Development of a gluten-free whole grain flour by combining soaking and high hydrostatic pressure treatments for enhancing functional, nutritional and bioactive properties.

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Abstract

Single and double high hydrostatic pressure (HHP) cycles combined with soaking pre-treatment were applied on buckwheat (BW) whole-grain to enhance the nutritional, functional and bioactive properties of BW flour. Results showed modifications in the microstructure of pre-soaked and HHP treated samples which were associated with the appearance of disruptions on the surface of the starch granules. Combined treatments significantly ($p<0.05$) affected the ability of the flours to produce a stable emulsion or foam layer. These treatments also resulted in a significant decrease ($p<0.05$) in the gelatinization enthalpy and in the peak viscosity of the flours, while increasing their stability versus heating and shearing. Combined treatments also enhanced significantly ($p<0.05$) the antioxidant properties with an increase of phenol content from 311mg GAE/100g in native flour to 361 and 347 mg GAE/100g in pre-soaked and HHP-treated samples at single and double cycle, respectively. Moreover, those samples also exhibited higher minerals retention than un-soaked ones. The data obtained from this study demonstrated the efficiency of the application of combined soaking and HHP treatments on BW grains to enhance the nutritional and functional response of resulting BW flours providing a value-added ingredient with interest in the gluten-free baked goods industry.

Keywords: High hydrostatic pressure; Whole-grain buckwheat; Functional and nutritional properties; Antioxidant capacity**.**

1. Introduction

In recent years, increased efficacy in the diagnosis of celiac disease (CD) along with growing interest in reducing the consumption of gluten have led to a fast-growing gluten-free market (King et al., 2020). As a response to this trend, food manufacturers have focused on the development of new gluten-free (GF) bakery products with enhanced nutritional and sensory properties. Processing non-gluten flours requires a number of technical constraints to be overcome as gluten strongly influences on dough handling and rheology. Compared with wheat bakery products gluten-free doughs results in breads with lower loaf volume, harder crumb, and poor texture properties and shorter shelf life (Ronda et al., 2017). In addition, gluten free bread (GFB) is usually considered poor nutritionally as it is formulated with refined flours or starches which promote a higher glycaemic index than commercial wheat-based bread. Moreover, GFB is low in protein, fibres, vitamins (B12, D, folate), minerals (calcium, iron, zinc and magnesium) and high on saturated lipids (Vici et al., 2016).

A current approach to improve the nutritional quality of GF baked goods is the use of nutrient-dense raw materials such as pseudocereals. Several authors have reported nutrient profile improvements in pseudocereals-containing GFB. Alvarez-Jubete et al. (2009) noted an increase of more than double of the protein and fibre levels in GFB by replacing 50% of potato starch with different pseudocereal flours. Moreover, significantly higher levels of monounsaturated and polyunsaturated fatty acids were also observed in those breads. In addition, the minerals content showed a significant increase, which contributed to reaching the recommended intake levels for these nutrients in coeliac population.

Among pseudocereals, buckwheat, one of the most employed ingredients in GFB formulations, offers a well-balanced amino-acid profile, and is a good source of dietetic fibre, resistant starch, vitamins and minerals. In addition, it is also notable for its content in the flavonoid rutin, a quercetin glycoside known for its anti-inflammatory and

antioxidant properties which is predominantly present in the outer layers of the grains. Increasing levels of protein and minerals (Cu and Mn) with increasing buckwheat flour (BW) proportion in GFB formulations have been reported by other authors (Conte et al., 2019). The use of BW flour has also been associated with improvements in dough structure and a reduction in the retrogradation degree of the crumb of GFB (Conte et al., 2019).

To improve the sensory and quality attributes of GFB, different technological strategies have been used to reproduce the gluten functionality. Physical treatments of flours and starches have emerged as a very interesting alternative to the use of hydrocolloids, gums and other additives and technological adjuvants in the formula of GFB. Physically modified flours and starches are safe and natural ingredients that do not have to be declared as food additives, resulting in clean labels. Particularly, high hydrostatic pressure (HHP) has drawn researchers interest as allows the functionality of food components to be modified (protein denaturation, starch gelatinization or increase in triglycerides fusion point) meanwhile keeps the freshness, colour, flavour and minimize micronutrients loss in final products (Balasubramaniam et al., 2016). HHP has also been proposed to improve ingredients functionality in GF products. Taylor et al. (2016) reported protein polymerisation in rice, sorghum and teff treated batters and the formation of cross-linked protein aggregates through disulphide bonds in oat batters. Protein aggregation helps to improve the viscoelastic properties of gluten-free batters and to retain the gas produced during proofing. The impact of HHP treatment on starch granules has also been assessed. HHP can modify the ordered crystalline structures of starch, as changes in the crystallinity pattern from A to the B-isomorph revealed (Balasubramaniam et al., 2016). A loss of birefringence and an increase in granule damage of barley and wheat starch has also been noted with increasing pressure levels (Estrada-Girón et al., 2005). Moreover, a number of studies of the impact of HHP on starch gelatinization have reported effects such as decreased granule swelling capacity,

ability to retain the granular shape and reduced amylose leaching. HHP-induced gelatinization has been reported to promote only partial disintegration of crystalline regions unless severe pressure conditions were achieved (Balakrishna et al., 2020). Critical factors affecting the extent of gelatinization have been proposed by Kim et al. (2012). Overall, the degree of gelatinization increases with increasing pressure, temperature and holding time. As in the case of heat-induced, pressure-induced gelatinization is also water-dependent. Ravichandran et al. (2018) showed a higher degree of gelatinization of rice grains when they previously underwent a soaking treatment. The use of pre-gelatinized starch has been proposed to overcome the detrimental effects of the absence of gluten and improvements in texture, volume and shelf-life of GFB have been reported (Vallons et al., 2011)

Most research conducted on the use of HHP to modify the functionality of gluten-free ingredients has focused on the modification of flours and starches. The HHP treatment of whole grains of gluten-free cereals and pseudocereals to modulate the functional and nutritional properties of the resulting flours has not been considered before. The objective of this research was to investigate the effects of HHP treatment of BW wholegrains on techno-functional, nutritional and bioactive properties of the resulting flours. The effect of a soaking pre-treatment or number of cycles in which the HHP treatment is applied were studied to assess the potential of this technology for the treatment of whole grains. The BW flours were evaluated for their granulometry, colour, functional pasting and thermal properties, microstructure, antioxidant capacity and mineral content.

2. Materials and methods

2.1. Grain treatment and flour obtention.

Buckwheat grains (BW) (Panda variety) (74.7% total carbohydrates, 28.3% dietary fibre, 11.3% protein, 3.2% lipid, 2.1% ash, and 8.7% moisture*)* were kindly provided by Grupo BC Servicios 2011 (Palencia, Spain). First, a cleaning process was applied to

ensure the safety of the grains. BW grains were packed with distilled water (grain to water ratio, 1:4) in co-extruded Polyethylene/Polyamyde pouch and sealed with minimum head space and separated in batches. Samples selected to soaking treatment were immersed in excess water for 4 hours at 40ºC. The above temperature was based on a preliminary study of soaking conditions (20ºC vs 40ºC). As small differences were observed regarding the combination of HHP treatment and soaking at 20ºC in comparison with the HHP and un-soaked treated sample (supplementary data, tables A1 and A2), the soaking temperature of 20ºC was excluded from the experimental design of this study.

In a second step, the effect of HHP was evaluated in soaked and un-soaked samples using a HHP unit (Wave 6000/135, NC Hyperbaric, Burgos, Spain) based in the Agrarian Technology Institute of Castilla y Leon (Spain) with a vessel of 135 L and 200 mm diameter. The temperature of the water inside the chamber was monitored during the processing, applying 6.08 x 10^8 Pa (600 MPa), with a single-cycle or double-cycle of 30 min total holding time. The pressure come-up speed was 120 mPa/min and after the holding time the pressure was instantaneously released.

After pressure treatment, grains were blotted, dehusked and ground using a Faribon mill (F6003 PH, Omas, Padova, Italia) and then milled with Fidibus Medium mill (Komo Grain Mills, Penninberg, Austria) making the final flour fraction went through a 250 µm mesh. All flours were stabilized to get a 12-13% of final moisture, and then packed and stored at 5ºC temperature until further used.

2.2. Particle size distribution of flours

The samples granulometry was determined by a Mastersizer 2000 laser diffraction particle size analyzer (Malvern Instruments Ltd, UK). The particle size distribution was characterized by the median diameter (D_{50}) and the size dispersion ((D_{90}-D_{10})/D_{50}) as described Abebe et al. (2015). All samples were measured in triplicate.

2.3. Scanning electron microscopy (SEM)

Analysis of the microstructure of BW flour samples were performed with a Quanta 200FEG scanning electron microscope (FEI, Oregon, U.S.A). Micrographs were taken with an accelerating voltage of 7keV) in low vacuum mode using a secondary electron detector.

2.4. Flour colour analysis

Colour results were obtained in the CIE L*a*b* and CIE L*C*h coordinates using the D65 iluminant (colour temperature of 6504 k) and the standard observer was 10º. Colour differences (ΔE) of each treated sample with respect to the control was obtained with the following equation: $\Delta E = \{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2\}^{1/2}$ Measures were collected by a colorimeter (Minolta spectrophotometer CN-508i, Minolta, Co. LTD, Japan). The colorimeter was calibrated using a light trap and a white tile. Flours were placed in a Petri dish and measurements were taken directly. Five measurements were evaluated in duplicate for the different flours.

2.5. Techno-functional properties.

Water holding capacity (WHC, g H_2O absorbed/g sample), water absorption capacity (WAC, g H_2O absorbed/g sample), oil absorption capacity (OAC, g oil absorbed/g sample), water absorption index (WAI, g gel/g sample), water solubility index (WSI, g soluble matter/100g sample), swelling power (SP, g gel/g insoluble matter), foaming capacity (FC, mL foam/g sample), foaming stability (FS, percentage of foam retained after 1 hour), emulsion activity (EA, percentage of emulsion layer to total volume) and emulsion stability (ES, percentage of emulsion layer after heating 30 min to total volume) were made following the procedure described by Rico et al. (2020). All outcomes were referred to dry matter (db.).

2.6. Pasting properties.

The pasting properties were determined following the procedure used by Vela et al. (2021). Each sample was analysed at least in duplicate to get the pasting profiles and

the following parameters: pasting temperature (PT), pasting time (Pt), peak viscosity (PV), trough viscosity (TV), breakdown (BD), final viscosity (FV) and setback (ST).

2.7. Rheological properties of gels

The rheological properties of the gel samples were obtained following the same procedure of Vela et al. (2021). Data obtained were adjusted to potential equations to get the rheology parameters as was described by Vela et al. All analyses were performed in duplicate.

2.8. Thermal properties.

Thermal properties of samples were assessed using the procedure followed by Vela et al. (2021). Data obtained after a first run were onset (T_O), peak (T_P) and endset (T_E) temperatures (ºC) and the enthalpy of gelatinization (ΔH) (J/g flour dm). A second run was performed after keeping the pan at 4 ± 2 ºC for 7 days using, the same procedure to record the retrogradation transition. Each sample was analysed at least in duplicate.

2.9. Total phenol content (TP) and total antioxidant capacitiy (TAC)

Total phenol content and total antioxidant capacity using DPPH assay were determined following the procedures described previously by Rico et al. (2020). Extracts from BW flours were prepared as following: one gram of each flour sample was mixed with 10 ml of an acidified aliquote (pH = 2 with 0,1 M HCL) of methanol:water (1:1, v:v) and maintained at 30ºC, 250 rpm, overnight. After incubation the samples were centrifugate at 2800 xg during 10 min and the supernatant was collected, filtered (Whatman paper nº1) and the final volume was adjusted to 25 ml using the extracting solvent. This extraction method was performed in duplicate and samples were also duplicated.

TPs were measured using the Folin-Ciocalteu method with modifications. The absorbance was measured at 765 nm with a microplate reader (Fluostar Omega, BMG, Ortenberg, Germany). Results were expressed as mg gallic acid equivalents (GAE) per

100 g of sample (dry basis) using a calibration curve with Gallic acid as standard (9.8–70 µM). Samples were evaluated in duplicate.

The antioxidant capacity of the extracts against the DPPH radical was estimated according to the procedure of Rico et al. An amount of 25 µL of extracts was mixed with 100 µL of MilliQ water and 125 µL of DPPH working solution (100 µM using methanol as solvent) in a 96-well microplate. Absorbance at 515 nm was recorded for 30 min in a microplate reader (Fluostar Omega, BMG Ortenberg, Germany). Results were corrected for each sample moisture (12%-13%) and expressed as mg Trolox equivalent/100 g sample.

A DPPH direct method (Q-DPPH) on solid samples without previous extraction was also carried out as following. Ten milligrams of powdered samples (particle size below 300 µm) were mixed with 1.5 mL of 60 µM DPPH methanolic solution. After incubation at 700 rpm for 30 min (Thermomixer Compact, Eppendorf AG, Hamburg, Germany), samples were centrifuged at 14,000 ×g for 2 min and the absorbance measured at 515 nm in a microplate reader (Fluostar Omega, BMG Ortenberg, Germany). Results were corrected for moisture and expressed as µmol Eq. Trolox/100 g of WB (db).

2.10. Minerals content

Flour samples were incinerated and the dry-way mineralized and ashes were diluted with 5 ml of HNO_3 (6,5%). After reaching and keep the boiling temperature for two minutes, chill and filtered using mili-Q water into 100 ml flask pouring HNO_3 (65 %), samples were set in a graphite furnace tube atomizer (Varian GTA 120Z, Agilent Technologies, California, USA) and the SpectrAA 240 FS atomic absorption espectrophotmeter (Varian, Agilent Technologies, California, USA) to analyze the content in B, Ca, Fe, Mn, Zn. Results were expressed in mg/100g (db).

2.11 Statistical Analysis

The effects of the number of HHP cycles applied and the pre-soaking treatment on the parameters studied were determined by multifactor analysis of variance of the data.

One-Way ANOVA was applied to evaluate significant differences ($p<0.05$) between samples. All statistical analyses were performed using Statgraphics Centurion v.18 (Bitstream, Cambridge, MN, USA).

3. Results and discussion

3.1. Particle size distribution of flours

Flour granulometry data can provide useful information about relevant functional and nutritional properties as those results could affect the processing performance of flours (Vela et al., 2021). In table 1 is presented the particle size distribution of flours. The soaking pre-treatment had a significant ($p<0.01$) effect on D_{50}. Native flours exhibited the lowest values, being significantly ($p<0.05$) different from all treated samples. Un-soaked HHP-treated flours (1C and 2C) showed a significant ($p<0.05$) average increase of 22,3% in this parameter, whereas pre-soaked HHP-treated samples (1C+S40 and 2C+S40) had an increase of 56,3% regarding the native flour. Size dispersion was significantly ($p<0.01$) affected by the number of HHP cycles and the soaking pre-treatment. Treated samples with single cycle showed significantly ($p<0.05$) lower values than samples with double cycles either with or without pre-soaking treatment. Moreover, all pre-soaked samples had significantly ($p<0.05$) lower values than those un-soaked. All flour samples including the control were obtained with the same milling procedure hence, HHP treatments could exert some structural changes to the buckwheat grain, promoting a greater shear milling resistance and modifying the grain fracturability. Structural modifications after HHP treatments have been previously observed by Ravichandran et al. (2018) in rice grains. Those authors observed a negative activation volume of high-pressured paddy grains by a compaction phenomenon.

Table 1

Granulometry, colour difference, hydration, emulsion and foaming properties of flour samples obtained from native and HHP-treated buckwheat grains.

SAMPLE	D_{50} (µm)	$(D_{90}-D_{10})/D_{50}$	ΔE	WHC (g/g)	WAC (g/g)	OAC (g/g)	WAI (g/g)	WSI (g/100g)	SP (g/g)	FC (ml/g)	FS (%)	EA (%)	ES (%)
Native	102.8 ^{a}	2.05 ^{c}	-	2.77 ^{a}	1.17 ^{a}	1.1 ^{a}	5.68 ^{b}	5.09 ^{a}	5.99 ^{b}	0.71 ^{d}	44.4 ^{b}	56 ^{e}	20.8 ^{d}
1C	125.8 ^{b}	2.04 ^{c}	2.1 ^{a}	2.98 ^{b}	1.23 ^{b}	1.2 ^{b}	5.54 ^{ab}	5.08 ^{a}	5.84 ^{ab}	0.26 ^{b}	0.0 ^{a}	44 ^{d}	11.1 ^{c}
2C	125.2 ^{b}	2.08 ^{d}	3.7 ^{b}	2.80 ^{a}	1.14 ^{a}	1.2 ^{b}	5.47 ^{a}	4.92 ^{a}	5.76 ^{a}	0.39 ^{c}	0.0 ^{a}	33 ^{c}	5.6 ^{b}
1C+S40	161.6 ^{c}	1.88 ^{a}	4.6 ^{b}	3.55 ^{c}	1.22 ^{b}	1.2 ^{b}	5.93 ^{c}	5.23 ^{ab}	6.25 ^{c}	0.00 ^{a}	0.0 ^{a}	6.0 ^{a}	3.8 ^{a}
2C+S40	160.8 ^{c}	1.93 ^{b}	4.0 ^{b}	3.46 ^{c}	1.31 ^{c}	1.3 ^{b}	6.18 ^{d}	6.14 ^{b}	6.58 ^{d}	0.19 ^{b}	0.0 ^{a}	18 ^{b}	3.1 ^{a}
SE	0.7	0.01	0.4	0.05	0.01	0.0	0.05	0.31	0.06	0.04	2.4	1.0	0.4
Number of HHP Cycles	ns	**	ns	*	ns	ns	ns	ns	ns	**	-	ns	**
Soaking pre-treatment	**	**	**	**	**	ns	**	ns	**	**	-	**	**
Cycles*Soaking	ns	ns	**	ns	**	ns	*	ns	*	ns	-	**	**

1C: HHP single-cycle treated sample; 2C: HHP double-cycle treated sample; 1C+S40: Pre-soaked and HHP single-cycle treated sample; 2C+S40: Pre-soaked and HHP double-cycle treated sample. D_{50}: median diameter; $(D_{90}-D_{10})/D_{50}$: Size dispersion; ΔE: Difference of colour between each treated sample and the control; WHC: Water holding capacity, WAC: Water absorption capacity, OAC: Oil absorption capacity, WAI: Water absorption index, WSI: Water solubility index, SP: Swelling power, FC: Foam capacity, FS: Foam stability, EA: Emulsion activity, ES: Emulsion stability. Samples with different small letters show significant differences between treatments ($p<0.05$). SE: Pooled standard error from ANOVA. ** $p<0.01$, *$p<0.05$, ns: not significant.

3.2. Scanning electron microscopy (SEM)

Effects of the HHP and combined treatments on the microstructure of the flour samples were investigated. Figure 1 shows the micrographs of starch granules of BW flour samples. Starch granules of native flours (A images) exhibited the typical spherical-polygonal appearance, ranged between 2-14 μm of diameter, with a smooth surface and small indentations (Zhu, 2016). Images of treated samples (B and C corresponding to 1C+S40 and 2C+S40, respectively) revealed noticeable changes in the starch granular structure such as some deformations and increased surface roughness of the granules. Furthermore, the well-defined boundaries observed in control granules seemed to be dimmer, although the integrity of most granules was retained. These effects were in agreement with the findings of previous studies on the effect of HHP on buckwheat starch structure (Vallons and Arendt, 2009). The appearance of visible effects on starch after HHP treatment is reasonable as buckwheat starch is associated with A-type crystalline polymorph which is more vulnerable than other types to HHP treatments (Balasubramaniam et al., 2016). The loss of the shape and surface integrity in the starch granule was increased in the case of pre-soaked samples treated with double cycle. In those samples extragranular material, was observed probably of a proteinic nature, entwining and connecting the starch granules. Accordingly with Cao et al. (2018) HHP treatment could exert some modifications on protein structure to produce higher protein particle specific surface area, protein aggregation and more surface roughness. These structural modifications would be more pronounced in pre-soaked HHP-treated flours as can be observed in Figure 1.

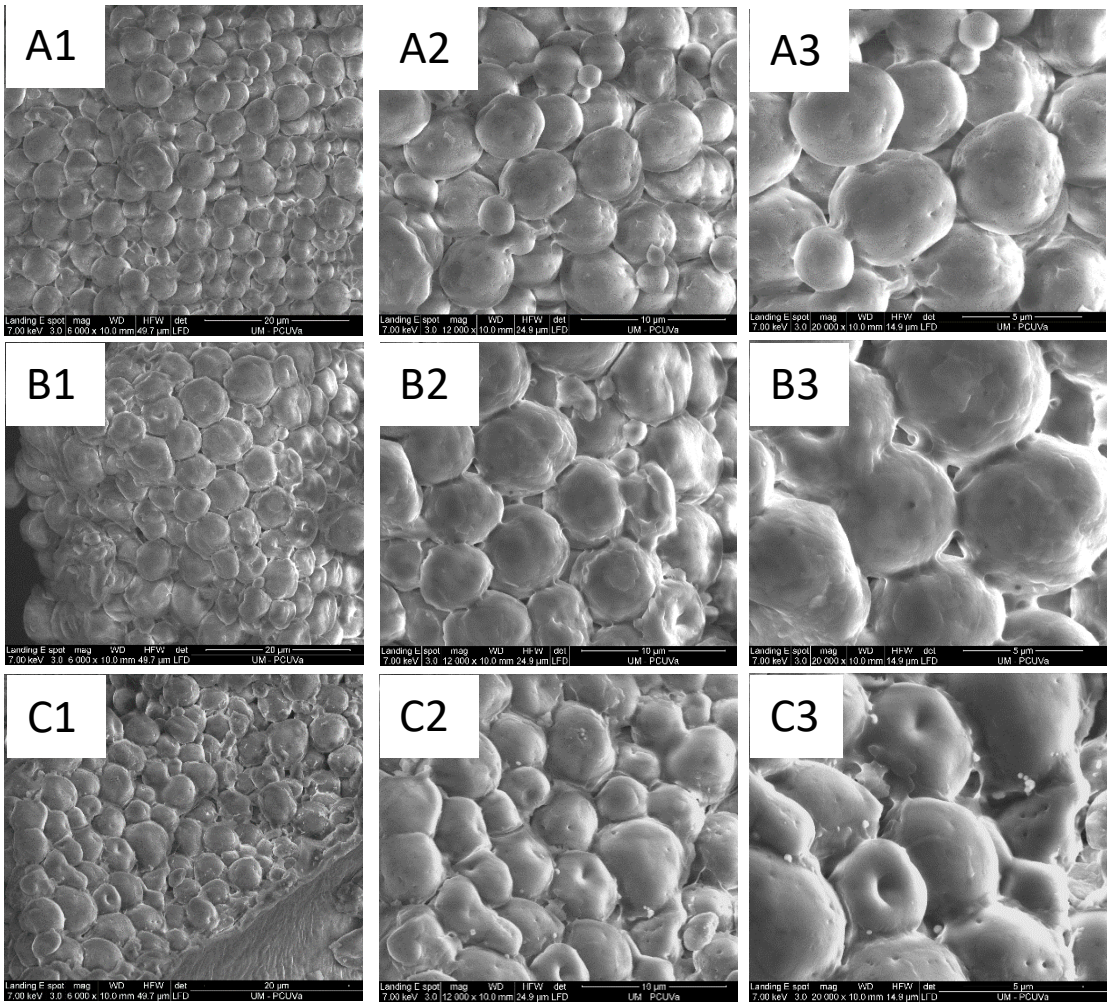

Figure 1. SEM images of flour samples obtained from native and HHP-treated buckwheat grains: A: Native; B: 1C+S40; C: 2C+S40, at different magnifications: 6000x (1), 12000x (2) and 20000x (3)

3.3. Flour colour analysis

Sample colour parameters are summarized in Table 1 (ΔE) and in supplementary material Table B1 (L*, a*, b*, C*, h). Soaking pre-treatment significantly ($p<0.05$) affected the luminosity (L*) of the samples. All treated samples showed a significant decrease ($p<0.05$) of the luminosity as compared to the control. Pre-soaked and HHP double-cycle treated samples (2C+S40) exhibited the lowest values. Colour differences between native and treated flours (ΔE) were lower to 5, so the changes in BW flour colour were not sensory noticeable. Only a slight but significant ($p<0.05$) variation of the hue (h) was found for the un-soaked and single-cycle treated sample (1C) regarding the native flour.

In contrast, significantly ($p<0.05$) bigger Chroma values (C*) for all treated samples in comparison with the native were found. Among the treated samples, significantly ($p<0.05$) increased values were observed for the double-cycled samples compared to the single-cycled ones irrespective of the soaking pre-treatment. Similar results have previously been reported by Yu et al. (2017), who evaluated the effect of HHP treatment with and without soaking on brown rice grains. Those authors stated the soaking treatment could help to diffuse pigments present in the outer layers of the grain to the water, so that the depressurization process could promote the migration of these components present in the pressure transmission water to the inner parts of the grains trough fissures. This phenomenon could be intensified when double pressurisation cycle is used and could explain the differences found in Chroma for HHP double-cycle treated samples compared to HHP single-cycle ones.

3.4. Functional properties.

Results of the flour hydration properties (WHC and WAC), oil absorption capacity (OAC), gel hydration properties (WAI, WSI and SP) and surfactant activity properties (emulsifying activity and stability –EA, ES-, foaming capacity and stability –FC, FS-) are displayed in Table 1.

The impact of the soaking pre-treatment in the water holding and absorption capacity was significant ($p<0.01$) as pre-soaked samples exhibited a substantial increase, especially in WHC (between 25-28%) regarding the native one. Significant ($p<0.01$) effect of the soaking pre-treatment was also found in WAC. In this case, the pre-soaked with double-cycle samples showed a significant increase ($p<0.05$) of 12% compared to the control. Being carbohydrates and proteins mainly responsible for a flour water binding capacity (Vela et al., 2021), modification of these components would explain variations in the BW flour hydration properties observed after treatments. It was reported HHP treatment led to cold gelatinization of starches and enhanced their ability to bind water (Balakrishna et al., 2020). In addition, Yu et al. (2017) reported an increase in hydrogen

bonding between water and starch after HHP processing which could promote water absorption.

Results showed a significant ($p<0.05$) increase in OAC (9%-14%) of treated samples regarding the native flour. However, none of the studied factors significantly affected this parameter. Changes in the mechanism of oil entrapment by proteins as consequence of HHP treatment have been reported by Cao et al. (2018) to explain this behaviour.

The effect of soaking pre-treatment on gel hydration properties was significant as HHP pre-soaked samples showed significantly ($p<0.05$) higher values in WAI and SP than un-soaked ones. As reported by Ravichandran et al (2018), a higher degree of gelatinization in paddy grains was promoted not only by HHP but also by a soaking pre-treatment. This effect could lead to an increase in the swelling volume, as well as an increase in the water absorption as the hydrogen bonding between starch molecules could be replaced by hydrogen bonding with water (Herlina, 2017).

Meanwhile, in WSI only the pre-soaked and HHP double-cycle treated sample had a significant ($p<0,05$) difference with the native one The protein agglomeration and increased solubility after HHP treatment of grains have been previously reported by Błaszczak et al. (2007).

Both cycle and soaking pre-treatment exerted a significant ($p<0.01$) effect on the FC of BW flours. A significant ($p<0.05$) and remarkable reduction in the foaming capacity of all studied samples regarding the native flour was observed. Pre-soaked samples showed the lowest FC values and these samples did not show any ability to retain the foam generated during the test. A similar trend was found in the emulsion activity and stability of tested samples, as a significant ($p<0.05$) and important drop was observed in these parameters, especially for the pre-soaked ones. Surface activity properties are mainly driven by the protein fraction of the BW flour. Balasubramaniam et al. (2016) reported that high-pressure promotes complex protein modifications which could impair

the tertiary structure of proteins, disfavouring hydrophobic interactions. This phenomenon might have led to changes in the surfactant properties of HHP treated BW flours hindering their capacity to generate stable emulsions and foams.

3.5. Pasting properties

Viscosity profiles and pasting properties of samples are shown in Figure 2 and in Table B2 of supplementary material respectively. Pasting viscosity profile is mainly determined by starch behaviour but in whole-flours, other components such as fibre, proteins and fats may affect their pasting properties (Vela et al., 2021). The impact of soaking pre-treatment was remarkable as viscosity profiles of pre-soaked samples showed an overall decrease. The multifactor analysis of variance also revealed a statistically significant effect for soaking pre-treatment factor in Peak (PV), Breakdown (BV) and Final (FV) viscosities ($p<0.01$), and in Trough (TV) and Setback (SV) viscosities ($p<0.05$). Meanwhile, the "Cycle" factor affected significantly to FV ($p<0.05$). No significant changes regarding pasting temperature (PT) were observed, as around 65ºC was the temperature at which starch granules started to swell in all tested samples. Opposite results were found by Liu et al. (2016), who reported an increase in PT of a BW starch:water mixture subjected to a HHP treatment. Those authors attributed this effect to changes in the starch structure induced by HHP, enhancing the granule thermostability. This effect might be limited in the present study due to the presence of hull and the complex nature of the BW grain matrix that could mitigate the effects of the pressure on starch granules. Conversely, combined treatments had significant effect on pasting properties as both pre-soaked and HHP single and double cycle treated samples showed lower PV values than native flour (18% for 1C+S40 and 16% for 2C+S40) and the un-soaked ones. This decrease was consistent with previous findings of Liu et al. (2016). Furthermore, all HHP treated samples showed a significant ($p<0.05$) decrease in BV, ranging up from 88% for 2C+S40 to 93% for 1C+S40. Higher stability of the swollen granules under continuous heating and shearing was also been noted by Liu et

al. (2016). Authors linked the reduction in BV with a limited granular swelling, as HHP promoted the granule's ability to withstand the continued heating and shearing, improving their stability. Similar results were obtained by Lin and Fernández-Fraguas, (2020) who stated this enhanced stability versus shearing and heating as a consequence of a reinforced crystalline structure and an entanglement between starch-protein/fibre caused by the high-pressure treatment. This increased stability in the starch granule might explained the significant ($p<0.05$) decrease in the SV results obtained in the HHP treated samples regarding the control (29% for 1C+S40 and 23% for 2C+S40). The increased rigidity of the starch granules might reduce amylose leaching during heat-paste cycle and hence, there would be less amylose free molecules able to retrogradate in the cool-paste cycle. Liu et al. (2016) also suggested the formation of stable amylose-lipid complexes could decrease starch retrogradation and SV parameter.

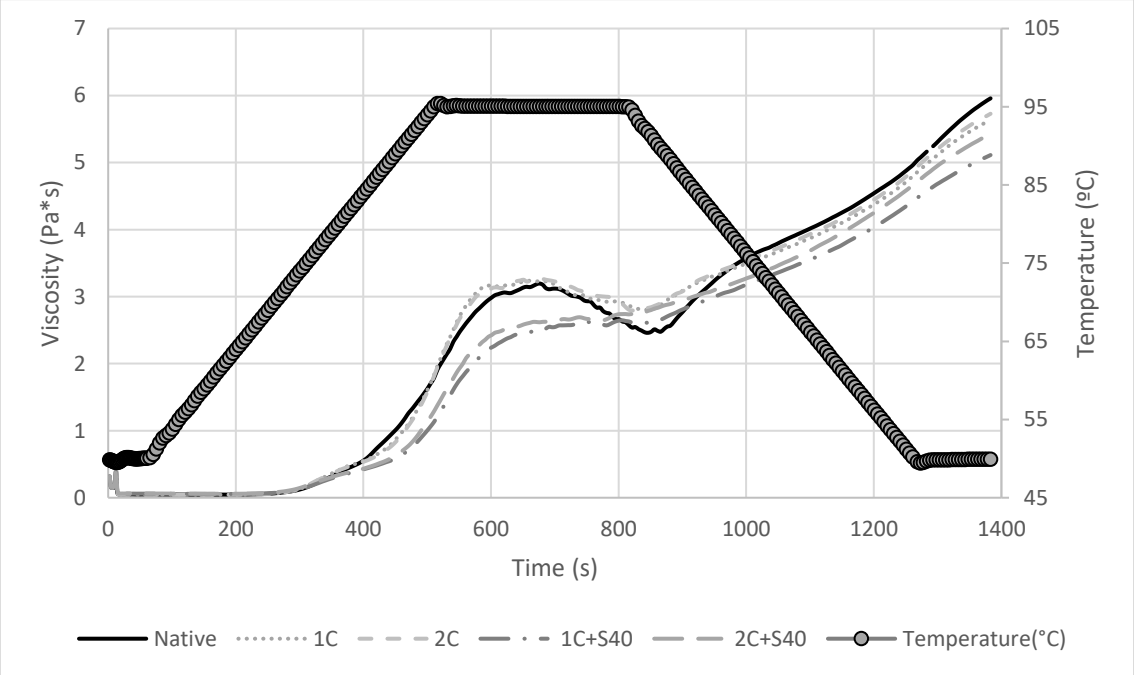

Figure 2. Pasting profiles of flour samples obtained from native and HHP-treated buckwheat grains: 1C: HHP single-cycle treated sample; 2C: HHP double-cycle treated sample; 1C+S40: Pre-soaked and HHP single-cycle treated sample; 2C+S40: Pre-soaked and HHP double-cycle treated sample.

3.6. Rheological properties of gels

The mechanical spectra of control and treated gel samples obtained in the dynamic oscillatory test is showed in Figure 3, while the rheology parameters are summarized in the supplementary material (Table B2). All treated samples showed a significant decrease ($p<0.05$) regarding the elastic and viscous moduli in comparison with the native gel sample, except the un-soaked HHP double-cycle sample in the elastic modulus. The $G_1`$ values of those treated samples decreased from 24 to 28% for 1C and 1C+S40 samples, respectively. The decreasing values for the viscous modulus compared to the control ranged from 30 to 36%. Therefore, the complex modulus values had significantly ($p<0.05$) lower outcomes for pre-soaked samples and 1C. On the basis of these results it can be stated that treatments given in this work led to a weakened gel structure. The loss tangent values of treated samples also showed significantly ($p<0.05$) lower values than the control except for 1C+S40. Although all samples exhibited a solid-like behaviour as Tan $(\delta)_1$ values were less than 1, results denoted a tendency to increase the weight of the viscous component of the gels after HHP treatment. This might be due to treatment enhanced granule structure resistance, so less amylose was available for gelling as suggested by Li and Zhu (2018).

All treated samples showed a significant ($p<0.05$) decrease in exponent value "a" denoting a reduction in the frequency dependence of G' comparing with the control sample. This agrees with the findings obtained by Li and Zhu (2018) after studying frequency sweeps of HHP treated starch gels of maize and quinoa. This lower value of the exponent “a” was associated by those researches with an enhance of the long-term stability of quinoa starch gel.

The soaking pre-treatment factor and its interaction with ”Cycle” factor were found significant ($p<0.01$ and <0.05, respectively) in the maximum stress which gels could withstand before disrupting their structure (τ_{max}). Significant ($p<0.05$) increased τ_{max} values was shown for almost all treated samples, although it was less pronounced for

pre-soaked samples. It was possible HHP treatment could promote some molecular interactions and/or starch structure modifications which raised the gel stability.

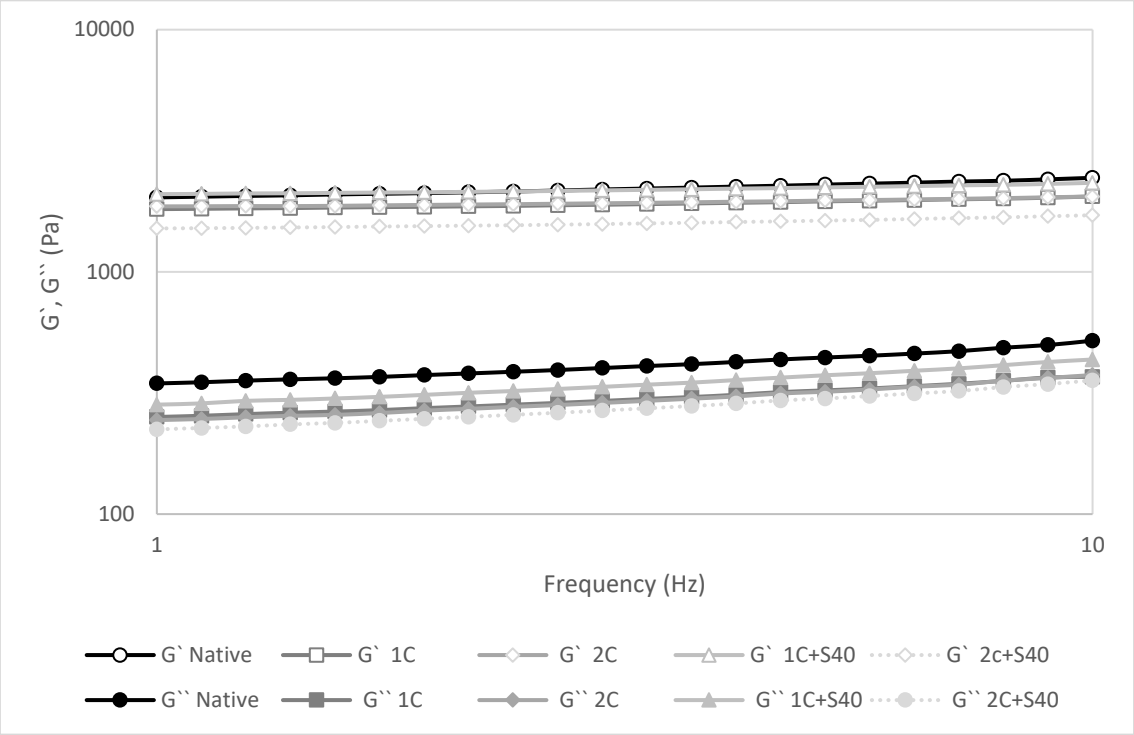

Figure 3. Effect of HHP treatment on viscoelastic moduli evolution *vs* frequency (Hz). Empty symbols represent the elastic modulus (G'); filled symbols represent the viscous modulus (G´´). 1C: HHP single-cycle treated sample; 2C: HHP double-cycle treated sample; 1C+S40: Pre-soaked and HHP single-cycle treated sample; 2C+S40: Pre-soaked and HHP double-cycle treated sample.

3.7. Thermal properties.

The thermal properties obtained from DSC tests and used to analyse the gelatinization (first scan) and retrogradation (second scan) transitions of the samples are shown in Table 2. Endothermic thermal transition data showed two peaks, the first has been related to starch gelatinization and the other has been associated with protein denaturation and amylose-lipid complex dissociation (Lin and Fernández-Fraguas, 2020). The gelatinization thermal parameters values of the native flour were found to be in the range of results reported by Zhu (2016). Soaking pre-treatment had a significant ($p<0.05$) effect in gelatinization enthalpy (ΔH_{gel}). Pre-soaked samples exhibited a

significant ($p<0.05$) reduction in ΔH_{gel} compared to the native one (20% for 1C+S40 and 25% for 2C+S40) and to the un-soaked samples. This result suggests that starch in pre-soaked samples could be partially gelatinized regardless of the number of cycles of HHP-treatment. Gelatinization could happen as buckwheat starch has an A-type crystalline structure, which has the highest sensitivity to cold-gelatinize in a high-pressure treatment (Balasubramaniam et al., 2016). Another key factor to trigger cold-gelatinization is the presence of water (Balakrishna et al., 2020). Kim et al. also reported that at least 50% of moisture content is necessary to partially gelatinize a starch-water suspension. Considering that the pressure was applied over whole buckwheat grains, probably the presence of the hull could provide a physical barrier limiting moisture diffusivity, mainly in not pre-treated by soaking samples. However, the soaking pre-treatment might provide enough moisture to the inner parts of buckwheat grains to produce some degree of gelatinization. Ravichandran et al. (2018) also confirmed that gelatinization degree for pre-soaked and HHP treated paddy grains was higher than that of un-soaked ones.

All pre-soaked samples also exhibited a significant ($p<0.05$) reduction in the gelatinization temperature range (ΔT), caused by an increase in the onset temperature ($T_{o\text{-}gel}$). The ΔT reduction of BW starch samples treated with HHP has been previously reported by Liu (2016). Lower values of the gelatinization peak width indicate higher starch crystallites homogeneity and a better organized granular structure, requiring a shorter temperature range to fully hydrate.

The amylose-lipid complex dissociation peak had an enthalpy of 0.9 J/g in the native sample, which is consistent with the data reported by Zhu (2016). All treated samples showed lower values, although not significantly different, than the native flour. Small differences were also found by Lin and Fernández-Fraguas (2020) in the peak temperature of the amylose-lipid complex dissociation.

No significant differences were observed in the retrogradation enthalpy (ΔH_{ret}) caused by amylopectin recrystallization after one week of storage at 4ºC. Li and Zhu

(2018) also reported minor variations in the retrogradation temperatures studying the thermal effect of HHP-treatment on quinoa and maize starch. In contrast, in this second scan, a significant ($p<0.05$) reduction in the amylose-lipid enthalpy ($\Delta H_{am\text{-}lip}$) was found for the treated samples compared to the native one. In particular, pre-soaked samples exhibited a significant ($p<0.05$) decrease in $\Delta H_{am\text{-}lip}$ compared to un-soaked ones. $\Delta H_{am\text{-}lip}$ in the second scan also showed increased values compared to the first scan. Vela et al. (2021) suggested that these higher values appeared because better complex formation conditions occurred as consequence of amylose leakage above the gelatinisation temperature.

Table 2

Thermal properties of flour samples obtained from native and HHP-treated buckwheat grains.

SAMPLE	First scan							Second scan						
	ΔH_{gel} (J/g)	$T_{o\text{-}gel}$ (ºC)	$T_{p\text{-}gel}$ (ºC)	$T_{e\text{-}gel}$ (ºC)	ΔT (ºC)	$\Delta H_{am\text{-}lip}$ (J/g)	$T_{p\text{-}am\text{-}lip}$ (ºC)	ΔH_{ret} (J/g)	$T_{o\text{-}ret}$ (ºC)	$T_{p\text{-}ret}$ (ºC)	$T_{e\text{-}ret}$ (ºC)	ΔT (ºC)	$\Delta H_{am\text{-}lip}$ (J/g)	$T_{p\text{-}am\text{-}lip}$ (ºC)
Native	8.9 ^{b}	56.9 ^{a}	66.4 ^{a}	75.0 ^{a}	18.1 ^{c}	0.9 ^{a}	96.9 ^{a}	2.4 ^{a}	33.6 ^{a}	48.7 ^{a}	59.9 ^{a}	26.3 ^{a}	2.15 ^{c}	95.6 ^{a}
1C	8.3 ^{b}	56.8 ^{a}	66.5 ^{a}	74.6 ^{a}	17.8 ^{bc}	0.8 ^{a}	96.1 ^{a}	2.6 ^{a}	25.5 ^{a}	48.5 ^{a}	60.3 ^{a}	34.9 ^{a}	1.48 ^{b}	95.5 ^{a}
2C	8.8 ^{b}	56.9 ^{a}	66.4 ^{a}	74.2 ^{a}	17.3 ^{bc}	0.4 ^{a}	96.3 ^{a}	2.9 ^{a}	37.9 ^{a}	47.2 ^{a}	58.9 ^{a}	21.0 ^{a}	1.54 ^{b}	94.0 ^{a}
1C+S40	7.1 ^{a}	58.5 ^{b}	66.6 ^{a}	74.3 ^{a}	15.8 ^{a}	0.5 ^{a}	96.9 ^{a}	2.3 ^{a}	38.2 ^{a}	47.8 ^{a}	58.4 ^{a}	20.2 ^{a}	1.13 ^{a}	93.9 ^{a}
2C+S40	6.7 ^{a}	58.2 ^{b}	66.4 ^{a}	74.0 ^{a}	15.8 ^{a}	0.3 ^{a}	97.7 ^{a}	3.1 ^{a}	28.4 ^{a}	48.4 ^{a}	67.8 ^{a}	39.4 ^{a}	1.02 ^{a}	93.0 ^{a}
SE	0.3	0.2	0.2	0.3	0.2	0.2	0.9	0.5	4.8	0.1	3.0	6.0	0.07	1.0
Number of HHP Cycles	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Soaking pre-treatment	*	*	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	*	ns
Cycles*Soaking	ns	ns	ns	ns	ns	ns	ns	ns	**	ns	ns	*	ns	ns

1C: HHP single-cycle treated sample; 2C: HHP double-cycle treated sample; 1C+S40: Pre-soaked and HHP single-cycle treated sample; 2C+S40: Pre-soaked and HHP double-cycle treated sample. ΔH_{gel}: Enthalpy of gelatinization, T_o, T_p, $T_{e\text{-}gel}$: Onset, peak and endset temperature of gelatinization, ΔT: $(T_e\text{-}T_o)_{gel/ret}$, $\Delta H_{am\text{-}lip}$: Enthalpy of amylose-lipid complex dissociation, $T_{p\text{-}am\text{-}lip}$: Peak temperature of the amylose-lipid complex dissociation, ΔH_{ret}: Enthalpy of retrograded amylopectin, T_o, T_p,$T_{e\text{-}ret}$: Onset, peak and endset temperatures of melted retrograded amylopectin. Samples with different small letters show significant differences between treatments ($p<0.05$). SE: Pooled standard error from ANOVA. ** $p<0.01$, *$p<0.05$, ns: not significant.

3.8. Total phenol and total antioxidant capacity

The total phenol content (TP) and total antioxidant capacity (TAC) of buckwheat flours were determined in extracts using DPPH and in the flour samples using direct DPPH method (Table 3). Control samples showed a TP value of 311 mg GAE/100g, similar to those reported by Quettier-Deleu et al. (2000), although variations of TP in buckwheat flour have been reported in the literature associated with variety, location and environmental conditions (Zhang et al., 2017). TP was significantly affected by the soaking pre-treatment ($p<0.01$) and the number of HHP cycles applied ($p<0.05$). A significant ($p<0.05$) increase was observed in TP in un-soaked and pressurized samples using one cycle (1C) meanwhile not significant differences with native flour were observed when a double cycle was applied to the sample (2C). Other authors have already reported an increase in phenol content in other vegetal matrixes after HHP treatment (Balasubramaniam et al., 2016). This effect was associated with the improvement in the extractability of bioactive compounds due to the physical effect of the pressure. The cell permeabilization and the pressure-driven mass transport triggering a mass-transfer phenomenon (osmosis) could be also taken as plausible explanation (Balakrishna et al., 2020). The use of soaking pre-treatment significantly ($p<0.05$) improved TP content over the native flour regardless of the number of HHP cycles. This pre-treatment combined with HHP probably promoted a better extractability of phenol content increasing the solubility of the phenols.

The evaluation of total antioxidant capacity showed that native sample showed higher DPPH values (373 mg TE/100g) than un-soaked and pressurized samples regardless of the use of single or double HHP treatment. However, the use of soaking pre-treatment produced an enhancement of the antioxidant properties of pressurized with one cycle samples reaching values not significantly different to the native flour. A similar behaviour was observed in the study when direct method (Q-DPPH) was used.

The multifactor analysis of variance showed a significant effect of the soaking pre-treatment ($p<0.01$). The application of HHP reduced the DPPH value (41%) although the use of soaking enhanced the antioxidant activity of both single or double HHP treated samples. The overall increase in the antioxidant markers of the pre-soaked samples respecting the un-soaked ones could be attributed to the increased extractability of phenolic compounds, already reported to be potent radical scavengers (Bautista-expósito et al., 2020). Q-DPPH values were higher compared to those observed in extracts DPPH. This difference has been associated to a higher proportion of non-extractable cell-wall bound phenols and hence, non-measurable by classical methods (Rico et al. 2020).

Few information is available on the impact of HHP treatment of whole grains on the antioxidant capacity of resulting flours. Zhou et al. (2015) observed a reduction in the percentage of DPPH inhibition of HHP treated buckwheat flour (600 MPa, 2 cycles, 15 min/cycle, room temperature). In addition, high retaining of the antioxidant capacity (DPPH) of hydrophilic extracts after high-pressure treatment (200 MPa during 4 and 9 min) of BW raw groats was previously reported by Błaszczak (2013). No significant changes ($p>0.05$) in the antioxidant capacity of the extracts against the DPPH radical were also observed by Xia and Li, (2018) when HHP-treated matrices of wholegrain brown rice were evaluated(150-450 MPa, 10 min). Balasubramaniam et al. (2016) have stated HHP affects only non-covalent bonds so bioactive compounds should be preserved after HHP treatment.

Table 3

Antioxidant properties and mineral content of flour samples obtained from native and HHP-treated buckwheat grains.

SAMPLE	TP (mg Eq. AG/ 100g)	DPPH (mg Eq. Trolox /100g)	Q-DPPH (mg Eq. Trolox /100g)	B (mg/100g)	Ca (mg/100g)	Fe (mg/100g)	Mn (mg/100g)	Zn (mg/100g)
Native	311 ^{a}	373 ^{b}	401 ^{b}	0.445 ^{bc}	35 ^{b}	7.00 ^{b}	0.50 ^{b}	1.93 ^{c}
1C	338 ^{b}	252 ^{a}	245 ^{a}	0.404 ^{ab}	30 ^{ab}	3.85 ^{a}	0.40 ^{a}	1.69 ^{ab}
2C	297 ^{a}	219 ^{a}	230 ^{a}	0.363 ^{a}	20 ^{a}	3.15 ^{a}	0.40 ^{a}	1.57 ^{a}
1C+S40	361 ^{b}	366 ^{b}	391 ^{b}	0.487 ^{c}	30 ^{ab}	7.10 ^{b}	0.50 ^{b}	1.84 ^{bc}
2C+S40	347 ^{b}	238 ^{a}	374 ^{b}	0.564 ^{d}	25 ^{ab}	3.45 ^{a}	0.45 ^{ab}	1.89 ^{c}
SE	7.4	24.0	18.0	0.016	3,0	0.37	0.02	0.05
Number of HHP Cycles	*	*	ns	ns	*	**	ns	ns
Soaking pre-treatment	**	ns	**	**	ns	**	*	**
Cycles*Soaking	ns	ns	ns	*	ns	*	ns	ns

1C: HHP single-cycle treated sample; 2C: HHP double-cycle treated sample; 1C+S40: Pre-soaked and HHP single-cycle treated sample; 2C+S40: Pre-soaked and HHP double-cycle treated sample. TP: Total phenols; DPPH: antioxidant capacity against the DPPH radical in sample extracts; Q-DPPH: antioxidant capacity against the DPPH radical in solid samples. Samples with different small letters show significant differences between treatments ($p<0.05$). SE: Pooled standard error from ANOVA. ** $p<0.01$, *$p<0.05$, ns: not significant.

3.9. Minerals content

Table 3 shows the minerals content of control and HHP treated flours. The data obtained in the present study for the control sample were similar to those reported by Steadman et al. (2001) for whole buckwheat groats fraction. The minerals content was significantly affected by the soaking pre-treatment, except for calcium, while the number of HHP cycles significantly affected the calcium and iron content. In general, un-soaked flour samples had a lower minerals content than native sample regardless of the number of cycles used and the mineral analysed, showing that the application of HHP treatment without soaking produced a negative effect on the mineral content. However, the soaking pre-treatment enhanced the concentration of B, Fe, Mn and Zn in the flours compared to un-soaked ones, probably due to the increase in their extractability. As it was observed in the case of TP, contradictory information has been published about the influence of HHP treatment on the minerals content of flours. Briones-Labarca et al. (2011) reported a loss of minerals content after HHP treatment while Xia and Li (2018) observed a retention of Zn and Fe after a mild HHP treatment (30-90 MPa, 5 min). Although Xia and Li (2018) reported hydrothermal treatments could release minerals to the medium, those probably could interact with other component such as phytic acid or protein, reducing the diffusion to the pressure transmission water. Therefore, variations in the minerals content could be related to changes in phytate content or protein hydrolysis during HHP treatment. In addition, those authors suggested other factors as enzyme activity could be involved in fluctuations of minerals content in the grains.

4. Conclusions

HHP treatment of whole BW grain (600MPa, 30 min) induced some structural changes in its protein-starch matrix. The extent of these changes became more significant after a soaking pre-treatment of 4 h at 40ºC. Resulting flours showed an enhanced water absorption capacity and a loss of emulsion activity and foaming

capacity. Combined treatments increased the flour thermal stability and the preservation of starch granule during its hydrothermal transformation, reducing the amylose leaching, as evidenced by the reduction of retrogradation observed in the pasting test. Changes in starch granule integrity were confirmed by the lower gelatinization enthalpy obtained that suggests the treatments induced a partial gelatinization of pre-soaked samples.

In addition, results observed in this study suggested that the soaking pre-treatment of grains prior a continuous HHP treatment contributed to enhance the phenol content, maintaining the antioxidant capacity and the minerals content of resulting flours. Therefore, the combination of both treatments could be used to modulate the bioactive and techno-functional properties of resulting flours in order to obtain new flour ingredients for the gluten-free bakery industry.

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