Pressurized liquid extraction of brewer's spent grain: kinetics and crude extracts characterization

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Abstract

In this study, extraction yield of valuable compounds from brewer's spent grain (BSG) using pressurized liquid extraction (PLE) was investigated varying temperature, solvent type, and flow rate at a constant pressure. The results were compared with Soxhlet extractions using the total phenolic compounds (TPC), total flavonoid compounds (TFC), antioxidant activity (AA), reducing sugars (RS), and total reducing sugars (TRS) as indices. The highest PLE extraction yield was 20.1 wt%, at 120°C, 2 mL/min using ethanol to water volume ratio of 0.5, at 10 MPa. The TPC, TFC, and AA content were favored by water and water/ethanol extractions and temperature increase. The highest AA was obtained with water at 120 °C and 4 mL/min achieving 9944, 4769 and 4096 µmol TE/100 g of BSG extract, respectively. PLE was capable of present high extraction yields maintaining the RS and TRS in the BSG matrix. In addition, BSG was defatted with compressed propane before the extraction with PLE, showing that

compressed propane is highly efficient in recovering the lipid fraction from BSG. The results demonstrated a great potential of water and different EtOH/Water solvent ratio at pressurized conditions for obtaining bio-compounds of BSG in a crude extract with expressive biological activity.

1. Introduction

One of the most significant challenges of the 21st century is the sustainable reuse and valorization of biomass from agro-industrial residue. Food and beverage industries are responsible for the production of large amount of residues, such as peels, seeds, pits, pulps, press cakes, and leaves that can be used as raw materials to obtain high added-value products [1]. Thus, agro-industrial residues' valorization has emerged as a critical strategy for an economic and environmental-friendly production within the concept of biorefinery [2]. In this context, the brewing sector with billions of liters of beer produced annually is responsible for large amounts of solid residues, among them brewer spent grain, solid hop residue, and surplus yeasts [3].

Brewer's spent grain (BSG) represents 85% of the by-products generated by the beer industry. It constitutes the solid residue from the initial stage of the brewing process, resulting from the grinding and barley grains cooking in the mashing stage. The residue consists of barley grains, husks, and endosperm, and it is classified as a heterogeneous material composed mainly of cellulose (12–25%), hemicellulose (19–42%), lignin (8–28%), proteins (14–31%) and extractable, such as lipids (5–13%) [4–8].

BSG is usually destined for animal feed due to its high nutritional value, low cost, and soil disposal. However, studies explored the possibility to enhance this residue as a matrix in the production of compounds such as xylitol, lactic acid and extractable bio-compounds such as sugars, proteins, antioxidants, phenolics, flavonoids, vitamins, and minerals for chemical, pharmaceutical, and food industries [3,9,10].

Studies have described the extraction of bio-compounds from BSG by conventional solid-liquid extraction processes, such as maceration, infusion, and Soxhlet extraction [9–11]. However, alternative extraction methodologies based on compressed fluids have been proposed to efficiently obtain and recovery of different

compounds, such as extraction of spent coffee grounds oil using high-pressure CO_2 [12], high-pressure extraction of caffeine [13], pressurized hot water extraction of pectin [14], pressurized liquid extraction combined with dispersive liquid–liquid micro extraction of endocrine disrupting compounds from cheese [15], compressed extraction of total phenolics and flavonoids content in hops [16].

Pressurized liquids are widely used in obtaining bio-compounds from natural sources and in the thermochemical fractionation of lignocellulosic biomass. At high temperatures, the solvents used in PLE have unique properties due to the change in the dielectric constant, density, viscosity, and diffusivity due to intermolecular forces' rupture, such as van der Waals, hydrogen bonds, and dipole interactions. High pressures are used to maintains the solvent at a liquid state while temperatures over the solvent boiling point are applied. However, several studies indicated that pressure had no significant effects on extract yields and bioactive compounds recovery. Even so, pressures between 5 to 10 MPa are applied to reduces the occurrence of air bubbles inside the solid matrix, increasing the analyte solubility and desorption kinetics [17–19]. Moreover, at high temperatures and elevated pressures, the mass transfer is improved, the solubility and diffusivity of solutes are increased causing the analyte-matrix bonds to break, and the surface tension of solvents and viscosity decrease allowing the solvent to penetrate the solid matrix easily, solvating the components of interest and accelerating the extraction rates [19–21]. The most used fluids are subcritical water and pressurized aqueous ethanol (PAE) since they do not generate residues from the extracts' neutralization, they are non-toxic and allow the preservation of chemical and thermolabile bio-compounds. Moreover, the combination of these two polar solvents provides a solvent-medium with thermodynamic excess properties (e.g., excess volume of mixture (density), dielectric constant, viscosity, etc.) that favor the interaction and

extraction of different classes of compounds when compared to the extracts obtained using the stand-alone solvents.

Alonso-Riaño et al. [22] proposed subcritical water as a hydrolytic medium to recover and fractionate the protein fraction and phenolic compounds from craft BSG, varying the temperature from 125 to 185 ºC at a constant flow rate of 4 mL/min. They obtained 78 % as the maximum yield of solubilized protein at 185 ºC and phenolic recovery, while the maximum level of free amino acids was reached at 160 ºC with a value of 55 mg free amino acids/$g_{protein-BSG}$. Torres-Mayanga et al. [23] studied the production of C-5 sugars from the BSG hydrolysis, varying the reaction temperature (140, 160, 180, and 210 °C), flow rate (10 and 20 mL min^{-1}), and the solvent/feed ratio (S/F: 64, 80 and 112), and they obtained a maximum yield of reducing sugars of 5.84 g per 100 g of feed and the maximum total reducing sugar yield of 35.11 g per 100 g of feed, at the optimal operating conditions of 210 °C, 20 mL min^{-1} water flow rate, and S/F of 64. Those authors reported that arabinose was the most abundant identified sugar product, with a maximum yield of approximately 3.1 g per 100 g of feed. Benito-Román et al. [6] evaluated pressurized aqueous ethanol extraction of β-glucans and phenolic compounds from waxy barley, under conditions of temperature (135–175 °C), extraction time (15–55 min), and ethanol content (5–20%) and they obtained 51% β-glucan extraction yield with a molecular weight of 500–600 kDa and 5 mg GAE/ g barley at mild conditions of 151 °C, 21 min of extraction using 16% ethanol in water. Therefore, the literature has reported an excellent potential for pressurized water and ethanol to obtain different add-value products from BSG. However, different parameters as solvent concentration, extraction kinetics, and the characterization of a broader class of compounds still need to be studied. In addition, as the BSG presents a high content of lipids, around 5-13% as above, a fractionating extraction strategy using a first extraction

stage with a nonpolar solvent can be interesting to produce crude extract fractions, e.g., lipid fraction and a phenolics-rich fraction that can be used in different industries with different and specific applications. Compressed propane has been successfully applied to extract lipids from different raw materials producing solvent-free crude extracts and allowing fast extraction rates [24–27]. However, to the best of our acknowledge, BSG defatting with compressed propane has not been reported in the literature. Thus, it was considered a valuable comparison to study PLE extractions of BSG using compressed propane as a prior defatting step.

This study aims to recover a broader class of compounds from BSG evaluating different extractions parameters using water, ethanol, and different ethanol to water ratios as pressurized solvents for the best operating condition to achieve the highest extraction yield and compounds recovery. In addition, compressed propane is also evaluated as an initial step to recover the lipid contend in the BSG. Total phenolic compounds (TPC), total flavonoids compounds (TFC), antioxidant activity (AA), reducing sugars (RS), and total reducing sugars (TRS) were also quantified in the BSG extracts to evaluate the potential use of the PLE technique to recovery from BSG compounds with bioactive properties.

2. Materials and methods

2.1. Raw material and sample preparation

Brewer's spent grains was provided by the OPA Bier microbrewery (Joinville, Santa Catarina, Brazil), with initial moisture and volatile compounds of 80.5 ± 0.1 wt%.

The raw material (BSG) was oven-dried with forced air circulation at 45 °C for 24 h until constant weight reaching the final moisture and volatile content of 4.9 ± 0.1 wt%. The average particle size was estimated using a Tyler series sieves in a vertical vibratory sieve shaker following the method presented by Gomide [28]. The milled weight material retained in each sieve was: mesh 8 (6.7 ± 0.8%), mesh 10 (24.6 ± 1.2%), mesh 14 (27.4 ± 0.2%), mesh 20 (30.0 ± 0.9%), mesh 28 (7.8 ± 0.5%), and mesh 35 (2.3 ± 0.3%). The different material fractions were then mixed and packed in polyethylene vacuum bags and stored at -4 °C until use.

2.2. Chemicals

All reactants and solvents used in this work were used as received. Analytical regents gallic acid, catechin, glucose, Trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid), Folin-Ciocalteu, ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-tri(2-pyridyl)-s-triazine), DNS (3,5-dinitro salicylic acid), MSTFA (n-trimethylsilyl-n-methyl trifluoroacetamide) and pyridine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sulfuric acid (98% purity) was purchased from Vetec. Ethanol (99.8 % purity), acetone (99.5% purity), ethyl acetate (99.5% purity) and n-hexane (99.5% purity) were purchased from Neon (Suzano, SP, Brazil). Deionized water was produced by a hermetic deionizer (Union, Model 50 L/hour).

2.3. BGS composition

The moisture, ash, total extractives, structural carbohydrates fractions, and lignin content were determined based on the methodologies recommended by the National Renewable Energy Laboratory (NREL). The moisture content was determined gravimetrically by oven-dried drying the samples at 105 ºC for 3 hours based on the NREL/TP-510-42621 [29]. The ash content was obtained by dried sample incineration at 575 ± 25 °C for 4 h based on the NREL/TP-510-42622 [30]. Total extractives in BSG were determined by sequential Soxhlet extractions with deionized water and 95 % ethanol based on the NREL/TP-510-42619 methodology [31], in which two consecutive extractions of 6 hours each are performed using the same raw material sample.

The structural carbohydrates fractions and lignin content were determined from the hydrolysis of BSG using sulfuric acid 72 % based on the NREL/TP-510-42618, proposed by Sluiter et al. [32].

2.4. Soxhlet extractions

Soxhlet extractions were performed to determine the total extractable solutes content, and the results were used as a reference in choosing the best extraction solvent for the pressurized fluid extractions. The selected solvents for the extractions were deionized water, ethanol, ethyl acetate, *n*-hexane, and acetone. The Soxhlet apparatus was loaded with 3 g of dried BSG and 175 mL of solvent, and the extractions were performed for 6 h. The solution was evaporated in a rotary vacuum evaporator (IKA, Model RV 10 digital) and then dried in an air circulation oven (IKA, Nova Ética, Model 400-2) until constant weight at 60 °C. The extracts were stored in amber flasks and kept over refrigeration at -4 °C. Sequential Soxhlet were also performed, and the sequence of solvents was water followed by ethanol, and in the opposite - ethanol followed by

extraction with water. The extraction yields (%) was calculated as (mass of extract (g) / mass of BSG sample (g)) x 100.

2.5. Pressurized liquid extraction (PLE)

Pressurized liquid extractions of BSG were performed in a semi-continuous system, which was presented in previous works [14,33]. Briefly, the experimental setup is composed of a jacketed extraction vessel of 20.44 cm^3 internal volume (17.2 cm height and 1.24 cm in diameter) with an electrical-heating and thermocouples and temperature controller. The solvents were pre-heated by a coil system inserted in an oven with electrical heating. Stainless steel filters with pore sizes of 0.5 mm were placed at the inlet and the reactor's outlet to minimize particle spillover. A high-pressure liquid pump (Eldex, model 2SM, EUA) was used to pressure the system and transfers the solvent in a constant volumetric flow through the extractor. The pressure in the extractor was controlled by a backpressure valve (Swagelok, model KPB1SOA) and a manometer located at the outlet of the extractor. The stream from the extraction vessel was cooled to 40 ℃ by cool water refrigeration system composed of a jacketed vessel and a thermostatic bath, and it was sampled periodically at the outlet of the backpressure.

Deionized water, ethanol, and a hydroalcoholic solutions at ethanol to water volume ratios of 0.25, 0.50, and 0.75 were used in pressurized liquid extractions at temperatures of 60 to 120 ℃. The experiments were performed at a constant pressure of 10 MPa. Previous works indicate that pressure is a minor effect variable on removing bio-compounds from plant matrices; thus, the selected pressure needs to ensure that the solvent remains in its liquid state throughout the extraction [19,34,35]. The extraction

1 chamber was filled with approximately 6 g of BSG. The filled extractor was kept

2 pressurized and static for 15 min before the dynamic extraction period to assure thermal

3 and mechanical equilibration of the system. The dynamic extractions were performed

4 with constant solvent flow of 2, 4, and 6 mL/min. The extracts were collected in 50 mL

5 round-bottom volumetric flasks periodically to build the overall extractions curves

6 during 60 min of extraction. Approximately 5 mL of solution was collected and stored

7 in amber flasks at -4 °C for analysis. The remaining amount of solution was oven-dried

8 with forced air circulation at 60 ºC for ethanol, and 80 ºC for aqueous solutions, until

9 constant weight.

10

11 2.5.1. Experimental design and statistical analysis

12

13   Pressurized liquid extractions were performed considering three variables,

14 temperature (ºC) (X_1), ethanol to water (EtOH/Water) volume ratio (X_2), and solvent

15 flow rate (mL/min) (X_3) at two levels: X_1: 60 to 120 ºC, X_2: 0 to 1, X_3: 2 to 6 mL/min,

16 respectively. The Box-Behnken (3^{k-p}) design was adopted for extractions with water,

17 ethanol, and EtOH/Water corresponded to a total of 15 experiments with triplicate at the

18 central point, as presented in Table 1. The experiments were conducted randomly. As

19 pointed out, the minimum and maximum temperature levels were 60 and 120 ºC. This

20 temperature range was established considering the possibility of obtaining higher yields

21 of extracts and preventing their degradation and the hydrolysis of BSG. According to

22 Bubalo et al. [36], it is possible to obtain extracts rich in various phenolic groups, such

23 as coumarins, cinnamic acids, quinones, flavonoids lignans by pressurized fluids under

24 conditions of 60 to 280 ºC and 10 to 103 bar. However, temperatures above 140 °C

1   might initiate the plant material hydrolysis, and temperatures above 280 °C the

2   degradation of thermolabile compounds.

3

4   **Table 1**

5   Box-Behnken factorial design of experiments with independent variables and their

6   levels with a central point (C) for pressurized liquid extractions.

Run	Coded levels			Actual levels		
	X_1	X_2	X_3	Temperature (ºC)	EtOH/Water	Flow rate (mL/min)
1	-1	-1	0	60	0,0	4
2	+1	-1	0	120	0,0	4
3	-1	+1	0	60	1,0	4
4	+1	+1	0	120	1,0	4
5	-1	0	-1	60	0,5	2
6	+1	0	-1	120	0,5	2
7	-1	0	+1	60	0,5	6
8	+1	0	+1	120	0,5	6
9	0	-1	-1	90	0,0	2
10	0	+1	-1	90	1,0	2
11	0	-1	+1	90	0,0	6
12	0	+1	+1	90	1,0	6
13 (C)	0	0	0	90	0,5	4
14 (C)	0	0	0	90	0,5	4
15 (C)	0	0	0	90	0,5	4

7

8

9   The extraction yield results were statistically evaluated by analysis of variance

10   (ANOVA) at a 95% level of confidence using the Statistica 10 software (Statsoft Inc.,

11   USA) to identify the variables' effect and their significance in the extraction of BSG by

12   PLE.

13

14   2.6. Characterizations of BSG extracts

15

16   The BSG extracts obtained in this work (approximately 50 mg) were firstly

17   solubilized using 2 mL of methanol 80 % (v/v). The methanol phase was analyzed by

18   spectrophotometric methods to determine the total phenolic compounds (TPC), total

flavonoid compounds (TFC), and antioxidant activity (AA). The absorbance was measured in a spectrophotometer (UV-Vis Global Analyzer; model GTA97). All analyses were replicated (2 samples of the same extract), and at the central point three replicates of experiment were performed to access the uncertainty of the experimental procedure.

2.6.1. Total phenolic compounds (TPC)

The TPC was determined by the Folin-Ciocalteu reagent as described by Singleton et al. [37]. 0.1 mL of extract solution, prepared as described above, and 0.4 mL of methanol, was mixed with 2.5 mL of Folin-Ciocalteu reagent (diluted 1:10 in distilled water) and was kept in the darkness for 3 min. Afterward, 2 mL of 7.5 % sodium carbonate were added, and the mixture was incubated in the dark for 120 min. Then, the absorbance was measured at 760 nm. The quantitative results were calculated using a standard curve of gallic acid and expressed as mg of gallic acid equivalent per 100 g of sample (mg GAE/100 g).

2.6.2. Total flavonoid compounds (TFC)

TFC present in extracts was determined based on the method proposed by Zhishen et al. [38]. Aliquots (0.1 to 0.3 mL) of samples, prepared as previously described, and methanol up to 0.4 mL, 1.6 mL of distilled water, and 0.12 mL of $NaNO_2$ (5 % w/v) were added to amber flasks and mixed. After 5 min, 0.12 mL of $AlCl_3$ (10 % w/v) was added; and after 5 min, 0.8 mL of NaOH (1 mol·L^{-1}) and 0.96 mL of distilled water were added. The absorbance of mixture was measured at 510 nm. The catechin

1 was used as the reference, and the results were expressed as mg of catechin equivalent

2 per 100 g of sample (mg CE/100 g).

3

4 2.6.3. Antioxidant activity (AA)

5

6 The antioxidant activity was performed by three methods, using the extract

7 solubilized in a methanol:water solution (80% v/v). All AA results were expressed as

8 µmol of Trolox equivalent (TE) antioxidant capacity per 100 g of sample (µmol TE/100

9 g). The ABTS radical cation decolorization assay was performed based on the

10 procedure described by Re et al. [39]. The DPPH radical scavenging assay was based on

11 the method proposed by Brand-Williams et al. [40]. The ferric reducing antioxidant

12 power (FRAP) assay was conducted according to Benzie and Strain [41]. More details

13 for AA determinations were previously described by Fetzer et al. [27].

14

15 2.7. Reducing sugar

16

17 The reducing sugars (RS) and total reducing sugars (TRS) content present in

18 extracts were determined by the DNS colorimetric method [42]. The extracts were

19 solubilized with distilled water, reacted with DNS reagent in a thermal bath at 100 °C

20 for 5 min. After the reaction, the mixture was cooled to room temperature and the

21 absorbance was measured at 540 nm. TRS was determined after acid hydrolysis with

22 hydrochloric acid (37 % w/w) at 60 °C for 20 min and neutralized with sodium

23 hydroxide (6 mol·L^{-1}) [23]. The quantification of RS and TRS was performed using a

24 calibration curve elaborated with glucose. Results were expressed as g of glucose

25 equivalent per 100 g of sample (g GE/100 g).

1

2 2.8. GC-MS analysis of Soxhlet extracts

3

4   The Soxhlet extracts were analyzed by GC-MS to identify compounds present in

5 the BSG extracts with different solvents. Firstly, the extracts were derivatized as

6 follows: 5 mg of samples were dissolved with 200 μL of pyridine and mixed with 200

7 μL of MSTFA and maintained at 30 °C by 20 min. A solution was made up to 1 mL

8 with dichloromethane and injected in a gas chromatograph. The scanning of Soxhlet

9 extracts was analyzed using VF-5MS column (30 m × 0.25 mm; 0.25 μm) in a

10 Shimadzu QP2010 SE gas chromatograph followed by detection through mass

11 spectrometry operating with electron impact ionizing source at 70 eV. The equipment

12 was operated in split ratio mode (10:1) with a 1 μL volume, which was injected into a

13 helium gas with total flow of 19.5 mL/min. The injector and mass spectrometer

14 interface were set at 200 °C and 260 °C, respectively, and the column temperature

15 program started at 60 °C, remained for 2 min, followed by heating at a rate of 4 °C/min,

16 up to 100 °C and remaining for 4 min. At this same rate, it reached 180 °C and at a rate

17 of 15 °C/min it reached 250 °C, after which was maintained isothermally during 10 min,

18 resulting in 51 min of analysis. The mass spectra were collected 4 min after to start the

19 chromatographic analysis and every 0.3 s in the range of 70-1000 m/z. The

20 identification of compounds was carried out with the mass spectra of each peak

21 considering the NIST (National Institute of Standards and Technology) library database.

22

23

24 **3. Results and discussion**

25

3.1. BSG characterization

Table 2 shows the characterization of BSG raw material. The ash content in the BSG is comparable to the range of 3-5 % reported in the literature [23,43,44]. The glucan content (mainly cellulose) of BSG was 25.4 ± 1.3 wt%, similar to values found by Paz et al. [45], who quantified the glucan content for two varieties of BSG obtaining a result in the order of 25 %. Hemicellulose quantified was 21.2 ± 1.2 wt%, also in agreement with the data presented by Paz et al. [45]. In addition to hemicellulose, also xylose and arabinose fractions observed in this study are in agreement with the literature [45].

Measured insoluble and acid-soluble lignin levels in the BSG raw material were 12.3 ± 1.0 wt% and 6.0 ± 0.3 wt%, respectively. These values are comparable to the range of 10.11 to 13.12 % for insoluble lignin and 3.69 to 6.10 % for soluble lignin, as reported in the literature [23,43,44].

Table 2

Composition of brewer's spent grain (BSG) raw material used in this study.

Component	Content (wt%)
Moisture	4.9 ± 0.1
Ash	3.3 ± 0.1
Protein	---
Glucose	25.4 ± 1.3
Hemicellulose	21.2 ± 1.2
Xylose	14.3 ± 0.8
Arabinose	6.9 ± 0.4
Acid insoluble lignin	12.3 ± 1.0
Acid soluble lignin	6.0 ± 0.3
Water extractives	9.4 ± 0.5
Ethanol extractives	11.1 ± 0.3

BSG composition was expressed in wt% (mean ± standard deviation) based on triplicate experiments.

Also, the BSG samples used in this work presented an average particle diameter of 1.2 ± 0.4 mm, according to the particle diameter profile, and real density of 1.368 g/cm³ ± 0.001 with an apparent density of 0.232 g/cm³ and porosity of 0.830.

3.2. Soxhlet extraction and extract characterization

Soxhlet extractions were performed as a preliminary assay to compare extraction yields and extract characterization with the PLE method proposed in this study. The overall Soxhlet yields were obtained after 6 h, using different solvents with different polarity indexes as presented in Table 3. As expected, the yield was higher following the increase in the solvent polarity; water followed by ethanol in a sequential extraction (S1–2) presented the highest extraction yield, 9.6 ± 0.6 wt%, and 10.5 ± 0.1 wt%, respectively. The extraction yield was statistically different using ethanol followed by water (S3-4), indicating that performing extraction with ethanol, different amounts of compounds can be recovered from BSG with these two polar solvents in Soxhlet. The extraction yield using water was higher than the value reported by Torres-Mayanga et al. [23] (5.7 ± 0.3 wt%); however, the yield obtained with ethanol in the present study was lower than the value reported by those authors [23] (13.6 ± 0.2 wt%) and also by Paz et al. [43] (14.00 ± 0.10 wt%). In sequential extractions (S1–2 and S3–4), the yield using water decreased around 1.9 points percent (p.p.) after the extraction with ethanol as the first solvent. In vegetable matrices rich in polysaccharides, such as BSG, with dehydration by ethanol such polysaccharides tend to condense forming a hard coating around each plant cell wall microfibril, making rehydration difficult and thus the extraction of some components is harrowing [46].

Table 3

Soxhlet extraction yields by different solvents.

Extraction	Solvent	Polarity[#]	Extraction cycles	Yield (%) *
S1-2	Sequential Water and Ethanol	10.2 - 5.2	4 – 10	9.6 ± 0.6 - 10.5 ± 0.1
S3-4	Sequential Ethanol and Water	5.2 - 10.2	10 – 4	10.1 ± 0.3 - 7.7 ± 0.3
5	Ethyl acetate	4.3	16	7.6 ± 0.1
6	Acetone	5.4	28	6.6 ± 0.1
7	n-Hexane	0.0	18	6.3 ± 0.3

[#]Ref. (Byers, 2003); *Extraction yield expressed in wt% (mean ± standard deviation) based on triplicate experiments.

The extraction yield using acetone was 6.6 ± 0.1 wt%, a lower value than presented by Del Rio et al. [48], who performed the extraction for 8 hours and obtained 8.3 ± 0.1 wt%. Such differences can be explained because the total extraction time and the number of cycles performed during the extraction lead to differences in extractives yield and the chemical composition of BSG that changes according to the barley variety, harvest, malting, and mashing process [4,5]. The extraction with n-hexane reached 6.3 ± 0.3 wt%, and this value agrees with the literature, where values within 6.41 to 7.50% are reported [49,50]. The extraction with ethyl acetate provided a yield of 7.6 ± 0.1 wt%, which are, from our best knowledge, the first data reported in the open literature regarding the Soxhlet extraction of BSG raw material with ethyl acetate as solvent. It is worth mentioning that ethyl acetate is considered a GRAS solvent [36] and it might be used in substitution of either a non-polar or low polar solvent to recover solutes with the same characteristic from lignocellulosic material, as BSG.

The Soxhlet extract characterization is presented in Figure 1, all results were obtained in triplicate. The total phenolic compounds (TPC) are presented in Figure 1(A), wherein the TPC increased through increasing the solvent polarity. Hence the highest TPC value was obtained by water (827 ± 36 mg GAE/100 g of BSG extract),

followed by the ethanol (249 ± 10 mg GAE/100 g of BSG extract) and the lowest one obtained with *n*-hexane (21 ± 5 mg GAE/100 g of BSG extract). Also, for total flavonoid compounds (TFC), presented in Figure 1(B), water was responsible for the highest recovery, 341 ± 1 mg CE/100 g of BSG extract, followed by acetone and ethanol with TFC values of 67 ± 1 and 61 ± 1 mg CE/100 g of BSG extract, respectively.

The antioxidant activity (AA) was analyzed using the ABTS, DPPH, and FRAP methodologies, and its values are presented in Figure 1(C). The maximum AA value was obtained by extraction with water related to ABST (3249 ± 46 µmol TE/100 g of BSG extract) and FRAP methodologies (1762 ± 14 µmol TE/100 g of BSG extract). In addition to water, ethanol also showed significant AA results of 81 ± 5, 489 ± 19 and 1129 ± 10 µmol TE/100 g of BSG extract for ABST, DPPH, and FRAP, respectively. The antioxidant activity increased as the concentration of total phenolics and flavonoids increased, evidencing that the AA response is due to the presence of these components in the BSG extracts. The same effect was reported by Meneses et al. [51], who evaluated the efficacy of different solvents for extracting antioxidant phenolic compounds from brewer's spent grains.

Figure 1(D) depicts the results of reducing sugars (RS) and total reducing sugars (TRS). The highest RS and TRS were 28.6 ± 0.4 and 32 ± 1 g GE/100 g of BSG extract, respectively, obtained by water as the solvent. In comparison, ethanol extraction presented RS and TRS values of 16.4 ± 0.1 and 18 ± 1 g GE/100 g of BSG extract.
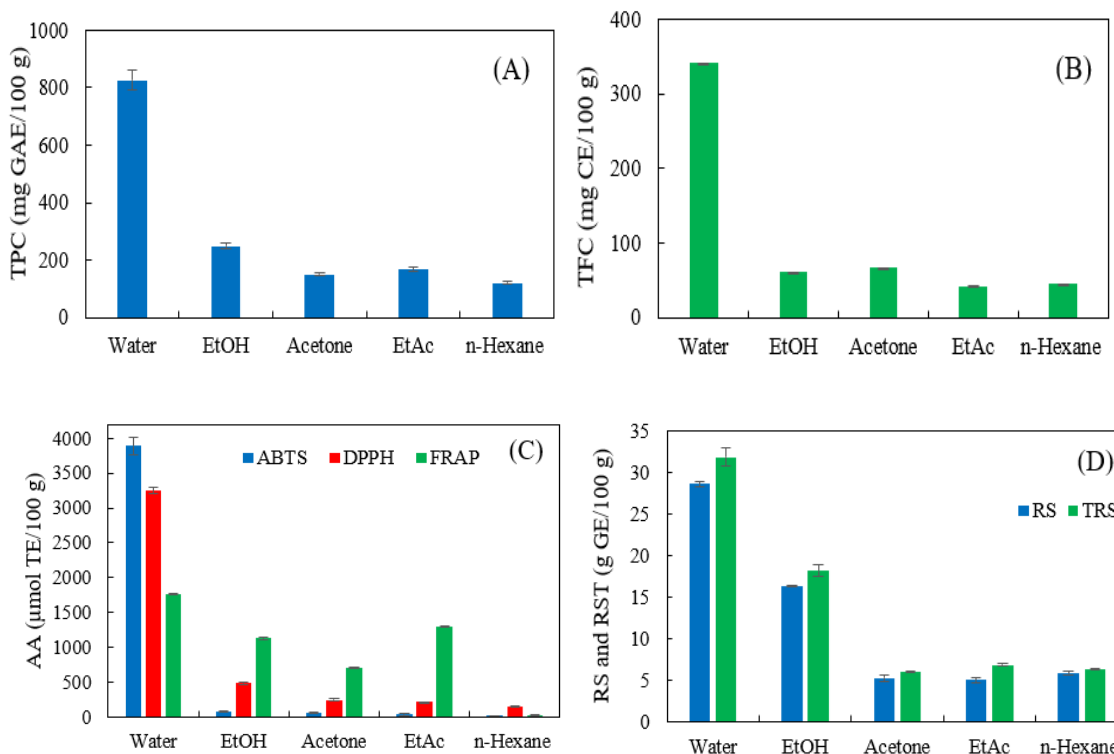
Fig. 1. Characterization of Soxhlet extracts obtained with different solvents. (A) Total phenolic compounds (TPC); (B) Total flavonoid compounds (TFC); (C) Antioxidant activity (AA); (D) Reducing sugars (RS) and total reducing sugars (TRS).

Table 4 shows the compounds identified by GC/MS for each extract obtained by Soxhlet using solvents with different polarities and physicochemical characteristics. The main compounds identified for water extracts are carbohydrates (D-erythrose, fructose, D-tagatofuranose, xylose, glucopyranose, maltose, D-turanose, and cellobiose). Some of these 'sugars' compounds are also present in extracts obtained by acetone, ethanol, and ethyl acetate. Lipids compounds have been identified when the solvent polarity decreases with *n*-hexane being the solvent that recovered exclusively lipid compounds, followed by acetone and ethyl acetate.

Figure 1 indicates that for all BSG extract characterization, increasing the solvent polarity also increases the amount of TPC, TFC, AA, and RS content. Besides

1   that, as previously highlighted and presented in Table 4, the reduction of solvent

2   polarity can favor the extraction of different classes of chemical compounds.

3

4   **Table 4**

5   Identified compounds in extracts obtained by Soxhlet with different solvents.

Extract	Water	Ethanol	Ethyl Acetate	Acetone	*n*-Hexane
	Propanoic acid	Lactic acid Propanoic acid	Propanoic acid	Propanoic acid	---
	---	---	---	Caproic acid	Caproic acid
	---	---	Myristic acid	Myristic acid	Myristic acid
	---	Palmitic acid	Palmitic acid	Palmitic acid	Palmitic acid
	---	Linoleic acid	Linoleic acid	Linoleic acid	Linoleic acid
	---	Stearic acid	Stearic acid	Stearic acid	Stearic acid
	---	Oleic acid	Oleic acid	Oleic acid	Oleic acid
	---	α-linolenic acid	α-linolenic acid	α-linolenic acid	α -linolenic acid
	---	---	11-eicosenoic acid	---	11-eicosenoic acid
	---	---	---	---	Behenic acid
	---	1-monopalmitin	1-monopalmitin	1-monopalmitin	1-monopalmitin
	---	---	1-monoolein	1-monoolein	1-monoolein
	D-erythrose	D-erythrose	D-erythrose	D-erythrose	---
	Fructose	---	Rhamnose	---	---
	Xylose	Xylose	---	---	---
	D-tagatofuranose	---	---	---	---
	Glucopyranose	---	---	---	---
	Maltose	Maltose	---	---	---
	D-turanose	---	---	---	---
	Cellobiose	Cellobiose	---	---	---
	D-mannitol	---	---	---	---
	D-arabinol	---	---	---	---

(Note: leftmost column vertical label "Compounds identified")

6

7   The recovery of those compounds is associated with the global Soxhlet

8   extraction yields since the highest yields were obtained by water and ethanol as

9   solvents. The results related to higher yields and higher biological activities for the BSG

10   extracts from Soxhlet were used to establish the solvents water and ethanol for the

extractions with pressurized liquids. Besides, these two solvents, water, and ethanol, are low-cost, non-toxic, environmentally friendly solvents and generally recognized as safe (GRAS) [36].

3.3. Pressurized liquid extractions

Pressurized liquid extractions (PLE) were performed with water, ethanol, and using mixtures of ethanol and water (EtOH/Water) at volume ratios of 0.25, 0.5, and 0.75. The overall extraction yields obtained with water, ethanol, and pressurized EtOH/water mixtures in their respective operating conditions of solvent ratios, temperature, and flow rates are presented in Table 5. The pressure was fixed at 10 MPa for all experiments, and the static extraction time of 15 min was set to assure the thermal and mechanical equilibrations, while the dynamic extraction time was set at 60 min. Runs 1 to 15 correspond to the experimental design described in section 2.5.2. The additional experiments 16 to 21 were added to complement the effect analysis of the variables on the extraction yield and other responses of interest in this study.

The highest extraction yield using pressurized water was 17.8 ± 0.3 wt% obtained at 120 ℃ and 4 mL/min (run 2). For pressurized ethanol, the highest yield was 11.6 wt% obtained at 120 °C and 2 mL/min (run 21), as well as for the mixture ethanol/water at a volume ratio of 0.5 EtOH/Water with the highest yield of 19.3 ± 0.8 wt% (run 6). All these results are higher than the yields obtained by Soxhlet extractions with the same solvents (water and ethanol). The condition 0.5 EtOH/Water, 120 °C and 2 mL/min, achieved around 97% of the yield obtained with the sequential Soxhlet extraction using water followed by ethanol (run S1−2, Table 2, a yield of 20.1 ± 0.6 wt%). However, the best result for pressurized water extraction, 17.8 ± 0.3 wt%, was

1 around 54% greater than the overall yield obtained with water in the Soxhlet apparatus

2 $(9.6 \pm 0.6$ wt%).

3

4 **Table 5**

5 Extraction yields obtained with different pressurized liquids at 10 MPa.

Run/Solvent	Solvent ratio (EtOH/Water)	Temperature (ºC)	Flow rate (mL/min)	Yield* (%)
Water				
1	--	60	4	6.6
2	--	120	4	17.8 ± 0.3
9	--	90	2	9.4
11	--	90	6	9.9
20	--	120	2	16.9
Ethanol				
3	--	60	4	7.3
4	--	120	4	11.5 ± 0.3
10	--	90	2	8.6
12	--	90	6	9.3
21	--	120	2	11.6
Ethanol/Water				
5	0.5	60	2	10.2
6	0.5	120	2	19.3 ± 0.8
7	0.5	60	6	11.4
8	0.5	120	6	19.4
13 (C)	0.5	90	4	13.0
14 (C)	0.5	90	4	12.7
15 (C)	0.5	90	4	12.4
16	0.5	80	2	11.1
17	0.5	100	2	13.6
18	0.25	120	2	18.6 ± 0.1
19	0.75	120	2	17.8 ± 0.7

6 (C) – central point; *Extraction yield expressed in wt% (mean ± standard deviation) based on triplicate
7 experiments.

8

9

10   A factorial design 3^2 was applied to evaluate the influence of extraction

11 variables, temperature, solvent ratio, and flow rate on the extraction yields of BSG

12 crude extracts at 60 min of extraction (Table 5). The Pareto chart of standardized effects

13 is shown in Figure 2, showing the significant effect ($p \geq 0.05$) of each variable studied

14 in this process.

15
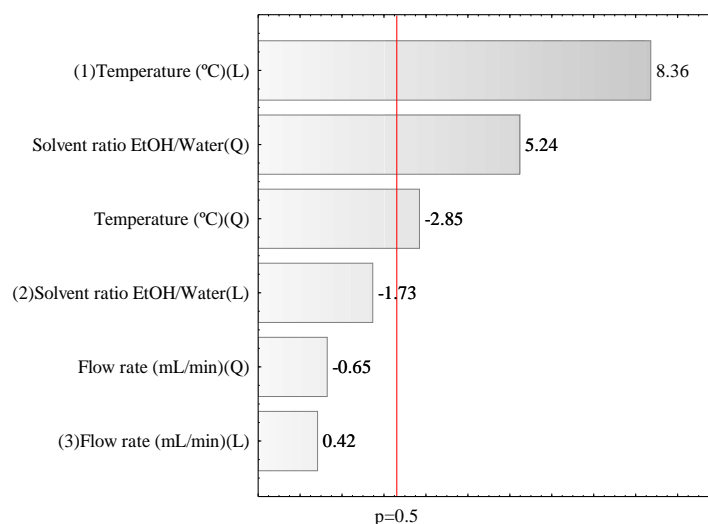
(L) – linear effects; (Q) – quadratics effects.

Fig. 2. Pareto chart for the extraction yield obtained in the pressurized liquid extractions.

The temperature had the most relevant effect, demonstrated by the 8.36 points percent (p.p.) when evaluating its linear effect. With the increase in temperature, there is a significant increase in the yield of extractions (as shown in Table 5), where the highest yields for the mixture EtOH/Water solvent was obtained at the highest operating temperature (120 °C). The solvent ratio EtOH/Water presented a decreasing linear effect of -1.73 p.p. related to the ethanol addition; hence the highest yield was obtained at 0.5 EtOH/Water, demonstrating that the combination of water and ethanol is efficient in recovering soluble compounds from BSG. The flow rate values used in this work (2 to 6 mL/min) had no significant effect, so the lowest flow rate was used to perform additional extractions with less solvent. Thus, the optimal extraction conditions for achieving maximum yield indicated by the statistical analysis were the temperature of 120 °C, a solvent ratio of 0.5 EtOH/Water, and a 2 mL/min flow rate.

1   For all evaluated conditions with different solvents (Table 5), the extraction

2   yield increased with temperature due to breaking of the hydrogen bonds and dipole-

3   dipole molecular interactions between the extractable compounds and the solid's

4   structure (vegetable matrix), thus reducing the activation energy required to their

5   desorption. Indeed, viscosity and surface tension of the solvents decrease with the

6   increase in the temperatures, enhancing the solvent transport into the matrix and

7   accelerating the dissolution of the soluble compounds (extracts). Therefore, the mass

8   transfer rate increases, resulting in higher overall extraction yields at higher

9   temperatures [52,53].

10   To better understand the kinetic behavior of the BSG extractions using

11   pressurized liquids, the overall extraction curves were measured at all conditions

12   presented in Table 5. Extractions using pressurized water as solvent are presented in

13   Figure 3(A), and pressurized ethanol in Figure 3(B), the yields of extractions at 120 °C

14   and 4 mL/min were obtained in triplicate. Figure 4(A)-(C) depicts the influence of

15   temperature, solvent flow rate, and the concentration of ethanol/water at different

16   conditions. For the extraction using pressurized water (Figure 3A), the comparison of

17   extraction curves at fixed solvent flow rate reveals that the initial extraction rate was

18   favored with increasing the temperature, e.g., results at 90 °C and 120 °C and fixed flow

19   rate at 2 mL/min (full red triangles and black circles, respectively), and curves at 60 °C

20   and 120 °C and flow rate at 4 mL/min (open blue squares and black circles,

21   respectively). In the same way, at a fixed temperature, the solvent flow rate also has

22   favored the initial extraction rate, indicating that the increase in solvent flow rate

23   favored the extraction of the easily accessible solutes, i.e., the compounds that were

24   obtained by breaking the cell walls when crushing and milling the raw material.

25   Moreover, the temperature contributed to an increase in the apparent solubility in

1 pressurized water, as the initial extraction rate systematically increased. For all

2 conditions presented in Figure 1(A), a constant extraction rate is observed until 8 min,

3 where the solubilization is the rate-limiting step. For the extraction at 60 °C and 90 °C, it

4 is observed that after 10 min the amount of the extract recovered rapidly decreased,

5 indicating that the diffusion rate has controlled the solute recovery from the raw

6 material until the end of the extraction (60 min), and also showing that at this condition

7 the process presented a very short time of falling extraction rate (FER) and reached the

8 diffusive controlled step (DC) after approximately 12 min.

9   On the other hand, at higher temperature (120 ºC) and solvent flow rate of 2 and

10 4 mL/min, the extraction followed a constant extraction rate-controlling step (CER) up

11 to the same time (around 8 min). However, different from the other conditions after this

12 constant rate step the extraction process followed to a long period still controlled by the

13 solubility of different compounds in water, showing that both FER and DC steps did not

14 end up in this process up 60 min of dynamic extraction at 120 ºC. This behavior is

15 related to the feature of the BSG, in which probably the analytes are in part adsorbed at

16 the surface by van der Waals forces, and some chemically bonded to the matrix.

17 Another factor affecting the extraction yield is the decrease in the water dielectric

18 constant, polarity, and solubility parameter as the temperature increases. Water at

19 temperatures above 100 ºC and below the critical temperature (374 ºC), under high

20 pressure, favors the extraction of polar and nonpolar compounds [36,54,55]. The

21 increase in temperature also decreases the water's surface tension, allowing the

22 compounds to dissolve more quickly, as the water can moisten more easily and

23 penetrate the solid matrix, enhancing the diffusion rate due to the decrease in viscosity

24 [36]. Therefore, these changes in water properties were responsible for increasing the

25 extraction yield from 6.6 wt% (60 °C and 4 mL/min) to 17.8 ± 0.3 wt% (120 °C and 4

mL/min). It also observed from Figure 3(A) that increasing the water flow rate, the

initial extraction rate increase, as earlier mentioned, confirming that the solutes are

strongly bonded with the matrix and required high activation energy to the overcome
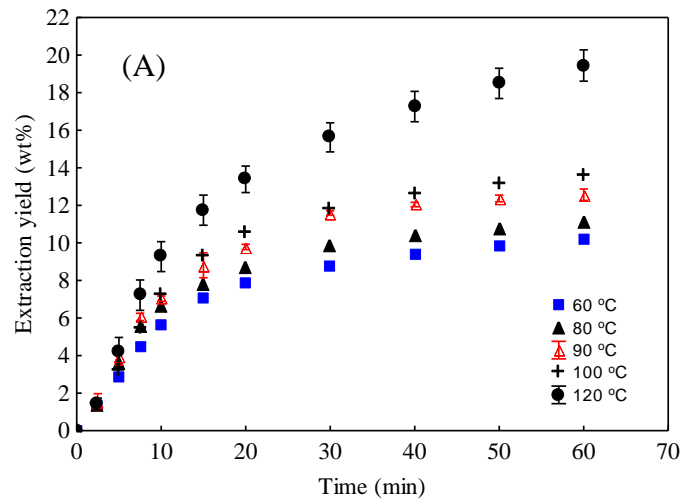
the desorption step.
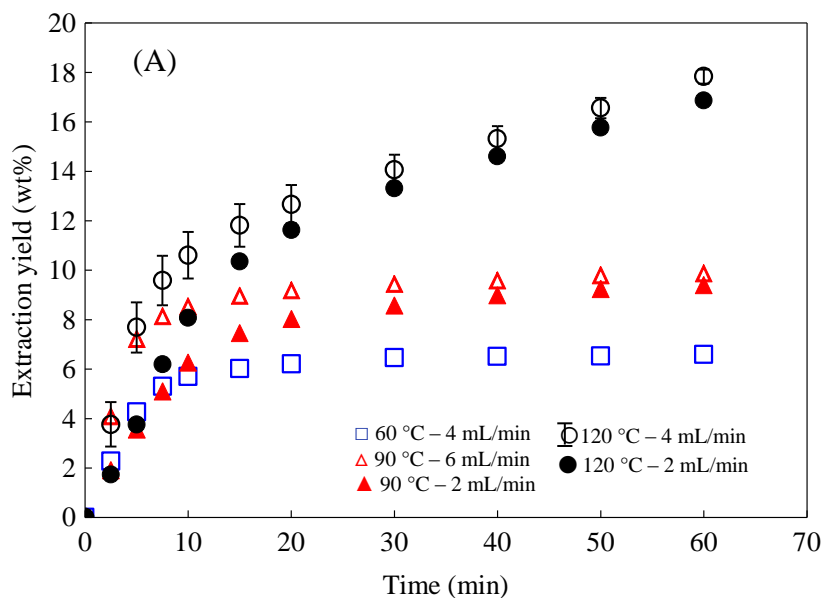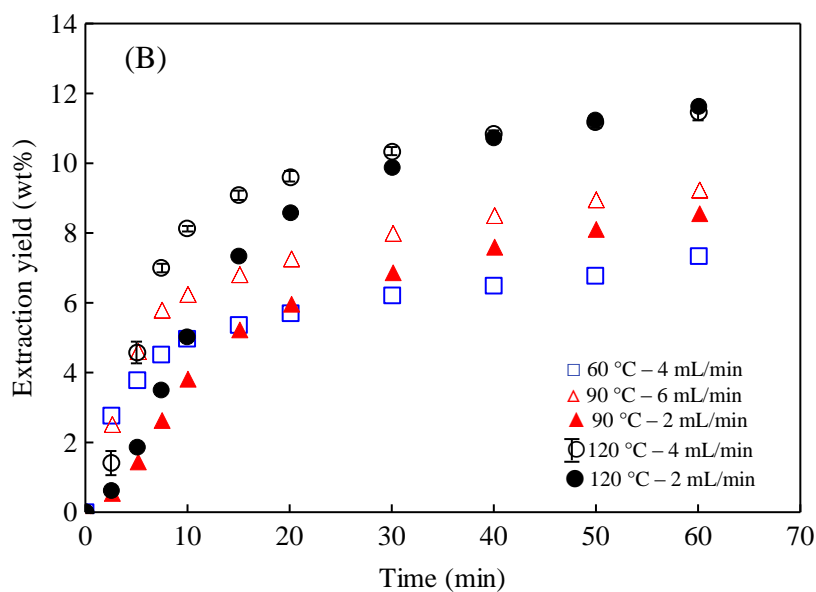
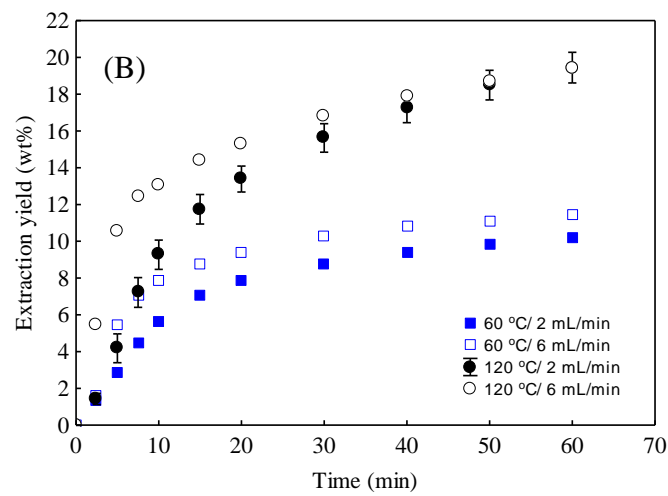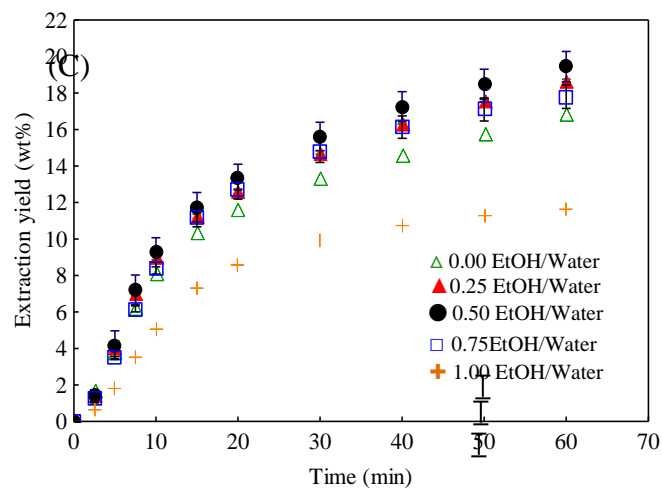**Fig. 3.** Overall extraction curves using pressurized liquids: (A) water; (B) ethanol.

1

2

3

4   **Fig. 4.** Overall extraction curves using mixtures of ethanol and water as pressurized

5   liquid solvent at different process conditions: (A) different temperatures at a fixed

6   solvent flow rate 2 mL/min and ethanol to water (EtOH/Water) volume ratio of 0.5; (B)

1 comparisons at different temperatures and solvent flow rates; (C) varying the solvent

2 concentrations, at 120 °C and 2 mL/min. All conditions were performed at 10 MPa.

3

4   For the extractions with pressurized ethanol, Figure 3(B), the temperature also

5 presented a positive effect in terms of the global extraction yield, and the kinetic

6 behavior was similar to those discussed about the water, where the increase in the

7 temperature and the solvent flow rate increased the initial extraction rates and yields.

8 However, using ethanol, the maximum extraction was lower than values found for

9 water, where the highest value for ethanol was 11.5 wt%, obtained at 120 °C and 4

10 mL/min; around 5 p.p. below the value obtained by water. These results suggest that the

11 ethanol presents lower cohesion forces and interaction with the solutes present in BSG.

12   Extractions using different solvent ratio of ETOH/Water are presented in Figure

13 4, the results expressed by whiskers were obtained in triplicate. Figure 4(A) depicts the

14 overall extraction curves for experiments at different temperatures using 0.5

15 EtOH/Water volume ratio with a fixed solvent flow rate of 2 mL/min, and Figure 4(B)

16 shows the comparison among different solvent flow rates and temperatures. The initial

17 extraction rate and the extraction yield increased from 60 ºC to 120 ºC and solvent flow

18 rate from 2 to 6 mL/min, with an expressive improvement from 100 to 120 °C

19 demonstrating that also for a mixture of solvents, both solubility and maximum yield

20 achieved were increased by increasing the energy available supplied to the system. It

21 can be noted that at 120 °C, the water fraction was responsible for the same effect

22 observed in pressurized water (Figure 3A) due to the decrease in the dielectric constant

23 and other molecular properties. Using a fixed mixture of ethanol and water, the

24 temperature effect combined with solvent ratio results in the highest pressurized liquid

25 extraction yield (19.4 ± 0.8 wt%), obtained at the condition of 0.5 EtOH/Water, 120 °C

and 2 mL/min. This response may be explained considering that mixtures of high and low polar solvents provide a suitable physicochemical medium for extracting a broader range of analytes [35]. The influence of solvent concentration is emphasized in Figure 4(C), where the effect of solvent ratio was evaluated at the best extraction condition (120 °C and 2 mL/min) that presented the highest overall extraction yield (presented in Table 3). The extractions were performed with different solvent ratios of 0.00 (100% water), 0.25, 0.50, 0.75, and 1.00 (100% ethanol), and it can be noted that the water addition to ethanol enhanced the initial extraction rate (related to the apparent solubility) and the total amount of solutes recovered, in which a mixture of an equal volume of ethanol and water lead to a condition enhanced the extraction efficiency.

3.4. Total phenolic compounds (TPC)

The total phenolic compounds (TPC) obtained in pressurized liquid extraction are present in Table 6. The highest TPC value was 2130 ± 1 mg GAE/100g of BSG extract, obtained by water extraction at 120 °C and 4 mL/min. For extraction with ethanol, the higher TCP value was 1227 ± 71 mg GAE/100 g of BSG extract also at 120 °C and 2 mL/min. The temperature was a significant factor in both extractions using water and ethanol (conditions represented as 0.00 and 1.00 EtOH/Water solvent ratios, in Table 5), where increasing the temperature from 60 to 120 °C also increased the TPC values. Setting the flow rate to 4 mL/min for water extractions, at 60 °C the TPC was 1579 ± 13 mg GAE/100 g of BSG extract, then increasing to 120 °C the TPC increase to 2130 ± 1 mg GAE/100 g of BSG extract. The same occurs for the ethanol extractions, even more sharply, from 167 ± 3 mg GAE/100 g of BSG extract at 60 °C to 1227 ± 71 mg GAE/100 g of BSG extract at 120 °C.

1

Table 6

Total phenolic compounds (TPC) and total flavonoid compounds (TFC) content in pressurized liquid extractions of BSG.

Solvent ratio (EtOH/Water)	Experimental condition	TPC (mg GAE/100 g) *	TFC (mg CE/100 g) *
0.00 (Water)			
	60 °C/4 mL/min	1579 ± 13	332 ± 15
	90 °C/2 mL/min	1254 ± 24	249 ± 6
	90 °C/6 mL/min	1577 ± 25	304 ± 2
	120 °C/4 mL/min	2130 ± 1	387 ± 7
1.00 (Ethanol)			
	60 °C/4 mL/min	167 ± 3	16 ± 1
	90 °C/2 mL/min	336 ± 4	36 ± 1
	90 °C/6 mL/min	289 ± 2	43 ± 2
	120 °C/2 mL/min	1228 ± 71	156 ± 1
	120 °C/4 mL/min	945 ± 7	138 ± 1
0.5	60 °C/2 mL/min	1860 ± 25	626 ± 8
	60 °C/6 mL/min	1828 ± 35	607 ± 24
	80 °C/2 mL/min	1783 ± 65	484 ± 10
	90 °C/4 mL/min	1840 ± 16	778 ± 32
	100 °C/2 mL/min	1320 ± 163	740 ± 8
	120 °C/2 mL/min	1471 ± 25	311 ± 2
	120 °C/6 mL/min	1358 ± 35	439 ± 3
0.25	120 °C/2 mL/min	1569 ± 25	304 ± 15
0.75	120 °C/2 mL/min	1384 ± 113	242 ± 2

Values are mean ± standard deviation considering triplicate experiments.

6

In the experiments at 0.5 EtOH/Water extractions, the highest TPC value was 1860 ± 25 mg GAE/100 g of BSG extract obtained at 60 °C and 2 mL/min. The effect of temperature was opposite to extractions with 0.00 (water) and 1.00 (ethanol). Setting the flow rate at 2 mL/min, the TPC value increased with decreasing temperature from 1471 ± 25 at 120 °C to 1860 ± 25 mg GAE/100 g of BSG extract at 60 °C.

For different ethanol concentrations, 0.25, 0.50, 0.75, and 1.00 EtOH/Water at the same condition of 120 ºC and 2 mL/min, the highest total phenolics content was 1569 ± 25 mg GAE/100 g of BSG extract at 0.25 EtOH/Water. The increasing of ethanol in pressurized liquid extractions decreases the amount of total phenolics content

due to the increase of hemicellulose sugars extraction by changes in dielectric constant, therefore changing the relative permittivity [6,20]. Huerta and Saldaña [20] performed barley and canola straws hydrolysis using subcritical water (sCW) and pressurized aqueous ethanol (PAE) at 140–220 °C, 50–200 bar and 20–100% (v:v) ethanol concentrations. The optimum process conditions for phenolic compounds recovery found by those authors (45.4 ± 1.8 mg GAE/g barley straw and 52.9 ± 2.0 mg GAE/g canola straw) were 180 °C, 50 bar, and 20% ethanol. They also observed that, with the temperature increase also increases the TPC value. Otherwise, the higher the ethanol concentration, the lesser TPC were removed.

Comparing the results of TPC obtained by PLE with those presented for Soxhlet extraction with ethanol and water (Figure 1A), it is observed that all PLE results for water (Table 6) were higher than Soxhlet, reaching around 1500 GAE/100 g of BSG extract at mild temperatures (60 and 90 °C) and 2130 ± 1 mg GAE/100 g of BSG extract at 120 °C, against around 800 mg GAE/100 g of BSG extract obtained in Soxhlet. TPC results for PLE with ethanol were significantly higher than the Soxhlet result (Figure 1A) only for PLE at 120 °C conditions. The most important results are the high TPC values in the extraction were kept for the extracts obtained with mixtures of ethanol and water, where the best relation between extraction yield and TPC value is obtained at 0.25 EtOH/Water, 120 °C, and 2 mL/min.

3.5. Total flavonoid compounds (TFC)

The total flavonoid compounds are presented in Table 6. For the extractions using pressurized solvents, 0.00 and 1.00 EtOH/Water, the highest TFC removal was obtained with the highest temperature investigated (120 °C), and its value was 387 ± 7 mg CE/100 g of BSG extract for water and 156 ± 1 mg CE/100 g of BSG extract for

ethanol. Moreover, when using a mixture of water/ethanol at 0.50 EtOH/Water solvent ratio, 90 °C and 4 mL/min, the TFC content increased to 778 ± 32 mg CE/100 g of BSG extract. Flavonoids are highly thermolabile phenolic compounds whose stability depends on the pH, generally require lower extraction temperatures, from 85 to 126 °C, and short holding extraction time [36,56]. Also, the significant increase in TFC by using the 0.5 EtOH/Water solvent ratio was also observed by Zang et al. [57], that performed pressurized liquid extraction of *Houttuynia cordata* Thunb using ethanol at different concentrations in water (0, 30, 50, 70%) and reported that an ethanol concentration of 50% results in both the highest flavonoid yield and highest flavonoids content.

The comparison between PLE results and the extracts obtained by Soxhlet with water and ethanol reveals that the extraction with pressurized water did not present more significant improvements in terms of TFC values. PLE with ethanol presented higher values only for conditions performed at 120 °C. However, when EtOH/Water was used as the solvent in PLE, the TFC values increased to seven-folds compared to the TFC values for the extract obtained in Soxhlet.

3.6. Antioxidant activity (AA)

The antioxidant activity (AA) for all pressurized liquid extraction conditions was performed according to ABTS, DPPH, and FRAP analyses, and these values are presented in Table 7. Pressurized water at 120 °C and 4 mL/min was responsible for the highest AA value of 9944 ± 391 µmol TE/100 g of BSG extract performed by ABTS methodology. Much higher than the value found by Soxhlet (Figure 1C). Also, water at the same condition was responsible for the highest values for DPPH and FRAP methodologies, of 4769 ± 330 and 4096 ± 111 µmol TE/100 g of BSG extract, respectively. For ethanol extractions, the highest AA value was 6578 ± 402 µmol

1   TE/100 g of BSG extract also obtained by ABTS methodology, at 120 °C and 2 mL/min

2   and for DPPH and FRAP 2367 ± 5 and 2805 ± 27 µmol TE/100 g of BSG extract,

3   respectively; values at least two-fold higher than values measured in the extracts

4   obtained with ethanol in Soxhlet.

5   **Table 7**

6   Antioxidant activity (AA) by ABTS, DPPH, and FRAP methods for brewer's spent

7   grain extracts by pressurized liquid extractions.

Solvent ratio (EtOH/Water)	Experimental condition	AA (µmol TE/100 g)		
		ABTS	DPPH	FRAP
0.00 (Water)				
	60 °C/4 mL/min	6544 ± 651	3495 ± 277	2321 ± 69
	90 °C/2 mL/min	6779 ± 134	2469 ± 79	1766 ± 124
	90 °C/6 mL/min	7717 ± 336	2972 ± 15	2074 ± 7
	120 °C/4 mL/min	9944 ± 391	4769 ± 330	4096 ± 111
1.00 (Ethanol)				
	60 °C/4 mL/min	16 ± 4	55 ± 25	25 ± 4
	90 °C_2 mL/min	243 ± 26	506 ± 63	1353 ± 39
	90 °C/6 mL/min	373 ± 83	360 ± 8	1040 ± 47
	120 °C/2 mL/min	6578 ± 402	2367 ± 5	2805 ± 27
	120 °C/4 mL/min	3653 ± 166	1691 ± 206	2296 ± 82
0.5	60 °C/2 mL/min	5334 ± 615	3311 ± 99	3907 ± 13
	60 °C/6 mL/min	5671 ± 458	3464 ± 116	3411 ± 163
	80 °C/2 mL/min	2163 ± 18	3224 ± 88	3453 ± 2
	90 °C/4 mL/min	6903 ± 265	3012 ± 190	3750 ± 42
	100 °C/2 mL/min	2016 ± 83	2496 ± 192	3055 ± 4
	120 °C/2 mL/min	6384 ± 444	3054 ± 140	3332 ± 123
	120 °C/6 mL/min	6911 ± 54	2652 ± 59	3231 ± 62
0.25	120 °C/2 mL/min	2764 ± 110	3273 ± 121	2343 ± 54
0.75	120 °C/2 mL/min	2073 ± 388	2861 ± 374	2702 ± 207

8 Values are mean ± standard deviation considering triplicate experiments.

9   The AA values obtained in PLE extracts with ethanol were lower than those

10   obtained by water as solvent due to the antioxidant activity increased with the increase

11   in the polarity of the solvent, the same occurred for the Soxhlet extractions, in which the

12   AA of the water solvent (3894 ± 125 µmol TE/100 g of BSG extract) was

13   approximately 50 times higher than values found for extracts obtained with ethanol (81

14   ± 5 µmol TE/100 g of BSG extract) for ABTS methodology (as presented in Figure 1).

The 0.5 EtOH/Water extractions provided extracts with intermediate AA values for extractions with water and ethanol (0.00 and 1.00 EtOH/Water). The highest AA was 6911 ± 54 µmol TE/100 g of BSG extract by ABTS at condition 120 °C and 6 mL/min. While for the methodologies DPPH and FRAP the best AA value was obtained at 60 °C in different flow ratios, at 6 mL/min to DPPH with 3464 ± 116 and at 2 mL/min to FRAP with 3907 ± 1 µmol TE/100 g of BSG extract.

In general, for PLE with water and ethanol (0.00 and 1.00 EtOH/Water), the increase in temperature also increased the antioxidant activity. However, for the 0.5 EtOH/Water extractions, the highest AA was obtained at a lower temperature, at 90 °C. The same behavior was observed for the analyses of total phenolic compounds (TPC), and total flavonoid compounds (TFC) since the antioxidant activity present in the BSG extracts is attributed to the presence of these compounds.

Figure 5 shows the correlation of the total amount of phenolic compounds in the extract with the antioxidant activity results for pure solvents (water and ethanol) and EtOH/Water mixtures. It is interesting to note that a linear correlation is observed only for pure solvents, water, and ethanol for both AA measurements, ABTS and DPPH. For the mixture of EtOH/Water, no correlation is observed for ABTS measurements with TPC, probably because this method is too sensitive to other classes of compounds extracted by the mixture of the solvents. It is worth recalling that the mixture EtOH/Water provided higher extraction yields for the three ratios EtOH/Water investigated. On the other hand, the DPPH method provided a much more straight correlation between AA and TPC values, as already reported in the literature by Trevisani Juchen et al. for parboiled rice bran oil extract with supercritical CO_2 and ethanol as co-solvent [58]. Since it is known that different groups of compounds affect the antioxidant activities of crude extracts, the correlation shown in Figure 5B is

interesting because it is indicating that phenolic compounds are the key factor for the

antioxidant properties of crude extracts from BSG obtained by PLE with water and

ethanol; since TPC (by DPPH mostly) direct correlate with the antioxidant activity.
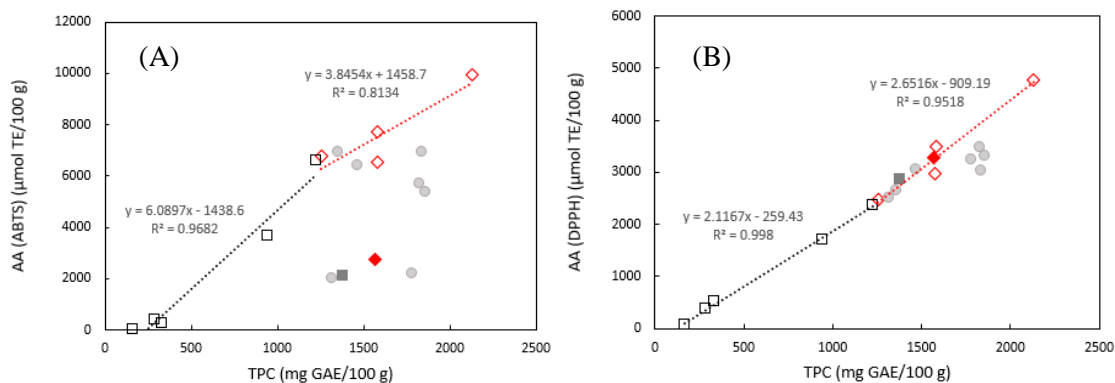
Fig. 5. Correlation of the total amount of phenolic compounds in the extracts with the antioxidant activity (AA) results for extracts obtained by PLE with different solvents: water: red open diamonds; ethanol: black open square; EtOH/Water 0.5: grey full circles;

3.7. Reducing sugars

Table 8 provides the reducing sugars (RS) and total reducing sugars (TRS) content analysis in BSG extracts obtained by PLE. The highest RS content was obtained by water extraction at 60 °C and 4 mL/min, 28.6 ± 0.5 g GE/100 g of BSG extract. Although, the condition at 90 °C and 6 mL/min was responsible for the highest TRS content (35.3 ± 1.4 g GE/100 g of BSG extract). Also, with 0.5 EtOH/Water, the maximum RS, and TRS content were quantified at 60 °C, 18.9 ± 1.0 g GE/100 g of BSG extract of RS at 6 mL/min, and 22.0 ± 0.6 g GE/100 g of BSG extract of TRS at 2 mL/min. While, for ethanol extractions, the maximum RS content was 16.4 ± 0.2 g

GE/100 g of BSG extract at 120 °C and 2 mL/min, and the TRS was 18.2 ± 0.9 at the same condition.

For water and the mixture 0.5 EtOH/Water, the temperature decrease caused an increase in the RS and TRS content, while for ethanol extractions, with the temperature increase the RS and TRS content was also increased. The glucose and hemicellulose (amount of xylose and arabinose fractions) content in BSG raw material (Table 2), $22.5 \pm 1.8\%$ and $33.8 \pm 1.7\%$, respectively, suggest a high potential of sugar removal. However, the RS and TRS results obtained for the extracts with the solvents water, ethanol, and 0.5 EtOH/Water at temperatures of 60 °C to 120 °C were low. These results were pretty nearly those obtained by the Soxhlet extraction. For water, the RS and TRS content were 28.6 ± 0.4 and 31.9 ± 1.1 g GE/100 g of BSG extract, respectively, and for ethanol 16.4 ± 0.1 and 18.2 ± 0.7 g GE/100 g of BSG extract. This behavior indicates that the extractions studied in this work could not release sugars from the matrix, just leaching the available sugars in the BSG. Thus, it is an important fact considering that PLE can be used to recovery soluble compounds to produce extracts with high values of TPC, TFC, and high antioxidant activity while keeping the carbohydrates in the matrix, and therefore it can be further used in second-generation chemical platforms, biofuels, or even to be used as animal fed. It is worth mentioning that temperatures higher than 120 ºC, especially between 140 – 210 °C, are indicated to hydrolysis of BSG matrix to sugars [23,51].

Torres-Mayanga et al. [23] reported significant differences between RS and TRS content when performed subcritical water hydrolyses of BSG at run temperatures of 140 to 210 ºC. The TRS was about 15-times higher than RS content at 140 °C, indicating oligomeric sugars' predominance in the hydrolysis products.

Table 8

Reducing sugar and total reducing sugar content in brewer's spent grain extracts.

Solvent ratio (EtOH/Water)	Experimental condition	RS (g GE/100 g)	TRS (g GE/100 g)
0.00 (Water)			
	60 °C/4 mL/min	28.6 ± 0.5	32.5 ± 0.2
	90 °C/2 mL/min	25.7 ± 0.5	32.7 ± 0.4
	90 °C/6 mL/min	26.2 ± 1.2	35.3 ± 1.4
	120 °C/4 mL/min	18.9 ± 0.4	29.3 ± 0.3
1.00 (Ethanol)			
	60 °C/4 mL/min	3.6 ± 0.3	3.9 ± 0.1
	90 °C/2 mL/min	7.1 ± 0.02	7.2 ± 0.1
	90 °C/6 mL/min	6.5 ± 0.3	7.7 ± 0.4
	120 °C/2 mL/min	16.4 ± 0.2	18.2 ± 0.9
	120 °C/4 mL/min	7.8 ± 0.3	8.6 ± 0.01
0.5			
	60 °C/2 mL/min	18.4 ± 0.6	22.01 ± 0.6
	60 °C/6 mL/min	18.9 ± 1.0	22.0 ± 0.5
	80 °C/2 mL/min	16.4 ± 0.1	17.1 ± 0.05
	90 °C/4 mL/min	15.9 ± 0.5	19.7 ± 0.9
	100 °C/2 mL/min	15.9 ± 0.1	17.6 ± 1.0
	120 °C/2 mL/min	11.3 ± 0.2	13.7 ± 0.4
	120 °C/6 mL/min	13.02 ± 0.3	15.3 ± 0.1
0.25	120 °C/2 mL/min	15.2 ± 0.2	18.9 ± 0.5
0.75	120 °C/2 mL/min	10.5 ± 0.4	11.1 ± 0.1

Values are mean ± standard deviation considering triplicate experiments.

However, the results of RS and TRS obtained in this work do not present significant differences, indicating that both analyzes detect only the presence of simple low molecular weight sugars.

3.8. General correlations and operating conditions

Aiming to identify the best operational condition of PLE for producing crude extracts from BSG, the measured features of all extracts obtained were correlated to the extraction yields, as presented in Figure 6. From this correlation, from the process point of view, it is observed that better conditions to recovery crude extracts from BSG with

1 PLE are those performed using water at 120 °C, which provides a higher extraction

2 yield (above 17 wt%) with the highest TPC and AA responses. Such conditions (water

3 at 120 °C) are followed by conditions using EtOH/Water at concentrations of ethanol at

4 0.25 to 0.75 (v:v) also at 120 °C, with the advantage that the sugar contents were lower

5 for the extracts obtained in EtOH/Water when compared to water as solvent.

6

7

8
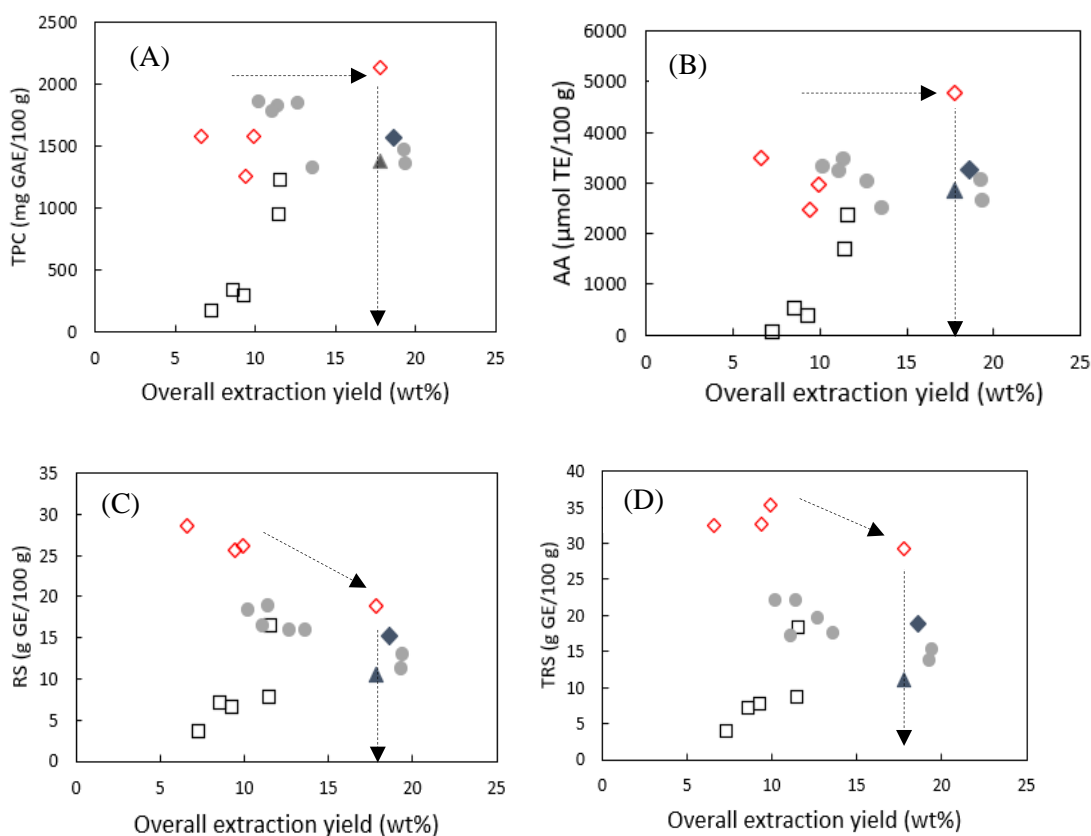
Fig. 6. Correlation of the (A) total amount of phenolic compounds (TPC), (B)

antioxidant activity by DPPH method, (C) reducing sugars (RS), and (D) total reducing

sugars in the extracts related to extraction yields obtained in each PLE condition and

solvents: water: red open diamonds; ethanol: black open square; EtOH/Water 0.5: grey

full circles; EtOH/Water 0.75: grey full square; EtOH/Water 0.25: gray full diamond.

14

15

1   3.9 Compressed propane extractions

2

3   Since the extraction using *n*-hexane (Table 4) demonstrated the high selectivity

4   of this non-polar solvent provided a selective extraction to lipids (represented by the

5   fatty acids identifications), with low sugars contend, antioxidant, TPC and TFC, a

6   sequential extraction was proposed aiming to firstly recover the lipid fraction present in

7   the BSG before the extraction with pressurized polar liquid solvents. Thus, in this work

8   extractions with compressed propane were performed at constant pressure of 10 MPa

9   and constant flow rate of 2.0 ± 0.2 cm^3/min during 90 min of dynamic extraction. The

10   experimental procedure and setup for the extractions with compressed propane was the

11   same as presented by Fetzer et al. [27].

12   Compressed propane was selected in this work for the extraction of defatting the

13   BSG because it has been used as an alternative process for oil recovery in substitution to

14   solvent extraction with *n*-hexane for different oilseeds [27,59,60]. As mentioned in the

15   literature [24], propane can be used as the solvent to produce an oil with high quality

16   and solvent-free, because it can extract unsaturated fatty acids and compounds with

17   antioxidant capacity from different types of oilseeds. Thus, considering that compressed

18   propane has shown to be an effective solvent for oil recovery form different raw

19   materials, we proposed propane as the non-polar solvent to recovery the lipids fraction

20   in the first extraction step. In addition, application of compressed for lipids extraction

21   from BSG has not been reported in the literature. Thus, this section aims present the

22   results of sequential extraction with compressed propane followed by the extraction

23   with the best solvent obtained in the previous sections.

24   After removing the lipid fraction, the solid residues were used in a subsequent

25   extraction with pressurized 0.5 EtOH/Water at 120 ºC and 2 mL/min. The results of

extractions yield are presented in Table 9. The highest sequential yield was obtained by compressed propane at 60 °C (P2), followed by the solid residue extraction (P2$_R$) reaching 23.6 ± 0.1 wt% (4.0 ± 0.1wt% of extraction with propane + 19.6 wt% of PLE extraction). Also, the extraction at 40 and 80 °C, presented a similar yield of 21.9 and 23.1 wt%, respectively. Compressed propane extraction presented values ranged between 3.2 – 4.4 wt%. At constant pressure, the increasing of temperature decreases the solvent density and the vapor pressure of the solutes increases, consequentially the extraction yield increases [27,61].

All the pressurized 0.5 EtOH/Water solid residues extractions (P1$_R$, P2$_R$ and P3$_R$) presented similar yield with the BSG 0.5 EtOH/Water at 120 °C and 2 mL/min extraction (Table 5) of 19.3 ± 0.8 wt%, showing that the priory compressed propane extraction for defatted the BSG is not affecting the solutes recovery in the PLE extraction step.

Figure 7 depicts the overall extraction curves of sequential extractions. The compressed propane extractions were performed at 90 first min, and the behavior of the lipid fraction extraction were analyzed in two periods: higher extraction rate and lower extraction rate. At the first 25 min of extraction, the convective mass transfer controls the extraction process allowing the higher extraction rate once the lipid fraction is in direct contact with the solvent. After this time, the extraction becomes slow due to the resistance to diffusion once the remaining lipid fraction is difficult to release and internal mass transfer controls the extraction process [27,61]. The pressurized 0.5 EtOH/Water extraction (90 min to 150 min) present the same kinetics behavior from the extractions of Figure 4 in section 3.3.
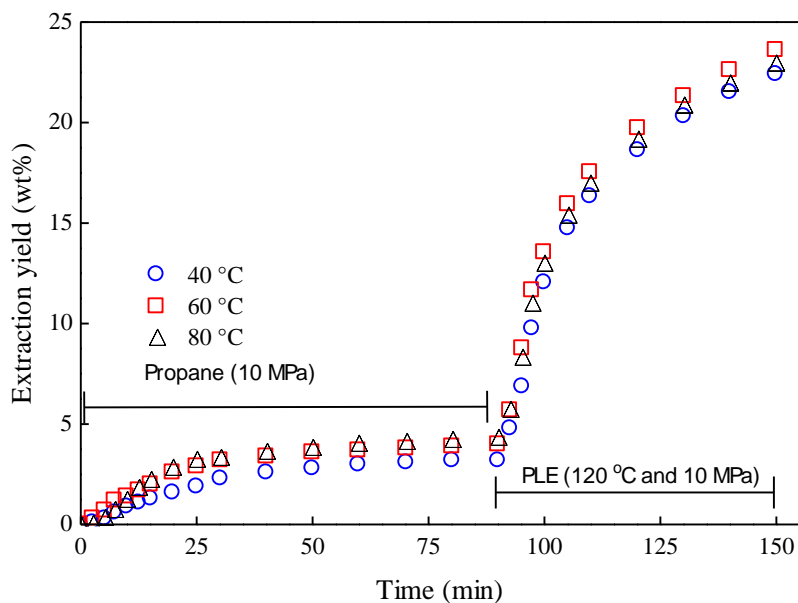
1

2 **Fig. 7.** Overall extraction curves of sequential compressed propane followed by 0.5

3 EtOH/Water at 120 °C and 2 mL/min extractions.

4

5   The characterization of extracts obtained by compressed propane shows low

6 results of biological activity. The results are compared to the Soxhlet extraction of *n*-

7 hexane (Figure 1), a nonpolar solvent. However, the TPC value is approximately 40%

8 lower than the value obtained by Soxhlet n-hexane (121 ± 5 mg GAE/100g), also for

9 AA and TFC values. Otherwise, increasing the temperature the biological activity

10 extracts also increased. These results show that it is possible to obtain different classes

11 of compounds using different extractions solvents, as also presented in Table 4, once

12 non-polar solvents as *n*-hexane and compressed propane are capable of solubilizing fat-

13 soluble compounds (as triglycerides, tocopherols, phytosterols and carotenoids), while

14 the pressurized 0.5 EtOH/Water extractions can obtain crude extracts with high

15 biological activity. Thus, sequential extractions using different solvents classes are

16 indicated to recovery and obtain different classes of compounds with different

17 properties.

Table 9

Results of extraction yields and extract characterization of sequential extraction of compressed propane and 0.5 EtOH/Water.

Run/Solvent	T (°C)	$\rho_{sol.}$[a] (g/ml)	Yield (wt%)	TPC (mg GAE/100 g)	TFC (mg CE/100 g)	AA (µmol TE/100 g)			RS	TRS
						ABTS	DPPH	FRAP	(g GE/100 g)	
Propane										
P1	40	0.493	3.2	44 ± 3	22 ± 2	2.3 ± 0.1	52 ± 2	12 ± 1	0.51 ± 0.02	0.79 ± 0.01
P2	60	0.467	4.0 ± 0.1	50 ± 2	40 ± 1	4.2 ± 0.3	55 ± 4	15 ± 1	0.52 ± 0.04	0.71 ± 0.04
P3	80	0.430	4.4	51 ± 5	39 ± 3	4.2 ± 0.3	57 ± 2	17 ± 1	0.50 ± 0.01	0.91 ± 0.07
0.5 EtOH/Water										
P1$_R$	120	-	19.2	753 ± 186	175 ± 8	1169 ± 499	1482 ± 805	1507 ± 363	13.9 ± 0.1	15.9 ± 0.2
P2$_R$	120	-	19.6	796 ± 6	150 ± 1	4006 ± 250	1672 ± 53	1849 ± 82	11.1 ± 0.3	14.1 ± 0.5
P3$_R$	120	-	18.7	1327 ± 91	203 ± 9	1951 ± 504	2353 ± 846	2399 ± 166	12.9 ±0.1	15.1 ± 0.1

4. Conclusions

In this study, the pressurized liquid extraction of brewer's spent grains using water, ethanol, and mixtures of both solvents were evaluated varying temperature and solvent flow rate. Furthermore, compressed propane was applied as a defatting step of BSG. All extracts obtained with different solvents were characterized by determination of total phenolic and flavonoids compounds, antioxidant activity and reducing sugars.

Results were compared to Soxhlet extractions with different solvents (polar and nonpolar) and water and ethanol were responsible for the highest extraction yields and high recovery of phenolic compounds.

The extraction yield in PLE experiments demonstrated that the significant variables are temperature and solvent composition. The recovery of extracts was favored by the increase in temperature and by the mixture of ethanol/water of 50% (v:v). The increase in the solvent flow rate favored the initial rate extractions for all solvents tested in PLE. The highest extraction yield reached approximately 20 wt% at 120 °C, 0.5 of EtOH/Water solvent ratio, 2 mL/min and 10 MPa., showing that the mixture of ethanol and water at 120 °C is technically feasible for recovering an important amount of soluble compounds from BSG.

BSG extracts showed expressive values of total phenolic, flavonoids compounds, and antioxidant activity. Water was responsible for the highest TPC value of 2130 ± 1 mg GAE/100g of BSG extract, at 120 °C and 4 mL/min. In comparison, the highest TFC content was 778 ± 32 mg CE/100 g of BSG extract obtained at 0.50 EtOH/Water solvent ratio, 90 °C and 4 mL/min. Water at the condition of 120 °C and 4 mL/min was also responsible for the highest AA value, of 9944 ± 391 µmol TE/100 g of BSG extract performed by ABTS methodology. These results were favored by water

and water/ethanol extractions and for the temperature increase. Moreover, the increase of ethanol decreases the sugar concentration in the extracts, which showed similar concentrations of reducing sugars and total reducing sugar, and the values obtained are also similar to those obtained by the Soxhlet methodology.

Compressed propane was an efficient lipids recovery as a defatting step in a sequential extraction approach before the PLE process with a polar solvent system.

Therefore, pressurized water and EtOH/Water mixtures were demonstrated to be suitable and promising environmental-friendly technology for the recovery of different bio-compounds BSG, and its technical feasibility was demonstrated by the results achieved in this study.

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