate, in no hydrolysed PJ samples, in order to identify the real free EA amount. The resistance of EA might be affected by the food-matrix, especially by the pH value, however in this case it is also reasonable to think that, despite the adequate mixing of the juice, the samples could contain different amounts of solid residue, resulting in different amounts of EA.

3.5. pH, Brix and Colour changes

In agreement with literature [57], the pH of non-treated PJ resulted to be 3.0. The measures taken after the different treatments didn't show any changes in the pH of the juice (data not shown). The Brix measured was

about 17°Brix according with literature [58] and even in this case, no difference was found between the non-treated fresh juice and the other treated samples (data not shown).

Colour is one of the main quality parameters for consumers food acceptance. Pomegranate is rich in anthocyanins compounds that confer a colour that varies from light pink to red-violet and the loss of the colour in juices is probably mostly due to a decrease of their content [58]. Analysis of the colour were performed at different times in order to quantify the loss that occur in the samples after the treatments and during the storage at 4°C (Figure 5). The lightness (L^*), redness (a^*) and yellowness (b^*) were evaluated (Figure 5). SC-CO₂ treated juice remained almost stable in the first two weeks of storage, afterward it was possible to observe an increase of L^* value, which resulted three fold higher at the end of the 28d and, similarly, a three-fold decrease of b^* value, while a^* remained stable. The heat treatment was the one that had undergone a great visible variation. Thermally-treated juice remained stable the first two week of storage, afterward the L^* value was found to be double at the end of 28d period as it was at time 0, on the other hand, the redness a^* was found to decrease similarly with a loss of 46.4% compared to time 0, instead, the yellowness lost a 25%. Compared with other thermal treatment used to concentrate the juice [58] we observed a different behaviour by the L^* value, where a decrease was found during the concentration.

Temperature seems to be the main responsible of the loss of the redness in PJ. Our results might be a consequence of the presence of ascorbic acid. Further studies comparing juice with and without ascorbic acid should be performed to better demonstrate the added value of ascorbic acid on the preservation of PJ.

4. Conclusions

This is the first study to investigate the use of SC-CO₂ as an innovative and alternative technology for the pasteurization of PJ. Results were compared to thermal and HHP pasteurization. The optimized process parameters of 12.7 MPa, 45°C and 40 minutes, assured microbial safety (total inactivation of mesophilic bacteria and yeasts and moulds) of the product until 28 days of storage at 4°C. The quality parameters were affected, especially during the storage. The SC-CO₂ treated juice, resulted to have intermediate quality characteristics between the thermally pasteurized and HHP juice. It is worth to underline that the mild process conditions (of temperature and pressure) and the use of SC-CO₂, already recognized as green solvent, could be an interesting solution for reducing the costs related to the production process. Additional tests for sensorial quality and consumer's acceptance should be performed to demonstrate the benefit of the SC-CO₂ process compared to thermal and HHP pasteurization of PJ.

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Figures and tables

Pomegranate juice samples				
	Non treated	SC-CO ₂	Non treated (+ AA)	SC-CO ₂ (+ AA)
PPO [$\Delta 485 \text{ min}^{-1} \text{ g}^{-1}$]	ND	ND	ND	ND
POD [$\Delta 485 \text{ min}^{-1} \text{ g}^{-1}$]	0.033 \pm 0.001	0.010 \pm 0.001	ND	ND

Table 1: Peroxidases (POD) and polyphenol oxidases (PPO) activities in fresh (non treated) and SC-CO₂ treated pomegranate juices before and after the addition of 0.1% (w/w) of Ascorbic Acid (AA). ND means Not Detected. SC-CO₂ process conditions: 16MPa, 45°C, 40 min.

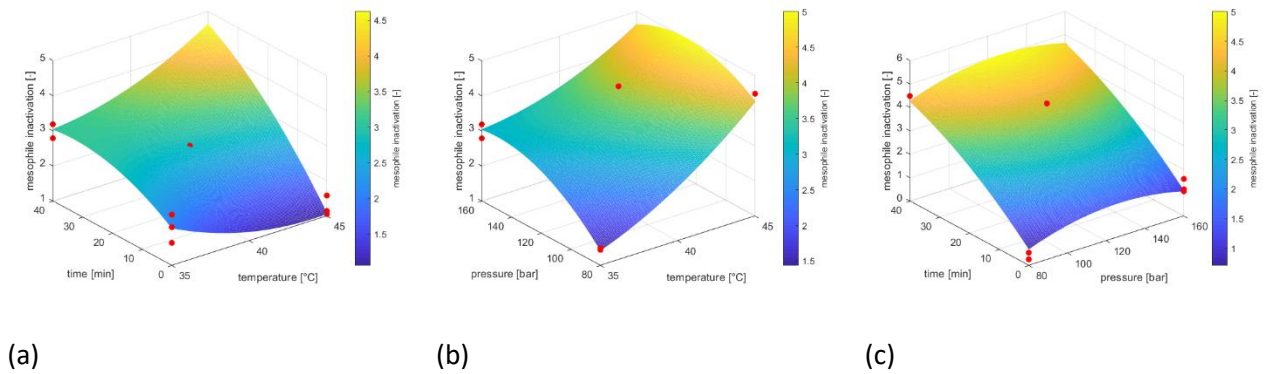


Figure 1. Response surfaces for logarithmic inactivation of mesophilic bacteria as function of temperature and time at a constant pressure $P=16 \text{ MPa}$ (a), temperature and pressure at constant time $t=40 \text{ min}$ (b) and pressure and time at a constant temperature of $T=45^\circ\text{C}$ (c)

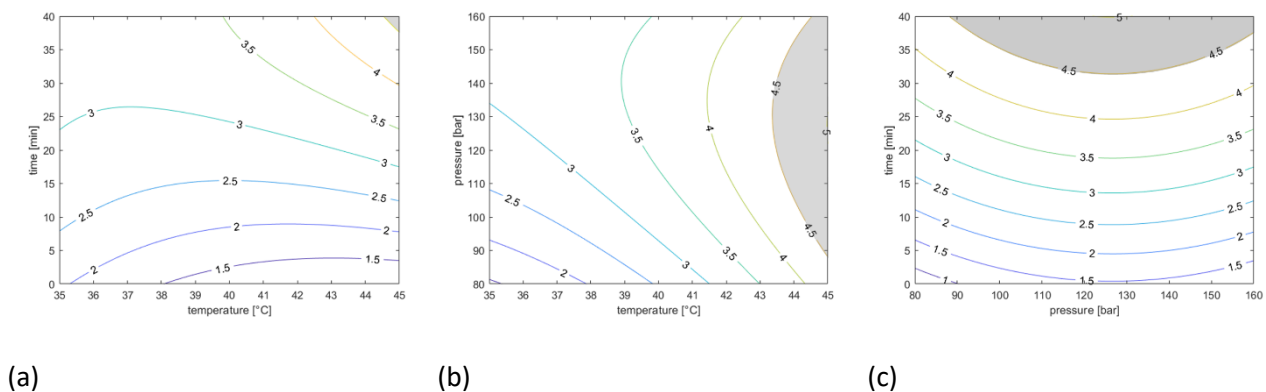


Figure 2. Response surfaces for mesophilic bacteria inactivation as function of temperature and process time, at the maximum pressure of 16 MPa (a), temperature and pressure, at the maximum process time of 40 min (b) and pressure and process time, at

the maximum temperature of 45 °C (c). Each line represents an equal inactivation value. The grey area represents the region with complete inactivation of mesophilic bacteria

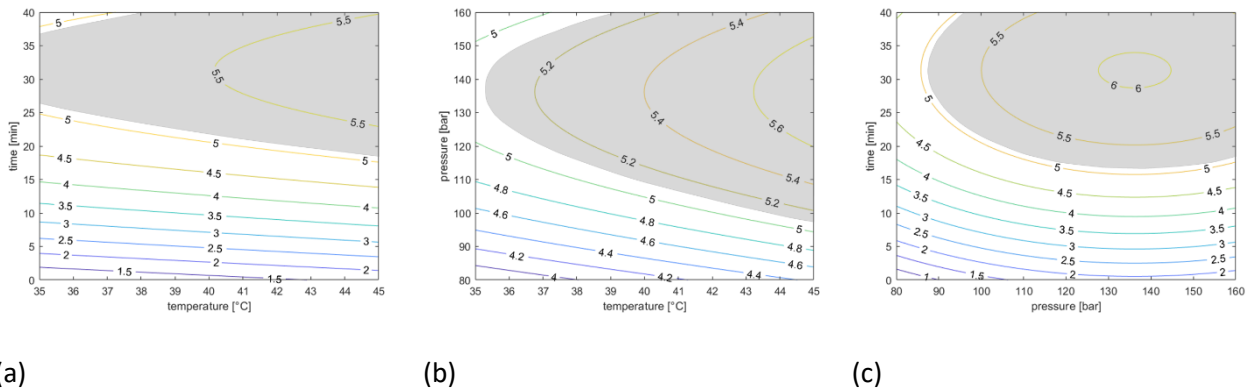


Figure 3. Response surfaces for yeast and moulds inactivation as function of temperature and process time, at the maximum pressure $P=16$ MPa (a), temperature and pressure, at the maximum process time of $t=40$ min (b) and pressure and process time, at the maximum temperature of $T=45$ °C (c). Each line represents an equal inactivation value. The grey area represents the region with complete inactivation of yeasts and moulds.

Microbial analysis

	T ₀		T ₇		T ₁₄		T ₂₁		T ₂₈	
	PCA	RBA	PCA	RBA	PCA	RBA	PCA	RBA	PCA	RBA
Non treated	4.3 ± 0.31	4.2 ± 0.02	4.2 ± 0.0	3.7 ± 0.75	4.5 ± 0.11	5.1 ± 0.52	4.4 ± 0.01	4.2 ± 0.62	4.6 ± 0.24	4.6 ± 0.02
SC-CO₂ treatment	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	1.8	< LoD
Thermal treatment	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD
HHP treatment	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD	< LoD

Table 2. Microbiological growth during shelf life at 4 °C up to 28 days for raw (non treated), SC-CO₂ treated samples and thermal and HHP treated samples. All the data are expressed as Log CFU/mL ± Standard Deviation. Abbreviations: PCA (Plate Count Agar), RBA (Rose Bengal Agar). SC-CO₂ process conditions: T=45 °C, t=40 minutes and P=12.7 MPa.

Phenolic compound analysis: TPC and AAt

	T ₀		T ₇		T ₁₄		T ₂₁		T ₂₈	
	TPC	AAt	TPC	AAt	TPC	AAt	TPC	AAt	TPC	AAt
	µg/mL ± SD	% ± SD	µg/mL ± SD	% ± SD	µg/mL ± SD	% ± SD	µg/mL ± SD	% ± SD	µg/mL ± SD	% ± SD
Non-treated	713.9 ± 0.14 _a	57.7 ± 0.007 _a	489.1 ± 0.14 _{a,b}	25.8 ± 0.1 _{a,b,c,d}	604.3 ± 0.18 _a	24.1 ± 0.01 _{a,b,c}	539.5 ± 0.18 _a	6.7 ± 0.007 _{a,b,c}	335 ± 0.006 _a	2.1 ± 0.05 _{a,b,c}
SC-CO₂ treatment	873.1 ± 0.001 _b	32.1 ± 0.007 _{b,c,d}	832.8 ± 0.015 _{b,a}	21.7 ± 0.004 _{b,a,d}	1135.7 ±0.005 _b	30 ± 0.01 _{b,a,c}	1142 ± 0.006 _b	ND	678 ± 0.006 _b	ND
Thermal treatment	605.6 ± 0.002 _c	44.5 ± 0.05 _{c,a,b,d}	812.8 ± 0.007 _{c,d}	39.2 ± 0.004 _{c,a,d}	979.1 ± 0.01 _c	31.5 ± 0.001 _{c,a,b}	994 ± 0.006 _c	1.7 ± 0.03 _{b,a,c}	719.2 ± 0.006 _c	ND
HHP treatment	686.8 ± 0.002 _{d,a}	68 ± 0.09 _{d,a,b,c}	985.7 ± 0.002 _{d,c}	54.2 ± 0.08 _{d,a,b,c}	1577 ± 0.004 _d	57.8 ± 0.01 _d	2112.4 ± 0.04 _d	34.4 ± 0.07 _d	1293 ± 0.04 _d	22.5 ± 0.07 _d

Table 3: Change in total phenols content (TPC) and antioxidant activity during the storage at 4 °C. different letters represent statistically significant differences (P<0.05) among individual time point of storage (abbreviations: TPC Total Polyphenols Content, GAE Gallic Acid Equivalent expressed in µg/mL, AA Antioxidant Activity, expressed in %; S.D. standard deviation, ND the antioxidant activity is not-detected in the sample). SC-CO₂ process conditions: T=45 °C, t=40 minutes and P=12.7 MPa.

Anthocyanins profile by HPLC

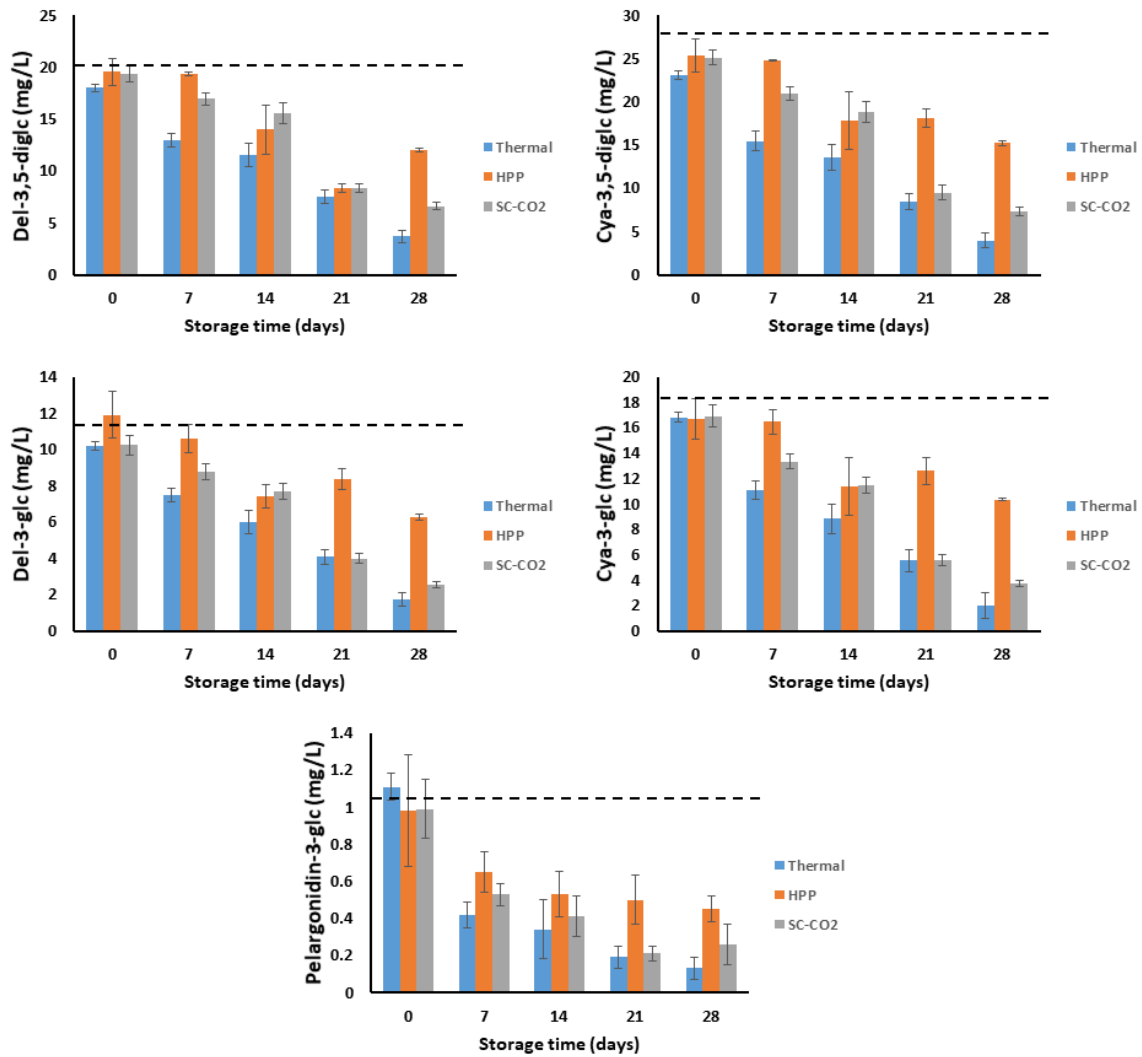


Figure 4: (HPLC-anthocyanins) Quantification ($\mu\text{g/L}$) of the major anthocyanins present in pomegranate juice. Del-3,5-Glc: delphinidin-3,5-diglucoside; Cy-3,5-Glc: cyanidin-3,5-diglucoside; Del-3-Glc: delphinidin-3-glucoside; Cy-3-Glc: cyanidin-3-glucoside; Pg-3-Glc: pelargonidin-3-glucoside. SC-CO₂ process conditions: T=45 °C, t=40 minutes and P=12.7 MPa.

Ellagic Acid by HPLC-DAD

		SC-CO ₂	Thermal	HHP	
Non treated		treatment	treatment	treatment	
		[$\mu\text{g/mL}$] \pm SD	[$\mu\text{g/mL}$] \pm SD	[$\mu\text{g/mL}$] \pm SD	
Ellagic Acid	T ₀	55.9 \pm 5.7	42.8 \pm 8.6	40.8 \pm 5.6	53.6 \pm 4.4
	T ₂₈	46.3 \pm 2.6	19.1 \pm 0.6	45.9 \pm 0.4	30.8 \pm 0.4

Table 4: Change in ellagic acid content, during the storage at 4 °C. Analyses performed by HPLC-DAD. For the calibration curve, ellagic acid standard was employed. Results are expressed in [$\mu\text{g/mL}$]. SC-CO₂ process conditions: T=45 °C, t=40 minutes and P=12.7 MPa.

Colour changes

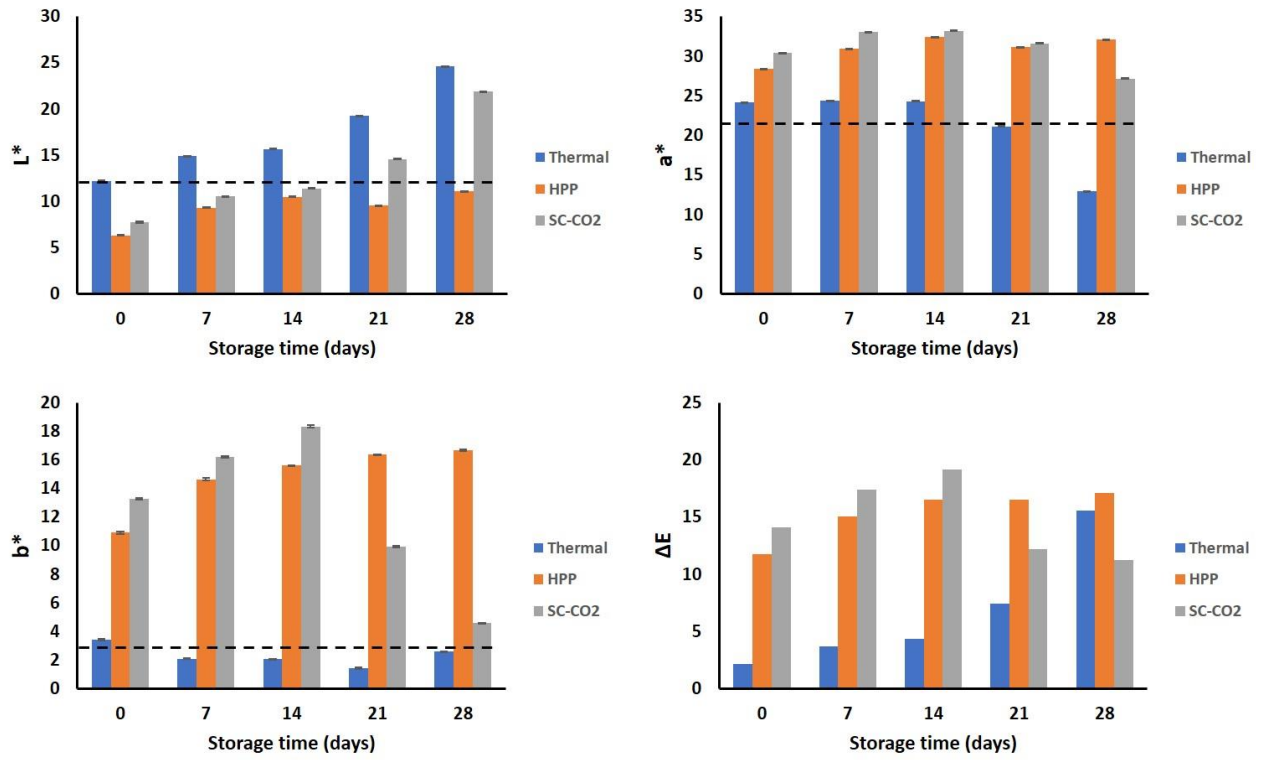


Figure 5: Change in colour in SC-CO₂, Thermal and HPP samples, during the storage at 4 °C. SC-CO₂ process conditions: T=45 °C, t=40 minutes and P=12.7 MPa.

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