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High hydrostatic pressure blanching of baby spinach (*Spinacia oleracea* L.)

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Highlights

- This study compares traditional hot water blanching with HHP blanching for spinach.
- PPO and POD activity were chosen as indicators of adequacy of blanching.
- POD demonstrated to be more baro-resistant than PPO.
- HHP performed well compared to thermal treatments in terms of keeping quality.

Abstract

Given the high susceptibility of baby spinach leaves to thermal processing, the use of high hydrostatic pressure (HHP) is explored as a non-thermal blanching method. The effects of HHP

were compared with thermal blanching by following residual activity of polyphenol oxidases and peroxidases, colour retention, chlorophyll and carotenoids content, antioxidant capacity and total polyphenols content. Spinach subjected to 700 MPa at 20 ºC for 15 min represented the best treatment among the conditions studied due to its balanced effect on target enzymes and quality indices. The latter treatment reduced enzyme activities of polyphenol oxidases and peroxidases by 86.4 and 76.7 %, respectively. Furthermore, leaves did not present changes in colour and an increase by 13.6 % and 15.6 % was found in chlorophyll and carotenoids content, respectively; regarding phytochemical compounds, retentions of 28.2 % of antioxidant capacity and 77.1 % of polyphenols content were found. Results demonstrated that HHP (700 MPa) at room temperature, when compared with thermal treatments, presented better retention of polyphenols, not significantly different chlorophyll and carotenoids content and no perceptible differences in the instrumental colour evaluated through ΔE value; therefore, it can be considered a realistic practical alternative to the widely used thermal blanching.

Key words

Non-thermal technology; Leafy vegetables; Polyphenol oxidases; Peroxidases.

Chemical compounds studied in this article

Polyvinylpyrrolidone (PubChem CID: 6917); Serum albumin (PubChem CID: 16132389); Catechol (PubChem CID: 289); Guaiacol (PubChem CID: 460); hydrogen peroxide (PubChem CID: 784); Ethanol (PubChem CID: 702); 2,2-diphenyl-1-picrylhydrazyl (DPPH) (PubChem CID: 2735032); Gallic acid (PubChem CID: 370); Ascorbic acid (PubChem CID: 54670067); Acetone (PubChem CID: 180).

1. Introduction

In recent years there has been an increasing consumer demand for nutritious products with high sensorial quality and acceptable shelf life. This demand has driven research and development in non-thermal food processing technologies, amongst which, the use of high hydrostatic pressure (HHP) processing is believed to have considerable potential with some innovative applications, such as improving the intake of nutrient and non-nutrient phytochemicals, and development of new products and ingredients with extended life and keeping quality (Rastogi, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 2007). Additionally, HHP has been increasingly investigated in the last decade for lowering enzyme activity.

Most vegetables that are canned, frozen and dehydrated cannot be stored for long periods without blanching which typically occurs in water at high temperatures (75-95 °C) for relatively short times (1-10 min) (Gökmen, 2010). Although thermal treatments are effective in terms of reducing enzymatic activity and microbial load, they affect levels of antioxidants, polyphenols, vitamins, carotenoids and flavonoids, and deteriorate sensory properties (Medina-Meza, Barnaba, Villani, & Barbosa-Cánovas, 2015). Rastogi et al. (2007) proposed HHP as a non-thermal blanching method which, contrary to thermal treatments, has limited influence on covalent bonds of low molecular weight components such as some nutrients, colour and flavour compounds (Oey, Van der Plancken, Van Loey, & Hendrickx, 2008). Among these attributes, colour has a strong influence on consumers´ acceptance and purchase intention (Gökmen, 2010; Medina-Meza et al., 2015). It is thought that enzymatic browning induced by peroxidases (POD) and polyphenoloxidases (PPO) contributes the most to colour deterioration (Steet & Tong, 1996). Enzymes selected as indicators of the blanching adequacy may vary from one product to another. Between these enzymes, POD and PPO activities are usually chosen to indicate the extent of thermal blanching (Whitaker, 1991), in addition these enzymes are amongst the most resistant to pressure.

Several studies have focused on the effects of HHP on enzyme activity in vegetables and vegetable-based products, but very few are *in situ* studies. Moreover, HHP processing as a blanching method for leafy vegetables, has only been studied in the case of white cabbage (Alvarez-Jubete, Valverde, Patras, Mullen, & Marcos, 2014). There is virtually no information on the effects of HHP on the quality characteristics of baby spinach leaves (*Spinacia oleracea* L.) despite its high commercial demand and increasing consumption. It is noteworthy that spinach leaf production doubled in Asia since 2002 and its worldwide production exceeded 20 Mt in 2013 (FAOSTAT, 2015). The present study compares traditional hot water blanching of baby spinach leaves with HHP blanching with respect to residual enzyme activities as well as other physico-chemical characteristics, with a view to establish whether HHP blanching is a realistic practical alternative to the widely used thermal blanching.

2. Materials and methods

2.1 Raw material

Baby spinach leaves (*Spinacia oleracea* L.), cv. Monza, were directly obtained from suppliers within the Dorset county, UK. The leaves were harvested in June-August and were transported at refrigerated temperatures to the University of Reading where they were stored at 4 ºC before being processed. Five leaves weighing 0.5-1.0 g each were selected and packaged (Multivac® A300, Germany) in PA-PE (Polyamide-Polyethylene) bags under vacuum (-85 kPa gauge).

2.2 HHP treatments

The HHP equipment (Stansted Fluid Power Ltd., UK) with canister dimensions: 37 mm diameter and 246 mm length, had a maximum working pressure of 900 MPa and used a 30 g/100mL solution of 1,2-Propanediol (Sigma-Aldrich, UK) for transmitting pressure. The temperature was controlled by a thermostatic device circulating distilled water through the jacket

and registered with an external sensor device. The samples were placed in the product canister, for processing, following which they were immediately cooled in a cold water bath (4 ± 2 ºC) and stored refrigerated (4 ± 2 ºC). The come up time for pressurisation was 30-60 s depending on pressure applied, and depressurization time was less than 30 s.

Processing conditions based on preliminary studies were selected: 700 MPa, 70 ºC, 15 min; 700 MPa, 20 ºC, 15 min; and 800 MPa, 20 ºC, 15 min. Temperature and pressure conditions were monitored during HHP processing (**Figure 1**), and temperatures mentioned are the average temperatures (± 3 °C) of the pressure transmitting fluid. Untreated samples were considered as controls.

2.3 Thermal blanching

Beakers, each containing spinach leaves dipped in distilled water (ratio 1:200 g:mL), were placed in a temperature controlled water bath for a given time, after which the samples were immediately cooled by immersion in cold water (0-4 ºC for 3 min). Based on preliminary experiments with spinach leaves, treatments at 70 ºC for 15 min and 90 ºC for 0.5 min were chosen because these conditions represent a mild thermal blanching and regular blanching treatment, respectively.

2.4 Chemical and physical analysis

2.4.1 Extraction method for PPO (E.C.1.10.3.1) and POD (E.C.1.11.1.7)

A combination of methods described by Arnnok, Ruangviriyachai, Mahachai, Techawongstien, & Chanthai (2010); Kim, Kim, Chung, & Moon (2014); and Wang et al. (2013) was used to extract PPO and POD from spinach leaves. All the enzyme extraction steps were carried out at 4 °C. Briefly, 5.00 ± 0.10 g of spinach leaves were chopped and ground with a mortar and a pestle, the mass was immediately transferred into 20 mL of cold (4 °C) sodium phosphate buffer (0.2 mol/L; pH 6.5-7.0) containing 0.8 g (4 g/100 mL) polyvinylpyrrolidone, average mol

weight 40,000 Da (Sigma-Aldrich, USA). The mixture was stirred for 1 h, centrifuged at 10,000 x g for 10 min (Sorvall^{TM} RC-6, Thermo Fisher Scientific, USA) and the supernatant separated through a Whatman No. 1 filter paper. The enzyme extract was collected in a caramel bottle and stored refrigerated until assayed.

2.4.2 Protein assay

Total soluble protein content of enzyme extracts was determined according to the method described by Bradford (1976). For the assays, the following reagents were used: Bradford protein assay dye reagent concentrate (Bio-Rad, USA) and bovine serum albumin (BSA), protein assay standard (Bio-Rad, USA).

2.4.3 PPO and POD activity assays

Polyphenol oxidase activity was assayed spectrophotometrically (PerkinElmer Inc., Lambda 25, UK), by following the method described by Wang et al. (2013). The stored extract (0.5 mL) was added to a reaction mixture consisting of 1 mL of sodium phosphate buffer (0.1 mol/L; pH 6.5-7.0) containing 0.1 mol/L catechol solution (Sigma-Aldrich, USA) and incubated in a water bath at 25 °C for 5 min. The absorbance at 420 nm was monitored at 25 °C for 3 min.

Peroxidase activity was also determined spectrophotometrically by following a combination of methods described by Wang et al. (2013) and Kim et al. (2014). The reaction mixture consisted of 1.4 mL of 25 mmol/L guaiacol solution (Sigma-Aldrich, USA), 25 mmol/L hydrogen peroxide solution 30 mL/100 mL of water (Sigma-Aldrich, USA) dissolved in sodium phosphate buffer (0.05 mol/L; pH 6.5-7.0), and 0.1 mL of enzyme extract. The activity of POD was assessed at 470 nm and 25 °C for 3 min.

The blank for both PPO and POD assays, was the same reaction mixture except that the enzyme extract was replaced with distilled water. One control assay for each untreated sample was performed without adding substrate to determine if there were any endogenous substrates present

and to prevent over counting of the enzyme activity in the extract. One unit of PPO and POD activity (AU) was defined as the change in absorbance of 0.001 units per min (based on the linear part of the curve). The residual activity was expressed as the ratio between treated and untreated samples (control).

2.4.4 Extraction and determination of antioxidant capacity and total phenolic content

Initially an extraction was conducted following the methodology proposed by Viacava, Roura, & Agüero (2015). Briefly, spinach leaves were chopped and samples of 1.0 ± 0.1 g were taken and placed in 150 mL Erlenmeyer flask containing 20 mL extracting solvent (ethanol solution in water 80 mL/100 mL, acidified with 2 g/100 mL citric acid). Extraction was carried out at 25 ºC under agitation for 1 h. Following extraction, the homogenates were centrifuged (10000 x g for 15 min). Supernatants were recovered and the pellet was re-extracted with 10 mL acetone (Sigma-Aldrich, USA) under the same conditions. Then, it was again centrifuged and the second supernatant was retained. Both supernatants formed the source of antioxidants.

The antioxidant capacity was evaluated through the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, following the methodology described by Viacava et al. (2015). A 0.4 mL aliquot of spinach leaves extract was placed in a cuvette containing 1.6 mL of 100 µmol/L DPPH solution (Sigma-Aldrich, USA). The mixture was shaken and the decrease in absorbance at 517 nm was measured after 1 h, in dark, using an UV-visible spectrophotometer. Blank solutions, without DPPH, were prepared to correct any influence due to the colour of spinach extract. The radical scavenging capacity (RSC) was calculated according to the equation (1):

$$RSC\ (\%) = (A_o - A_t)/(100/A_o) \quad (1)$$

where A_0 is the initial absorption of the mixture and $A_t = A_s - A_b$, where A_s is the absorption of the mixture at the end of reaction and A_b is the absorption of the blank. Additionally, a standard curve with ascorbic acid was made.

The total phenolic content (TPC) was determined using the Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventos, 1999) with gallic acid as standard. Spinach leaves extracts (0.2 mL) were added to 1 mL Folin-Ciocalteu reagent (diluted 1:10) and 0.5 mL of distilled water. After 5 min incubation, 0.3 mL of saturated solution of sodium carbonate was added (sodium carbonate anhydrous, Fisher Scientific, USA). The mixture was left at room temperature (20 ºC) in the dark for 2 h and the absorbance was read at 765 nm using a UV-visible spectrophotometer. Antioxidant capacity and TPC after thermal treatments were corrected by a water loss factor.

2.4.5 Determination of chlorophylls and carotenoids

Chlorophyll and carotenoid contents were determined according to AOAC methodology 942.04 (AOAC International, 1995). Briefly, samples were ground in a mortar with a pestle, 0.30 ± 0.03 g of each sample was weighed and homogenized with 20 mL of cold acetone. Next, solutions were poured into a separator funnel with 20 mL of diethyl ether (Sigma-Aldrich, USA), and then washed thrice with 10 g/100 mL sodium chloride solution (20ml). The extract was recovered in an Erlenmeyer flask, filtered through Whatman No. 1 filter paper, diluted two-fold with cold acetone and kept refrigerated in a caramel bottle.

The absorbance of the extracts was measured at 660.0 and 642.5 nm for chlorophyll determination, and at 450 nm for the determination of carotenoids. The following equations (2) (AOAC International, 1995) and (3) (Scott, 2001) were used to determine total chlorophyll ($mg.L^{-1}$) and carotenoid contents ($mg.100\ g^{-1}$ fresh tissue):

$$\text{Total chlorophyll} = 7.12 \cdot A_{660\ nm} + 16.8 \cdot A_{642.5\ nm} \quad (2)$$

$$\text{Carotenoids} = A_{450\ nm} \cdot [(FV \cdot 1000) / (SW \cdot 2500)] \quad (3)$$

where A is the absorbance at different wavelengths, FV is the extract filtered volume in millilitres and SW is the sample weight in grams. Measurements were done in duplicate and corrected by a water loss factor in the case of thermal treatments.

2.4.6 Measurement of instrumental colour

Instrumental colour was measured using a colourimeter (ColorLite sph 850, Germany) calibrated with a white standard. The illuminating/viewing geometry was D65/10º, the probe head´s measuring spot diameter was 9 mm and the colour space used was the CIELab system. Leaf colour was measured on the upper side and each leaf´s value was the average of 3 individual measurements in different parts of the same leaf, colourimeter operating with 3 cycles. For evaluating colour changes after treatments, chroma or saturation index (C*), hue angle (Hº) and ΔE, which expresses colour differences when comparing with a control sample, were calculated with the equations (4) (Koukounaras, Siomos, & Sfakiotakis, 2009), (5) (Koukounaras et al., 2009), and (6) (Chisari, Todaro, Barbagallo & Spagna, 2010):

$$C^*=[(a^*)^2+(b^*)^2]^{0.5} \quad (4)$$

$$H°=180+(\tan\text{-}1(b^*/a^*))\cdot(180/\pi) \quad (5)$$

$$\Delta E=[(L_c^*-L^*)^2+(a_c^*-a^*)^2+(b_c^*-b^*)^2]^{0.5} \quad (6)$$

where L_s^*, a_c^* and b_c^* are the parameters L* (lightness), a* (redness and greenness), and b* (yellowness and blueness) for the control sample, respectively.

2.4.8 Determination of water loss during thermal blanching

Water loss (WL) was determined in triplicates and then averaged. Samples were weighed before and after processing (w_b and w_a, respectively) for each thermal treatment selected. WL percentage was calculated as follows (7):

$$WL(\%)=[1-(w_a / w_b)]\cdot 100 \quad (7)$$

2.5 Statistical analysis

Data were analysed using SAS 9.0 software (SAS, 2002). For all experiments, General Linear Model procedure was used for analysis of variance (ANOVA) with different variation sources

depending on the experiment. For all cases, differences between levels of factors under analysis were assessed by multiple comparison Tukey-Kramer test (with a significance level of 5%).

3. Results and discussion

3.1 Effect of selected HHP and thermal treatments

3.1.1 PPO and POD activities

Confidence intervals at 95% level (CI95) characterizing enzyme extracts (n=20) were 1.07-1.26 $mg.mL^{-1}$ for protein content, and 376.93-508.09 activity units $(AU).min^{-1}.g^{-1}$ and 12.89-17.66 $AU.min^{-1}.mg^{-1}$ for PPO and POD activities based on fresh tissue, respectively. The reduction in enzyme activity achieved by selected HHP and thermal treatments is presented in **Table 1**. The highest reduction achieved amongst HHP treatments was when pressure and high temperature were combined. The effect of high temperatures for enhancing the denaturation of enzymes by HHP is well reported (Krebbers et al., 2003; Sila et al., 2007). In the case of treatments at room temperature (20 ºC), an increase in pressure from 700 MPa to 800 MPa did not cause a significant decrease in the activities of PPO and POD. On the other hand, thermal treatments achieved total inactivation of PPO and POD at the highest temperature assessed (90 °C); meanwhile at 70 °C, PPO was fully inactivated but POD presented a low residual activity.

Regarding barostability of the target enzymes, the high resistance to HHP treatments demonstrated by POD could be associated with the presence of more stable isoenzymes, as described in the case of thermal treatments by Gökmen (2010). Additionally, the isoenzymes can vary in content within spinach leaves depending on variety, age and environmental factors (Gökmen, 2010). Wang et al. (2013) also reported that the baro-resistance of POD is higher than PPO, and as some authors have hypothesized, the baro-resistance of POD is linked to its lower molecular weight (35 kDa) given that high pressure has little effect on lower molecular weight compounds (Rastogi, Eshtiaghi, & Knorr, 1999).

3.1.2 Chlorophyll and carotenoid contents and instrumental colour

The effect of selected treatments on colour was evaluated through chlorophyll (Chl) and carotenoids content, as well as instrumental colour (**Figure 2** and **Table 2**¸ respectively). Control samples presented CI95 of 79.51-107.53 mg .100 g^{-1} of fresh tissue (FT) and 26.81-37.92 mg .100 g^{-1} FT, respectively for Chl and carotenoids content (n=4). As shown in **Figure 2,** Chl and carotenoids contents decreased significantly when combined pressure and high temperature were applied (treatment P1). Chlorophylls are stable under high pressures, but as reported by Oey et al. (2008) they are significantly reduced at temperatures greater than 50 ºC which is similar to the results of the present study. Regarding carotenoid content, similar trends were found by Kim, Park, Cho, & Park (2001) who subjected carrot juice to 500 MPa at 70 ºC for 10 min. The increases detected in Chl and carotenoids contents of samples subjected to 700 MPa and 800 MPa at 20 ºC for 15 min (treatments P2 and P3 in **Figure 2**), could be associated with a severe cell damage caused by high pressure resulting in increased Chl and carotenoids extraction. Higher contents of Chl were also observed after applying HHP to oil-based spinach sauce (Medina-Meza et al., 2015). On the other hand, Wang et al. (2013) and Kim et al. (2001) did not find increased contents in Chl and carotenoids after subjecting spinach to HHP, probably because the leaves were previously processed causing cell damage and consequent release of these components. Finally, Chl and carotenoids content after treatment P2 (700 MPa; 20 ºC; 15 min) were not significantly different compared to those after thermal blanching (T1 and T2 shown in **Figure 2**).

Green colour in spinach is mainly determined by the CIELab parameter (-a*): the higher its value the greener the leaves, but C*, Hº and ΔE also contribute to the evaluation of instrumental colour. Differences in colour expressed by ΔE value, as a general rule, need to be higher than 1.8 (suprathreshold) in order to be perceptible by human eye and values greater than 5.0 are considered to be remarkable differences (Melgosa, Pérez, Yebra, Huertas, & Hita, 2001). In this investigation, all treated samples presented perceptible changes in colour (**Table 2**). Samples subjected to HHP

combined with high temperature not only gave the highest ΔE but also presented a significantly lower (-a*) value which indicates that leaves were less green; in addition, hue angle was close to 90º indicating that colour turned from green to yellow green. This marked effect on colour of the leaves by the combination of high pressure and high temperature would make the product unacceptable for consumers, and correlates well with the low retention of Chl exhibited (treatment P1 in **Figure 2**), possibly due to the formation of pheophytins from the degradation of Chl. On the other hand, even though the ΔE values for samples treated with HHP at room temperature and those thermally blanched were detectable (ΔE > 1.8) they presented better green colour, with similar or higher values of (-a*), C* and Hº than in control samples. Alvarez-Jubete et al. (2014), who evaluated colour in HHP treated white cabbage (200, 400 and 600 MPa at 20 and 40 ºC for 5 min) found similar results. In the present work, for both HHP and thermal blanching, texture modifications may had resulted in changes in the nature and extent of internally scattered light and the distribution of surface reflectance (MacDougall, 2002), resulting in greener leaves; with the exception of treatment with combined pressure and high temperature. Moreover, cell damage with consequent pigment release could have benefitted pressure treated samples. And water loss with values of 22 ± 6% and 25 ± 7%, respectively for samples subjected to thermal treatments at 70 ºC and 90 ºC, enhanced their green colour due to the concentration of pigments.

High retention and improved extractability of pigments, Chl and carotenoids, correlated well with colour retention of spinach leaves subjected to HHP at room temperature (20 ºC) and thermal treatments; meanwhile, the reduction in pigment content in samples treated with high pressure and high temperature is in accordance with the instrumental colour evaluation. The latter negative effect was also observed in green beans and basil by Krebbers, Matser, Koets, & Van den Berg (2002a) and Krebbers, Matser, Koets, Bartels, & Van Den Berg (2002b), respectively. Green colour retention after HHP treatments at room temperature is in accordance with the results found by Krebbers et al. (2002a), Wang et al. (2013), and Medina-Meza et al. (2015). Chlorophyll and carotenoids are very important phytochemicals and their presence is not only related to the

characteristic colour of vegetables but also to the beneficial effects on consumers´ health (Tang, 2010).

3.1.3 Antioxidant capacity and total phenolic content

Spinach leaves are amongst the most nutritious vegetables in terms of antioxidant capacity and total phenolic content (TPC) (Zhou & Yu, 2006). Initial values found in the raw material studied, expressed as CI95 (n=4), were 46.35-50.81 mg of ascorbic acid per 100 g FT and 87.28-101.90 mg of gallic acid per 100 g FT, respectively for antioxidant capacity and TPC. Reductions of 55-73% in antioxidant capacity and 12-23% in TPC were found after HHP processing, and no significant differences were detected between treatments (**Figure 3**). Additionally, in these components the effect of HHP combined with high temperature did not cause further reductions, contrary to the observed effect on Chl and carotenoids. On the other hand, thermal treatments reduced antioxidant capacity to values not significantly different to those after HHP, but TPC reached values of roughly 16% lower than those found in pressure treated samples. It is noteworthy that inevitably a certain amount of compounds present in spinach leaves was lost due to leaching in water after traditional blanching, and this might have affected antioxidant capacity and TPC, as well as Chl and carotenoids. The latter is not possible during HHP processing, therefore it represents an advantage over traditional blanching.

Regarding high pressure sensitivity, polyphenols demonstrated to be more baro-resistant than antioxidant compounds determined by DPHH methodology. Similar results were reported by Barba, Esteve, & Frigola (2013) who evaluated antioxidant compounds and TPC in blueberry juice and Alvarez-Jubete et al. (2014) in white cabbage. Possible explanations for this phenomenon could be a loss of antioxidant capacity due to vitamin C enzymatic degradation during pressurization (Barba et al., 2013) or after HHP due to residual activity of quality deteriorating enzymes. Supporting the explanation of loss of antioxidant capacity due to a loss of vitamin C, a remarkable decrease in ascorbic acid content was found in white cabbage after applying HHP (Alvarez-Jubete et al., 2014).

Meanwhile for polyphenols, multiple mechanisms for their degradation might have been involved. However, the lower reductions, comparing with control samples, found in the present study could be mainly attributed to thermal effects during pressurization with consequent activation of PPO and POD, and these effects might have been more severe in thermal treatments.

4. Conclusion

Spinach subjected to 700 MPa at 20 ºC for 15 min represented the best treatment among the HHP conditions studied; because a further increase in pressure (up to 800 MPa) did not cause a significant reduction in the activities of PPO and POD, and its performance in terms of colour, chlorophyll and carotenoids retention was superior comparing with the treatment where high pressure and high temperature were combined. The quality indices studied indicate that HHP (700 MPa) processing at room temperature (20 ºC) performed well compared to thermal treatments; presenting better retention of polyphenols, not significantly different chlorophyll and carotenoids content and no perceptible differences in the instrumental colour evaluated through ΔE value. Therefore, high pressure at room temperature must be considered as a realistic and promising alternative either for non-thermal blanching of spinach leaves or for pre-treating those which will be further processed (canned, frozen, dehydrated, etc.).

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References

Alvarez-Jubete, L., Valverde, J., Patras, A., Mullen, A. M., & Marcos, B. (2014). Assessing the impact of high-pressure processing on selected physical and biochemical attributes of white cabbage (*Brassica oleracea* L. var. capitata alba). *Food and Bioprocess Technology*, *7*(3), 682-692.

AOAC (Association of Official Analytical Chemists) International (1995). Method 942.04: Chlorophyll in plants: Spectrophotometric method of total chlorophyll and the a and b components. In *Official Method of Analysis* (16th ed.). Arlington, VA: AOAC International.

Arnnok, P., Ruangviriyachai, C., Mahachai, R., Techawongstien, S., & Chanthai, S. (2010). Optimization and determination of polyphenol oxidase and peroxidase activities in hot pepper (*Capsicum annuum* L.) pericarb. *International Food Research Journal*, *17*, 385-392.

Barba, F. J., Esteve, M. J., & Frigola, A. (2013). Physicochemical and nutritional characteristics of blueberry juice after high pressure processing. *Food Research International, 50*(2), 545-549.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1), 248-254.

Chisari, M., Todaro, A., Barbagallo, R. N., & Spagna, G. (2010). Salinity effects on enzymatic browning and antioxidant capacity of fresh-cut baby Romaine lettuce (*Lactuca sativa* L. cv. Duende). *Food Chemistry*, *119*(4), 1502-1506.

FAOSTAT, FAO. (2015). Statistics of the Food and Agriculture Organization of the United Nations [electronic document]. *URL http://faostat3.fao.org/browse/Q/QC/E [last access: November 13th, 2015]*.

Gökmen, V. (2010). Chapter 5: Selection of the indicator enzyme for blanching of vegetables. In A. Bayindirli (Ed.), *Enzymes in fruit and vegetable processing: Chemistry and engineering applications* (pp. 123-144). Boca Raton, FL: CRC Press.

Kim, D. H., Kim, H. B., Chung, H. S., & Moon, K. D. (2014). Browning control of fresh-cut lettuce by phytoncide treatment. *Food Chemistry*, *159*, 188-192.

Kim, Y. S., Park, S. J., Cho, Y. H., & Park, J. (2001). Effects of combined treatment of high hydrostatic pressure and mild heat on the quality of carrot juice. *Journal of Food Science, 66*(9), 1355-1360.

Koukounaras, A., Siomos, A. S., & Sfakiotakis, E. (2009). Impact of heat treatment on ethylene production and yellowing of modified atmosphere packaged rocket leaves. *Postharvest Biology and Technology*, *54*(3), 172-176.

Krebbers, B., Matser, A. M., Hoogerwerf, S. W., Moezelaar, R., Tomassen, M. M., & van den Berg, R. W. (2003). Combined high-pressure and thermal treatments for processing of tomato puree: evaluation of microbial inactivation and quality parameters. *Innovative Food Science & Emerging Technologies, 4*(4), 377-385.

Krebbers, B., Matser, A. M., Koets, M., & Van den Berg, R. W. (2002a). Quality and storage-stability of high-pressure preserved green beans. *Journal of Food Engineering, 54*(1), 27-33.

Krebbers, B., Matser, A., Koets, M., Bartels, P., & Van Den Berg, R. (2002b). High pressure-temperature processing as an alternative for preserving basil. *International Journal of High Pressure Research, 22*(3-4), 711-714.

MacDougall, D. (2002). Colour measurement of food: Principles and practice. In D. MacDougall (Ed.), *Colour in food improving quality* (pp. 33-60). Boca Raton, FL: Woodhead Publishing Limited and CRC Press.

Medina-Meza, I. G., Barnaba, C., Villani, F., & Barbosa-Cánovas, G. V. (2015). Effects of thermal and high pressure treatments in color and chemical attributes of an oil-based spinach sauce. *LWT-Food Science and Technology, 60*(1), 86-94.

Melgosa, M., Pérez, M. M., Yebra, A., Huertas, R., & Hita, E. (2001). Algunas reflexiones y recientes recomendaciones internacionales sobre evaluación de diferencias de color. *Óptica Pura y Aplicada, 34*(1), 1-10.

Oey, I., Van der Plancken, I., Van Loey, A., & Hendrickx, M. (2008). Does high pressure processing influence nutritional aspects of plant based food systems?. *Trends in Food Science & Technology, 19*(6), 300-308.

Rastogi, N. K., Eshtiaghi, M. N., & Knorr, D. (1999). Effects of combined high pressure and heat treatment on the reduction of peroxidase and polyphenoloxidase activity in red grapes. *Food Biotechnology, 13*(2), 195-208.

Rastogi, N. K., Raghavarao, K. S. M. S., Balasubramaniam, V. M., Niranjan, K., & Knorr, D. (2007). Opportunities and challenges in high pressure processing of foods. *Critical Reviews in Food Science and Nutrition, 47*(1), 69-112.

Scott, K. (2001). Detection and measurement of carotenoids by UV/VIS spectrophotometry. In R. Wrolstad (Ed.), *Current protocols in food analytical chemistry*. New York: Wiley.

Sila, D. N., Smout, C., Satara, Y., Truong, V., Van Loey, A., & Hendrickx, M. (2007). Combined thermal and high pressure effect on carrot pectinmethylesterase stability and catalytic activity. *Journal of Food Engineering, 78*(3), 755-764.

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology*, (299C), 152-178.

Steet, J. A., & Tong, C. H. (1996). Degradation kinetics of green color and chlorophylls in peas by colorimetry and HPLC. *Journal of Food Science, 61*(5), 924-928.

Tang, G. (2010). Spinach and Carrots: Vitamin A and Health. In R. Watson & V. Preedy (Eds.), *Bioactive foods in promoting health* (pp. 381-392). San Diego, CA: Academic Press.

Viacava, G.E., Roura, S.I., & Agüero, M.V. (2015). Antioxidant activity of Butterhead lettuce: factors significantly affecting antioxidant extraction and quantification. *Journal of Food Measurement & Characterization, 9*(2), 206-214.

Wang, R., Xu, Q., Yao, J., Zhang, Y., Liao, X., Hu, X., Wu, J., & Zhang, Y. (2013). Post-effects of high hydrostatic pressure on green color retention and related properties of spinach puree during storage. *Innovative Food Science & Emerging Technologies, 17*, 63-71

Whitaker, J. R. (1991). Enzymes: monitors of food stability and quality. *Trends in Food Science & Technology, 2,* 94-97.

Zhou, K., & Yu, L. (2006). Total phenolic contents and antioxidant properties of commonly consumed vegetables grown in Colorado. *LWT-Food Science and Technology, 39*(10), 1155-1162.

Figures and Tables

Figure 1

Pressure (P) and temperature (T) change with respect to time during HHP processing. **A.** Treatment **P1** (700 MPa; 70 ºC; 15 min) **B.** Treatment **P2** (700 MPa; 20 ºC; 15 min) **C.** Treatment **P3** (800 MPa; 20 ºC; 15 min). Solid and dotted lines represent pressure and temperature changes, respectively.

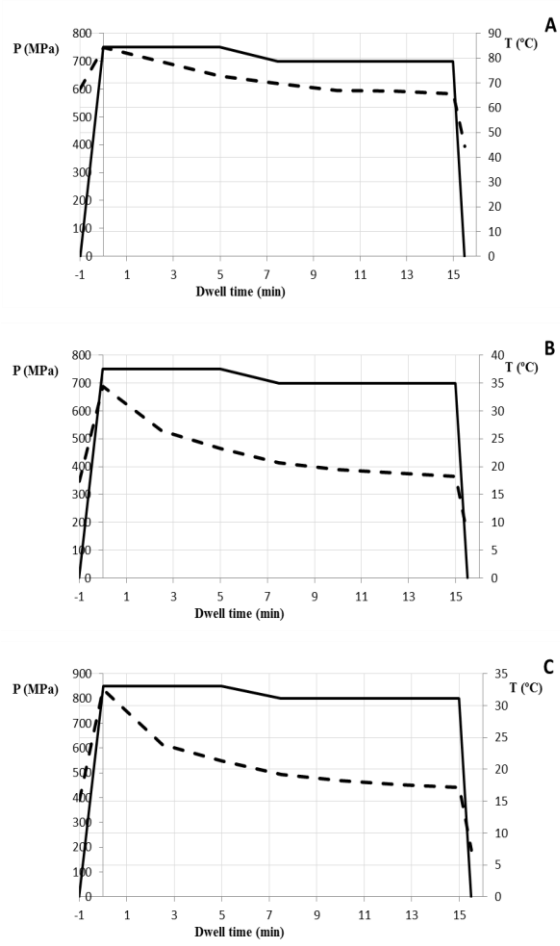

Figure 2

Residual content of total chlorophyll (dark-grey bars) and carotenoids (light-grey bars) after selected treatments. **P1** (700 MPa; 70 °C; 15 min), **P2** (700 MPa; 20 °C; 15 min), **P3** (800 MPa; 20 °C; 15 min), **T1** (0.1 MPa; 70 °C; 15 min), and **T2** (0.1 MPa; 90 °C; 0.5 min). Data are presented as the means ± standard errors expressed as vertical segments (n=2). [a, b, c]: Different letters in each type of bar indicate significant differences (Tukey's test, $p<0.05$).

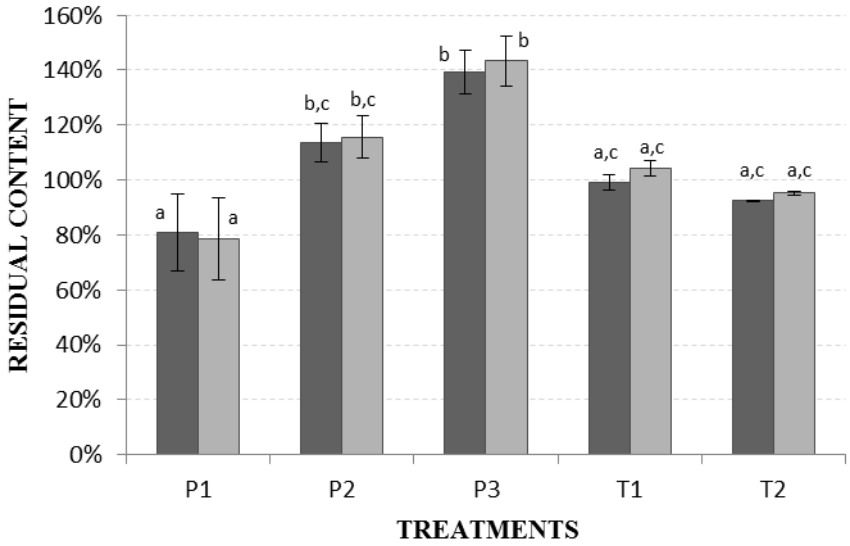

Figure 3

Residual content of antioxidant capacity (dark-grey bars) and total polyphenols (light-grey bars) after selected treatments. **P1** (700 MPa; 70 °C; 15 min), **P2** (700 MPa; 20 °C; 15 min), **P3** (800 MPa; 20 °C; 15 min), **T1** (0.1 MPa; 70 °C; 15 min), and **T2** (0.1 MPa; 90 °C; 0.5 min). Data are presented as the means ± standard errors expressed as vertical segments (n=2). [a, b, c]: Different letters in each type of bar indicate significant differences (Tukey's test, $p<0.05$).

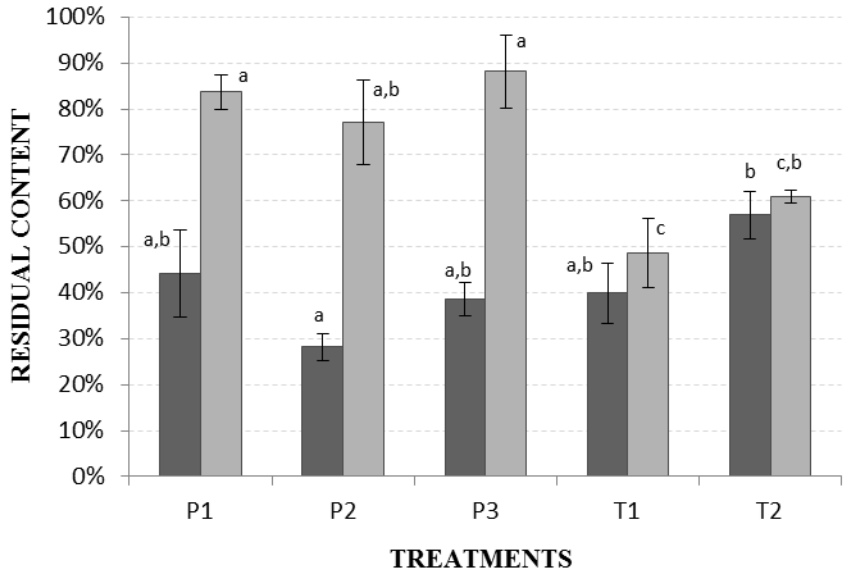

Table 1

Residual enzymatic activity (RA) of PPO and POD after HHP and thermal treatments.

Treatment	P (MPa)	T (°C)	t (min)	RA PPO (%)	RA POD (%)
P1	700	70	15	n.d.	0.9 ± 0.6^{a}
P2	700	20	15	13.6 ± 1.8^{a}	23.3 ± 7.6^{b}
P3	800	20	15	17.1 ± 0.3^{a}	27.4 ± 1.1^{b}
T1	0.1	70	15	n.d.	2.0 ± 0.8^{a}
T2	0.1	90	0.5	n.d.	n.d.

Data are presented as the means ± standard errors (n=2). [a, b]: Different letters in the same column indicate significant differences (Tukey's test, $p<0.05$). n.d.: Not detected.

Table 2

Instrumental colour in control and treated samples. **C** (Control, untreated samples), **P1** (700 MPa; 70 °C; 15 min), **P2** (700 MPa; 20 °C; 15 min), **P3** (800 MPa; 20 °C; 15 min), **T1** (0.1 MPa; 70 °C; 15 min), and **T2** (0.1 MPa; 90 °C; 0.5 min).

Treatment	**INSTRUMENTAL COLOUR**			
	(-a*)	***C****	***H°***	***ΔE****
C	7.95 ± 0.17^{a}	18.66 ± 0.75^{a}	115.71 ± 0.65^{a}	-
P1	1.93 ± 0.17^{b}	17.89 ± 1.51^{a}	96.65 ± 0.68^{b}	10.96 ± 0.99^{a}
P2	8.53 ± 0.31^{a}	$20.64 \pm 1.23^{a,b}$	115.06 ± 0.85^{a}	6.06 ± 0.99^{b}
P3	8.54 ± 0.31^{a}	$18.99 \pm 1.32^{a,c}$	117.70 ± 1.09^{c}	6.55 ± 1.05^{b}
T1	10.99 ± 0.26^{c}	23.40 ± 0.77^{b}	118.19 ± 0.39^{c}	6.67 ± 0.48^{b}
T2	11.32 ± 0.27^{c}	$22.01 \pm 0.82^{b,c}$	121.27 ± 0.63^{d}	6.34 ± 0.54^{b}

Data are presented as the means ± standard errors (n=20 and 15 for control and treated samples, respectively). $^{a, b, c, d}$*: Different letters in the same column indicate significant differences (Tukey's test, $p<0.05$).*