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Subcritical water as hydrolytic medium to recover and fractionate the protein fraction and phenolic compounds from craft brewer's spent grain

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ABSTRACT

The valorization of the brewer's spent grain (BSG) generated in a craft beer industry was studied by subcritical water hydrolysis in a semi-continuous fixed-bed reactor. Temperature was varied from 125 to 185 °C at a constant flow rate of 4 mL/min. Biomass hydrolysis yielded a maximum of 78% of solubilized protein at 185 °C. Free amino acids presented a maximum level at 160 °C with a value of 55 mg free amino acids/$g_{protein\text{-}BSG}$. Polar amino acid presented a maximum at lower temperatures than non-polar amino acids. The maximum in total phenolic compounds was reached at 185 °C. This maximum is the same for aldehyde phenolic compounds such as vanillin, syringic and protocatechuic aldehyde; however, for hydroxycinnamic acids, such as ferulic acid and p-coumaric, the maximum was obtained at 160 °C. This allows a fractionation of the bioactive compounds. Subcritical water addresses opportunities for small breweries to be incorporated within the biorefinery concept.

1. Introduction

Currently, in the linear economic system, most of the by-products or wastes generated in the food industry are usually discarded. Integration of these waste material flows as raw material to another industrial process constitutes the main principle of the circular economy concept to reach the zero waste objective. Brewer's spent grain (BSG) is a lignocellulosic solid by-product generated in the brewing industry that remains after the mashing and wort filtration process. BSG is generated in large, but also small companies (Mussatto, Dragone & Roberto, 2006). Valorisation of the by-products generated by small local companies addresses also opportunities for small breweries to be incorporated within the biorefinery concept. Especially, taking into account that the size of the Europe craft beer market was worth US$ 42.52 million in 2020 and it is expected to grow up to 91.26 million by the end of 2025 (Market Data Forecast, 2020).

BSG accounts approximately for 20 kg per 100 L of beer produced (Mussatto et al., 2006). BSG contains also a considerable amount of proteins, in the mass percent range from 10 to 30 % and a small amount of lipids, around 6% (Alonso-Riaño et al., 2020). BSG is also a valuable source of phenolic compounds, with approximately 1.2% of mono- and di-meric phenolic acids, being hydroxycinnamic acids, such as ferulic and p-coumaric acids, the primary class of phenolic compounds present in the BSG that have demonstrated antioxidant effects (McCarthy et al., 2013). Therefore, based on the chemical composition of BSG, integration of this by-product within a biorefinery concept is of great interest to obtain different high value biocompounds.

To convert BSG into valuable added products, the first step should be the extraction of the bioactive compounds. Different extraction techniques can be applied, being the most commonly used extraction system the solid–liquid extraction by maceration of the biomass in a solvent. However, long extraction times are needed and, in most cases, low extraction yield is achieved. BSG as lignocellulosic biomass contains important amounts of insoluble lignin. This lignin is connected to the cell wall polysaccharides by phenolic acids, being necessary a hydrolytic method to release them (Alonso-Riaño et al., 2020; Birsan, Wilde, Waldron, & Rai, 2019). In a previous work, ultrasound assisted extraction (UAE) was compared with chemical/enzymatic hydrolysis to obtain extracts rich in hydroxycinnamic acids (Alonso-Riaño et al., 2020). The best results were obtained by using alkaline hydrolysis in terms of productivity and final extraction yield due to higher solubility of the lignin under basic conditions. Therefore, bioactive compounds extraction from biomass is still a major challenge to the biorefinery concept.

The use of subcritical water (subW) has been proposed in literature to fractionate and separate the different components of the lignocellulosic biomass (Cocero et al., 2018). subW is pressurized water in its liquid state in the temperature range from 100 °C to 374 °C. Under these conditions, water presents unique properties such as higher ionic

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product and lower dielectric constant than at ambient conditions (Cocero et al., 2018). Due to the tuneable properties with temperature, different selectivity can be achieved in the release of the bioactive compounds from the biomass. For instance, the different phenolic compounds obtained from decomposition of rice bran under subW treatment showed the maximum production of p-coumaric acid and vanillin at 165 °C and 190 °C, respectively, while protocatechuic acid and vanillic acid presented the highest level at 230 and 295 °C, respectively (Pourali, Asghari & Yoshida, 2010). Therefore, the temperature in subW treatment is a critical parameter.

A few studies on subW have been conducted with BSG. Torres-Mayanga et al. (2019) focused on the production of C-5 sugars observing that total carbohydrate yields were dependent on the hydrolysis temperature in the range from 160 to 210 °C. On the other hand, Qin, Johansen and Mussatto (2018) evaluated different pretreatment strategies for protein extraction from BSG including hydrothermal treatment, in the range from 30 up to 135 °C at two different extraction times, 1 and 24 h. It must be noticed that subW is water in its liquid state in the range from 100 °C to 374 °C. These authors determined that the maximum extraction yield was obtained at 60 °C (ca 60%). This extraction yield was surprisingly high, since, according to literature, albumins (water-soluble), and globulins (salt-soluble) compose only around 20% of the total protein content in BSG as a result of their unavoidable extraction during brewing process (Arauzo et al., 2019), being hordeins (alcohol soluble) and glutelins (acid/alkali soluble) proteins the main proteins in BSG. Therefore, it is also worth exploring subW treatment further to produce hydrolysates from proteins from BSG, since by controlling temperature and reaction time it is possible to control the production of small peptides and free amino acids from different food wastes (Marcet, Álvarez, Paredes, & Díaz, 2016).

The aim of this work was to verify if subW treatment is a suitable technology to extract/hydrolyse the protein fraction from BSG. One of the main goals of this work was to perform a detailed extract characterization considering also the release into the extraction medium of the bound phenolic compounds of BSG. Antioxidant capacity of the subW extracts and the presence of inhibitors, such as furfural and hydroxymehtyl furfural was also explored. The solid residue generated after subW treatment would be also characterized to check mass balance of the process.

2. Material and methods

2.1. Raw material

BSG was supplied by Brebajes del Norte S.L. (Dolina, craft beer), a local brewery located in Burgos (Spain). This raw material was first preconditioned by washing it with water (until neutral pH) and drying in an air convection oven (45 °C, 3 h) until a humidity value of 8% (w/w) was reached. No size reduction was carried out.

2.2. Subcritical water equipment

SubW experiments have been carried out in a semi-continuous fixed-bed reactor (see Figure S-1) built by our research group (Trigueros et al., 2021). An HPLC pump (Gilson 305, SC-10 head with a maximum flow rate of 10 mL / min) was used for pressurization and water pumping. Water was heated up to the desired treatment temperature by circulating it through a heat exchanger (60 cm of 0.317 cm AISI 316 piping) placed inside an oven (Selecta T 204A) together with the fixed bed reactor (length 20.6 cm and internal diameter of 2.8 cm). Two metallic filters (10 μm pore size) were placed at the top and the bottom of the reactor to avoid loss of solid particles and clogging of the system. Pressure was controlled by a pressure regulating valve (pressure Tech 6784 V962 max 41.4 MPa).

subW experiments were performed at a fixed water flow rate of 4 mL/min and operating pressure of 5 MPa. Approximately 12 g of dry BSG were placed in the reactor and inserted in the oven. The experiments were performed in the temperature range from 125 to 185 °C. Temperature and pressure were steadily increasing up to the selected treatment conditions during a static holding time of 30 min, in order to have enough time to reach the operation temperature. The time zero was taken as the time at which the first drop of liquid extract was obtained at the outlet pipe. Effluents were cooled and periodically collected for further characterization. The solid residue that remained in the reactor after subW treatment was washed, dried in an oven at 45 °C until constant weight, weighted and analyzed.

2.3. Enzymatic and basic hydrolysis

Firstly, 7.5 g of BSG were introduced into a 500 mL jacketed reactor provided with magnetic agitation together with 150 mL of the reaction medium. Enzymatic and basic hydrolysis were performed at 50 °C for 4 h. For basic hydrolysis, different concentrations of NaOH were assayed, 0.01 M, 0.1 M and 1 M. For enzymatic hydrolysis, the enzyme was added to the water medium in a mass percentage of 6% (enzyme:BSG ratio, w/w). Three different enzymes were used: protease, proteinase EC 3.2.1.6, from *Bacillus subtilis* (Biocon), xylanase, a mixture of xylanase *endo*-1,4β (EC 3.2.1.8) and xylanase *endo*-1,3β (EC 3.2.1.32) from *Trichoderma longibrachiatum* (Biocon) and cellulase, 1,4-(1,3:1,4)-β-D-Glucan 4-glucanohydrolase, EC 3.2.1.4, from *Aspergillus niger* (Sigma-Aldrich). After 4 h, the enzyme was inactivated by heating the sample at 100 °C for 5 min and immediately cooled in ice and kept in the refrigerator until analysis.

2.4. Analytical methods

2.4.1. Brewer's spent grain characterization

A complete chemical characterization of BSG was performed according to the National Renewable Energy Laboratory standard protocols (NREL Laboratory Analytical Procedures) to determine structural carbohydrates (cellulose and hemicellulose), lignin, moisture, total solids, ash, total extractives, and protein. Duplicate analysis was done for characterization of the composition of BSG. Detailed description can be found in the NREL protocols. Extractives free BSG was subjected to two steps acid hydrolysis. Firstly, 0.3 g of dry BSG were mixed with 3.0 mL of 72% (w/w) H_2SO_4 and incubated at 30 °C for 1 h. Then, the sample was diluted to 4% (w/w) H_2SO_4 by adding 84 mL of deionized water and was autoclaved at 121 °C for 1 h. Then, the hydrolysate was cooled down to room temperature and vacuum filtered through a 0.7 μm pore size hydrophilic glass fiber filter (Millipore). The acid insoluble residue (AIR) was rinsed with 50 mL of deionized water, dried 4 h at 105 °C and weighed. Ash content was determined by weight difference after placing AIR in a muffle furnace at 575 °C for 24 h. Klason lignin (KL) was calculated as the difference between AIR and ash. Acid soluble lignin in the hydrolysate was estimated by absorbance reading of the sample at 240 nm and using 25 $L \cdot g^{-1} \cdot cm^{-1}$ as absorptivity constant.

For cellulose and hemicellulose determination, sugar recovery standards (SRSs) were used to account for sample sugar degradation during the dilute sulfuric acid step. SRSs and BSG hydrolysates were neutralized with $CaCO_3$ to pH 5–6 and filtered through a 0.2 μm syringe filters before HPLC determination.

Monosaccharides were determined by HPLC equipped with a Biorad Aminex-HPX-87H column and its corresponding pre-column with two detectors, a variable wavelength detector (VWD) and a refractive index detector (RID), using 0.005 M sulfuric acid as mobile phase. The temperature of the column and the refractive index detector was 40 °C. The injected sample volume was 20 μL. Cellulose was estimated as the difference between glucose determined after hydrolysis and glucose due to starch and β-glucans. Hemicellulose was estimated from the xylan and arabinan content in the sample.

Ash content was determined by weight difference after placing samples in a muffle furnace at 575 °C for 24 h until constant weight.

Starch and β-glucans. Starch and β-Glucan content was determined by

using the total starch (amyloglucosidase/α-amylase method) and the mixed linkage beta-glucan assay kit, according to the manufacturer's (Megazyme International Ltd.) instructions.

Elemental composition. Elemental composition (C, H, N, S, O) of the raw material was determined by an organic elemental micro-analyzer equipment (Thermo Scientific Model Flash 2000).

Lipid fraction. The oil content of the BSG was determined by Soxhlet extraction (Buchi B-811) using hexane as solvent.

Protein content. Protein in the raw material was estimated from the nitrogen content present in the samples as measured by the elemental analysis and verified by the Kjeldhal method. The *N*-factor was calculated from the amino acid profile and nitrogen data, according to the NREL standard protocols (NREL Laboratory Analytical Procedures). The *N*-factor of the sample was calculated as the average of the upper and lower limits, k_A and k_p respectively, according the following equations:

$$k_A = \frac{\sum E_i}{\sum D_i} \tag{1a}$$

$$k_P = \frac{\sum E_i}{\sum N} \tag{1b}$$

where E_i are the grams of the i_{th} amino acid/100 g of dry sample; Di are the grams of nitrogen of the i_{th} amino acid/100 g of dry sample and N are the grams of nitrogen/100 g of dry sample. A range of highest probability (k_1, k_2) was calculated as follows:

$$k_1 = avg(k_A, k_p) + 0.25(avg(k_A, k_p)) \tag{2a}$$

$$k_2 = avg(k_A, k_p) - 0.25(avg(k_A, k_p)) \tag{2b}$$

Amino acid profile. The amino acid profile of BSG was obtained according to AOAC Official Method 982.30 (AOAC) with some modifications. A sample of 100 mg of milled BSG was hydrolyzed for 1 h in a boiling water bath and then for 23 h at 110 °C in an oven, in 1 mL of 6 N HCl, dissolved in 1 mL of 0.1 M HCl: EtOH (1:1, v: v) and neutralized with Na_2CO_3 until the pH was greater than 1.5 but less than 5. Tryptophan and cysteine are lost by acid hydrolysis, and methionine can be partially destroyed by acid hydrolysis, so, an alkaline hydrolysis was performed to determine these amino acids. In this case, a sample of 100 mg of milled BSG was hydrolyzed for 1 h in a boiling water bath and then for 23 h at 110 °C in an oven, in 7 mL of 4.2 M NaOH, and neutralized with 6 N HCl until pH greater than 1.5 but less than 5. Final solutions were analyzed by gas chromatography after derivatization by using the EZ:faast™ kit (Phenomenex). The derivatized amino acids were analyzed using a GC-FID instrument (Hewlett Packard; HP, 5890 Series II) equipped with an auto-sampler (Avondale, PA, USA). Aliquots of the derivatized amino acids (4 μL) were injected at 1:15 split ratio at 250 °C into a Zebron column (ZB –AAA, 10 m and 0.25 mm in diameter) programmed from 110 to 320 °C at 32 °C/min. Helium was used as a carrier gas at 60 kPa and nitrogen was used as a make-up gas. The detector temperature was 320 °C. Amino acids were identified by standards included in the mentioned kit. Norleucine was used as internal standard.

2.4.2. Characterization of liquid and solid streams from subcritical water treatment

2.4.2.1. Liquid extracts. Protein content. Protein content in the subW extracts was quantitatively analyzed by two different methods: 1) from the nitrogen content by using a Shimadzu TOC-V CSN analyzer using KNO_3 as standard and a convenient NF and 2) by using the kit RC DC™ (Bio Rad Laboratories) based on the Lowry assay but modified to allow protein determination in the presence of reducing agents and detergents using bovine serum albumin as standard.

Degree of hydrolysis. Degree of hydrolysis (DH) is defined as the proportion of cleaved peptide bonds in a protein hydrolysate (Adler-Nissen, 1979):

$$DH = \frac{h}{h_{tot}} \cdot 100 \tag{3}$$

where h_{tot} is the sum of the millimoles of individual amino acids per gram in the unhydrolyzed protein that was calculated from the amino acid profile, and h is the free amino acid content in the subW extracts, expressed as meq/g protein and was determined by the ninhydrin assay. The ninhydrin reaction method was carried out according to the Sigma Aldrich protocol. First, 2 mL of the sample were gently mixed with 1 mL of ninhydrin reagent solution and placed into a boiling water bath for 10 min. Samples were cooled and 5 mL of 95% ethanol were added. Absorbance was measured at 570 nm. Ninhydrin reagent solution was purchased from Sigma-Aldrich and leucine was used as standard.

Free amino acids. Free amino acids were analyzed by gas chromatography after derivatization by using the EZ:faast™ kit (Phenomenex) as described in section 2.4.1. Free amino acids yield of the aqueous extracts was obtained by the Equation 4.

$$Yield, i = \frac{E_i}{R_i} \cdot 100 \tag{4}$$

where E_i are the grams of the i_{th} amino acid/100 $g_{dry\text{-}BSG}$ in the subW extracts collected and Ri are the grams of the i_{th} amino acid/100 $g_{dry\text{-}BSG}$ in the raw material.

Total organic carbon (TOC) A Total Organic Carbon Analyzer Shimadzu (TOC-V CSN) was used to quantify the concentration of total carbon (TC) and inorganic carbon (IC). Potassium hydrogen phthalate and sodium hydrogen carbonate were used as standards. The TOC concentration was then calculated by subtracting the IC concentration from the obtained TC concentration.

Total Polyphenols Content (TPC) and Antioxidant Activity. TPC was determined by using the Folin–Ciocalteu reagent following the method described by Singleton, Orthofer, and Lamuela-Raventós (1999). A calibration curve was prepared with standard solutions of gallic acid by following the same colorimetric method and results were expressed as mg of gallic acid equivalent (GAE) per gram of dry BSG.

The FRAP method was performed according to Benzie and Strain (1996). Absorbance was read at 593 nm. As standard, a solution of $FeSO_4 \cdot 7H_2O$ (0.1 M) was used. Results were expressed in μmoles of Fe^{2+} per gram of dry BSG.

Individual phenolic compounds. The identification and quantification of individual phenolic compounds was performed according to the method previously described by Alonso-Riaño et al. (2020). This method was already applied to identify and quantify individual phenolic compounds from other raw materials such as olive leaves (Kashaninejad, Sanz, Blanco, Beltrán, & Niknam, 2020) and onion peels (Benito-Román, Blanco, Sanz & Beltrán, 2020). The sample was filtered through a 0.2 μm syringe filters. After that, 80 μL of sample were injected in the HPLC system. The separation was performed at 25 °C on a Kinetex® μm Biphenyl 100 Å, 250 × 4.6 mm column (Phenomenex). The mobile phase consisted of ammonium acetate 5 mM with acetic acid (1%; v/v) in water (solvent A) and ammonium acetate 5 mM with acetic acid (1%; v/v) in acetonitrile (solvent B). The composition of the mobile phase varied during the run according to a nonlinear gradient as follows: from 0 to 7 min 2% of solvent B (isocratic), from 7 to 20 min from 2% to 8% solvent B, from 20 to 35 min from 8% to 10% solvent B and from 35 to 55 min 10% to 18% solvent B and post time of 10 min, at a flow rate of 0.8 mL/min. Detection and quantification was performed at 240, 280, 330, 340, 350, and 370 nm. The HP ChemStation software was employed to collect and analyse the chromatographic data delivered by the diode array detector and own library was used to identify the different phenolic compounds by comparing retention times and spectral data with those of authentic standards: syringic aldehyde, protocatechuic aldehyde, vanillin, p-coumaric acid, ferulic acid, catechin, vanillic acid, 4-vinylphenol and 4-vinylguaicol standards (Sigma-Aldrich). Peak purity was checked to exclude any contribution from

interfering peaks. Individual stock solutions of the above phenolic compounds, and their mixtures, were prepared in methanol to plot the calibration curves.

Furfural and hydroxymethylfurfural (HMF). Furfural and HMF analysis were performed by HPLC-RID-VWD Agilent 1260 at 284 and 275 nm respectively, by using the same column and method as described in section 2.4.1. Calibration was performed with standards of 5-hydroxymethyl-2-furaldehyde (HMF), 97%, and furfural, 99%, purchased from Alfa Aesar and Sigma-Aldrich, respectively. The sample was filtered through a 0.2 μm syringe filters. After that, 20 μL of sample were injected in the HPLC system.

2.4.2.2. Solid residues. Solid residues after subW treatment were washed, dried for 24 h at 45 °C and weighted. Elemental composition (C, H, N, S, O) was determined. The high heating value (HHV) of the solid residue and raw material was evaluated by the following equation (Friedl, Padouvas, Rotter & Varmuza, 2005)

$$HHV(kJ/kg) = 3.55C^2 - 232C - 2230H + 51.2C{\cdot}H + 131N + 20600 \quad [5]$$

2.5. Statistical analysis

All values were expressed as mean ± standard deviation of at least three replicates. The significance of the differences was determined based on an analysis of the variance with the Fisher's Least Significant Difference (LSD) method at p-value ≤ 0.05. Correlation between antioxidant activity and the different bio-compounds released in the medium, TPC, free amino acids and protein fraction, was determined using Person's Correlation Test. The software Statgraphics X64 was used.

3. Results and discussion

3.1. Breweŕs spent grain characterization

Chemical composition of the BSG is showed in Table S-1. The protein content was 17.7 ± 0.1% (w/w). This value was within the range of other values found in the literature, from 10 to 31% (w/w) (Lynch, Steffen, & Arendt, 2016). The protein content has been traditionally calculated by multiplying the total nitrogen determined by the Kjeldahl or Dumas methods by a standard nitrogen conversion factor of 6.25, which assumes that 1 kg of material contents 160 g of N. However, the relation between protein and nitrogen can vary depending on the amino acid composition and the presence of other nitrogenous organic compounds such as nucleic acids, urea, ammonia, phospholipids, nitrates, and purine derivatives (Sriperm, Pesti & Tillman, 2011). In this work, the N:P conversion factor was calculated by using the amino acid profile showed in Table 1, yielding a value of 6.11. According to the equations 2a and 2b, the range of highest probability (k_1, k_2) was 5.8–6.4. Since 6.25 was within the range of highest probability calculated and being the standard value widely used in the literature, this value was used as N:P in this work. No conversion factor for BSG was found in the literature, although Merrill & Watt (1955) reported a value of 5.83 as the specific factor for the conversion of nitrogen content to protein content for barley.

Extractives in water were 24.3% (w/w), which included 5.02 g/100 $g_{dry\text{-}BSG}$ of soluble proteins (28.4% of the total protein content). This

Table 1
Amino acid profile of BSG. Final accumulative free amino acid yields in the subW extracts obtained at different temperatures, expressed as mg aa/$g_{protein}$ and percent yield as the ratio of individual amino acids in the extracts and in the raw material, Eq 4). F = 4 mL/min.

Amino acid	BSG	SubW 125 °C		SubW 145 °C		SubW 160 °C		subW 185 °C	
	mg aa/g protein	mg aa/g protein	Yield, %	mg aa/g protein	Yield, %	mg aa/g protein	Yield, %	mg aa/g protein	Yield, %
Alanine	48.1 ± 0.2	2.46 ± 0.01^{a}	5.13 ± 0.03^{a}	3.08 ± 0.01^{b}	6.41 ± 0.01^{b}	3.29 ± 0.01^{c}	6.84 ± 0.01^{c}	3.62 ± 0.01^{d}	7.52 ± 0.02^{d}
Aspartic acid	69 ± 3	3.32 ± 0.01^{a}	4.8 ± 0.2^{a}	10.2 ± 0.1^{c}	15.6 ± 0.8^{c}	15.4 ± 0.2^{d}	22 ± 1^{d}	4.4 ± 0.1^{b}	6.3 ± 0.4^{b}
Cysteine	4.7 ± 0.2	n.d.	–	n.d.		0.33 ± 0.01^{b}	7.1 ± 0.4^{b}	0.22 ± 0.01^{a}	4.8 ± 0.3^{a}
Glutamic acid	117 ± 2	6.3 ± 0.1^{c}	5.4 ± 0.2^{c}	6.15 ± 0.02^{c}	5.3 ± 0.1^{c}	4.26 ± 0.09^{b}	3.65 ± 0.1^{b}	1.15 ± 0.06^{a}	0.98 ± 0.07^{a}
Glycine	42 ± 1	0.84 ± 0.01^{a}	2.01 ± 0.07^{a}	1.28 ± 0.01^{b}	3.1 ± 0.1^{b}	1.91 ± 0.01^{c}	4.6 ± 0.1^{c}	3.16 ± 0.01^{d}	7.6 ± 0.2^{d}
Histidine^{e}	23 ± 2	1.47 ± 0.05^{b}	6.4 ± 0.8^{b}	1.92 ± 0.01^{c}	8.4 ± 0.8^{c}	1.9 ± 0.1^{c}	8 ± 1^{c}	1.08 ± 0.05^{a}	4.7 ± 0.6^{a}
Hydroxylysine	n.d.	n.d.		n.d.		5.0 ± 0.3^{b}		0.17 ± 0.01^{a}	
Hydroxyproline	4.3 ± 0.2	n.d.		0.14 ± 0.01^{c}	3.3 ± 0.2^{c}	0.09 ± 0.01^{b}	2.25 ± 0.3^{b}	0.023 ± 0.001^{a}	0.53 ± 0.03^{a}
Isoleucine^{e}	69.4 ± 0.1	0.74 ± 0.01^{a}	1.07 ± 0.01^{a}	1.18 ± 0.01^{b}	1.69 ± 0.02^{b}	1.42 ± 0.01^{d}	2.04 ± 0.02^{d}	1.40 ± 0.01^{c}	2.01 ± 0.02^{c}
Leucine^{e}	87 ± 2	1.39 ± 0.01^{a}	1.59 ± 0.05^{a}	2.13 ± 0.01^{b}	2.45 ± 0.08^{b}	2.35 ± 0.01^{c}	2.70 ± 0.08^{c}	2.43 ± 0.01^{d}	2.79 ± 0.08^{d}
Lysine^{e}	83 ± 1	2.08 ± 0.01^{a}	2.51 ± 0.05^{a}	6.9 ± 0.3^{c}	8.4 ± 0.5^{c}	4.7 ± 0.3^{b}	5.7 ± 0.4^{b}	1.57 ± 0.03^{a}	1.90 ± 0.07^{a}
Methionine^{e}	18.9 ± 0.8	0.24 ± 0.01^{b}	1.3 ± 0.1^{b}	0.85 ± 0.01^{c}	4.5 ± 0.2^{c}	1.25 ± 0.01^{d}	6.6 ± 0.3^{d}	0.17 ± 0.01^{a}	0.89 ± 0.9^{a}
Phenylalnine^{e}	68 ± 3	1.46 ± 0.01^{a}	2.1 ± 0.1^{a}	1.83 ± 0.02^{c}	2.7 ± 0.2^{c}	1.61 ± 0.01^{b}	2.4 ± 0.1^{b}	1.37 ± 0.06^{a}	2.0 ± 0.2^{a}
Proline	123 ± 5	1.93 ± 0.01^{a}	1.56 ± 0.07^{a}	2.25 ± 0.01^{c}	1.83 ± 0.08^{c}	2.28 ± 0.01^{d}	1.85 ± 0.08^{d}	1.94 ± 0.01^{b}	1.57 ± 0.07^{b}
Serine	44.1 ± 0.6	1.96 ± 0.03^{b}	4.4 ± 0.1^{b}	2.86 ± 0.02^{d}	6.5 ± 0.1^{d}	2.43 ± 0.01^{c}	5.5 ± 0.1^{c}	1.21 ± 0.04^{a}	2.7 ± 0.1^{a}
Threonine^{e}	41 ± 2	0.73 ± 0.01^{a}	1.8 ± 0.1^{a}	1.07 ± 0.01^{b}	2.6 ± 0.1^{b}	1.12 ± 0.01^{c}	2.7 ± 0.1^{c}	0.70 ± 0.03^{a}	1.7 ± 0.1^{a}
Tryptophan^{e}	14.7 ± 0.2	0.26 ± 0.01^{b}	1.79 ± 0.07^{b}	0.40 ± 0.01^{c}	2.7 ± 0.1^{c}	0.67 ± 0.05^{d}	4.6 ± 0.4^{d}	0.016 ± 0.004^{a}	0.11 ± 0.03^{a}
Tyrosine	22.5 ± 0.6	2.20 ± 0.02^{b}	9.8 ± 0.3^{b}	2.80 ± 0.03^{c}	12.5 ± 0.4^{c}	1.85 ± 0.05^{a}	8.2 ± 0.4^{a}	2.4 ± 0.1^{b}	10.5 ± 0.8^{b}
Valine^{e}	123 ± 6	1.56 ± 0.02^{a}	1.27 ± 0.07^{a}	2.30 ± 0.02^{b}	1.9 ± 0.1^{b}	3.25 ± 0.01^{c}	2.7 ± 0.1^{c}	4.76 ± 0.06^{d}	3.9 ± 0.2^{d}
TAA	1002 ± 31	28.9 ± 0.3^{a}	2.9 ± 0.1^{a}	47.4 ± 0.6^{c}	4.7 ± 0.2^{c}	55 ± 1^{d}	5.5 ± 0.3^{d}	31.7 ± 0.6^{b}	3.2 ± 0.2^{b}
TEAA	527 ± 17	9.9 ± 0.1^{a}	1.88 ± 0.09^{a}	18.6 ± 0.4^{c}	3.5 ± 0.2^{c}	18.3 ± 0.5^{c}	3.5 ± 0.2^{c}	13.5 ± 0.3^{b}	2.6 ± 0.1^{b}
TEAA/TAA (%)	53 ± 3^{e}	34.3 ± 0.8^{b}	–	39.4 ± 1^{c}		33 ± 2^{a}		43 ± 2^{d}	
non-P/TAA (molar ratio)	0.63^{c} ± 0.04	0.43 ± 0.01^{b}		0.37 ± 0.01^{a}		0.38 ± 0.01^{a}		0.66 ± 0.02^{d}	

n.d., not detected. Values are expressed as mean ± standard deviation from triplicate determination. Values with different letters in each raw are significantly different when applying the Fisher's least significant differences (LSD) method at p-value ≤ 0.05 for mg aa/$g_{protein}$ and yield values. Aspartic acid includes asparagine. Glutamic acid includes glutamine.
TAA, total amino acids. TEAA, total essential amino acids. TEAA/TAA: ratio essential amino acids to total amino acids.
non-P/TAA: molar ratio of non-polar amino acids to total amino acids.

result agrees with literature that reported around 20% of soluble proteins from the total protein content in BSG, being hordeins over 50% of the total amount of proteins, followed by glutelins (Arauzo et al., 2019). Hordeins are rich in glutamic acid and proline and these amino acids constituted 24% of the BSG amino acids determined in this work, 117 ± 2 and 123 ± 5 mg aa/$g_{prot\text{-}BSG}$, respectively (see Table 1). It was found that 53 ± 2% of the total BSG amino acids were essential amino acids (EAA), being valine, leucine and lysine the three most abundant. These results agree with those in literature that also reported glutamine/glutamate, proline and leucine as the most abundant amino acids (Arauzo et al., 2019; Ikram, Huang, Zhang, Wang, & Yin, 2017)

Elemental composition of the BSG is showed in Table 2, with a mass percent of C, H, N and O of 47.0 ± 0.2, 6.9 ± 0.1, 2.8 ± 0.1 and 39.5 ± 0.1, respectively. The values obtained were similar to the values found in literature that reported values of C (47.18–51.3%, w/w), H (6.02–8.20%, w/w), N (3.32–4.9%, w/w), O (32.9–41.04%, w/w) and S (0.26–0.45%, w/w) (Arauzo, Olszewski & Kruse, 2018; Jackowski et al., 2019). However, in this work, sulphur was not found. Elemental composition led to molar ratios H:C and O:C of 1.75 ± 0.04 and 0.63 ± 0.01, respectively and an HHV of 19.14 ± 0.08.

3.2. Protein extraction

Fig. 1a shows the accumulative total protein fraction obtained in the subW extracts, expressed as g protein/100 $g_{dry\text{-}BSG}$, calculated by multiplying the total nitrogen determined by TOC-N by 6.25. The highest protein extraction level was 13.8 g/100 $g_{dry\text{-}BSG}$, 78% of the total protein in the BSG, and it was achieved at the maximum temperature level covered in this work, 185 °C. At 185 °C a plateau was reached at 150 min of extraction, while longer times were needed at lower temperatures (see Fig. 1a). Du, Arauzo, Meza Zavala, Cao, Olszewski and Kruse (2020) concluded that time was a crucial factor to obtain high protein extraction yields. These authors reported a low protein extraction yield of 6.7% from BSG by subW treatment at 200 °C (F = 6 mL/min), but only 20 min of extraction time. Sereewatthanawut, Prapintip, Watchiraruji, Goto, Sasaki and Shotipruk (2008) studied the effect of temperature on the extraction of protein from deoiled rice bran between 100 and 220 °C by subW. These authors reached the highest protein yield at 200 °C, recovering nearly all the protein from the original bran.

Protein extraction by subW treatment was compared with other

Table 2
Elemental analysis, ash content, H:C, O:C and N:C molar ratios and estimated heating value (HHV) of BSG and the solid residues after subW treatment (SWR).

Sample	Raw material	SWR 125 °C	SWR 145 °C	SWR 160 °C	SWR 185 °C
C (%, w/w)	47.0 ± 0.2[a]	52.3 ± 0.9[b]	53.7 ± 0.2[c]	54.8 ± 0.9[c]	60.3 ± 0.5[d]
H (%, w/w)	6.9 ± 0.1[a]	7.02 ± 0.09[a]	7.3 ± 0.2[b]	7.5 ± 0.1[b]	7.34 ± 0.04[b]
N (%, w/w)	2.8 ± 0.1[b]	3.33 ± 0.05[c]	2.45 ± 0.02[b]	1.67 ± 0.01[a]	1.79 ± 0.01[a]
O (%, w/w)	39.5 ± 0.1[c]	37 ± 2[c]	33.2 ± 0.3[b]	32 ± 2[b]	29 ± 1[a]
Ash (%, w/w)	2.92 ± 0.02 [d]	1.62 ± 0.01[b]	1.67 ± 0.01[b]	1.26 ± 0.09 [a]	2.41 ± 0.04[c]
H:C	1.75 ± 0.04[c]	1.61 ± 0.01[b]	1.63 ± 0.05[b]	1.65 ± 0.05[b]	1.46 ± 0.02[a]
O:C	0.63 ± 0.01[d]	0.54 ± 0.04[c]	0.468 ± 0.003[b]	0.46 ± 0.02[b]	0.37 ± 0.02[a]
N:C	0.052 ± 0.002[c]	0.055 ± 0.005[c]	0.039 ± 0.006[b]	0.026 ± 0.004[a]	0.0255 ± 0.0003[a]
HHV (MJ/kg)	19.14 ± 0.08[a]	21.7 ± 0.5[b]	22.5 ± 0.2[c]	23.1 ± 0.5[d]	26.0 ± 0.3[e]

Values with different letters in each raw are significantly different when applying the Fisher's least significant differences (LSD) method at p-value ≤ 0.05.

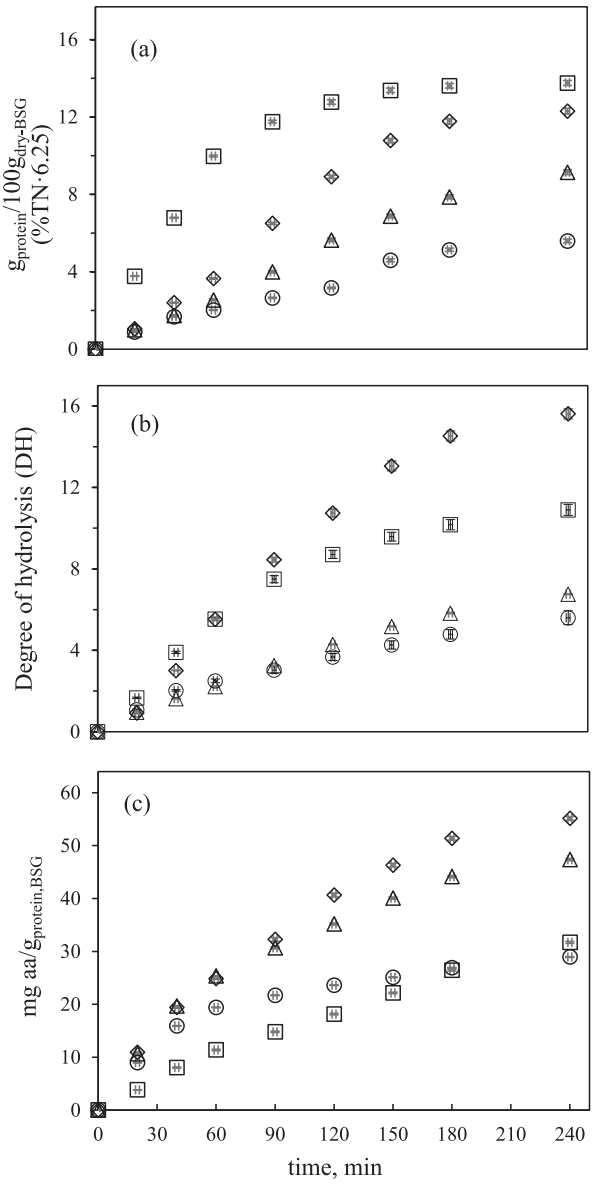

Fig. 1. Accumulative profile in the subW extracts of (a) protein fraction, $g_{protein}/100g_{dry\text{-}BSG}$ (b) degree of hydrolysis and (c) free amino acids (mg aa/$g_{protein,BSG}$) at different temperatures. (○ 125 °C, △ 145 °C, ◇ 160 °C and □ 185°). (F = 4 mL/min).

hydrolytic methods such as alkaline and enzymatic hydrolysis at the same treatment time, 4 h (Table S-2). At the highest temperature levels essayed in this work, 160 and 185 °C, subW treatment achieved much higher extraction yields of the protein fraction than enzymatic treatment by protease (47.1 ± 0.6%, enzyme loading 6%) or basic hydrolysis (50.5 ± 0.2%, 0.1 M NaOH). Table S-2 also includes previous results obtained by ultrasound assisted extraction (UAE) of BSG that also yielded lower extraction yield than subW treatment, 46.6 ± 0.7% (Alonso-Riaño et al., 2020). Under subcritical conditions, the physical–chemical properties of water change drastically with temperature. The ionic product of water increases in the subcritical range increasing the concentration of H^+ and OH^- that favors biomass hydrolysis. Moreover, the dielectric constant decreases allowing water to interact with non-polar substances, thus decreasing their binding force and dissolving them. Therefore, biopolymers, like proteins, are released from the matrix and broken down into valuable peptides and free amino acids.

Total protein content was also determined by the kit RC DC™ and

compared with the TOC-N method. The results obtained with the spectrophotometric method were around 30% higher than those obtained by nitrogen measurements. A linear correlation was stablished between the protein content determined by both methods:

$$\%\ \text{Protein (kit } RC\ DC^{TM}) = 1.3735\ \%\ \text{Protein (\%TN·6.25)},\ R^2 = 0.9846 \quad (6)$$

The slope of this correlation shows the overestimation in the protein content by the spectrophotometric method. This overestimation can be also appreciated in literature. Sereewatthanawut et al. (2008) reported a protein yield of 219 mg/$g_{\text{rice bran}}$ by subW as determined by the Lowry method, while the composition of the feedstock showed a mass percent of protein of 18.56%. In literature, it is reported that the Cu^{2+} ion present in the reagent is overly sensitive to some amino acids such as tryptophan and tyrosine (Lourenço, Barbarino, Lavín, Lanfer Marquez and Aidar, 2004). The different content of these amino acids in the BSG and in the BSA standard could be the reason that justifies the difference in the protein fraction content by both methods. Furthermore, Niamke, Kouame, Kouadio, Koffi, Faulet and Dabonne (2006) studied the influence of 57 chemicals (mineral and organic acids, organic solvents, phenolic compounds, mineral and organic salts) on the efficiency of protein determination by the Lowry method. These authors found that the influence of phenolic compounds was governed by the structure of their molecules. This influence caused a high overestimation of the protein level.

3.3. Degree of hydrolysis and free amino acids

The degree of hydrolysis (DH) has been plotted in Fig. 1b. The value of h_{tot}, 8.0 ± 0.2 mmol/g protein was calculated using the amino acid profile of the BSG (see Table 1). The results show that the lowest DH was obtained at 125 °C (5.5 ± 0.3), and a maximum DH was reached at 160 °C (15.6 ± 0.2). This result correlated with the highest content of total free amino acids in the subW extracts, as determined by the sum of individual free amino acids determined by gas chromatography, with a maximum level at 160 °C (see Fig. 1c). At 185 °C a significant decrease of the free amino acids content took place.

The lower DH and free amino acid content at 185 °C could be attributed to amino acid decomposition. According to literature, high temperatures and/or high residence time could cause amino acid degradation producing different carboxylic acids and other nitrogen containing compounds such as ethanolamine and the non-proteinogenic amino acid ornithine (Rogalinski, Herrmann, & Brunner, 2005). Furthermore, the temperature at which degradation of protein or its hydrolysis products take place depends of the protein source. For instance, animal wastes require higher temperatures or longer reaction times than vegetable wastes (Marcet et al., 2016). Considering the disrupted lignocellulosic structure during beer production, BSG protein could be even more accessible during extraction (Du et al., 2020). Other subW studies reported higher temperatures for the optimum amino acids release (Marcet et al., 2016) but lower temperatures have been also observed in the literature for subW treatment of oysters at 150 °C (Lee, Saravana, Cho, Haq & Chun, 2018). In any case, it must be highlighted that the high residence time used in this work, around 28–29 min, could cause amino acid degradation even at not so high temperature.

Final free amino acid content of subW extracts is shown in Table 1 together with the free amino acid yields evaluated according to Equation 4. The highest amino acid yield was obtained at 160 °C with a value of 55 ± 1 mg amino acid/ $g_{\text{protein-BSG}}$ (9.7 ± 0.2 mg amino acid/ $g_{\text{dry-BSG}}$,) that corresponds to 5.5 ± 0.3% of the total amino acids of the BSG. These values are comparable to the values reported for other authors for free amino acids release by subW treatment. Sereewatthanawut et al. (2008) obtained 8.0 ± 1.6 mg free amino acids/$g_{\text{rice bran}}$ while Watchararuji, Goto, Sasaki and Shotipruk (2008) recovered 5% of the total protein content as free amino acids from two different sources, deoiled rice bran and raw soybean.

Although, the concentration of total free amino acids at 160 °C was at maximum level, this maximum was achieved at different temperature for each individual amino acid. For a better understanding, the yields of the different amino acids have been plotted in Fig. 2. It can be observed that polar amino acids (serine, threonine, aspartic and glutamic acids, lysine, histidine, tyrosine and cysteine) reached the maximum level at lower temperatures with higher amino acid yields. Aspartic acid showed the highest yield at 160 °C but also a dramatic degradation at 185 °C. Glutamic acid yield continuously decreased by increasing operating temperature. Both amino acids conform the negative charged amino acids group, the strong polar nature of their residues incomes that they are normally found on the surface of globular proteins and interact favourably with solvent molecules, nevertheless this fact also means that they are very labile (Abdelmoez & Yoshida, 2013). Positive charged amino acids group includes lysine and histidine, which showed a maximum yield at 145–160 °C. These amino acids are highly hydrophilic and are usually involved in reactions with negatively charged groups. Furthermore, lysine has a high tendency to be involved in Maillard reactions with the carbonyl groups or reducing carbohydrates (Lamp, Kaltschmitt & Lüdtke, 2020). Serine, threonine, tyrosine and cysteine are hydrophilic neutral amino acids with an aliphatic chain. All of them presented the highest yield at 145–160 °C, except cysteine, which was found at negligible concentration due to its very low stability. On the other hand, for most of the non-polar amino acids, individual yields increased by increasing the operation temperature, except for methionine. Hydrophobic amino acids tend to reside in the interior of a protein to minimise contact with water, as this conformation stabilises the protein in the aqueous solution (Widyarani, Sari, Ratnaningsih, Sanders and Bruins, 2016). The decrease of the dielectric constant of subW with temperature may favour its affinity for these amino acids due to their hydrophobic character. In addition, small aliphatic amino acids could be formed during the decomposition of the other amino acids (Esteban, García, Ramos & Márquez, 2008). Similar trend in the extractability of amino acids has been reported by Abdelmoez & Yoshida (2013).

The different behaviour of polar and non-polar amino acids with temperature was reflected in the selectivity value towards non-polar amino acids, defined as the amount of free non-polar amino acids released in the subW extracts relative to the total amount of free amino acids on a molar base, in a similar way as the hydrophobic selectivity defined by Widyarani et al. (2016). The non-polar selectivity remained constant in the temperature range from 125 to 160 °C, but it greatly increased at 185 °C. As an example, Figure S-2 shows the accumulative free amino acid content in the subW extracts collected at 160 °C.

3.4. Total phenolic content and antioxidant activity of subW extracts

Fig. 3a shows TPC release in the temperature range from 125 to 185 °C (F = 4 mL/min) as determined by the Folin Ciocalteu method. An

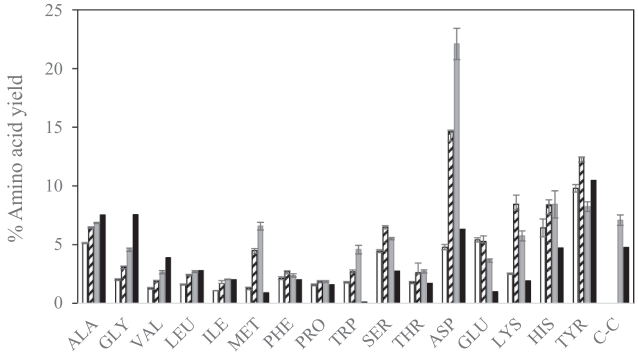

Fig. 2. Final yield of individual amino acids as a function of temperature: 125 °C □; 140 °C ▨; 160 °C ▩, 185 °C ■.

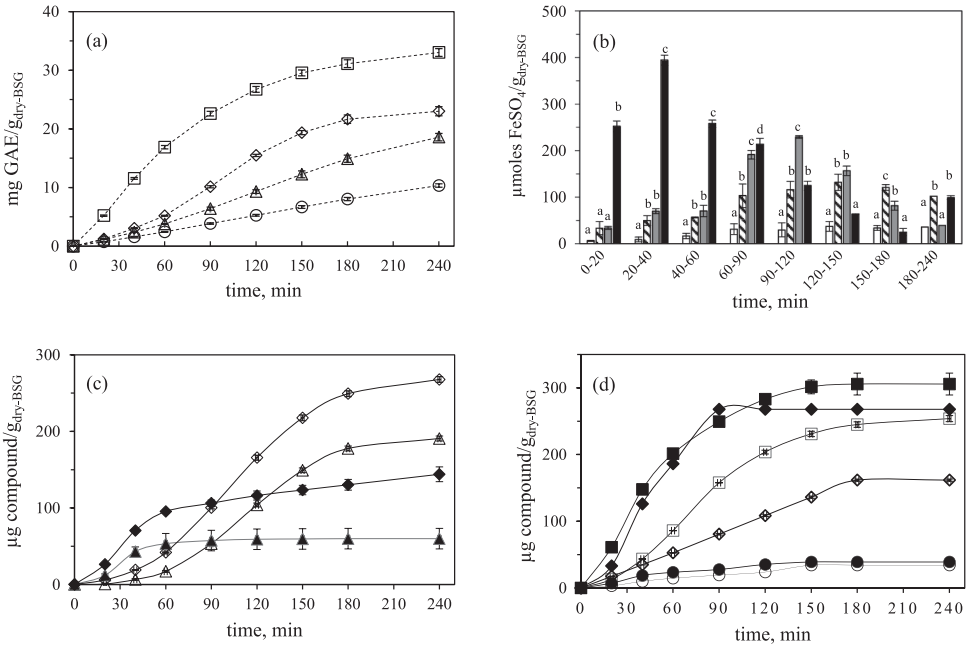

Fig. 3. (a) Accumulative TPC, mg GAE/$g_{dry,BSG}$, at different temperatures (○ 125 °C, △ 145 °C, ◇ 160 °C and □ 185 °C) (b) Antioxidant activity (µm $FeSO_4/g_{dry,BSG}$) collected at the different time intervals at different temperatures: 125 °C □; 140 °C ▨; 160 °C ■, 185 °C ■. Values with different letters at each interval time are significantly different when applying the Fisher's least significant differences (LSD) method at p-value ≤ 0.05. (c) (d) Accumulative individual phenolic compounds at 160 °C (open symbols) and 185 °C (filled symbols): (c) ▲△ p-coumaric; ◇◆ ferulic acid (d) □ ■ vanillin; ○ ● Syringic aldehyde; ◇◆ protocatechuic aldehyde. (F = 4 mL/min).

increase in the operating temperature led to an increase in TPC in the extraction medium, with values after 240 min of extraction, from 10.3 ± 0.3 to 33.0 ± 0.3 mg GAE/$g_{dry-BSG}$ at 125 and 185 °C, respectively. SubW treatment resulted in higher amounts of TPC from BSG than the values obtained in previous studies by using other hydrolysis technics, such as UAE and acid/alkaline hydrolysis, with TPC values of 3.3 ± 0.1, 30 ± 5 and 16.2 ± 0.2 mg GAE/$g_{,dry-BSG}$, respectively (Alonso-Riaño et al., 2020). In that work, a maximum TPC release of 42.0 ± 0.4 mg GAE/$g_{dry-BSG}$ was achieved by enzymatic hydrolysis with 6% (w/w) of xylanase for 24 h of incubation. However, productivity by enzymatic hydrolysis was lower, 0.087 mg GAE/($g_{dry-BSG}$·min) than for subW, 0.28 mg GAE/($g_{dry-BSG}$.min) at 185 °C, evaluated from the initial linear extraction curve.

Fig. 3b shows the results of the antioxidant capacity of the extracts collected at the different time intervals, as determined by the FRAP assay. The results showed that increasing the temperature up to 185 °C significantly increased the antioxidant activity (p less than 0.05). A positive correlation ($R^2 = 0.9511$) between antioxidant activity and TPC was stablished according to the Pearson product moment correlation. According to Marcet et al. (2016), antioxidant capacity increased when small peptides are obtained. In this work, it was also found a positive correlation ($R^2 = 0.7629$) between antioxidant activity and the amount of protein in the subW extracts, while the amount of free amino acids was not significantly correlated with the antioxidant activity (R^2 = 0.1189). The high TPC value and antioxidant capacity of the extracts obtained at the highest temperatures could be due to newly formed compounds related to Maillard reactions. The increase in browning is directly associated with advanced phases of the Maillard reaction. This browning was visually observed in the colour of the extracts from BSG since they become darker by increasing temperature (Figure S-3). The colour change would indicate the increase of the amount of hydrolysis and decomposition products in the aqueous phase. The formation of hydroxymethyl furfural (HMF) and furfural can be also considered as indicators of Maillard reactions. It is well known that these compounds take part in reactions leading to the formation of melanoidins and other polymers and aromatic substances. The concentration of HMF and furfural in subW extracts has been reported in Figure S-4. Both compounds significantly increased with temperature and showed a positive correlation with antioxidant activity (p less than 0.05) with Pearsons correlation coefficients of 0.6887 and 0.8091, respectively. In any case, it must be highlighted that synthetic aqueous dilutions of HMF and furfural standards, at similar concentrations to the concentrations found in the subW extracts, exhibited no response to the TPC and antioxidant activity assays. The effect of the Maillard reaction products on the response of the spectrophotometric methods used to determine the TPC and the antioxidant activity was determined by a simple experiment. A mixture of an amino acid, glutamine (0.05 M), and glucose (0.05 M) was placed in a vessel immersed in a boiling water bath for 4 h at a pH value of 5.8 adjusted with phosphate buffer. Aliquots were taken each hour and TPC and FRAP assays were performed (Figure S-5). The TPC and FRAP responses increased with time proving that both measurements, TPC and antioxidant activity, in subW extracts could be influenced by the Maillard reaction products.

Fig. 3c and 3d show the accumulative release of the identified individual phenolic compounds in the subW extracts obtained at 160 °C and 185 °C, respectively. Five phenolic compounds were identified at both temperatures: two hydroxycinnamic acids, ferulic and p-coumaric acids, and three aldehydes, vanillin (4-hydroxy-3-methoxybenzaldehyde), protocatechuic aldehyde (3,4-dihydroxybenzaldehyde) and syringic aldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde). Other phenolic compounds were also identified in subW extracts at 160 °C, such as catechin (112 ± 3 µg/$g_{dry-BSG}$), vanillic acid (17.9 ± 0.4 µg/$g_{dry-BSG}$), 4-vinylphenol (19 ± 5 µg/$g_{dry-BSG}$) and 4- vinylguaiacol (56 ± 2 µg/$g_{dry-BSG}$), but they could not be determined at 185 °C. Higher release of hydroxycinnamic acids was found at 160 °C than at 185 °C. On the contrary, the highest concentration of aldehydes was determined at 185 °C. In a previous work, the release of individual phenolic compounds was determined by UAE and chemical/enzymatic hydrolysis (Alonso-Riaño et al., 2020). A comparison of these techniques with the results obtained by subW yielded the following conclusions. Among these techniques, the highest amount of vanillin was achieved by subW, 306 ± 16 µg/$g_{dry-BSG}$ and 254 ± 5 µg/$g_{dry-BSG}$, at 185 °C and 160 °C respectively, followed by enzymatic hydrolysis with 6% of xylanase (g enzyme/$g_{dry-BSG}$). Doctor, Parker, Vang, Smith, Kayan and Yang (2020) studied the stability of the vanillin under subW conditions and reported that no degradation of vanillin took place up to 200 °C. However, subW was not as effective as basic hydrolysis to release hydroxycinnamic acids at the operation conditions used in this work. By basic hydrolysis, 1305.7 ± 0.5 µg/$g_{dry-BSG}$ and 538 ± 4 µg/$g_{dry-BSG}$ of ferulic and p-coumaric were obtained, respectively. These values were higher than the highest level obtained at 160 °C by subW (see Fig. 3c). In any case, it

must be highlighted, that neither chemicals nor enzymes are needed by subW treatment. At subW conditions, the ionic product of water increases making subW appropriate for hydrolysis reactions. Hydroxycinnamic acids are directly esterified or etherified to the lignin surface, so, higher temperatures could be necessary to improve the hydroxycinnamic acids extraction. However, higher temperatures could lead to degradation of these compounds. Fabian, Tran-Thi, Kasim and Ju (2010) reported that thermal decomposition of ferulic and coumaric acids started at about 172 °C and the amount extracted from defatted rice bran started to decrease at 175 °C in the study of the release of phenolic acids from defatted rice bran by subW treatment. In this work, a similar behaviour was observed since higher content of these two phenolic acids was determined at 160 °C than a 185 °C.

It must be highlighted that in the subW extracts from BSG, the total amount of individual phenolic compounds that could be successfully determined was 1.21 mg/$g_{dry\text{-}BSG}$ and 0.82 mg/$g_{dry\text{-}BSG}$ at 160 °C and 185 °C, respectively. On the other, hand, 23.01 mgGAE/$g_{dry\text{-}BSG}$ and 33.03 mgGAE/$g_{dry\text{-}BSGy}$ were obtained by the Folin–Ciocalteu method, at 160 °C and 185 °C respectively. These differences could be attributed to the fact that some of the individual phenolic compounds could not be properly identified by our system due to lack of standards. Furthermore, subW extracts contain carbohydrates, proteins and amino acids, which are considered as interfering substances for TPC analysis. Additionally, Maillard reaction products showed signal at Folin–Ciocalteu analysis as previously shown. This behaviour was also observed in other subW systems in the literature. Fabian et al. (2010) also reported a significant difference between TPC, as determined by the Folin–Ciocalteu method (with the highest value of 19.48 g GAE·(kg bran)$^{-1}$ at 200 °C) and by the HPLC analysis, showing also a different trend with temperature by both methods. The highest HPLC values reported by Fabian et al. (2010) was 2 g (kg bran)$^{-1}$ at 175 °C, and an abrupt decrease at 200 °C, 0.38 g (kg bran) $^{-1}$, was observed.

3.5. Organic carbon in the subcritical water extracts

The total organic carbon (TOC) presented in the subW extracts has been plotted in Fig. 4. TOC extraction rate and yield increased with temperature, although, at 160 °C and 185 °C, the same final TOC value was achieved. TOC would include monomer and oligomer carbohydrates, but also protein and amino acids and their degradation products, such as different organic acids (Kang et al., 2001). Degradation of carbohydrates and proteins can release at high temperatures NH_3 and CO_2 or other gas products that would led to similar TOC yield at 185 °C and 160 °C since gas products were not determined in this work.

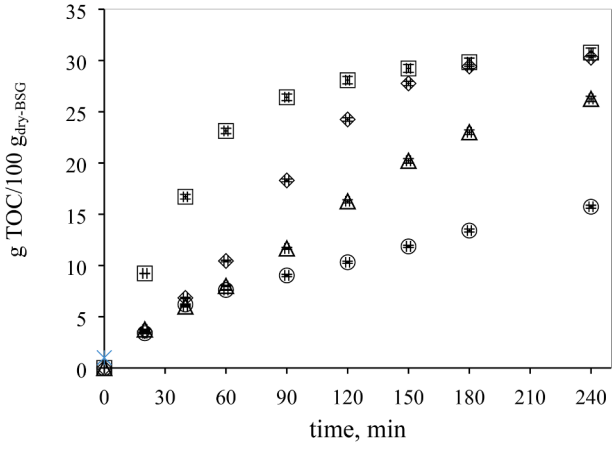

Fig. 4. Accumulative total organic carbon (g TOC/100 $g_{dry.BSG)}$ on subW extracts at different temperatures (○ 125 °C, △ 145 °C, ◇ 160 °C and □ 185 °C), F = 4 mL/min.

3.6. Solid residue after subW treatment

The total extraction yield was evaluated according to Equation 7:

$$Yield(\%) = \frac{w - w_i}{w} \cdot 100 \quad (7)$$

where w is the weight of the dry BSG placed into the reactor and w_i is the weight of the dry solid residue remaining after the subW treatment. The results are collected in Table S-3 and show significantly increasing values of the total extraction yield with temperature from 50% to 81% at 125 and 185 °C, respectively. This result agrees with the increase of TOC and the protein fraction in the subW extracts with temperature.

The elemental composition of the solid residues after extraction was listed in Table 2. The C content in the solid residue increased with temperature, while the oxygen content decreased; therefore, the molar ratio O:C decreased from 0.63 to 0.37 by increasing temperature. The decrease of the O:C ratio is associated with a decrease in the amount of OH, carboxyl (COOH) and carbonyl (C═O) groups. According to Jackowski et al (2019), hydrothermal carbonization (HTC) treatment made BSG much more similar to hard coal with higher HHV (26.5 MJ/kg) and C content (58.6% w/w), as well as lower O:C ratio, from 0.65 to 0.37, after HTC. In this work, HHV also increased by increasing temperature with a maximum value of 26.04 MJ/kg at 185 °C. The values of both H:C and N:C ratios in the solid residue decreased gradually with temperature. This trend suggests that carbon and nitrogen components of the solid have gradually moved to the aqueous phase in the form of their hydrolysis products or their decomposition products (Abdelmoez & Yoshida, 2013)

Table S-3 also shows the mass balance for C and N. C_{inlet} and N_{inlet} have been calculated from the elemental composition of the BSG and C_{outlet} and N_{outlet} from the C and N remaining in the solid residues (see Table 2) and the C and N present in the subW extracts (Fig. 1a and 4). The mass balance deviation for C and N was calculated according to the Equation 8:

$$C(or\ N)balance = \frac{C(or\ N)_{outlet}}{C(or\ N)_{inlet}} \cdot 100 \quad (8)$$

In general, mass balances present deviations around 10% that can be considered acceptable mass balance errors in a semi-continuous system. It must be also highlighted that at severe operation conditions, high temperature and long residence time, part of the biomass may also be converted to volatile compounds that were not measured in this work.

4. Conclusions

SubW treatment was confirmed as an efficient extraction/hydrolysis method to recover the protein fraction of the BSG generated in a local brewery factory. Around of 78% of the protein fraction was recovered by working in a semi continuous fix-bed reactor at 185 °C and 4 mL/min. Polar amino acids showed a maximum at lower temperature level than non-polar amino acids. Therefore, fractionation of bioactive compounds can be achieved by working under different conditions. This fact was also observed for phenolic compounds. Hydroxycinnamic acids were more sensitive to temperature than aldehyde phenolic compounds. Subcritical water is a suitable technology to extract and fractionate the different biocompounds extracted from BSG allowing to incorporate this by-product within the biorefinery concept.

CRediT authorship contribution statement

P. Alonso-Riaño: Investigation, Formal analysis, Writing - original draft. **M.T. Sanz:** Writing - original draft, Supervision. **O. Benito-Román:** Writing - review & editing. **S. Beltrán:** Investigation. **E. Trigueros:** Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.129264.

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