

## Nutritional Quality, Potential Health Promoting Properties and Sensory Perception of an Improved Gluten-Free Bread Formulation Containing Inulin, Rice Protein and Bioactive Compounds Extracted from Coffee Byproducts

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The present study aimed to improve the formulation of a gluten-free commercial bread-making premix by adding inulin, rice protein and extracts of coffee by-products (silverskin and husk) to obtain healthier products enriched in protein and dietary fiber with the potential to reduce the risk of chronic diseases. The new formulation combines well-known and novel food ingredients. To validate the novel ingredients, we determined the physicochemical characterization, caffeine and chlorogenic acid contents, and food safety of coffee silverskin (CSE) and husk (CHE) extracts. Sensory and nutritional quality, bioactive compounds (chlorogenic acid, total phenolic compounds, melanoidins and browning), *in vitro* bioaccessibility, and health-promoting properties (overall antioxidant capacity and  $\alpha$ -glucosidase activity) of the breads were evaluated as well.

Results support the feasibility of CSE and CHE as natural sustainable sources of antioxidants,  $\alpha$ -glucosidase inhibitors and colorants. The observed health-promoting properties suggest that coffee by-product extracts could potentially be used as functional food ingredients or supplements to reduce the risk of chronic diseases associated with oxidative stress and to control postprandial glucose levels. Based on consumers' preferences, we obtained new bread formulations with a high nutritional and sensorial quality, suitable for celiacs and with the potential to reduce the risk of gastrointestinal disease related to oxidative stress. Data on *in vitro* digestion indicated a significant ( $p < 0.05$ ) decrease in the bioaccessibility of sugars and a significant increase ( $p < 0.05$ ) in antioxidants.

### ABBREVIATIONS

CD, celiac disease; CGA, chlorogenic acid; CHE: coffee husk extract; CR, commercial recipe; CSE, coffee silverskin extract; GI, glycaemic index; IDF, insoluble dietary fibre; NF, new formulation; NFS, new formulation with 25 g CSE/kg dry matter CSE; NFH, new formulation with 25 g CHE/kg dry matter; OTA, Ochratoxin A; SDF, soluble dietary fibre; TAC, total antioxidant capacity; T2D, type 2 diabetes, TDF, total dietary fibre; TPC, total phenolic compounds.

### INTRODUCTION

Gluten-free products present a major challenge for the food industry in terms of organoleptic, technological and nutritional characteristics [Conte *et al.*, 2019]. Among gluten-free foods, bread is the most consumed in Europe [Padalino *et al.*, 2016]. The absence of gluten in these products affects starch digestibility increasing the postprandial glycaemic response. Consequently, people on a gluten-free diet have a higher risk of suffering from chronic diseases

like type 2 diabetes (T2D) [Giuberti *et al.*, 2015]. However, the addition of dietary fibre and protein has been found to reduce the glycaemic response of gluten-free products such as bread [Scazzina *et al.*, 2015].

Coffee husk is the outer skin and pulp obtained from the wet processing of coffee berries [del Castillo *et al.*, 2019]. It has a high content of carbohydrates (35–85%), soluble fibers (30.8%), minerals (3–11%), and proteins (5–11%). It is also rich in insoluble dietary fiber and can be a source of phytochemicals such as tannins (5–9%) and cyanidins (20%) for the food industries [del Castillo *et al.*, 2019].

Coffee silverskin is a thin tegument of the outer layer of the two beans forming the green coffee seed and represents 4.2% (w/w) of the coffee cherry. It is obtained when green coffee beans are roasting, being the unique byproduct of the roasting process. Coffee silverskin has high contents of dietary fiber (68–80%) and polysaccharides (60–70%), and presents a very high antioxidant activity [Pourfarzad *et al.*, 2013; Bresciani *et al.*, 2014]. Prior studies have proposed the use of coffee silverskin as an innovative food ingredient [Ballesteros *et al.*, 2014; Bresciani *et al.*, 2014].

The aqueous extracts of coffee silverskin (CSE) and coffee husk (CHE) have recently been proposed as a source of two safe food ingredients: aqueous extracts enriched in phyto-

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chemicals and antioxidant dietary fiber [Iriondo-DeHond *et al.*, 2018]. CSE was used in the preparation of biscuits with no added sugar, obtaining a product with a good nutritional quality and improved texture and colour [Garcia-Serna *et al.*, 2014]. Besides, CSE can be used to prevent or treat age-related chronic diseases caused by oxidation and inflammation, such as T2D. The bioactive compounds present in CSE affect several pathways involved in the pathogenesis of T2D, thereby reducing the risk of this disease [del Castillo *et al.*, 2016].

This study aimed to evaluate the use of CSE and CHE as functional ingredients in the formulation of gluten-free breads with a high nutritional value and the potential to control postprandial glucose. To the best of our knowledge, the use of CHE for diabetes has not been previously reported.

## MATERIALS AND METHODS

### Reagents

The reagents used in this study were: intestinal acetone powders, 4-methylumbelliferyl  $\alpha$ -D-glucopyranoside (4-MUG), acarbose,  $\alpha$ -amylase from human saliva (type IX-A), porcine pepsin from gastric mucosa (3200–4500 U/mg protein), pancreatin from porcine pancreas, porcine bile extract, cholestyramine resin, chlorogenic acid (CGA), caffeine, phloroglucinol, salicylic acid; 2,2'-azinobis [3-ethylbenzothiazoline-6-sulphonic acid] (ABTS), Folin-Ciocalteu reagent, N $\alpha$ -Acetyl-L-lysine, ortho-phthalaldehyde (OPA), methanol HPLC-grade, phenol, concentrated sulphuric acid (93–98%), and ammonium chloride were from Sigma-Aldrich (St. Louis, MO, USA). Sucrose, D-fructose, D-glucose kit, and the dietary fibre kit were from Megazyme (International Ireland Ltd, Bray, Ireland).

Buffered peptone water (BPW) was from Biocult, BD™ Difco™ plate count agar (PCA) medium and brain BD™ Bacto™ heart infusion (BHI) agar from BD (New York, NJ, USA) and Sabouraud dextrose agar (SDA) with chloramphenicol from CONDA (Torrejón de Ardoz, Madrid, Spain). Water was purified using a Milli-Q and Elix system (Millipore, Molsheim, France). All other chemicals and reagents were of analytical grade.

### Apparatus

Microplate spectrometers BioTek powerWave™ XS (BioTek Instruments, Winooski, VT, USA) and FP-6200 (JASCO, Easton, MD, USA) were used for the spectrophotometric assays. Bloc-digest 12 (Selecta, Barcelona, Spain) was employed for the digestion process in the Kjeldahl method. Accela liquid chromatograph (Thermo Scientific, San Jose, CA, USA) equipped with a DAD and coupled to a TSQ Quantum (Thermo Scientific) triple quadrupole analyser via an electrospray ionisation (ESI) interface was used to determine caffeine and CGA content. CertoCLAV A-4050 autoclave (CertoCLAV, Traun, Austria), Stomacher® 400 Circulator (Seward, UK), horizontal laminar flow bench Mini-H (Telstar, Madrid, Spain), Nüve EN120 incubator (Nüve, Ankara, Turkey) and SANYO Mir154 incubator (SANYO Electric Biomedical Co., Ltd., Osaka, Japan) were used for microbiological analysis. Bread was made using a PAN 850 bread machine (Fagor, San Sebastián, Spain).

### Commercial food ingredients

Bread was prepared using a gluten-free commercial baking pre-mix. The composition of the pre-mix was: 753 g/kg of corn starch, 52 g/kg of dietary fibre, 20 g/kg of salt, 13 g/kg of fat, and 10 g/kg of protein. ORAFIT® inulin and Remypro N80+ rice protein from Beneo Ibérica (Barcelona, Spain) were used to fortify the breads.

### Coffee by-products ingredients

Arabica coffee husk (*Coffea arabica*) was provided by Delikia (Pontevedra, Spain) while Robusta coffee silverskin (*Coffea canephora*) was provided by Fortaleza S.A. (Vitoria, Spain). Raw materials were stored at room temperature in a dry and dark place until the preparation of extracts.

### Aqueous coffee byproduct extracts

Robusta CSE and Arabica CHE aqueous extracts were obtained with hot water (50 g/L) at 100°C for 10 min as described in the patent WO 2013/004873 [delCastillo *et al.*, 2013]. Extracts were freeze-dried and stored at -20°C until use.

### Microbiological analyses of CSE and CHE

The safety of coffee byproduct extracts as food ingredients was evaluated by assaying counts of: (1) total aerobic microorganisms, (2) aerobic microorganisms forming endospores, and (3) moulds and yeasts. All assays were performed in sterile conditions with previous solubilization of the samples (10 g) in BPW (90 mL) using a stomacher (230 rpm, 1 min). Different conditions were set for each analysis: (1) pour plate method, PCA medium, incubation at 30°C 72 h; (2) pour plate, BHI agar medium, preincubation (80°C, 10 min) and incubation at 37°C 48 h; and (3) spread method, SDA with chloramphenicol and incubation at 25°C 120 h. Results were expressed as colony-forming units per gram (CFU/g).

### Physicochemical analyses of CSE and CHE

For the analyses, each freeze-dried extract (250 mg) was dissolved in Milli-Q water (10 mL) for 5 min using a vortex mixer at room temperature and then centrifuged at 4,000×g for 10 min. The supernatants were filtered through a 13 mm PTFE filter (pore size 0.45  $\mu$ m). Samples were kept at -20°C until analysis. The following components of CSE and CHE were analyzed: browning, content of total carbohydrates, glycemic sugars, total proteins, total dietary fibre (TDF), total lipids, total phenolic compounds (TPC), CGA and caffeine content, melanoidins, total antioxidant capacity (TAC), and  $\alpha$ -glucosidase activity.

### Bread-making

The studied bread formulations are shown in Table 1. Control breads were prepared using a gluten-free baking pre-mix (commercial recipe, CR) following the manufacturer's instructions. To reduce the bioaccessibility of sugars and increase the nutritional value of the commercial preparation, new bread formulations (NF) containing inulin and rice protein with no sugar were proposed. These basic formulations were supplemented with 25 g of CSE per kg of dry matter (d.m.) (NFS) or 25 g of CHE per kg d.m. (NFH). The breads were made with a domestic bread-maker using the gluten-free programme, obtaining 1000 g of the product.

TABLE 1. Bread formulations.

Ingredients (g)	CR	NF	NFS	NFH
Gluten-free baking pre-mix	410	385	372	372
Sourdough	20	13	13	13
Sunflower oil	90	19	19	19
Sugar	40	0	0	0
Rice protein	0	55	55	55
Inulin	0	40	40	40
CSE	0	0	13	0
CHE	0	0	0	13
Water	240	500	500	500
Estimated calories (kcal/100 g bread)	241	180	178	177
Estimated protein energy value (% on total kcal)	1.2	26.2 <sup>‡</sup>	26.5 <sup>‡</sup>	26.6 <sup>‡</sup>
Estimated fibre content (g fibre/100 g bread)	3.8 <sup>*</sup>	11.7 <sup>‡</sup>	11.6 <sup>‡</sup>	11.6 <sup>‡</sup>

CR – commercial recipe; NF – new formulation; NFS – new formulation with 25 g of coffee silverskin extract (CSE)/kg d.m.; NFH – new formulation with 25 g of coffee husk extract (CHE)/ kg d.m.

\*CR might be “source of fibre” ( $\geq 3$  g fibre/100 g bread); <sup>‡</sup>NF, NFS and NFH might be “high in protein” ( $\geq 20$  % energy value of bread provided by protein); <sup>‡</sup> NF, NFS and NFH might be “high fibre content” ( $\geq 6$  g fibre/100 g bread).

Homemade loaves were sliced (10 mm thick), and the slices were frozen and subsequently freeze-dried. The freeze-dried slices (approximately 50 g) were ground and sieved through a 40-mesh screen to obtain a powder. For the analyses, the bread powder (1 g) was dissolved in Milli-Q water (10 mL) for 5 min using a vortex mixer at room temperature and then centrifuged at  $4,000\times g$  for 10 min. The supernatants were filtered through a 13 mm PTFE filter (pore size 0.45  $\mu\text{m}$ ). Samples were kept at  $-20^\circ\text{C}$  until analysis.

### Sensory analysis

A preliminary sensory analysis of the NFS and NFH breads found that the maximum amount of CSE and CHE considered acceptable was 25 g/kg d.m. (data not shown). A sensory analysis of NF bread was carried out to evaluate its acceptance and of NFS and NFH breads to estimate the influence of extract supplementation on their sensory perception. Colour, texture, taste, and overall acceptance were evaluated in a hedonic sensory test involving 30 untrained panellists. Results of the verbal scale test were converted to a 7-point scale from 1 (lowest) to 7 (highest). Breads were considered acceptable if the average of the panellists' scores for all the parameters was equal to or above 4.

### Physicochemical analyses

#### Total carbohydrate content

Total carbohydrate content was determined using the phenol-sulphuric method described by Masuko *et al.* [2005]. Samples (0.1 mL) were mixed with concentrated sulphuric

acid (0.3 mL; 93–98% purity) and phenol (0.09 mL; 50 g/L) in a glass flask. The mixtures were incubated at  $90^\circ\text{C}$  for 5 min and cooled at room temperature. Absorbance was measured at 490 nm. A calibration curve was constructed using glucose (0.1–0.85 g/L). Reagent blank and sample blank were also analysed in each set of samples. All measurements were performed in triplicate, and results were expressed as g/100 g.

#### Glycaemic sugars

Glucose, fructose, and sucrose contents were determined with a K-SUFRG kit (Megazyme) adapted to a microplate format following the manufacturer's instructions. Analysis was carried out in triplicate. Results were expressed as g/100 g.

#### Dietary fiber

Insoluble (IDF), soluble (SDF), and TDF contents were determined using an enzymatic-gravimetric assay based on the AOAC-991.43 [1995] and AACC-32.07.01 [1995] methods. Analysis was carried out in duplicate for each sample. Results were expressed as g/100 g.

#### Total proteins

Total protein content was determined following the Kjeldahl method [AOAC-920.87, 32.1.22, 1995]. This procedure was carried out in triplicate. Results were expressed as g/100 g.

#### Free amino groups

An OPA assay was carried out to determine free amino groups content [Michalska *et al.*, 2008] in the soluble fraction of digested bread. Quantitative analysis was performed using a calibration curve of  $N\alpha$ -acetyl-L-lysine (0.01–1 mmol/L). All measurements were performed in triplicate. Results were expressed as mg Lys eq./kg of digested bread.

#### Total lipids

Total lipid content was determined according to Toschi *et al.* [2014] with minor modifications. Briefly, the sample (1 g) was mixed with 50 mL of *n*-hexane, homogenized by Ultra-Turrax for 3 min and then subjected to ultrasound treatment for 20 min. The organic layer (containing the lipid matter) was separated by centrifugation ( $1,620\times g$  for 20 min). The lipid fraction was collected and dried in a rotary evaporator. The fat content was determined gravimetrically. The analysis was carried out in triplicate. Results were expressed as g/100 g.

#### Total phenolic compounds

TPC content in the samples was analyzed using Folin-Ciocalteu adapted to a micromethod [Contini *et al.*, 2008]. The reaction was initiated by mixing 10  $\mu\text{L}$  of the sample with 150  $\mu\text{L}$  of the Folin-Ciocalteu reagent. After incubation at room temperature for 3 min, 50  $\mu\text{L}$  of sodium bicarbonate solution were added. The kinetics of the reaction at  $37^\circ\text{C}$  was followed for 120 min by measuring absorbance at 735 nm once each minute. Sample blank and reagent blank were also analyzed in each set of samples. The CGA calibration curve was used for quantification (0.01–0.6 g/L). All measurements were performed in triplicate, and results were expressed as mg CGA eq./g freeze-dried sample.

### Caffeine and CGA content

Caffeine and CGA content were determined with UPLC-MS/MS as described by Fernandez-Gomez *et al.* [2016]. Samples were diluted with Milli-Q water. For CGA quantification, 50 µg/mL of phloroglucinol were added as an internal standard, and 50 µg/mL of salicylic acid as an internal standard for caffeine. All the analyses were performed in triplicate, and results were expressed as mg caffeine or CGA/g freeze-dried sample.

### Browning

Browning was measured at 405 nm. All measurements were made in triplicate, and results were expressed as absorbance (Abs)/g freeze-dried sample.

### Melanoidins

Melanoidins content was determined according to Silván *et al.* [2010]. Samples were subjected to ultrafiltration using a Microcon YM-10 regenerated cellulose 10 kDa (Millipore, Bedford, MA, USA) at 12,000×g for 10 min. Melanoidins were measured spectrophotometrically at 405 nm. The analysis was carried out in triplicate and results were expressed as absorbance (Abs)/g freeze-dried sample.

## Health-promoting properties of bread

### Antioxidant capacity

An indirect ABTS<sup>•+</sup> decolourisation assay was performed according to Oki *et al.* [2006]. An ABTS<sup>•+</sup> stock solution was prepared by adding 44 µL of potassium persulfate (140 mmol/L) to a 2.5 mL ABTS<sup>•+</sup> aqueous solution (7 mmol/L). The mixture was allowed to stand for 16 h at room temperature. The working solution of the radical ABTS<sup>•+</sup> was prepared by diluting the stock solution 1:75 (v/v) in a sodium phosphate buffer (5 mmol/L, pH 7.4) to obtain an absorbance value of 0.7±0.02 at 734 nm. Samples (30 µL) were added to 270 µL of the working solution of ABTS<sup>•+</sup> in a microplate. Absorbance was measured at 734 nm and 30°C for 18 min, every 2 min. The reaction was complete after 5 min. CGA calibration (0.15–2 mmol/L) was used. All measurements were performed in triplicate. Results were expressed as mg CGA eq/g freeze-dried sample.

The antioxidant capacity of the insoluble fraction of the digested breads was determined by a direct ABTS<sup>•+</sup> assay or QUENCHER assay [Açar *et al.*, 2009]. Briefly, 10 mg of the sample were mixed with 70 mg of cellulose and stirred. After that, 10 mg of the mixture were mixed with 1.7 mL of the ABTS<sup>•+</sup> solution in a thermomixer (600 rpm, 37°C, 5 min) and centrifuged (4500×g, 25°C, 2 min). Absorbance (734 nm) of the supernatant was measured in a microplate. A CGA calibration curve (0.01–0.25 g/L) was used. Measurements were performed in triplicate and results expressed as g CGA eq/100 g.

### α-Glucosidase activity

Alpha-glucosidase activity was determined following Berthelot & Delmotte [1999] with slight modifications. Briefly, 0.1 g of rat intestine powder was dissolved in 3 mL of NaCl (90 g/L), sonicated in an ice bath for 6 min and then centrifuged at 10,000×g for 30 min to extract the enzyme contained

in the supernatant. In a 96-well microplate, 100 µL of the sample dissolved in phosphate buffered saline (100 mmol/L, pH 6.9) were mixed with 100 µL of α-glucosidase (diluted 1/10) and 100 µL of 4-MUG (2 mmol/L). Fluorescence was then monitored at an excitation wavelength of 360 nm and an emission wavelength of 460 nm at 37°C for 30 min. Blank of sample and negative control (buffer, enzyme and 4-MUG) were included. Acarbose was used as a positive control of the inhibition of enzymatic activity. Curves of samples and acarbose were assayed to cover the whole range of inhibition of the enzyme (~ 0.5–96 %). The percentage of α-glucosidase inhibition was calculated using the equation: % α-glucosidase = [(F<sub>n.c.</sub> - F<sub>s</sub>) / F<sub>n.c.</sub>] × 100, where F<sub>n.c.</sub> is the fluorescence of the negative control (without inhibitor) and F<sub>s</sub> is the fluorescence of the sample. All measurements were performed in triplicate, and results were expressed as sample concentration (g/L) causing 50% α-glucosidase inhibition (IC<sub>50</sub>).

### Release of nutrients and bioactive compounds during bread digestion

Bioaccessibility of the nutrients and bioactive compounds composing the breads was estimated applying an *in vitro* oral-gastrointestinal digestion system according to Hollebeek *et al.* [2013] with minor modifications. One g of sample was digested as follows: salivary step (pH 6.9, 10 mL, 5 min, 3.9 U-α-amylase/mL, aerobic), gastric step (pH 2, 13 mL, 90 min, 71.2 U pepsin/mL, aerobic), and duodenal step (pH 7, 16 mL, 150 min, 9.2 mg pancreatin and 55.2 mg bile extract/mL, aerobic). The obtained digests were centrifuged (1700×g, 4°C, 20 min) and separated into soluble and insoluble fractions. The soluble fraction was treated to mimic human intestinal reabsorption of bile salts with cholestyramine resin (100 g/L) [Martinez-Saez *et al.*, 2017]. The soluble and insoluble fractions were freeze-dried and stored in a dry cold place until analysis.

The soluble fractions of the digested breads were analyzed for contents of glycemic sugars, free amino acids, TPC, CGA and caffeine content, TAC, and α-glucosidase activity as described in the section of physicochemical analyses and health-promoting properties of bread, respectively. The insoluble fraction was recovered, and its antioxidant capacity was analyzed by direct ABTS<sup>•+</sup> assay as described in the section of health-promoting properties of bread.

### Statistical analysis

Data were expressed as mean ± standard deviation. Statistical analyses were performed using Statistica 7.1 (Stat Software Inc., California, USA). Experimental data were analyzed using ANOVA and significant differences among means from triplicate analyses at *p* ≤ 0.05 were determined using post hoc Duncan's test.

## RESULTS AND DISCUSSION

### Validation of CSE and CHE as food ingredients

#### Food safety

The microbiological analyses of CSE showed values of 3.25×10<sup>5</sup> CFU/g of endospores, 4.3×10<sup>3</sup> CFU/g of total aerobic microorganisms, and a yeast and mould con-

tent of less than  $10^2$  CFU/g. The analyses of CHE showed values below  $10^2$  CFU/g for all the microorganisms under study. The main safety hazard is the presence of ochratoxin A (OTA), a mycotoxin released by *Aspergillus* potentially nephrotoxic, carcinogenic, teratogenic and genotoxic. OTA limits have been defined for roasted coffee ( $5 \mu\text{g}/\text{kg}$ ) and soluble coffee ( $10 \mu\text{g}/\text{kg}$ ) [European Parliament regulation (EC) N.123/2005, 2005]. Since CSE and CHE are extracts of two coffee by-products, there is no specific OTA regulation limit. However, the absence of moulds in these extracts reduces the risk of OTA contamination. Adding CSE and CHE to the bread formulation did not increase microorganism content, suggesting good microbiology quality of the food ingredients.

#### Physicochemical analyses

Data on the physicochemical characterization of CSE and CHE are shown in Table 2. Differences in the composition of CSE and CHE can be explained by their nature, as they come from different anatomic parts of the coffee fruit with different chemical components [del Castillo *et al.*, 2019].

Total carbohydrate contents were  $17.95 \text{ g}/100 \text{ g}$  and  $24.18 \text{ g}/100 \text{ g}$  in CSE and CHE, respectively. Results obtained for CSE are in line with those reported by del Castillo *et al.* [2019]. As in the coffee silverskin raw material, CSE had a low content of simple sugars like glucose ( $0.25 \text{ g}/100 \text{ g}$ ), fructose ( $0.83 \text{ g}/100 \text{ g}$ ) and sucrose ( $1.93 \text{ g}/100 \text{ g}$ ). CHE had a higher glucose content ( $8.88 \text{ g}/100 \text{ g}$ ), while the contents of sucrose ( $0.12 \text{ g}/100 \text{ g}$ ) and fructose ( $0.01 \text{ g}/100 \text{ g}$ ) were lower than in CSE. Both extracts, especially CSE, presented low amounts of glycemic sugars.

The results of TDF content determination in CSE ( $36.06 \text{ g}/100 \text{ g}$ ) agree with those reported by del Castillo *et al.* [2019]. CSE dietary fibre is mainly composed of SDF ( $27 \text{ g}/100 \text{ g}$  of TDF). CHE presented a high amount of TDF ( $68.43 \text{ g}/100 \text{ g}$ ), mainly SDF ( $67.45 \text{ g}/100 \text{ g}$  of TDF). Both extracts can be considered good sources of SDF, especially CHE (Table 2).

The amount of soluble proteins present in CSE was  $17.54 \text{ g}/100 \text{ g}$  (Table 2), which is similar to the soluble protein content reported by Narita & Inouye, 2014 for CSE obtained under subcritical water conditions. CHE had a lower soluble protein content of  $5.07 \text{ g}/100 \text{ g}$  close to the  $7 \text{ g}/100 \text{ g}$  described by Gouvea *et al.* [2009] for coffee husk raw material. Therefore, CSE may be a better natural source of protein than CHE.

Similarly, caffeine content was also higher in CSE ( $53.25 \text{ mg}/\text{g}$ ) than in CHE ( $13.93 \text{ mg}/\text{g}$ ) with values similar to those reported by Mesías *et al.* [2014] and Murthy & Naidu [2012]. Since the safety limits of caffeine intake fixed by the European Food Safety Authority [2015] are  $400 \text{ mg}$  per day for non-pregnant adults and  $200 \text{ mg}$  per day for pregnant women, the daily intake of CSE considered to be safe would be  $\sim 7.5 \text{ g}$  for non-pregnant adults and  $\sim 4 \text{ g}$  for pregnant women. Consequently, the safe daily intake of CHE would be 4 times higher than for CSE. Caffeine content should be considered when applying these extracts for human nutrition and, in particular, considering certain members of the population.

TABLE 2. Physicochemical characterization of coffee silverskin (CSE) and coffee husk (CHE) extracts.

Analysis	CSE	CHE
Total carbohydrates (g/100 g)	$17.95 \pm 1.62^b$	$24.18 \pm 1.64^a$
Glucose (g/100 g)	$0.25 \pm 0.03^b$	$8.88 \pm 0.10^a$
Fructose (g/100 g)	$0.83 \pm 0.02^a$	$0.01 \pm 0.01^b$
Sucrose (g/100 g)	$1.93 \pm 0.21^a$	$0.12 \pm 0.01^b$
TDF (g/100 g) <sup>#</sup>	$36.06 \pm 1.67^b$	$68.43 \pm 4.50^a$
SDF (g/100 g) <sup>#</sup>	$27.90 \pm 1.14^b$	$67.45 \pm 4.65^a$
IDF (g/100 g) <sup>#</sup>	$8.17 \pm 0.53^a$	$0.99 \pm 0.15^b$
Total proteins (g/100 g)	$17.54 \pm 0.82^a$	$5.07 \pm 0.38^b$
Lipids (g/100 g)	$1.83 \pm 0.58^a$	$1.14 \pm 0.02^a$
Caffeine (mg/g)	$53.25 \pm 1.65^a$	$13.93 \pm 0.80^b$
CGA (mg/g)	$21.30 \pm 6.17^a$	$1.71 \pm 0.27^b$
Browning (abs/g)	$374.93 \pm 18.09^a$	$58.27 \pm 3.72^b$
Melanoidins (abs/g) <sup>*</sup>	$306.13 \pm 4.00^a$	$52.13 \pm 2.00^b$
TPC (mg CGA eq./g)	$81.03 \pm 5.56^a$	$19.67 \pm 1.24^b$
TAC (mg CGA eq./g)	$190.86 \pm 4.25^a$	$52.30 \pm 0.93^b$
Estimated calories kcal/100 g extract	158.4	127.3
$\alpha$ -Glucosidase (IC <sub>50</sub> , mg/mL)	$1.42 \pm 0.32^b$	$2.46 \pm 0.21^a$

Data are expressed as mean ( $n=3$ )  $\pm$  standard deviation. Values in each row having different letters indicate significant differences at  $p < 0.05$  (Duncan's test). CGA, chlorogenic acid; IDF, insoluble dietary fibre; SDF, soluble dietary fibre; TAC, total antioxidant capacity; TDF, total dietary fibre; TPC, total phenolic compounds.

<sup>#</sup>Results are expressed as mean ( $n=2$ )  $\pm$  standard deviation; <sup>\*</sup>Analyses performed on aqueous extracts fraction  $> 10 \text{ kDa}$ .

CGA content was significantly higher in CSE ( $21.30 \text{ mg}/\text{g}$ ) than in CHE ( $1.71 \text{ mg}/\text{g}$ ). These values are lower than those described by other authors [Mesías *et al.*, 2014; Murthy & Naidu, 2012] but higher than those reported by Bresciani *et al.* [2014]. These differences might be attributed to solvent extraction, set conditions, quantification method or origin of the by-products.

Differences in browning and melanoidin were also significant between the coffee by-product extracts (Table 2). The high browning and melanoidins values of CSE are associated with the Maillard reaction occurring during the roasting process. Data on browning and melanoidins suggest that CSE may be a good source of natural colorants to be applied in the food industry. The use of natural pigments is the current marketing trend because of consumers' concern about the safety of artificial food dyes, which is reinforced by the possible health benefits of natural pigments [Rodríguez-Amaya, 2016]. In this regard, coffee melanoidins exert several beneficial effects on human health due to their antioxidant, antimicrobial, anticarcinogenic, anti-inflammatory, antihypertensive, and antiglycative properties [Moreira *et al.*, 2012].

A TPC content of  $81.03 \text{ mg CGA eq}/\text{g}$  was found in CSE (Table 2). This value was 4 times higher than in CHE

(19.67 mg CGA eq/g). This difference in TPC content can be explained on the basis of CGA content, one of the main phenolic compounds in coffee by-products [del Castillo *et al.*, 2019]. Similarly, TAC was 3.6 times higher in CSE than in CHE (Table 2). This great difference may be associated with their different contents of melanoidins and phenolic compounds such as CGA which has been described as the main contributor to the overall antioxidant power of coffee by-products [Mesías *et al.*, 2014].

#### Effects of CSE and CHE on $\alpha$ -glucosidase activity

As seen in Table 2, the  $IC_{50}$  values obtained for each extract were significantly different. An  $IC_{50}$  value of 1.42 mg/mL was obtained for CSE which is comparable to values previously described by our group [del Castillo *et al.*, 2016]. Differences in the effect of the extracts on  $\alpha$ -glucosidase activity might be associated with their CGA content. This bioactive compound previously showed an  $IC_{50}$  value of 0.25 mg/mL [del Castillo *et al.*, 2016]. Other authors have proposed that CGA strongly inhibits  $\alpha$ -glucosidase activity and reduces postprandial blood glucose [Obloh *et al.*, 2015]. Data on the inhibitory effect on  $\alpha$ -glucosidase activity pinpoint CSE and CHE as ingredients to be used for better control of postprandial glucose level.

#### Food application of CSE and CHE

##### Sensory analysis of innovative breads

Breads were considered acceptable if their average score was equal to or higher than 4.0 (Figure 1). The sensory analysis indicated that the quality of NF made with inulin and rice protein was good (all values  $>5.1$ ), and no significant differences were observed between NF and NFS or NFH breads. Therefore, the addition of these two coffee by-products extracts (25 g/kg d.m.) did not affect the sensory quality of the product. Comparing NFS and NFH with NF, color was the most appreciated parameter, especially for NFH. The appearance (color) of the breads containing CSE and CHE supports its feasibility as a natural colorant, providing the typical color of wholemeal breads. These results highlight the potential application of these extracts as natural colorants for gluten-free bread formulations, since the darkening of the crumb color is desirable in the consumer choice of a bread [Matos & Rosell, 2012].

##### Nutritional analysis of innovative breads

Total carbohydrate content of the innovative breads (NF) and those containing CSE or CHE (25 g/kg d.m.) and inulin (NFS and NFH) were significantly different from the commercial pre-mix gluten-free bread (CR) (Table 3). Fructose, glucose, and sucrose contents were significantly lower in all new bread formulations (NF, NFS and NFH). As seen in Table 1, the reduction of starch and the substitution of sugar by inulin in the innovative breads decreased glycemic carbohydrates, as described by Rizzello *et al.* [2016].

TDF content of NF, NFS and NFS breads was significantly higher than CR. Adding inulin to the NF duplicated the TDF content compared to CR. CSE and CHE accounted for approximately 13% of TDF in the new bread formulations (NFS and NFH). Coffee silverskin has previously been proposed as an ingredient for reducing caloric density in biscuits

[García-Serna *et al.*, 2014]. The new bakery products could be labelled as having a “high fibre content”, as they contain at least 6 g of fibre per 100 g [European Parliament Regulation [EC] N. 1924/2006 [2006]. Therefore, the use of inulin as dietary fibre improved the nutritional value of the bread.

Total protein values were significantly higher in the innovative breads (NF, NFS and NFH) compared to CR due to the addition of rice protein (Table 3). CSE provided 7% extra protein in NFS breads compared to NF in accordance with the higher protein content of CSE (Table 2). Therefore, NF, NFS and NFH breads can be considered products that are “high in protein”, containing at least 20% of the energy value of the food provided by protein [European Parliament Regulation [EC] N. 1924/2006]. Several studies have demonstrated that the consumption of rice protein can be associated with a lower risk of oxidative stress preventing the occurrence of several diseases such as hyperlipidemia [Wang *et al.*, 2016].

Caffeine content of NFS and NFH breads was determined to evaluate the safety of consuming these products (Table 3). The daily bread intake recommended by WHO [2003] for European countries is 250–350 g. Considering the safety limits of the intake fixed by EFSA [2015], both products can be considered safe for non-pregnant adults. In the case of pregnant women, NFH consumption is safe if the daily consumption of NFS does not exceed 250 g.

CGA content in the NFS and NFH breads supplemented with coffee by-products extracts (25 g/kg d.m.) degraded by 68–70% (based on initial CGA concentration in CSE and CHE) (Table 2). Other studies have also found high thermal degradation of CGA during the baking process [Rupasinghe *et al.*, 2008]. The low sugar content in NF bread produced the highest reduction in browning compared to CR bread (Table 3). Browning values of NFS and NFH breads were higher and similar to CR, respectively. As reported in Figure 2, the addition of the extracts provided a natural color and the typical appearance of wholemeal breads. As shown in Table 3, the inclusion of extracts as food ingredients in NFS and NFH breads significantly improved

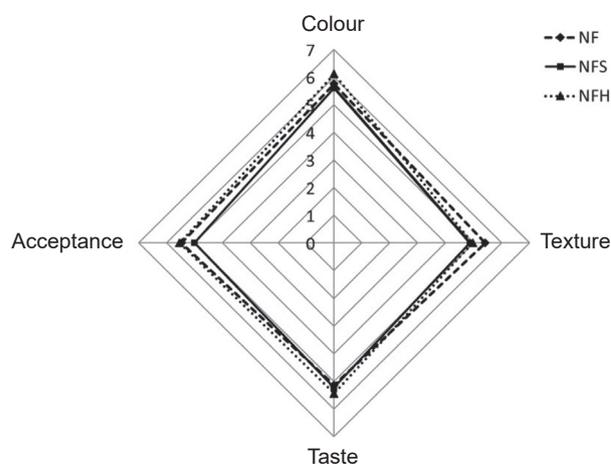


FIGURE 1. Mean scores (n=30) of 1–7 scale for overall acceptance of innovative gluten-free breads NF, NFS and NFH. NF – new formulation, NFS – new formulation with 25 g of coffee silverskin extract (CSE)/kg d.m.; NFH – new formulation with 25 g of coffee husk extract (CHE)/ kg d.m.

TABLE 3. Chemical characterization of CR, NF, NFS and NFH breads.

Analysis	CR	NF	NFS	NFH
Total carbohydrates(g/100 g)	27.74±2.08 <sup>a</sup>	18.76±2.78 <sup>b</sup>	18.21±1.26 <sup>b</sup>	18.38±1.40 <sup>b</sup>
Glucose (g/100 g)	2.70±0.06 <sup>a</sup>	0.26±0.01 <sup>c</sup>	0.28±0.01 <sup>c</sup>	0.61±0.02 <sup>b</sup>
Fructose (g/100 g)	5.10±0.06 <sup>a</sup>	1.52±0.10 <sup>b</sup>	1.53±0.02 <sup>b</sup>	1.47±0.12 <sup>b</sup>
Sucrose (g/100 g)	0.42±0.02 <sup>a</sup>	0.06±0.02 <sup>c</sup>	0.04±0.02 <sup>c</sup>	0.10±0.02 <sup>b</sup>
TDF (g/100 g)	5.73±0.91 <sup>b</sup>	11.28±0.37 <sup>a</sup>	12.72±0.52 <sup>a</sup>	12.82±1.23 <sup>a</sup>
SDF (g/100 g)	4.20±0.33 <sup>b</sup>	5.90±0.20 <sup>ab</sup>	6.20±0.20 <sup>ab</sup>	6.80±0.80 <sup>a</sup>
IDF (g/100 g)	1.53±0.58 <sup>c</sup>	5.30±0.10 <sup>b</sup>	6.50±0.30 <sup>a</sup>	6.00±0.40 <sup>ab</sup>
Total proteins (g/100 g)	1.21±0.25 <sup>c</sup>	8.36±0.21 <sup>b</sup>	9.03±0.40 <sup>a</sup>	7.36±0.98 <sup>b</sup>
Lipids (g/100 g)	14.89±0.47 <sup>a</sup>	2.14±0.38 <sup>c</sup>	3.22±0.22 <sup>b</sup>	3.30±0.31 <sup>b</sup>
Caffeine (mg/g)	nd	nd	0.72±0.05 <sup>a</sup>	0.36±0.02 <sup>b</sup>
CGA (mg/g)	nd	nd	0.25±0.01 <sup>a</sup>	0.02±0.002 <sup>b</sup>
Browning (Abs <sub>405</sub> /g)	1.63±0.09 <sup>b</sup>	0.90±0.05 <sup>c</sup>	2.22±0.08 <sup>a</sup>	1.21±0.02 <sup>b</sup>
Melanoidins* (Abs <sub>405</sub> /g)	1.05±0.05 <sup>b</sup>	0.64±0.05 <sup>c</sup>	1.54±0.03 <sup>a</sup>	1.11±0.13 <sup>b</sup>
TPC (mg CGA/g)	175.86±2.43 <sup>b</sup>	54.69±0.81 <sup>d</sup>	254.92±7.73 <sup>a</sup>	121.12±6.12 <sup>c</sup>
TAC (mg CGA/g)	87.24±1.82 <sup>c</sup>	76.10±1.28 <sup>d</sup>	288.27±3.57 <sup>a</sup>	129.39±1.80 <sup>b</sup>
Estimated calories (kcal/100 g bread)	250	128	138	133
α-Glucosidase (IC <sub>50</sub> ,mg/mL)	38.10±2.10 <sup>b</sup>	108.60±1.76 <sup>a</sup>	27.00±1.95 <sup>d</sup>	32.70±2.56 <sup>c</sup>

CR – commercial recipe; NF – new formulation; NFS – new formulation with 25 g of coffee silverskin extract (CSE)/kg d.m.; NFH – new formulation with 25 g of coffee husk extract (CHE)/ kg d.m.

Data are expressed as mean (n=3) ± standard deviation. Values in each row having different letters indicate significant differences at p<0.05 (Duncan's test). CGA, chlorogenic acid; IDF, insoluble dietary fibre; nd, not detected; SDF, soluble dietary fibre; TAC, total antioxidant capacity; TDF, total dietary fibre; TPC, total phenolic compounds. \*Analyses performed on aqueous extracts fraction >10 kDa.

antioxidant capacity (3.78 and 1.70-times higher compared to NF control bread for NFS and NFH breads, respectively). This higher antioxidant capacity could be related to phenolic compounds such as CGA and melanoidins. Antioxidants can play a double role as food preservatives and health-promoting compounds.

The highest TPC content was found in NFS bread (Table 3). Differences in TPC may be due to bread composition. CR bread had highest content of gluten-free baking pre-mix (Table 1) which had corn flour. Soong *et al.* [2014] have reported that baked muffins made with corn flour had a higher TPC content than those baked with rice flours. Consequently, NF had the lowest TPC content which increased to 79% and 55% with the addition of CSE and CHE, respectively. These results are in line with TPC values shown by CSE and CHE (Table 2).

#### Effect of bread components on α-glucosidase activity

As seen in Table 3, the addition of CSE and CHE to NF inhibited α-glucosidase activity in accordance with the results obtained for CSE and CHE ingredients (Table 2). The inhibitory activity of CSE was related to the presence of CGA [del Castillo *et al.*, 2016]. IC<sub>50</sub> values of NFS (27.00 mg/mL) and NFH (32.70 mg/mL) breads are comparable to those reported for

similar products such as pasta enriched with faba bean flour [Turco *et al.*, 2016].

#### Bioaccessibility of nutrients and bioactive compounds composing the innovative breads

As reported in Table 4, substituting the sugar and starch of the gluten-free baking pre-mix with inulin significantly reduced glycemic sugar content in digested NF, NFS and NFH compared to CR. Total glucose and fructose content was by at least 35% lower in NFH and NFS. A low content of free glucose available for absorption is an important factor in the prevention of diabetes.

CGA was not detected in NFS and NFH digested breads (Table 4). Previous studies have reported a significant decrease in CGA concentration during CSE digestion, especially after the hydrolysis reaction of the duodenal step [Fernandez-Gomez *et al.*, 2016]. Part of the CGA could be linked to the maillardized dietary coffee fibre structure which could protect against oxidative damages in the large intestine. The inclusion of phenolic groups, especially CGA, in the coffee melanoidin skeleton has been reported [Moreira *et al.*, 2012].

The antioxidant capacity of digested fractions of NFS and NFH breads was significantly higher than CR but was

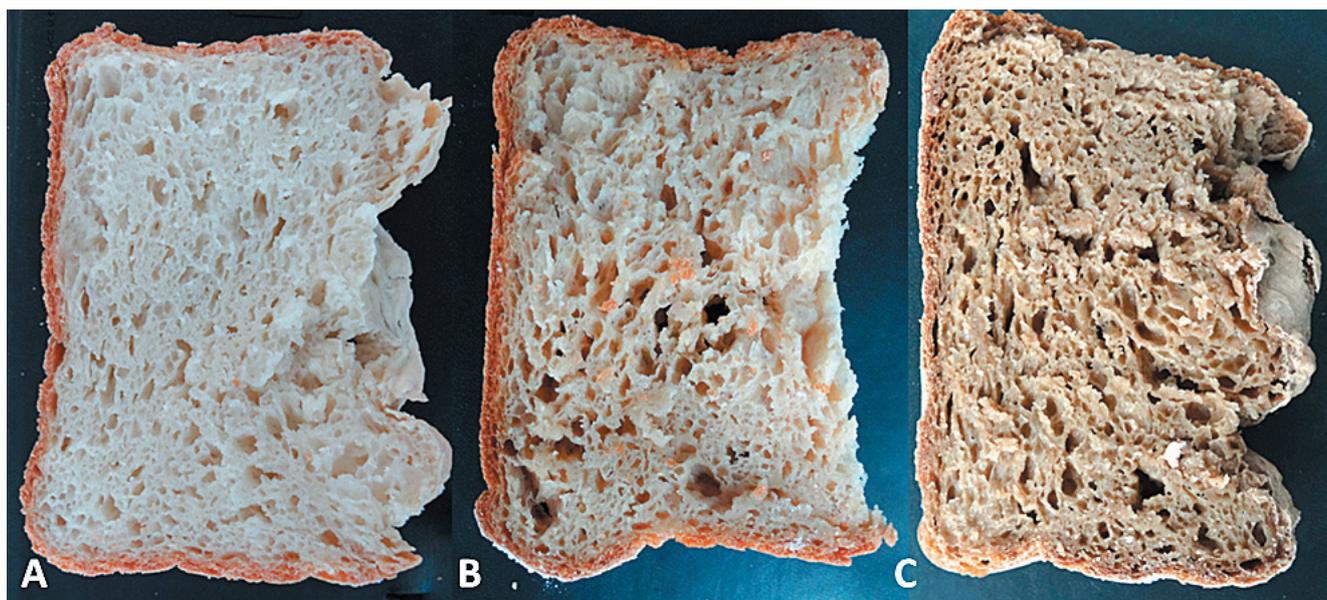


FIGURE 2. Cross section of innovative breads: (A) NF – new formulation, (B) NFS – new formulation with 25 g of coffee silverskin extract (CSE)/kg d.m.; (C) NFH – new formulation with 25 g of coffee husk extract (CHE)/ kg d.m.

similar to NF. This might be related to the release of several peptides with antioxidant properties caused by the hydrolysis of the rice protein during the digestion process. The rice protein used in this study is enriched in sulfur-amino acids which exert an antioxidative effect by scavenging free radicals [Métayer *et al.*, 2008].

As seen in Table 4, the antioxidant capacity of the insoluble fraction recovered from the digestion process of NFS and NFH breads was significantly higher than that found for

NF samples. Maillardized dietary coffee fibre may be responsible for the higher antioxidant power of the non-bioaccessible fraction [Silván *et al.*, 2010].

No significant differences were found among the  $IC_{50}$  values of the bioaccessible fractions of the digested breads (Table 4). The reduced inhibitory effect may be associated with the loss of free CGA during the digestion process. CGA inhibited  $\alpha$ -glucosidase activity in a dose-dependent manner [Oboh *et al.*, 2015].

TABLE 4. Chemical characterization of CR, NF, NFS and NFH digested breads.

Analysis	CR	NF	NFS	NFH
<i>Soluble fraction</i>				
Glucose (g/100 g)	7.47±0.13 <sup>a</sup>	7.05±0.19 <sup>b</sup>	5.86±0.08 <sup>c</sup>	5.72±0.28 <sup>c</sup>
Fructose (g/100 g)	3.42±0.11 <sup>a</sup>	3.37±0.08 <sup>a</sup>	1.15±0.01 <sup>c</sup>	1.58±0.10 <sup>b</sup>
Sucrose (g/100 g)	0.42±0.02	nd	nd	nd
FAG (mg lys eq/1000 g)	0.20±0.01 <sup>b</sup>	0.26±0.07 <sup>b</sup>	0.33±0.12 <sup>ab</sup>	0.45±0.05 <sup>a</sup>
Caffeine (mg/g)	nd	nd	0.43±0.01 <sup>a</sup>	0.17±0.02 <sup>b</sup>
CGA (mg/g)	nd	nd	nd	nd
TPC (mg CGA/g)	134.86±6.05 <sup>c</sup>	227.92±17.56 <sup>b</sup>	265.70±18.02 <sup>a</sup>	222.18±7.89 <sup>b</sup>
TAC-I (mg CGA eq/g)	573.96±30.70 <sup>b</sup>	742.62±23.55 <sup>a</sup>	756.29±22.76 <sup>a</sup>	721.34±27.86 <sup>a</sup>
$\alpha$ -Glucosidase ( $IC_{50}$ , mg/mL)	5.40±0.85 <sup>a</sup>	5.20±1.20 <sup>a</sup>	5.20±0.87 <sup>a</sup>	5.90±1.45 <sup>a</sup>
<i>Insoluble fraction</i>				
TAC-D (g CGA eq/100 g)	nd	22.88±2.80 <sup>c</sup>	35.01±0.97 <sup>a</sup>	30.68±0.94 <sup>b</sup>

CR – commercial recipe; NF – new formulation; NFS – new formulation with 25 g of coffee silverskin extract (CSE)/kg d.m.; NFH – new formulation with 25 g of coffee husk extract (CHE)/ kg d.m.

Data are expressed as mean (n=3) ± standard deviation. Values in each row having different letters indicate significant differences at  $p < 0.05$  (Duncan's test). CGA, chlorogenic acid; nd, not detected; FAG, free amino groups; TAC-D, total antioxidant capacity direct method; TAC-I, total antioxidant capacity indirect method; TPC, total phenolic compounds.

## CONCLUSIONS

CSE and CHE are sustainable natural sources of antioxidants and  $\alpha$ -glucosidase inhibitors. Furthermore, the extracts can be employed as natural colorants in the high dietary content formulation to provide the typical appearance of wholemeal bread. The new gluten-free bread formulations developed during this study may provide additional health benefits in people with specific nutritional requirements. Proof-of-concept human intervention studies are needed to verify the positive effects of these breads in controlling postprandial glucose. In conclusion, the nutritional properties of the new gluten-free bread formulations were greatly improved compared to the gluten-free commercial breads.

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