Bioaccessibility, physicochemical, sensorial, and nutritional characteristics of bread containing broccoli co-products

Tomás Lafarga [a], Eimear Gallagher [b], Ariadna Bademunt [c], Inmaculada Viñas [c], Gloria Bobo [a], Silvia Villaró [a], and Ingrid Aguiló-Aguayo [a]*

[a] IRTA, XaRTA-Postharvest, Parc Científic i Tecnològic Agroalimentari de Lleida, Parc de Gardeny, Edifici Fruitcentre, 25003, Lleida, Catalonia, Spain.

[b] Teagasc Food Research Centre Ashtown, Department of Food Chemistry and Technology, Dublin, Ireland.

[c] University of Lleida, Food Technology Department, XaRTA-Postharvest, Agrotecnio Center, Lleida, Spain.

[*]**Corresponding author**:
Dr Aguiló-Aguayo, phone: (+34) 973003431 Ext. 1551, email: Ingrid.Aguilo@irta.cat

Abbreviations

a_w: Water activity; DW: Dry weight; FRAP: Ferric reducing ability of plasma; DPPH: 2,2-diphenyl-1-picrylhydrazyl; C^*_{ab}: Chroma; δE: Difference from the control; S.D.: Standard deviation; ANOVA: Analysis of variance; TPC: Total phenolic content; CKB: Control bread; BLB: bread containing broccoli leaves; BSB: Bread containing broccoli stalks.

Abstract

The effects of the inclusion of industrial broccoli co-products namely stalks and leaves into bread on physicochemical and nutritional properties were evaluated. Incorporation of powdered broccoli co-products at a concentration of 2% (w/w) into bread formulations resulted in decreased weight and specific volumes when compared to the control ($p<0.05$). Broccoli-containing breads showed an increased green hue and a higher crust and crumb colour intensity ($p<0.05$). Incorporation of broccoli co-products into bread formulations significantly increased the total phenolic content and antioxidant capacity of the breads ($p<0.05$). The overall acceptance and appearance of the breads were not affected by broccoli incorporation. The phenolic content and antioxidant capacity of the three formulations increased after the gastric and intestinal phases of digestion when compared to the initial stage, measured from a methanol:water extract ($p<0.05$). Results obtained herein could open novel commercial opportunities for food processors and reduce the amount of food discarded as waste.

Practical applications

In recent decades, functional foods have gained increased consumer interest and are currently one of the hot trends in the food industry. In addition, sustainable food production and waste valorisation are becoming important issues in the food industry, where high amounts of co- products are generated. A large percentage of the total mass of broccoli plants is discarded as waste or used for low value purposes. Broccoli co-products are rich in bioactive compounds. Therefore, enrichment of bread with bioactive compounds obtained from broccoli co-products is both an economic and environmentally attractive option for food processors.

Keywords: functional bread, antioxidant activity, baked goods, broccoli co-products, phenolic compounds, bioaccessibility

1. Introduction

The generation of waste in the food processing industry is unfortunately unavoidable. However, a large amount of the food and food co-products currently discarded as waste are rich in valuable compounds which could be reincorporated into the food chain. Broccoli co-products such as leaves and stalks contain large amounts of health-promoting compounds including dietary fibre, vitamins, glucosinolates, and phenolic compounds (Domínguez-Perles et al., 2010; Hwang and Lim, 2015). Indeed, Wijngaard et al. (2009) reported that co-products generated during broccoli processing showed the highest antioxidant potential when compared to a large number of food-derived co-products generated in Ireland. In addition, Lafarga et al. (2018d) recently reported a similar nutritional profile between the stems and florets or leaves of several *Brassica* vegetables including different broccoli varieties. Therefore, the utilization of edible broccoli-derived co-products as novel ingredients for the development of functional foods would not only reduce the amount of food discarded as waste or used for low value purposes but also promote health and open novel commercial opportunities for food processors.

Functional foods deliver additional or enhanced benefits over their basic nutritional value and the functional foods market is currently one of the top trends in the food industry (Grasso et al., 2014). Besides being considered as safe and being encouraged as part of a healthy diet, bread and other baked products are of particular interest due to their popularity and widespread consumption. In addition, public confidence in the safety of food products has been damaged by numerous scares. However, a special study within the framework of a Eurobarometer survey, which asked respondents to state how they felt about the safety of a number of food products, concluded that bread and baked products were considered as the safest by the majority of the respondents (Gracia and Albisu, 2001).

Bread and baked products are ideally suited as delivery vehicles and have been repeatedly used as food vehicles for healthy compounds including folic acid (Crider et al., 2011), chitosan (Lafarga et al., 2013), lutein (Read et al., 2015), and enzymatic hydrolysates with antihypertensive properties (Fitzgerald et al., 2014). Glucosinolates and other health-promoting compounds found in broccoli co-products can be heavily lost during thermal processing (Lafarga et al., 2018a). However, the temperature achieved in the core of bread and the mild pH variations that occur during the breadmaking process suggest that these compounds could be retained in the end product. For example, Lafarga et al. (2016) recently demonstrated antihypertensive effects in spontaneously hypertensive rats after ingestion of a bread manufactured using an enzymatic hydrolysate with renin (EC 3.4.23.15) and angiotensin-I-converting enzyme (EC 3.4.15.1) inhibitory activities. Similar results were obtained by Alashi et al. (2018) after incorporation of leafy vegetables into bread formulations. In both cases, the *in vivo* bioactivity of the added ingredients was resistant to the breadmaking process.

The objective of this work was to produce functional breads with enhanced concentrations of fibre and phenolic compounds using broccoli co-products and to study the influence of their inclusion on the overall quality and acceptance of the product. The effects of the inclusion of broccoli stems or leaves into bread on parameters including colour, texture, moisture, water activity (a_w), antioxidant capacity, overall acceptance, or total phenolic content (TPC) were studied over a 7-day period. In addition, the resistance of these compounds to a simulated gastrointestinal digestion was also assessed.

2. Materials and methods

2.1 Chemicals and reagents

Methanol and ferric chloride were purchased from Panreac (Barcelona, Spain). Gallic acid, ascorbic acid, hydrochloride, 2,4,6-tris(2-pyridyl)-s-triazine, 2,2-diphenyl-1-picrylhydrazyl (DPPH), tris(2-carboxyethyl)phosphine hydrochloride, potassium phosphate monobasic, potassium phosphate dibasic, calcium chloride, α-amylase (EC 3.2.1.1), pepsin (EC 3.4.23.1), and sodium carbonate were purchased from Sigma-Aldrich (Steinheim, Germany). Folin-Ciocalteu's reagent was purchased from VWR (Llinars del Vallès, Spain). All reagents used were of analytical grade. Dried yeast (Nurture, Granada, Spain), vegetable fat (Hacendado, Valencia, Spain), wheat flour and other baking ingredients were all purchased locally.

2.2 Breadmaking

Broccoli processing co-products were kindly provided by Congelados de Navarra S.A.U (Navarra, Spain). Stems were cut into 10 × 10 × 10 mm cubes, sanitized in 100 ppm sodium hypochlorite for 2 min, rinsed with tap water, and left to dry at room temperature to reduce surface contamination. Leaves were sanitized in 100 ppm sodium hypochlorite for 2 min, rinsed with tap water, and left to dry at room temperature. Both stems and leaves were frozen, freeze-dried, milled to a thin powder, and stored at -20 ºC until further use.

Preliminary baking trials (data not shown) were carried out to establish the maximum flour substitution level and most appropriate baking times. Following these trials, formulations containing broccoli co-products at a concentration of 2% (w/w) obtained the highest acceptability scores. Bread loaves were produced following a straight dough baking procedure. Bread doughs were prepared for mixing according to the formulations listed in Table 1 and were mixed using an AM-7000 bread dough mixer (Orbegozo,

Murcia, Spain) equipped with a dough hook. Breads containing broccoli stalks and broccoli leaves at a concentration of 2% (w/w) were labelled as BSB and BLB, respectively. Control loaves were labelled as CKB. The amount of water added and the optimal mixing times (3.5 min) were calculated using a Chopin MixOlab (Chopin Technologies, Villenueve la Garenne, France). After mixing, the doughs were placed in a Rational SCC WE-101 oven (Rational AG, Landsberg am Lech, Germany) at 30 ºC and 80% relative humidity for 15 min. The bread pieces were then divided into 60 g pieces, moulded by hand, placed in tins (9 × 6 × 4 cm), and proofed for a further 45 min period. The loaves were baked at 200 ºC for 20 min in an industrial oven. Bread loaves were allowed to cool at room temperature for 2 h before being stored in sealed polyethylene bags at room temperature. The breads were analysed at days 1 and 7 post-baking.

2.3 Bread analysis

Crust and crumb colour recordings were taken using a Minolta CR-200 colorimeter (Minolta INC, Tokyo, Japan). CIE values were recorded in terms of L^* (lightness), a^* (redness, greenness), and b^* (yellowness/blueness). Calibration was carried out using a standard white tile (Y:92.5, x:0.3161, y:0.3321) provided by the manufacturer and the D65 illuminant, which approximates to daylight. Chroma (C^*_{ab}) and difference from the control (δE) were calculated as described previously (Wibowo et al. 2015). The results are the average of 10 measurements per formulation and replicate taken on day 1 post-baking.

Texture profile analysis was measured using a TA.XT2 Texture Analyzer (Stable Micro Systems Ltd., Surrey, England) connected to Exponent software v. 5.0.6.0 and equipped with a P/20 aluminium compression probe. The results are the average of 10 measurements per formulation and replicate taken on days 1 and 7 post-baking.

142 Bread loaf volume was calculated using a BVM-L370 volume measurer (TexVol
143 Instruments, Vike, Sweden). The results are the average of four samples and were
144 measured at day 1 post-baking for all loaves. Density was calculated dividing the weight
145 of each bread loaf into its volume. Specific volume was calculated by dividing the bread
146 loaves volume into their weight.
147 Moisture content of the breads was carried out using AACC method 44-15.02 and was
148 measured on days 1 and 7 post-baking.
149 The a_w of all samples was measured using an AquaLab meter (Decagon Devices Inc.,
150 WA, USA) and approximately 2 g of ground sample. Three measurements were taken for
151 each formulation and replicate on days 1 and 7 post-baking.
152 The pH of 1g of ground bread added to 10 g of distilled water was measured in a Basic
153 20 pH meter (Crison Instruments S.A., Barcelona, Spain) as previously described by
154 O'Shea et al. (2017). pH measurements were carried out in triplicate for each formulation
155 and replicate at day 1 post-baking.

156 **2.4 Total phenolic content**

157 The TPC was determined by the Folin Ciocalteu method following the modifications
158 described by Altisent et al. (2014). Briefly, for the extraction, the milled breads were
159 homogenized with methanol 70% (v/v) at a sample:methanol ratio of 1:4 (w/v) at 4 ºC.
160 Homogenization was performed using a T-25 ULTRA-TURRAX® homogenizer (IKA,
161 Staufem, Germany) operating at 12,000 rpm for 30 s. Homogenized samples were
162 immediately place in a stirrer at room temperature for 2 h and centrifuged using a Sigma-
163 3-18 KS centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at
164 10,000 × *g* for 20 min.
165 The assay was performed by adding 4.3 mL of MilliQ water and 0.5 mL of Folin-
166 Ciocalteu's reagent to 0.7 mL of extract. After incubation for 5 min at room temperature

in the dark, 2 mL of saturated sodium carbonate solution was added. The mixture was shaken and further incubated for 1 h at room temperature and in the dark. Absorbance was read at 760 nm using a GENESYS^{TM} 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, MA, USA). TPC was determined in triplicate for each treatment, sampling day, and replicate and results were expressed as mg of gallic acid equivalents per 100 g of dry weight (DW). Standard curves were prepared daily.

2.5 *In vitro* antioxidant activity: FRAP and DPPH· scavenging activity

Antioxidant activity was assessed using two different methods: the ferric reducing antioxidant power (FRAP) and the DPPH scavenging activity assays following the modifications described by Altisent et al. (2014). Both determinations were performed on the same extract used for the determination of TPC.

2.5.1 FRAP assay

The FRAP reagent was freshly prepared by mixing 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM hydrochloric acid, and 20 mM ferrous chloride in the proportion 10:1:1 (v/v/v). Determinations were carried out by mixing 1.4 mL of the FRAP reagent and 0.1 mL of the methanolic extract obtained following the methodology described above. After 20 min of incubation in the dark at 37 ºC and constant shaking, the absorbance was read at 593 nm using a GENESYS^{TM} 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, MA, USA). Antioxidant activity assessed using the FRAP assay was determined in triplicate per sample and replicate and expressed as mg of ascorbic acid equivalents per 100 g of DW. Standard curves were prepared daily.

2.5.2 DPPH assay

The assay was performed by adding 1.4 mL of 0.1 mM DPPH· solution to 0.1 mL of the methanolic extract obtained as described above. After 60 min of incubation at room temperature and in the dark, the absorbance was read at 515 nm using a using a GENESYS^{TM} 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, MA, USA). Antioxidant activity assessed using the DPPH assay was determined in triplicate per sample and replicate and expressed as mg of ascorbic acid equivalents per 100 g of DW. Standard curves were prepared daily.

2.6 *In vitro* gastrointestinal digestion

A simulated gastrointestinal digestion of the control and broccoli-containing breads was performed following the methodology previously reported by Minekus et al., (2014) and the modifications described by Zudaire et al., (2017). This methodology consists of three sequential stages including an oral (α-amylase, pH 7.0), gastric (pepsin, pH 3.0), and intestinal (pancreatin and fresh bile, pH 7.0) phase. Briefly, 5 g of milled sample were mixed with 3.5 mL of simulated salivary fluid and further mixed with 0.5 mL of salivary α-amylase solution (1500 U/mL), 0.025 mL of 0.3 M $CaCl_2$, and 0.975 mL of distilled water. The mixture was thoroughly mixed at 1,250 rpm and 31 ºC for 2 min. For the gastric phase, 10 mL of oral bolus were mixed with 7.5 mL of simulated gastric fluid, 1.6 mL of porcine pepsin (2,500 U/mL), 0.010 mL of 0.15 M $CaCl_2$, 0.2 M HCl (to reach pH 3.0) and 0.690 mL of distilled water. The mixture was incubated at 150 rpm and 37 ºC for 2 h. After the gastric phase, 10 mL of the mixture were collected and centrifuged at 12,000 rpm for 15 min and the supernatant was frozen using liquid nitrogen and stored at -80 ºC until further use. Moreover, 10 mL of gastric chime were mixed with 5.5 mL of simulated intestinal fluid, 2.5 mL of pancreatin (800 U/mL), 1.25 mL of 0.16 M bovine bile, 0.020 of 0.3 M CaCl2, 0.145 mL of 1 M NaOH (to reach pH 7.0), and 0.585 mL of distilled water. The mixture was incubated at 150 rpm and 37 ºC for 2 h. After this period,

10 mL of the mixture were collected, centrifuged at 12,000 rpm for 15 min, and the supernatant was frozen using liquid nitrogen and stored at -80 ºC until further use. Determinations of TPC and antioxidant capacity using both the FRAP and DPPH· methods were carried out after both gastric and intestinal phases. Results were expressed as percentage of either TPC or antioxidant activity retention.

2.7 Sensory evaluation

Sensory evaluation was undertaken at day 1 post-baking with 40 semi-trained panellists recruited from IRTA Fruitcentre who would be willing to buy broccoli-containing bread. Selected panellists were familiar with fruit, vegetable, and baked products quality and were capable of discriminating differences and communicating their reactions, though they were not formally trained. Sensory evaluation was conducted in a sensory laboratory with separate booths following the methodology described by Millar et al. (2017) with some modifications. Briefly, samples were placed on white polystyrene plates labelled with random codes and presented to consumers in a randomised order. A 60-s time laps was employed between each sensory palate, to reduce sensory fatigue, and mineral water was used as a palate cleaner between tastings. Each panellist assessed all three samples and was asked to indicate his or her opinion on the crust crunchiness, crumb sponginess, flavour, overall visual appearance, and overall acceptability of the products using a 9-point hedonic scale (from 1: dislike extremely to 9: like extremely). The samples could be re-tasted as often as desired.

2.8 Statistical analysis

Results are expressed as mean ± standard deviation (S.D.). Difference between samples were analysed using analysis of variance (ANOVA) with JMP 13 (SAS Institute Inc., NC, USA). Where significant differences were present, a Tukey pairwise comparison of the means was conducted to identify where the sample differences occurred. The criterion for

239 statistical significance was $p<0.05$. To identify relationships between nutritional
240 parameters, bivariate Pearson's correlation analysis was carried out.

3. Results and discussion

Previous studies suggested that broccoli co-products, which are used for low-value purposes or discarded as waste, are excellent sources of dietary fibre, glucosinolates, polyphenols, and other compounds with antioxidant activity (Domínguez-Perles et al., 2010). Indeed, Campas-Baypoli et al. (2009) reported the protein, ash, lipid, total carbohydrate, and crude fibre content of broccoli stalks as 22.4 ± 0.4, 7.8 ± 0.1, 4.5 ± 0.3, 65.1, and 11.6 ± 0.2, respectively. Similar values were reported in that study for broccoli stalks, which were calculated as 8.7 ± 0.7, 9.2 ± 0.3, 6.5 ± 0.4, 75.42, and 15.7 ± 0.7, respectively. In the current study broccoli stems used for breadmaking showed an antioxidant potential of 317 ± 29 and 239 ± 14 mg/100 g DW calculated using the FRAP and DPPH· methods, respectively. The TPC of the broccoli stems and leaves was calculated as 312 ± 41 and 205 ± 12 mg/100 g DW, respectively. Lower values obtained for broccoli leaves, which showed an antioxidant capacity of 108 ± 5 and 33 ± 2 mg/100 g DW when assessed using the FRAP and DPPH· method, respectively. In addition, the moisture content of the broccoli stems and leaves used for breadmaking was 90.1 ± 1.4 and 82.1 ± 0.6%, respectively. Results compared well in terms of TPC and antioxidant capacity to those recently obtained for the inflorescences and stems of different broccoli varieties (Lafarga et al., 2018b; Lafarga et al., 2018d).

3.1 Bread quality

A number of preliminary proofing and baking trials were carried out (data not shown). Following these baking trials, the standard breadmaking procedure was not significantly modified, apart from increased intermediate and final proofing times. Volume and density are key quality parameters in bread products. Weight, maximum height, specific volume, and density of BSB, BLB, and CKB are listed in Table 2. Incorporation of powdered

broccoli leaves at a concentration of 2% (w/w) into bread formulations resulted in increased weight when compared to the control (p<0.05). In addition, both the maximum height and specific volume of BSB and BLB were lower when compared to the control (p<0.05). The observed reduction in loaf height was higher in BSB when compared to BLB (p<0.05). Results compared well to those previously reported by Lee (2015) who observed a significant reduction in both volume and specific volume of bread loaves after inclusion of broccoli at concentrations ranging from 2.5 to 10.0% (w/w). Similar results were recently reported by Anwar et al. (2017) after inclusion of broccoli powder into wheat bread at concentrations ranging from 1 to 7% (w/w). Addition of fibrous ingredients resulted in decreased loaf specific volume previously (Filipovic et al., 2007; Lafarga et al., 2013). This could be attributed to a dilution of starch and gluten after addition of broccoli powder and to a decrease in the amount of fully hydrated starch granules caused by the powders competing for water with starch. Broccoli co-products are rich in glucosinolates (Domínguez-Perles et al., 2010), which are strong antimicrobials (Saladino et al., 2016), and their inclusion into bread formulations could alter yeast activity affecting bread volume and texture even at low concentrations.

Moisture content and a_w values of the control and broccoli-containing breads were determined at days 1 and 7 post-baking and results are shown in Figure 1. Results were comparable to those obtained previously in breads containing added functional ingredients (Lafarga et al., 2016). Anwar et al. (2017) observed no effect on the moisture content of breads after inclusion of broccoli at concentrations ranging from 1 to 7% (w/w). In the current study, addition of broccoli at a concentration of 2% (w/w) resulted in no differences in the moisture content of the broccoli-containing breads (BSB and BLB) and the control (CKB) at day 1 post-baking. However, when assessed at day 1, the moisture content of BSB was significantly higher than that of BLB (p<0.05). When assessed at day

7, BLB showed a higher moisture content when compared to CKB (p<0.05). Previous studies observed an increase in the moisture content of breads after inclusion of powdered tomato co-products into their formulation (Majzoobi et al., 2011). In turn, Odunlade et al. (2017) observed a decrease in the moisture content of breads containing dried leafy vegetables at flour substitution levels ranging between 1 and 3% (w/w). As expected, a decrease in the moisture content was observed at day 7 because of bread staling (p<0.05). The observed decrease at day 7 was higher in the control bread, CKB, when compared to BLB (p<0.05).

BLB had higher a_w values when compared to the BSB and CKB (p<0.05). At day 7, a_w values were lower when compared to day 1 post-baking (p<0.05) and no differences were observed between different bread formulations. This same trend was observed previously (Lafarga et al., 2016). In the current study, pH values which are listed in Table 2 showed no significant differences in BSB when compared to CKB. However, addition of powdered broccoli leaves into bread formulations at a concentration of 2% (w/w) resulted in a reduction in the pH (p<0.05).

Colour is another key quality parameter in baked products. Colour has a striking effect on the perception of the product by consumers and influences them on other factors such as aroma or flavour. Crust and crumb colour parameters are listed in Table 2. The parameter L^* defines lightness. An L^* value between 0 and 50 indicates dark, and a value ranging between 51 and 100 indicates light. Moreover, positive a^* and b^* values indicate red and yellow, respectively, and negative a^* and b^* values indicate green and blue. The L^* values suggest a lighter crust colour of BLB when compared to CKB (p<0.05), which could be caused by a reduced number of Maillard reactions. Results did not correlate to those obtained by Lee (2015), who observed a reduction in the L^* value of breads containing broccoli powder when compared to the negative controls. In addition,

315 incorporation of powdered broccoli leaves at a concentration of 2% (w/w) into bread
316 formulations resulted in reduced L^* crumb values when compared to the control and stalk-
317 containing breads ($p<0.05$). Incorporation of powdered broccoli leaves, which had a
318 strong green colour, resulted in an increased green hue when compared to both, CKB and
319 BSB ($p<0.05$). Incorporation of powdered broccoli stalks also resulted in lower a^* values
320 when compared to the control ($p<0.05$). Similar results were obtained by Gawlik-Dziki
321 et al. (2014) who reported that wheat breads containing broccoli sprouts at concentrations
322 ranging from 1 to 5% (w/w) were greener when compared to the control.
323 As expected, inclusion of broccoli co-products into bread formulations increased both
324 crust and crumb C^*_{ab} values, a quantitative indicator of colourfulness ($p<0.05$). This
325 indicates that the broccoli-containing breads had a higher colour intensity. Moreover, δE
326 combines the change in L^*, a^*, and b^* values to quantify the colour deviation from a
327 standard reference sample, in this case, wheat bread. Those samples with $\delta E > 3$ display
328 a visible colour deviation (Wibowo et al., 2015). Broccoli-containing breads had a $\delta E >$
329 3 in crust and crumb, exhibiting a visible colour deviation when compared to the control
330 ($p<0.05$). δE values in BLB were higher when compared to BSB ($p<0.05$). Results
331 suggest that the observed changes in colour between both formulations and the control
332 were higher after inclusion of broccoli leaves.
333 Figure 1 shows the texture profile of the CKB, BSB, and BLB at days 1 and 7 post-baking.
334 Hardness is the peak force that occurs during the compression of a product. Addition of
335 fibrous materials into bread formulations, even at low concentrations such as 1% (w/w),
336 resulted in increased hardness at day 1 post-baking previously (Lafarga et al., 2013).
337 However, in the current study no differences were observed in crumb hardness values of
338 BSB or BLB and CKB at day 1. Results contrast to those reported by Ranawana et al.
339 (2016) who formulated different breads containing powdered vegetables and observed

that firmness of breads containing broccoli was significantly higher at day 1 post-baking when compared to the control. However, the authors of that study reported that at day 4 post-baking all the vegetable-containing breads were similar in hardness to the control and the amount of flour substituted by powdered broccoli in that study was 10% (w/w). Similar results were obtained herein as no differences were observed between the hardness of the three breads at day 7 post-baking. The observed increase in hardness during storage ($p<0.05$) was attributed to moisture loss and bread staling. In addition, no differences were observed in the springiness or chewiness values of BSB or BLB and CKB at day 1 suggesting a similar mouthfeel for the control and the broccoli-containing breads. This same trend was observed at day 7 post-baking. Incorporation of powdered broccoli leaves into bread resulted in increased cohesiveness and resilience when compared to the control at day 1 post-baking ($p<0.05$). Resilience can be defined as how well the product "fights to retain its original height" and cohesiveness as how well a product withstands a second deformation relative to its resistance under the first deformation. Therefore, we can conclude that, at day 1 post-baking, no significant textural differences were observed between CKB and BSB and that BLB can retain better its textural properties after compression without being harder. Addition of powdered tomato into bread resulted in better retention of textural properties during storage previously (Majzoobi et al., 2011). In the current study, no differences were observed between the three samples at days 1 or 7 post-baking, except for a lower resilience at day 7 for BLB when compared to CKB ($p<0.05$).

3.2 Total phenolic content and antioxidant activity

The antioxidant capacity of CKB, BSB, and BLB assessed using the DPPH· and FRAP assays is shown in Figure 2. Post hoc analyses showed that both broccoli-containing breads had significantly higher antioxidant capacities compared to the plain bread

(p<0.05). Odunlade et al. (2017) observed an increase in the antioxidant potential of breads after inclusion of leafy vegetables into their formulation. The maximum increase in antioxidant activity assessed using the FRAP assay was observed after substitution of 3% (w/w) of the flour with powdered *Telfairia occidentalis* and varied from 126 µg/g in the control sample to 134 µg/g in the enriched bread. In addition, addition of broccoli leaves into gluten-free mini sponge cakes significantly increased their antioxidant capacity previously (Drabińska et al., 2017). Gawlik-Dziki et al. (2014) also obtained increased antioxidant capacity after inclusion of broccoli sprouts into wheat bread formulations at concentrations ranging from 1 to 5% (w/w) and similar results were reported by Lee (2015) who obtained DPPH· scavenging activity values ranging from 17.6 to 45.5% after inclusion of broccoli at concentrations ranging from 2.5 to 10.0% (w/w). Although powdered broccoli stems showed a higher antioxidant activity *in vitro* when compared to powdered broccoli leaves, the antioxidant capacity of the breads containing these co-products were not significantly different to each other. This could be caused by a higher stability towards breadmaking of antioxidant compounds found in broccoli leaves in comparison to those found in broccoli stalks. Indeed, the TPC and the DPPH· activity of BLB at day 7 was higher when compared to that of BSB and CKB (p<0.05). The same trend was observed for antioxidant activity assessed using the FRAP method, although it was not statistically significant. Antioxidant activity assessed at day 7 was lower when compared to that measured at day 1 post-baking for all three bread formulations (p<0.05), suggesting a degradation of antioxidant compounds throughout storage.

In addition, the TPC of CKB, BSB, and BLB measured at days 1 and 7 post-baking is shown in Figure 2. Results correlate well to those obtained for antioxidant activity as an increase in the TPC was observed for both, BSB and BLB when compared to CKB

(p<0.05). Indeed, a positive correlation was observed between the antioxidant capacity as assessed using the FRAP and DPPH· methods and the TPC at days 1 (r^2 = 0.977 and r^2 = 0.962 respectively) and 7 post-baking (r^2 = 0.741 and r^2 = 0.748 respectively). Similar results were reported by Odunlade et al. (2017) and Alashi et al. (2018) who obtained breads with increased TPC after incorporation of leafy vegetable powders, namely *Amaranthus viridis*, *Solanum macrocarpon*, and *Telfairia occidentalis*, at flour substitution levels ranging from 1 to 3% (w/w). Increased TPC was also observed in breads containing broccoli sprouts (Gawlik-Dziki et al., 2014). In the current study, the TPC of BLB at day 7 was higher than that of BSB (p<0.05). This explains the differences identified in DPPH· values at day 7 post-baking and suggests that polyphenols found in broccoli leaves are more resistant to breadmaking, which includes a thermal processing step, than those found in broccoli stems.

3.3 Resistance to *in vitro* digestion

Resistance of phenolic compounds and antioxidant capacity to a simulated gastrointestinal digestion is shown in Table 3. The *in vitro* gastrointestinal digestion strategy suggests which compounds survive the gastrointestinal tract conditions and are likely to reach the colon where they can act or be absorbed into the blood stream (McDougall et al., 2007). Bioavailability, which was not calculated in the current study, indicated the fraction of a given compound or its metabolite that reaches the systemic circulation and is determined *in vivo* in animals or humans (Carbonell-Capella et al., 2014). Results shown in Table 3 were comparable in magnitude for each bread formulation. The TPC of the three formulations increased after the gastric phase when compared to the initial stage, that measured from a methanol:water extract (p<0.05). Polyphenols are found in the free and bound forms in cereals and the products derived thereof. Conventional extraction using organic solvents, in this case methanol, can be

used to extract free polyphenols. However, the majority of the polyphenols found in cereals are in the insoluble bound form. Indeed, Abdel-Aal et al. (2013) recently reported the content of free and bound phenolic acids in wheat bread as 0.6 and 11.7 mg per serving, respectively. Thus, the higher content of polyphenols after digestion could be caused by the liberation of phenolic compounds which were bound to the cell wall material. Similar results were reported by Gawlik-Dziki et al. (2009) at the different stages of a simulated gastrointestinal digestion of wheat bread containing either 2.5 or 5.0% (w/w) buckwheat flavones. In that study, the authors observed a significant increase in the TPC of the breads during the different stages of digestion from 0.50 and 0.72 mg/mL at the initial stage and 0.78 and 0.79 mg/mL, and 0.72 and 0.90 mg/mL after the gastric and intestinal phases, respectively. Pérez-Jiménez and Saura-Calixto (2005) also reported that TPC and antioxidant activity of the digestive enzymatic extracts was significantly higher when compared to that of the water-methanol or water-acetone extracts. Moreover, results obtained for antioxidant activity were comparable to those obtained for TPC. The antioxidant potential of all samples increased during digestion ($p<0.05$). The observed increase in antioxidant activity could be attributed to a higher concentration of polyphenols as strong pH variations and pepsin may affect the integrity of cell walls, facilitating the liberation of phenolic and antioxidant compounds not detected in initial phases. In addition, hydrolysis of wheat- and broccoli-derived proteins could result in peptides with antioxidant properties (Cian et al., 2015; Niu et al., 2013). Also, different pH values can affect racemization of molecules creating two different chiral enantiomers and altering their biological activities, rendering antioxidants more reactive early in the digestive process (Jamali et al., 2008). In addition, the longer extraction process, if compared to values prior to digestion (those obtained after the methanol:water extraction), may partially explain these findings. Overall, results obtained

herein suggest that the amount of phenolic compounds released by the bread matrix during digestion, especially during the intestinal phase of digestion, may be higher than the one expected from common water-organic extracts. No differences were observed in the bioaccessibility of phenolic compounds of the different bread formulations. This could suggest that polyphenols derived from broccoli are not as resistant to degradation as those derived from wheat, although further studies are needed to confirm this hypothesis. However, broccoli-containing breads showed a higher antioxidant activity, assessed using both the FRAP and the DPPH· assays, when compared to the control ($p<0.05$).

3.4 Sensory analysis

Breads enriched with vegetables are generally accepted by consumers as a vehicle to increase vegetable consumption (Hobbs et al., 2014). Previous studies suggested that wheat bread containing broccoli sprouts at concentrations ranging from 1 to 2% (w/w) had the highest liking scores (Gawlik-Dziki et al., 2014). In that study, the authors reported that breads containing higher concentrations of broccoli sprouts were unacceptable in terms of aroma, taste, and texture. Similar results were observed by Lafarga et al. (2018c), who reported higher overall acceptability scores for crackers containing broccoli co-products at concentrations ranging between 12 and 15% (w/w) when compared to the controls. Anwar et al. (2017), obtained maximum overall acceptability scores in broccoli-containing breads at concentrations ranging from 1 to 2% (w/w) and unacceptable textural and sensory properties at higher concentrations. Similar results were obtained in the current study during preliminary baking trials (data not shown). The results of hedonic tests on the control and broccoli-containing breads are given in Figure 3. Overall, no significant differences were observed in any of the parameters evaluated for any of the three bread formulations. A big variation was

observed in the overall appearance scores for BLB. This was probably caused by the green color of BLB, as many panelists commented that they disliked colored breads.

Results suggest that overall, addition of powdered broccoli stalks or leaves into bread formulations at a concentration of 2% (w/w) did not affect the overall acceptability, appearance or texture of bread.

4. Conclusions

Addition of powdered broccoli stalks or leaves into bread formulations at a concentration of 2% (w/w) did not affect the overall acceptability, appearance or texture of the bread. Bigger colour and texture differences were observed after incorporation of powdered broccoli leaves, at a concentration of 2% (w/w), when compared to powdered broccoli stalks at the same concentration. Addition of broccoli into baked goods at the optimum concentrations can result not only in increased physicochemical quality but also in increased nutritional quality and bioactive properties. In the current study, antioxidant activity and phenolic compounds derived from broccoli were found to be resistant to the breadmaking process and to a simulated gastrointestinal digestion. Phenolic compound and antioxidant activity of breads containing powdered leaves were higher at day 7 post-baking, suggesting that phenolic and antioxidant compounds found in leaves could be more resistant to thermal processing and storage than those found in broccoli stems.

Conflict of interests

The authors declare no conflict of interests.

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Figure captions

Figure 1. (A) Moisture, (B) a_w, (C) crumb hardness, (D) crumb springiness, (E) crumb cohesiveness, (F) crumb resilience, and (G) crumb chewiness of the control and broccoli-containing breads

Values represent the mean of three independent experiments ± S.D. Capital letters indicate significant differences between different formulations at the same day. Lower case letters indicate significant differences between sampling days for the same bread formulation. The criterion for statistical significance was $p<0.05$.

Figure 2. Antioxidant activity assessed using the (A) FRAP and (B) DPPH· scavenging assay and (C) total phenolic content of broccoli-containing breads

Values represent the mean of three independent experiments ± S.D. Capital letters indicate significant differences between different formulations at the same day. Lower case letters indicate significant differences between sampling days for the same bread formulation. The criterion for statistical significance was $p<0.05$.

Figure 3. Sensory evaluation of broccoli-containing breads

Panellists scored overall acceptance and overall visual appearance using a 9-point hedonic scale: 9, like extremely; 5, neither like or dislike; 1, dislike extremely. Crust crunchiness, crumb sponginess, and flavour were scored using a 5-point hedonic scale: 1, nothing; 2, a little bit; 3, regular; 4: moderate; 5: a lot.

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