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

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22 **Abstract**

23 Given the high susceptibility of baby spinach leaves to thermal processing, the use of high
24 hydrostatic pressure (HHP) is explored as a non-thermal blanching method. The effects of HHP
25 were compared with thermal blanching by following residual activity of polyphenol oxidases and
26 peroxidases, colour retention, chlorophyll and carotenoids content, antioxidant capacity and total
27 polyphenols content. Spinach subjected to 700 MPa at 20 ºC for 15 min represented the best
28 treatment among the conditions studied due to its balanced effect on target enzymes and quality

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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

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55 products and ingredients with extended life and keeping quality (Rastogi, Raghavarao,
56 Balasubramaniam, Niranjan, & Knorr, 2007). Additionally, HHP has been increasingly investigated
57 in the last decade for lowering enzyme activity.

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

73 Several studies have focused on the effects of HHP on enzyme activity in vegetables and
74 vegetable-based products, but very few are *in situ* studies. Moreover, HHP processing as a
75 blanching method for leafy vegetables, has only been studied in the case of white cabbage (Alvarez-
76 Jubete, Valverde, Patras, Mullen, & Marcos, 2014). There is virtually no information on the effects
77 of HHP on the quality characteristics of baby spinach leaves (*Spinacia oleracea* L.) despite its high
78 commercial demand and increasing consumption. It is noteworthy that spinach leaf production
79 doubled in Asia since 2002 and its worldwide production exceeded 20 Mt in 2013 (FAOSTAT,
80 2015). The present study compares traditional hot water blanching of baby spinach leaves with

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81 HHP blanching with respect to residual enzyme activities as well as other physico-chemical
82 characteristics, with a view to establish whether HHP blanching is a realistic practical alternative to
83 the widely used thermal blanching.
84

85 **2. Materials and methods**

86

87 *2.1 Raw material*

88 Baby spinach leaves (*Spinacia oleracea* L.), cv. Monza, were directly obtained from suppliers
89 within the Dorset county, UK. The leaves were harvested in June-August and were transported at
90 refrigerated temperatures to the University of Reading where they were stored at 4 ºC before being
91 processed. Five leaves weighing 0.5-1.0 g each were selected and packaged (Multivac® A300,
92 Germany) in PA-PE (Polyamide-Polyethylene) bags under vacuum (-85 kPa gauge).
93

94 *2.2 HHP treatments*

95 The HHP equipment (Stansted Fluid Power Ltd., UK) with canister dimensions: 37 mm
96 diameter and 246 mm length, had a maximum working pressure of 900 MPa and used a 30
97 g/100mL solution of 1,2-Propanediol (Sigma-Aldrich, UK) for transmitting pressure. The
98 temperature was controlled by a thermostatic device circulating distilled water through the jacket
99 and registered with an external sensor device. The samples were placed in the product canister, for
100 processing, following which they were immediately cooled in a cold water bath (4 ± 2 ºC) and
101 stored refrigerated (4 ± 2 ºC). The come up time for pressurisation was 30-60 s depending on
102 pressure applied, and depressurization time was less than 30 s.
103 Processing conditions based on preliminary studies were selected: 700 MPa, 70 ºC, 15 min;
104 700 MPa, 20 ºC, 15 min; and 800 MPa, 20 ºC, 15 min. Temperature and pressure conditions were
105 monitored during HHP processing (**Figure 1**), and temperatures mentioned are the average

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106 temperatures (± 3 °C) of the pressure transmitting fluid. Untreated samples were considered as
107 controls.
108
109 *2.3 Thermal blanching*
110 Beakers, each containing spinach leaves dipped in distilled water (ratio 1:200 g:mL), were
111 placed in a temperature controlled water bath for a given time, after which the samples were
112 immediately cooled by immersion in cold water (0-4 ºC for 3 min). Based on preliminary
113 experiments with spinach leaves, treatments at 70 ºC for 15 min and 90 ºC for 0.5 min were chosen
114 because these conditions represent a mild thermal blanching and regular blanching treatment,
115 respectively.
116
117 *2.4 Chemical and physical analysis*
118 *2.4.1 Extraction method for PPO (E.C.1.10.3.1) and POD (E.C.1.11.1.7)*
119 A combination of methods described by Arnnok, Ruangviriyachai, Mahachai,
120 Techawongstien, & Chanthai (2010); Kim, Kim, Chung, & Moon (2014); and Wang et al. (2013)
121 was used to extract PPO and POD from spinach leaves. All the enzyme extraction steps were
122 carried out at 4 °C. Briefly, 5.00 ± 0.10 g of spinach leaves were chopped and ground with a mortar
123 and a pestle, the mass was immediately transferred into 20 mL of cold (4 °C) sodium phosphate
124 buffer (0.2 mol/L; pH 6.5-7.0) containing 0.8 g (4 g/100 mL) polyvinylpyrrolidone, average mol
125 weight 40,000 Da (Sigma-Aldrich, USA). The mixture was stirred for 1 h, centrifuged at 10,000 x g
126 for 10 min (Sorvall™ RC-6, Thermo Fisher Scientific, USA) and the supernatant separated through
127 a Whatman No. 1 filter paper. The enzyme extract was collected in a caramel bottle and stored
128 refrigerated until assayed.
129
130 *2.4.2 Protein assay*

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

135

136 *2.4.3 PPO and POD activity assays*

137 Polyphenol oxidase activity was assayed spectrophotometrically (PerkinElmer Inc., Lambda
138 25, UK), by following the method described by Wang et al. (2013). The stored extract (0.5 ml) was
139 added to a reaction mixture consisting of 1 mL of sodium phosphate buffer (0.1 mol/L; pH 6.5-7.0)
140 containing 0.1 mol/L catechol solution (Sigma-Aldrich, USA) and incubated in a water bath at 25
141 °C for 5 min. The absorbance at 420 nm was monitored at 25 °C for 3 min.

142 Peroxidase activity was also determined spectrophotometrically by following a combination
143 of methods described by Wang et al. (2013) and Kim et al. (2014). The reaction mixture consisted
144 of 1.4 mL of 25 mmol/L guaiacol solution (Sigma-Aldrich, USA), 25 mmol/L hydrogen peroxide
145 solution 30 mL/100 mL of water (Sigma-Aldrich, USA) dissolved in sodium phosphate buffer (0.05
146 mol/L; pH 6.5-7.0), and 0.1 mL of enzyme extract. The activity of POD was assessed at 470 nm and
147 25 °C for 3 min.

148 The blank for both PPO and POD assays, was the same reaction mixture except that the
149 enzyme extract was replaced with distilled water. One control assay for each untreated sample was
150 performed without adding substrate to determine if there were any endogenous substrates present
151 and to prevent over counting of the enzyme activity in the extract. One unit of PPO and POD
152 activity (AU) was defined as the change in absorbance of 0.001 units per min (based on the linear
153 part of the curve). The residual activity was expressed as the ratio between treated and untreated
154 samples (control).

155

156 *2.4.4 Extraction and determination of antioxidant capacity and total phenolic content*

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157 Initially an extraction was conducted following the methodology proposed by Viacava, Roura,
158 & Agüero (2015). Briefly, spinach leaves were chopped and samples of 1.0 ± 0.1 g were taken and
159 placed in 150 mL Erlenmeyer flask containing 20 mL extracting solvent (ethanol solution in water
160 80 mL/100 mL, acidified with 2 g/100 mL citric acid). Extraction was carried out at 25 ºC under
161 agitation for 1 h. Following extraction, the homogenates were centrifuged (10000 x g for 15 min).
162 Supernatants were recovered and the pellet was re-extracted with 10 mL acetone (Sigma-Aldrich,
163 USA) under the same conditions. Then, it was again centrifuged and the second supernatant was
164 retained. Both supernatants formed the source of antioxidants.

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

172 $$RSC\ (\%) = (A_o - A_t)/(100/A_o) \qquad (1)$$

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

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

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184 *2.4.5 Determination of chlorophylls and carotenoids*

185 Chlorophyll and carotenoid contents were determined according to AOAC methodology
186 942.04 (AOAC International, 1995). Briefly, samples were ground in a mortar with a pestle, 0.30 ±
187 0.03 g of each sample was weighed and homogenized with 20 mL of cold acetone. Next, solutions
188 were poured into a separator funnel with 20 mL of diethyl ether (Sigma-Aldrich, USA), and then
189 washed thrice with 10 g/100 mL sodium chloride solution (20ml). The extract was recovered in an
190 Erlenmeyer flask, filtered through Whatman No. 1 filter paper, diluted two-fold with cold acetone
191 and kept refrigerated in a caramel bottle.

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

196 $$\text{Total chlorophyll} = 7.12\cdot A_{660\ nm} + 16.8\cdot A_{642.5\ nm} \quad (2)$$

197 $$\text{Carotenoids} = A_{450\ nm}\cdot\ [(FV\cdot 1000) / (SW\cdot 2500)] \quad (3)$$

198

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

202

203 *2.4.6 Measurement of instrumental colour*

204 Instrumental colour was measured using a colourimeter (ColorLite sph 850, Germany)
205 calibrated with a white standard. The illuminating/viewing geometry was D65/10º, the probe head´s
206 measuring spot diameter was 9 mm and the colour space used was the CIELab system. Leaf colour
207 was measured on the upper side and each leaf´s value was the average of 3 individual measurements
208 in different parts of the same leaf, colourimeter operating with 3 cycles. For evaluating colour

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209 changes after treatments, chroma or saturation index (C*), hue angle (Hº) and ΔE, which expresses
210 colour differences when comparing with a control sample, were calculated with the equations (4)
211 (Koukounaras, Siomos, & Sfakiotakis, 2009), (5) (Koukounaras et al., 2009), and (6) (Chisari,
212 Todaro, Barbagallo & Spagna, 2010):

213 $C^*=[(a^*)^2+(b^*)^2]^{0.5}$ (4)

214 $H°=180+(\tan-1(b^*/a^*))\cdot(180/\pi)$ (5)

215 $\Delta E=[(L_c^*-L^*)^2+(a_c^*-a^*)^2+(b_c^*-b^*)^2]^{0.5}$ (6)

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

218

219 *2.4.8 Determination of water loss during thermal blanching*

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

223 $WL(\%)=[1-(w_a / w_b)]\cdot 100$ (7)

224

225 *2.5 Statistical analysis*

226 Data were analysed using SAS 9.0 software (SAS, 2002). For all experiments, General Linear
227 Model procedure was used for analysis of variance (ANOVA) with different variation sources
228 depending on the experiment. For all cases, differences between levels of factors under analysis
229 were assessed by multiple comparison Tukey-Kramer test (with a significance level of 5%).

230

231 **3. Results and discussion**

232

233 *3.1 Effect of selected HHP and thermal treatments*

234 *3.1.1 PPO and POD activities*

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235 Confidence intervals at 95% level (CI95) characterizing enzyme extracts (n=20) were 1.07-
236 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
237 AU.min^{-1}.mg^{-1} for PPO and POD activities based on fresh tissue, respectively. The reduction in
238 enzyme activity achieved by selected HHP and thermal treatments is presented in **Table 1**. The
239 highest reduction achieved amongst HHP treatments was when pressure and high temperature were
240 combined. The effect of high temperatures for enhancing the denaturation of enzymes by HHP is
241 well reported (Krebbers et al., 2003; Sila et al., 2007). In the case of treatments at room temperature
242 (20 ºC), an increase in pressure from 700 MPa to 800 MPa did not cause a significant decrease in
243 the activities of PPO and POD. On the other hand, thermal treatments achieved total inactivation of
244 PPO and POD at the highest temperature assessed (90 °C); meanwhile at 70 °C, PPO was fully
245 inactivated but POD presented a low residual activity.

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

254

255 *3.1.2 Chlorophyll and carotenoid contents and instrumental colour*

256 The effect of selected treatments on colour was evaluated through chlorophyll (Chl) and
257 carotenoids content, as well as instrumental colour (**Figure 2** and **Table 2**¸ respectively). Control
258 samples presented CI95 of 79.51-107.53 mg .100 g^{-1} of fresh tissue (FT) and 26.81-37.92 mg .100
259 g^{-1} FT, respectively for Chl and carotenoids content (n=4). As shown in **Figure 2,** Chl and
260 carotenoids contents decreased significantly when combined pressure and high temperature were

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261 applied (treatment P1). Chlorophylls are stable under high pressures, but as reported by Oey et al.
262 (2008) they are significantly reduced at temperatures greater than 50 ºC which is similar to the
263 results of the present study. Regarding carotenoid content, similar trends were found by Kim, Park,
264 Cho, & Park (2001) who subjected carrot juice to 500 MPa at 70 ºC for 10 min. The increases
265 detected in Chl and carotenoids contents of samples subjected to 700 MPa and 800 MPa at 20 ºC for
266 15 min (treatments P2 and P3 in **Figure 2**), could be associated with a severe cell damage caused by
267 high pressure resulting in increased Chl and carotenoids content. Higher contents of Chl were also
268 observed after applying HHP to oil-based spinach sauce (Medina-Meza et al., 2015). On the other
269 hand, Wang et al. (2013) and Kim et al. (2001) did not find increased contents in Chl and
270 carotenoids after subjecting spinach to HHP, probably because the leaves were previously
271 processed causing cell damage and consequent release of these components. Finally, Chl and
272 carotenoids content after treatment P2 (700 MPa; 20 ºC; 15 min) were not significantly different
273 compared to those after thermal blanching (T1 and T2 shown in **Figure 2**).

274 Green colour in spinach is mainly determined by the CIELab parameter (-a*): the higher its
275 value the greener the leaves, but C*, Hº and ΔE also contribute to the evaluation of instrumental
276 colour. Differences in colour expressed by ΔE value, as a general rule, need to be higher than 1.8
277 (suprathreshold) in order to be perceptible by human eye and values greater than 5.0 are considered
278 to be remarkable differences (Melgosa, Pérez, Yebra, Huertas, & Hita, 2001). In this investigation,
279 all treated samples presented perceptible changes in colour (**Table 2**). Samples subjected to HHP
280 combined with high temperature not only gave the highest ΔE but also presented a significantly
281 lower (-a*) value which indicates that leaves were less green; in addition, hue angle was close to
282 90º indicating that colour turned from green to yellow green. This marked effect on colour of the
283 leaves by the combination of high pressure and high temperature would make the product
284 unacceptable for consumers, and correlates well with the low retention of Chl exhibited (treatment
285 P1 in **Figure 2**), possibly due to the formation of pheophytins from the degradation of Chl. On the
286 other hand, even though the ΔE values for samples treated with HHP at room temperature and those

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287 thermally blanched were detectable (ΔE > 1.8) they presented better green colour, with similar or
288 higher values of (-a*), C* and Hº than in control samples. Alvarez-Jubete et al. (2014), who
289 evaluated colour in HHP treated white cabbage (200, 400 and 600 MPa at 20 and 40 ºC for 5 min)
290 found similar results. In the present work, for both HHP and thermal blanching, texture
291 modifications may had resulted in changes in the nature and extent of internally scattered light and
292 the distribution of surface reflectance (MacDougall, 2002), resulting in greener leaves; with the
293 exception of treatment with combined pressure and high temperature. Moreover, cell damage with
294 consequent pigment release could have benefitted pressure treated samples. And water loss with
295 values of 22 ± 6% and 25 ± 7%, respectively for samples subjected to thermal treatments at 70 ºC
296 and 90 ºC, enhanced their green colour due to the concentration of pigments.

297 High retention and improved extractability of pigments, Chl and carotenoids, correlated well
298 with colour retention of spinach leaves subjected to HHP at room temperature (20 ºC) and thermal
299 treatments; meanwhile, the reduction in pigment content in samples treated with high pressure and
300 high temperature is in accordance with the instrumental colour evaluation. The latter negative effect
301 was also observed in green beans and basil by Krebbers, Matser, Koets, & Van den Berg (2002a)
302 and Krebbers, Matser, Koets, Bartels, & Van Den Berg (2002b), respectively. Green colour
303 retention after HHP treatments at room temperature is in accordance with the results found by
304 Krebbers et al. (2002a), Wang et al. (2013), and Medina-Meza et al. (2015). Chlorophyll and
305 carotenoids are very important phytochemicals and their presence is not only related to the
306 characteristic colour of vegetables but also to the beneficial effects on consumers´ health (Tang,
307 2010).

308

309 *3.1.3 Antioxidant capacity and total phenolic content*

310 Spinach leaves are amongst the most nutritious vegetables in terms of antioxidant capacity
311 and total phenolic content (TPC) (Zhou & Yu, 2006). Initial values found in the raw material
312 studied, expressed as CI95 (n=4), were 46.35-50.81 mg of ascorbic acid per 100 g FT and 87.28-

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313 101.90 mg of gallic acid per 100 g FT, respectively for antioxidant capacity and TPC. Reductions of
314 55-73% in antioxidant capacity and 12-23% in TPC were found after HHP processing, and no
315 significant differences were detected between treatments (**Figure 3**). Additionally, in these
316 components the effect of HHP combined with high temperature did not cause further reductions,
317 contrary to the observed effect on Chl and carotenoids. On the other hand, thermal treatments
318 reduced antioxidant capacity to values not significantly different to those after HHP, but TPC
319 reached values of roughly 16% lower than those found in pressure treated samples. It is noteworthy
320 that inevitably a certain amount of compounds present in spinach leaves was lost due to leaching in
321 water after traditional blanching, and this might have affected antioxidant capacity and TPC, as well
322 as Chl and carotenoids. The latter is not possible during HHP, therefore it represents an advantage
323 over the traditional treatment.

324 Regarding high pressure sensitivity, polyphenols demonstrated to be more baro-resistant than
325 antioxidant compounds determined by DPHH methodology. Similar results were reported by Barba,
326 Esteve, & Frigola (2013) who evaluated antioxidant compounds and TPC in blueberry juice and
327 Alvarez-Jubete et al. (2014) in white cabbage. Possible explanations for this phenomenon could be
328 a loss of antioxidant capacity due to vitamin C enzymatic degradation during pressurization (Barba
329 et al., 2013) or after HHP due to residual activity of quality deteriorating enzymes. Supporting the
330 explanation of loss of antioxidant capacity due to a loss of vitamin C, a remarkable decrease in
331 ascorbic acid content was found in white cabbage after applying HHP (Alvarez-Jubete et al., 2014).
332 Meanwhile for polyphenols, multiple mechanisms for their degradation might have been involved.
333 However, the lower reductions, comparing with control samples, found in the present study could
334 be mainly attributed to thermal effects during pressurization with consequent activation of PPO and
335 POD, and these effects might have been more severe in thermal treatments.

336

337 **4. Conclusion**

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

350

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352

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359

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1 **Table 1**

2 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.

3 *Data are presented as the means ± standard errors (n=2).* [a, b]*: Different letters in the same column*

4 *indicate significant differences (Tukey's test, p<0.05). n.d.: Not detected.*

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1 **Table 2**

2 Instrumental colour in control and treated samples. **C** (Control, untreated samples), **P1** (700 MPa;
3 70 °C; 15 min), **P2** (700 MPa; 20 °C; 15 min), **P3** (800 MPa; 20 °C; 15 min), **T1** (0.1 MPa; 70 °C;
4 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 ± 1.23[a,b]	115.06 ± 0.85[a]	6.06 ± 0.99[b]
P3	8.54 ± 0.31[a]	18.99 ± 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 ± 0.82[b,c]	121.27 ± 0.63[d]	6.34 ± 0.54[b]

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

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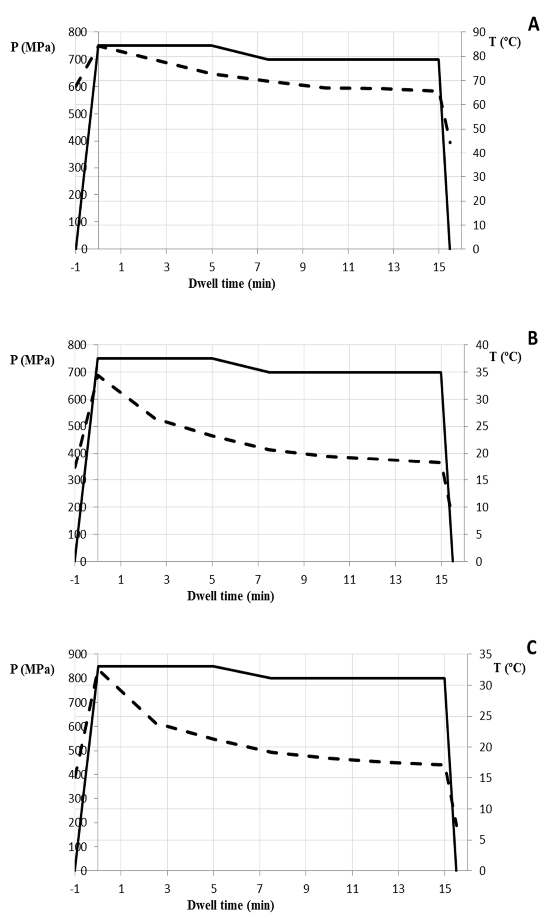
1 **Figure 1**

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



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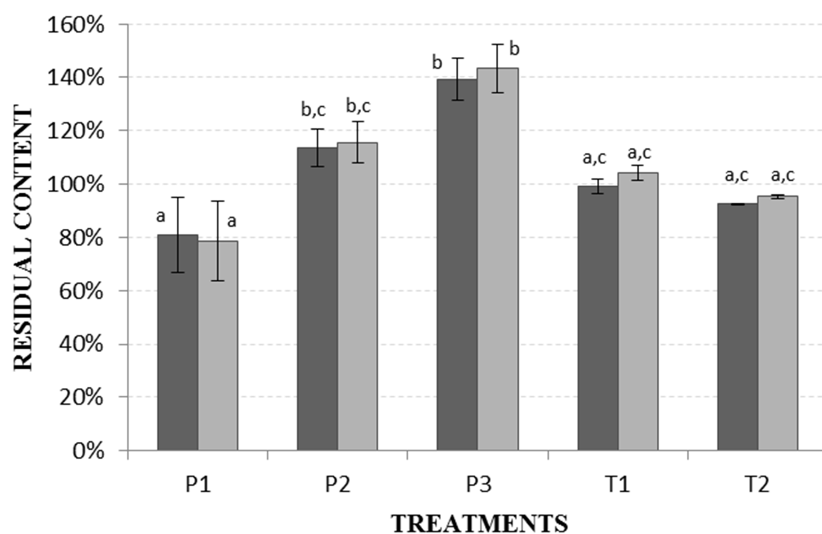
1 **Figure 2**

2 Residual content of total chlorophyll (dark-grey bars) and carotenoids (light-grey bars) after
3 selected treatments. **P1** (700 MPa; 70 °C; 15 min), **P2** (700 MPa; 20 °C; 15 min), **P3** (800 MPa; 20
4 °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
5 the means ± standard errors expressed as vertical segments (n=2). [a, b, c]: Different letters in each
6 type of bar indicate significant differences (Tukey's test, $p<0.05$).



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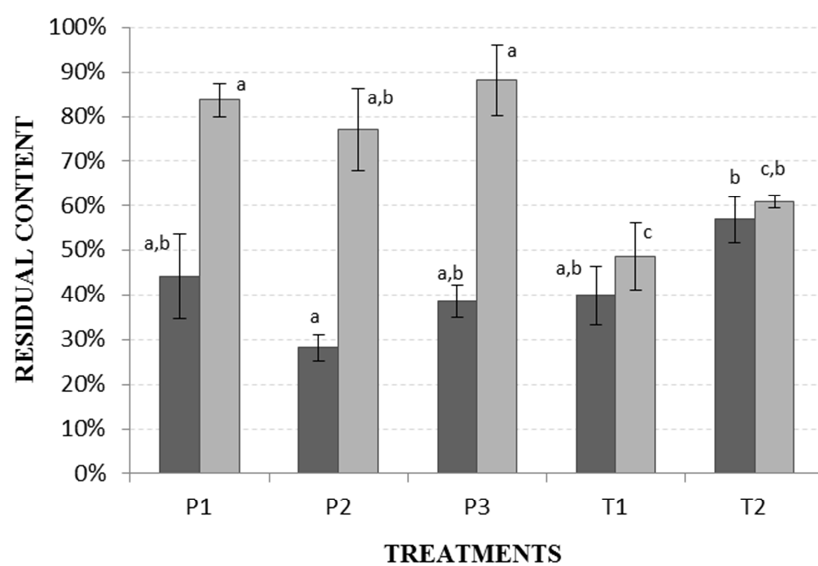
1 **Figure 3**

2 Residual content of antioxidant capacity (dark-grey bars) and total polyphenols (light-grey bars)
3 after selected treatments. **P1** (700 MPa; 70 °C; 15 min), **P2** (700 MPa; 20 °C; 15 min), **P3** (800
4 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
5 presented as the means ± standard errors expressed as vertical segments (n=2). [a, b, c]: Different
6 letters in each type of bar indicate significant differences (Tukey's test, $p<0.05$).



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1 **Highlights**

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

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