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Determination of free and bound phenolic compounds and their antioxidant activity in buckwheat bread loaf, crust and crumb

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Abstract

This study demonstrated the role of buckwheat flour in improving phenolic compounds in white wheat bread. Three bread samples were obtained by using different buckwheat levels (10, 20 and 30%) in formulations. HPLC-ESI-MS was used to detect the presence of free and bound phenolic compounds in bread loaf, crust and crumb. The phenolic profile changed thanks to the addition of buckwheat flour; in fact, flavan-3-ols and flavonols compounds (i.e. rutin, catechin, etc.) were identified in enriched buckwheat. As expected, the phenolic content increased proportionally to buckwheat flour quantity in bread formulations. The total free phenolic amounts ranged from 109 to 235 mg/kg d.w. in control bread and 30 % enriched buckwheat bread, respectively. Bread crusts showed the highest total free and bound phenolic content; however, flavan-3-ols, flavonols and flavones are more concentrated in crumb than crust. Moreover, enriched breads showed higher *in vitro* antioxidant properties (evaluated by DPPH and ABTS assays) than control one.

Keyword: *Fagopyrum esculentum* Moench, bread, phenolic compounds, radical scavenging activity

1. Introduction

Wheat bread is considered to be a good source of energy for the human body. It is known that bread obtained with natural raw ingredients such as cereals and seeds, spices, herbs and parts of green plants, fruit or vegetable products and waste products from the food industry can be enriched in antioxidant compounds (Blandino et al., 2013; Dziki, Rozyło, Gawlik-Dziki & Swieca, 2014, Balestra, Cocci, Pinnavaia & Romani, 2011).

Foods based on wholegrain cereals, including bread, play an important role in human health and well-being. It has been demonstrated that the regular consumption of wholegrain cereals can contribute to reduce the risk of cardiovascular disease (CVD), type 2 diabetes mellitus and certain types of cancer, as well as several gastrointestinal pathologies (Gil, Ortega & Maldonado, 2011). The healthy properties of whole grains are linked to the presence of bioactive compounds such as dietary fiber and phenolic compounds. Phenolic acids and flavonoids represent the most common form of phenolic compounds found in whole grains, and they are among the major and most complex groups of phytochemicals with a number of types that exist as soluble free compounds, soluble conjugates that are esterified to sugars and other low molecular mass compounds, and insoluble, bound forms (Zilic et al., 2011). Among cereals and pseudo-cereals, buckwheat represents a good source of bioactive compounds. These compounds are strictly related to the health benefits attributed to buckwheat including plasma cholesterol level reduction, neuroprotection, anticancer, anti-inflammatory, antidiabetic effects, and improvement of hypertension conditions (Gimenez-Bastida, & Zielinski, 2015). Moreover, buckwheat is a gluten-free pseudocereal, for this reason, it can be used for gluten-free products formulation. As reported by Gimenez-Bastida, Piskula and Zielinski (2015), the buckwheat flour incorporation into a bread gluten-free experimental formula affected positively the technological quality of the product, enriching its protein and microelement contents.

Among the microelements, buckwheat is a source of several phenolic compounds such as flavonols, flavan-3-ols, propelargonidins and phenolic acids (Verardo et al., 2010; Inglett, Chen, Berhow & Lee, 2011; Verardo, Gomez-Caravaca, Segura-Carretero, Caboni & Fernàndez-Gutièrrez, 2011) with antioxidant activity. Recently, Stokić and co-workers (Stokìc et al. 2015) stated that buckwheat-enriched wheat bread had higher content of dietary fibers and phenolic compounds than wheat bread; moreover the same authors noticed that the consumption of buckwheat enriched bread caused a significant decrease in total cholesterol, LDL-cholesterol as well as the ratio of LDL/HDL cholesterol in statin treated patients.

Several studies have been developed to evaluate the phenolic content in buckwheat bread; however, to our knowledge, literature lacks of information about phenolic distribution in buckwheat bread. Because of that, and due to the health effects attributed to the phenolic compounds of buckwheat, the aim of this study was to study in depth the content of free and bound phenolic compounds in whole, crust and crumb of bread samples formulated with different level of buckwheat flour (10, 20 and 30 %).

2. Materials and methods

2.1. Samples

Control bread and three bread samples obtained with wheat and different buckwheat flour (var. Lileja from Umbria, Italy) levels (10, 20 and 30% on wheat flour quantity) were formulated. All used ingredients were supplied by a local bakery company (Cesena, Italy). The list of ingredients and related amount used for each kind of bread are reported in Table 1. In particular, the wheat flour (type 0) used in this research, was a refined flour made from soft wheat (extraction rate ~ 700 g/kg). The physicochemical characteristics of the wheat flour used to develop the dough were: W= 270 10^{-4} J; P/L 0.5; moisture: 140 g/kg; ash: 6.5 g/kg;

dry gluten: 96 g/kg; protein content: 129 g/kg. The physicochemical characteristics of the buckwheat flour were instead: moisture: 120 g/kg; ash: 20 g/kg; protein content: 105 g/kg. Moreover, the buckwheat percentages used were chosen after preliminary trials, carried out in order to obtain products with good technological properties, comparable to those of a standard bread formulation (control bread).

2.1.1 Sponge Preparation

The sponge was prepared with 775 g of flour, 352 g of water and 7.75 g of brewer's yeast. The main ingredients of the dough were kept constant: sponge (1136 g), salt (37 g), brewer's yeast (39 g), improver (5 g) and water (510 g).

All ingredients were mixed in a kneading professional machine (Tauro –Sigma s.r.l, Brescia, Italy). As a first step, refined wheat flour was mixed with water for 3 minutes at minimum speed of 40 rpm, after that, sourdough was added and mixed for other 7 minutes, at the same speed. After mixing, the obtained "sponge dough" was stored in a thermoclimatic chamber (Constant Climate Chambers with Peltier technology, model HPP 108/749, Hemmert, Germany) at 28°C for 24 hours, before to add it into the dough.

2.1.2 Bread preparation

Bread enriched with buckwheat was obtained by mixing the sponge previously prepared, with the other ingredients: refined wheat flour, water, sodium chloride (NaCl), alpha-amylase and the different percentages of buckwheat. All ingredients were mixed for 7 minutes at minimum speed by using the same mixer used for the sponge preparation and let to leaven for 1 hour at 32°C and 70% of relative humidity in a thermoclimatic chamber (Constant Climate Chambers with Peltier technology, model HPP 108/749, Hemmert, Germany). The baking of bread samples was carried out in a professional oven for 20 minutes at 210°C (FC61, Angelo Po e

Grandi Cucine S.p.A, Carpi, Italy). After baking, bread samples were left to cool at room temperature for 2 hours, before performing analysis. The volume of the resulting bread (mL/g) was 3.3±0.9 for the control; 3.2±0.5 for the bread obtained with the addition of 10% of buckwheat flour; 2.9±0.1 for the product obtained with the addition of 20% of buckwheat flour and 2.5±0.2 for the bread with the 30% of buckwheat flour.

Each type of bread was produced in triplicate. After baking, crust and crumb were separated from each bread sample, frozen in encoded plastic bags at -20° C and then freeze-dried (Thermo HETO, powerdry LYOLAB 3000; Waltham, USA). Dried samples were ground to a fine powder in a blender mixer (Ika-Werke M20; Staufen, Germany) and used for the analyses.

2.2. Reagents and chemicals

HPLC-grade acetonitrile, ethanol and methanol were purchased from Labscan (Dublin, Ireland). Acetic acid analytical grade (assay > 99.5%) was purchased from Fluka (Buchs, Switzerland). Water was purified by using a Milli-Q system (Millipore, Bedford, MA, USA). Other reagents were purchased from VWR (Denver, CO, USA). Analytical standards were from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Extraction of free and bound phenolic compounds from control and buckwheat bread

To determine the free and bound phenolic fraction of bread samples, the method developed by Verardo et al. (2011) was applied.

Briefly, two grams of bread were extracted twice in an ultrasonic bath with a solution of ethanol/water (4:1 mL/mL). The supernatants were collected, evaporated and reconstituted with 2 mL of methanol/water (1:1 mL/mL). The extracts were stored at -18 °C until use.

To obtain the bound phenolic fraction, residues of free phenolic extraction were digested with 200 mL of 2 mol/L NaOH at room temperature for 4 h by shaking under nitrogen gas. The

hydrolyzed solution was acified to pH 2-3 by adding 10 mol/L hydrochloric acid in a cooling ice bath and extracted with 500 mL of hexane to remove the lipids. The final solution was extracted five times with 100 mL of 1/1 diethyl ether/ethyl acetate (mL/mL). The organic fractions were pooled and evaporated to dryness. The phenolic compounds were reconstituted in 2 mL of methanol/water (1:1 mL/mL).

2.4. HPLC-ESI -MS analysis of phenolic compounds

HPLC analysis was performed by an Agilent 1100 series LC system (Agilent Technologies, CA, USA) consisting of a vacuum degasser, autosampler, and a binary pump equipped with a reversed-phase Kinetex^{TM} C18 (100 mm × 4.6 mm, 2.6 µm) column (Phenomenex Inc, Torrance, CA, USA). The mobile phase and gradient program were used as previously described by Gomez-Caravaca, Verardo, Berardinelli, Marconi and Caboni (2014). All solvents were filtered with a 0.45 µm filter disk. The RP-HPLC system was coupled to a HP-Mass Spectrometer Detector (MSD, model G1946A) equipped with an ESI interface peak integration and data elaboration were performed using Chemstation software (Hewlett Packard, Wilmington, DE, USA). Parameters for analysis were set using negative ion mode with spectra acquired over a mass range from m/z 50–1300.

2.5. DPPH radical scavenging activity

The free radical scavenging activity of extracts (FRSA) was determined using the DPPH assay according to Parejo, Codina, Petrakisand and Kefalas (2000). Briefly, 0.1 mL of extract was added to 2.9 mL of 100 µmol/L DPPH methanol solution. The absorbance was determined at 517 nm after 30 minutes (at 25 °C). To assess the FRSA a Trolox calibration curve was performed and the results were expressed as µmoles of Trolox equivalent/100 g of bread d.w (dry weight).

The spectrophotometric analyses were performed using a UV-1601 spectrophotometer from Shimadzu (Duisburg, Germany).

2.6. ABTS Radical Cation Decolorization Assay

The ABTS assay, was performed by using the method previously described by Re et al. (1999) where the radical monocation $ABTS^{\bullet+}$ is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of hydrogen-donating antioxidants or with a standard. Briefly, $ABTS^{\bullet+}$ was obtained by reaction of 7.0 mmol/L ABTS and 2.45 mmol/L potassium persulfate (stand in the dark at room temperature for 16 h). The $ABTS^{\bullet+}$ stock solution was diluted with water in order to obtain an absorbance of 0.700 ± 0.02 (λ = 734 nm). After that, 0.01 mL of sample extract was added to 1 mL of $ABTS^{\bullet+}$ and stored in a dark room for 10 minutes. The absorbance was measured at 734 nm (at 30 °C). A Trolox calibration curve was performed and the results were expressed as µmoles of Trolox equivalent/100 g of bread d.w.

2.7. Statistical analysis

All analyses were carried out in triplicate (n=3) for each sample. Tukey's honest significant difference multiple comparison (one-way ANOVA) at a $p < 0.05$ level, and Pearson correlations were evaluated using Statistica 8.0 software (2007, StatSoft, Tulsa, OK, USA).

3. Results and discussion

HPLC-ESI-MS has been used to identify and quantify the single phenolic compounds in refined wheat and buckwheat flours (Supplementary table), and bread loaf, crust and crumb of both control and buckwheat enriched bread samples.

3.1. Determination of free phenolic compounds in control and buckwheat breads

Table 2 reports the free phenolic contents of control and buckwheat enriched bread loaf samples. A total of twenty-seven phenolic compounds were identified in the analyzed samples; however, control bread loaf showed the presence of only twelve phenolic compounds.

Among them, three hydroxybenzoic and seven hydroxycinnamic acid derivatives and two flavones isomers were identified.

The total amount of phenolic acids and flavonoids increased from 37 to 97 mg/kg bread d.w. and from 72 to 139 mg/kg bread d.w. from control to 30 % enriched buckwheat bread, respectively.

As expected, control bread formulated with refined wheat flour showed the apigenin-6-C-arabinoside-8-C-hexoside ((iso)-shaftoside) as principal free phenolic compound followed by *trans*-ferulic acid. These data agreed with those reported by Gianotti et al. (2011). The content of ferulic acid and other phenolic acids was in the same order of magnitude of that reported by several authors (Abdel-Aal & Rabalski, 2013; Lu et al., 2014; Yu & Beta, 2015).

Buckwheat enriched breads, as control sample, showed the apigenin-6-C-arabinoside-8-C-hexoside isomers, however their content decreased when buckwheat flour ratio increased. This trend is justified by the presence of these compounds in wheat flour but not in buckwheat flour. Similar trend has been observed for the ferulic acid isomers (*cis* and *trans*).

Contrary to control sample, buckwheat breads showed the presence of other phenolic bioactive compounds (Verardo, et al. 2010; Inglett et al. 2011; Verardo et al. 2011) such as flavan-3-ols, propelargonidins, flavonols among others.

The flavan-3-ols content increased from 10.4 to 18.3 % according to the increase of buckwheat flour amount used in the bread formulations. In particular, catechin and epicatechin epimers, and their mono- and di-galloylated derivatives are the main flavan-3-ols detected in buckwheat bread.

Propelargonidins, such as afzelchin-catechin and afzelchin-catechin-dimethylgallate were detected only in bread samples enriched with 20 and 30 % of buckwheat flour. Their content represented the 1.4 % of total free phenolic compounds in both samples; however, their amounts in bread were very low if compared with their content in buckwheat flours. This suggested that this kind of compounds is thermally labile.

As expected, flavonols content increased from 8.4 to 12.1 % of total phenolic compounds in enriched buckwheat breads; rutin was the main flavonol followed by isoquercitrin, quercetin and hyperin. Rutin content was in the same order of magnitude of those reported by Lin, Liu, Yu, Lin and Mau (2009) for buckwheat bread; however, the present results showed quercetin amounts that were ten times higher than the data reported by Lin and co-workers (Lin et al. 2009); this could be justified by the different origin of the buckwheat flours. These results encourage the use of buckwheat flour in bread formulation because, as reported by several authors (Szawara-Nowak, Koutsidis, Wiczkowski & Zielinski, 2014; Giménez-Bastida, Zielinski, Piskuła, Zielinska, & Szawara-Nowak, 2017), rutin and other buckwheat phenolic compounds could be involved in the possible beneficial roles on the prevention of glycation-associated diseases.

Flavones content decreased when buckwheat flour content increased; nevertheless, this trend is due to the lower content of apigenin-6-C-arabinoside-8-C-hexoside, which is the principal flavone in control bread. However, if the buckwheat flavone contribution has been considered, it is very clear that orientin, isorientin, vitexin and isovitexin were determined only in buckwheat breads and their content reached the high level when higher amounts of buckwheat were used in bread formulation.

Finally, phenolic acid derivatives increased from 34% (control bread) to 41 % in bread enriched with the highest amount of buckwheat flours. The total phenolic amounts improved from 1.9 to 2.6 times in enriched breads compared with control. The main phenolic acid

243 derivatives that contributed to this improvement were syringic acid, *p*-hydroxybenzaldehyde
244 and swertiamacroside. Vanillic, syringic, *p*-coumaric, sinapic, dehydroferulic and sinapoyl-
245 hexose acid derivatives were detected in free form in formulated breads, but they are present
246 only in bound form in refined wheat and buckwheat flours. This confirmed the hypothesis of
247 other authors that mixing, sourdough fermentation and baking process facilitate the release of
248 bound phenolic compounds to its free form (Angeloni & Collar, 2011; Yu & Beta, 2015)
249 To better evaluate the distribution of phenolic compounds in control and buckwheat enriched
250 breads, the crumb was separated from the crust in each loaf samples; free phenolic fraction of
251 the two section of loaf is reported in **Table 3**.
252 Control and enriched samples showed that crust has the higher phenolic content compared to
253 the crumb. These data totally fitted with the results reported in other works (Balestra et al.,
254 2011; Lu et al. 2014; Yu & Beta, 2015). According to Vitali, Dragojevic, and Sebecic (2009)
255 the high content of phenolic compounds in crust should be due to the effect of baking
256 temperature that probably hydrolyzes some complex phenols resulting in an increase of
257 extractable phenolic content. Anyway, Gélinas and McKinnon (2006) hypothesized that also
258 Maillard reactions are involved to some extent in the content of phenolic compounds in bread
259 crust.
260 Only four phenolic compounds were determined in control crumb; basically, apigenin-6-C-
261 arabinoside-8-C-hexoside isomers represented more than 95 % of its total phenolic content;
262 minor phenolics were *trans* ferulic acid and *p*-hydroxybenzaldehyde. Twelve phenolic
263 compounds were quantified in control crust. Apigenin-6-C-arabinoside-8-C-hexoside was the
264 principal phenolic compound followed by the *trans* ferulic acid and their sum corresponds to
265 81.9% of total phenolic compounds.
266 To our knowledge, no studies on the distribution of phenolic compounds in buckwheat crumb
267 and crust was published; because of that, the comparison with literature is difficult.

The total phenolic content in the two loaf sections increased when higher buckwheat flour ratio were used for formulation.

The first phenolic class in bread crumbs was the flavone; it represented the 69, 46 and 39 % of total phenolic compounds in 10, 20 and 30 % buckwheat enriched bread, respectively. It is important to underline that swertiamacroside was determined only in buckwheat bread crumbs; probably, the high temperature in crust caused its degradation. Flavan-3-ols and flavonols were the second and third phenolic fraction, respectively, and their content increased when high amounts of buckwheat were used. Rutin amounts in the enriched breads increased from 5 to 11 mg/kg of crumbs (corresponding to 4.2 and 9.6 % of total phenolic compounds, respectively) when the buckwheat ratio reached the 30 % of flour formulation.

Higher amounts of flavonols such as rutin, quercetin, hyperin and isoquercitrin were determined in crumb than in crust. This trend could result from the low thermal stability of flavonol compounds as reported by several authors (Cho and Lee, 2015). Moreover, as reported by Buchner, Krumbein, Rohn, & Kroh (2006), the presence of oxygen accelerates the degradation of rutin and quercetin, because of that we could suppose that, due to the structure of the crumb, the concentration of oxygen in crust is higher than in crumb thus low amounts of rutin and quercetin were detected in the upper section of loaf. However further analyses are needed to corroborate this aspect.

The main phenolic class in crust was the phenolic acid group that increased with the increase of buckwheat flour quantity. The same trend was reported by flavan-3-ols that was the second phenolic class ranged from 4.7 to 11.2 % of total phenolic fraction.

3.2. Determination of bound phenolic compounds in control and buckwheat breads

Twenty-four bound phenolic compounds were identified in the loaf of control and buckwheat breads (**Table 4**). Flavonoids were the most abundant phenolic compounds in bread samples

ranging from 70.5 (control bread) to 97.4 (30 % buckwheat bread) mg/kg bread d.w.; phenolic acid derivatives increased from 31 (in control bread) to 89 mg/kg bread d.w. (30 % buckwheat bread).

As expected, the increase of the level of substitution of buckwheat flour was followed by the increase of bound flavonols and flavan-3-ols content. Contrary, flavones decrease from 10.9 to 20 % compared to control according to the buckwheat level of substitution; however, as reported for the free phenolic fraction, increased isovitexin, vitexin, orientin and isorientin contents resulted proportionally to the quantity of buckwheat flour used in bread formulations; in fact, these flavones are not characteristic of wheat flours.

With regards to individual bound phenolic compounds, apigenin-6-C-arabinoside-8-C-hexoside resulted as the most abundant compounds in control and buckwheat breads, and their content decreased when buckwheat flour levels increased. Syringic and *trans* ferulic acids, and *p*-hydroxybenzaldehyde were other main bound phenolic compounds.

Ferulic acid content in control bread was in agreement with the data found by Yu, Nanguet and Beta (2013) in bread made from refined flour.

It is important to underline that some compounds, such as quercetin and propelargonidins, were not detected in bound form, probably due to their degradation and/or hydrolysis.

The results of the bound phenolic compounds determined in crumb and crust are given in **Table 5**.

The substitution of wheat flour with buckwheat one caused, in general, significant increase of phenolic acids content in bread crust; contrary, flavonoids content decreased.

Apigenin-6-C-arabinoside-8-C-hexoside was the main phenolic compounds in crust and crumb, and its content decreased when higher amounts of buckwheat flour have been added in the formulation.

317 *Trans* ferulic acid was the second phenolic compound in bread crust; its content in crumb was
318 from 16 to 25 times lower than in crust. Moreover, the ferulic content in buckwheat breads
319 was lower than control bread. Similar trend has been showed by *p*-hydroxybenzaldehyde.
320 Crumbs of breads formulated with buckwheat flours contained increasing amounts of flavan-
321 3-ols (from 12 to 29 mg/kg bread d.w.) according to the buckwheat flour ratio added during
322 bread formulation.
323 Rutin was also detected in bound form and its content was higher in buckwheat bread crumb
324 than crusts confirming the low thermal stability of this compound as previously reported for
325 its free form.
326
327 *3.3. Antioxidant activity of bread samples*
328 The results of antioxidant activity measured by DPPH and ABTS radicals, and total phenolic
329 content, expressed as sum of each phenolic compound determined by HPLC-ESI-MS, are
330 reported in **Table 6**.
331 Total free phenolic content in bread loaf varied between 109 and 235 mg/kg bread d.w.,
332 buckwheat bread samples showed higher amounts of these compounds compared to control.
333 These data confirm the results showed in previous works (Angioloni, & Collar, 2011;
334 Szawara-Nowak, Bączek & Zieliński, 2016) that demonstrate as the multigrain bread
335 exhibited increased polyphenol content, higher polyphenol bioaccessibility and higher
336 antioxidant power than bread obtained with single grains.
337 Significant correlations have been found between total phenolic content and DPPH and ABTS
338 assay results. Briefly, DPPH assay showed a positive correlation ($r = 0.9354$, $p < 0.001$) with
339 total phenolic amounts, and according to Yu et al. (2013) and Yu and Beta (2015), crusts
340 showed the highest scavenging activity due to the high phenolic content and probably to the
341 high presence of Maillard reaction products that could react with DPPH radical.

342 Significant differences ($p<0.05$) were found among the buckwheat bread samples; in fact high
343 ratio of buckwheat flour correspond to high phenolic content and radical scavenging activity.
344 Similarly, ABTS assay reported a positive correlation ($r = 0.9338$, $p < 0.001$) with free total
345 phenolic content in breads. ABTS scavenging capacity was comprised between 118 and 899
346 µmoles TEAC/100 g bread d.w.; according to results obtained by Yu et al. (2013) and Yu and
347 Beta (2015), crust samples showed the highest antioxidant capacity, and buckwheat breads
348 scavenging capacity increased when high buckwheat ratio was used during the bread
349 formulation.
350 High correlation was found between DPPH and ABTS assay results ($r = 0.9950$, $p< 0.001$).
351 Total bound phenolic compounds content ranged between 77 and 213 mg/kg bread d.w. These
352 values are apparently in contrast with the data of other authors (Abdel-Aal & Rabalski, 2013;
353 Yu & Beta, 2015) which showed that bound phenolic content was higher than free phenolic
354 content in bread obtained with wheat flour; according to Yu et al. (2013), the bound phenolic
355 content in refined flour could be more than ten times lower than in whole wheat flours.
356 Antioxidant activity, measured by DPPH and ABTS assays, decreased with the diminution of
357 buckwheat flour quantity. As noticed for free phenolic fraction, in each kind of bread crust
358 samples showed higher antioxidant activity than in loaf, and the last one presented higher
359 antioxidant activity than crumb. These results could be justified by a strong Maillard reaction
360 development in crust than in crumb.
361 Positive correlations were found between total bound phenolic content and DPPH ($r = 0.7765$,
362 $p < 0.05$) and between total bound phenolic content and ABTS ($r = 0.8361$, $p < 0.05$).
363

364 **4. Conclusions**

365 The phenolic composition of bread samples enriched with buckwheat flour was compared
366 with refined wheat flour bread. The addition of buckwheat flour allowed the introduction of

367 flavan-3-ols, propelargonidins and flavonols (i.e. rutin among others) in bread. In this way,
368 the buckwheat breads could represent “functional” breads, permitting to introduce these
369 bioactive phenolic classes in the diet.

370 Bread crust contains higher amounts of phenolic compounds and higher antioxidant activity
371 than bread crumb; however, the phenolic classes distribution varied between the two zone of
372 bread loaf. In fact, flavonoids were more concentrated in crumb than crust probably due to
373 their low thermal stability.

374 Finally, this work improves the information about the phenolic content of buckwheat
375 enhanced wheat bread and, to our knowledge, the phenolic composition of crust and crumb of
376 buckwheat breads has been showed for the first time. However, further researches are needed
377 to explore the neo-formation/degradation/hydrolysis reactions of phenolic compounds during
378 baking process in order to clarify the effect of temperature on single phenolic compound.

379

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Table 1. Amounts (kg) of the different ingredients used for bread formulation

Ingredients	Control	BB 10%	BB 20%	BB 30%
Wheat flour (Type 0)	1.25	1.125	1.00	0.875
Buckwheat flour	-	0.125	0.25	0.375
Water	0.83	0.83	0.83	0.83
Brewer's Sourdough	0.06	0.06	0.06	0.06
NaCl	0.06	0.06	0.06	0.06
Alpha-amylase	0.01	0.01	0.01	0.01
Sponge	1.84	1.84	1.84	1.84

Table 2. Content of single free phenolic compounds and relative classes in control (white bread) and buckwheat enriched bread loaf samples expressed as mg/kg d.w.

Compound	Control	BB 10%	BB 20%	BB 30%
Vanillic acid	0.9±0.1 ab	0.8±0.05 a	1.5± 0.1c	1.9±0.2 d
Syringic acid	0.9±0.1 a	26.5±1.3 b	24.7±1.1 b	31.6±1.0 c
p-hydroxy-benzaldehyde	4.1±0.3 a	15.8±0.7 b	21.6±0.9 c	32.0±1.1 d
p-coumaric acid	2.2±0.2 a	2.3±0.3 a	3.8±0.6 b	5.2±0.5 c
Sinapic acid	4.7±0.6 d	3.6±0.2 c	2.0±0.2 b	1.9±0.1 a
trans ferulic acid	20.1±0.3 d	17.7±0.8 bc	16.4±0.5 b	14.8±0.4 a
cis ferulic acid	2.5±0.2 b	2.2±0.3 b	1.7±0.1 a	1.5 ±0.2a
Dihydroferulic acid Isomer I	0.3±0.03 c	0.2±0.01 b	0.1±0.02 a	0.1±0.01 a
Sinapoyl hexose	0.9±0.05 b	1.3±0.2 c	1.2±0.2 c	0.6±0.1 a
Dihydroferulic acid Isomer II	0.5±0.1 b	0.1±0.02 a	0.1±0.01 a	0.1±0.01 a
Apigenin-6-C-arabinoside-8-C-hexoside Isomer I	68.5±1.2 d	60.5±1.4 c	49.5±1.1 b	43.3±0.8 a
Apigenin-6-C-arabinoside-8-C-hexoside Isomer II	3.4±0.1 b	2.9±0.2 a	2.7±0.1 a	2.5±0.2 a
Catechin	-	3.7±0.2 a	12.8±0.9 b	15.7±1.0 c
Epicatechin	-	6.4±0.5 a	8.2±0.3 b	10.3±0.7 c
Afzelchin-catechin	-	-	2.2±0.3 a	1.8±0.1 ab
Swertiamacroside	-	2.3±0.4 a	7.9±0.2 b	8.9±0.5 c
Orientin	-	1.6±0.2 a	2.5±0.1 b	2.8±0.05 c
Isorientin	-	0.9±0.04 a	1.3±0.1 b	1.7±0.1 c
Rutin	-	5.0±0.1 a	6.3±0.6 b	9.7±0.4 c
Isoquercitrin	-	3.9±0.4 a	5.6±0.2 b	6.9±0.6 c
Vitexin	-	2.9±0.3 a	7.1±0.6 b	9.2±0.8 c
Epigallocatechin	-	5.4±0.3 a	7.1±0.1 b	8.1±0.4 c
Isovitexin	-	1.3±0.2 a	3.5±0.3 b	4.4±0.1 c
Hyperin	-	2.5±0.03 a	2.9±0.1 b	3.6±0.3 c
Afzelchin-catechin-dimethylgallate	-	-	0.7±0.2 a	1.5±0.4 b
Epicatechin-dimethylgallate		2.6±0.6 a	6.9±0.9 b	9.0±0.5 c
Quercetin		3.5±0.2 a	5.9±0.4 b	8.2±0.7 c
Sum phenolic acids	37.1±0.3 a	72.8±0.8 b	81.1±0.6 c	98.5±0.5 d
Sum flavones	71.9±0.6 d	70.0±0.7 c	66.6±0.7 b	63.9±0.5 a
Sum flavan-3-ols	-	18.2±0.5 a	37.9±1.2 b	46.3±1.1 c
Sum flavonols		14.8±0.6 a	20.7±0.9 b	28.4±1.3 c

BB= buckwheat enriched bread

Analyses were carried out in triplicate (n=3). Different letters in the same line indicate significantly different values ($p < 0.05$).

Table 3. Content of single free phenolic compounds and relative classes in crust and crumb of formulated breads expressed as mg/kg d.w.

Compound	Control		BB 10%		BB 20%		BB 30%	
	Crumb	*Crust*	*Crumb*	*Crust*	*Crumb*	*Crust*	*Crumb*	*Crust*
Vanillic acid	-	0.8±0.1 b	0.6±0.05 a	0.9±0.1 b	0.6±0.03 a	1.4±0.1 d	1.1±0.1 c	2.9±0.3 e
Syringic acid	-	0.9±0.1 a	-	25.0±0.9 d	1.9±0.1 b	45.6±1.3 e	2.3±0.1 c	58.8±1.6 f
p-hydroxy-benzaldehyde	1.5±0.1 a	6.7±0.4 e	1.7±0.05 b	30.0±1.4 f	2.7±0.2 c	40.6±1.6 g	3.1±0.1 d	61.5±1.8 h
p-Coumaric acid	-	2.0±0.6 d	0.1±0.03 a	4.6±0.8 e	0.5±0.05 b	7.1±0.4 f	0.7±0.1 b,c	9.7±0.3 g
Sinapic acid	-	4.6±0.3 c	-	3.4±0.1 b	-	2.9±0.2 a	-	2.4±0.4 a
trans Ferulic acid	2.1±0.04 b	38.0± e	1.9±0.05 a	33.5±0.8 d	2.4±0.1 b	30.1±1.4 c	2.5±0.1 b	28.6±1.3 c
cis Ferulic acid	-	2.3±0.1 d	-	1.8±0.04 c	-	1.5±0.02 b	-	1.4±0.03 a
Dihydroferulic acid Isomer I	-	0.1±0.01 a,b	-	0.04±0.001 a	-	0.1±0.01 a,b	-	0.1±0.01 a,b
Sinapoyl hexose	-	0.8±0.02 b	-	1.0±0.05 c	-	1.1 ±0.1 c,d	-	0.7±0.03 a
Dihydroferulic acid Isomer II	-	0.5±0.05 c	-	0.03±0.005 a	-	0.04±0.001 a	-	0.1±0.03 b
Apigenin-6-C-arabinoside-8-C-hexoside Isomer I	74.1±1.2 h	62.8±1.3 f	66.7±0.9 g	54.3±1.0 e	46.3±0.8 c	50.0±0.6 d	42.6±0.4 b	40.4±0.6 a
Apigenin-6-C-arabinoside-8-C-hexoside Isomer II	3.2±0.2 c	3.7±0.2 d	2.6±0.1 a	3.2±0.1 c	2.5±0.1 a	2.9±0.04 b	2.6±0.1 a	2.4±0.2 a
Catechin	-	-	0.4±0.03 a	3.1±0.2 b	8.7±0.6 c	14.9±0.4 e	12.2±0.8 d	17.2±0.9 f
Epicatechin	-	-	5.1±0.2 a	-	11.7±0.6 b	4.8±0.2 a	15.8±0.4 c	4.8±0.3 a
Afzelchin-Catechin	-	-	-	-	-	2.0±0.1 b	1.1±0.1 a	2.5±0.1 c
Swertiamacroside	-	-	2.7±0.2 a	-	7.5±0.1 b	-	8.1±0.3 c	-
Orientin	-	-	1.4±0.1 b	-	4.1±0.2 c	0.9±0.1 a	4.5±0.2 c	1.0±0.05 a
Isorientin	-	-	0.7±0.1 a	-	1.9±0.2 b	0.8±0.1 a	2.5±0.1 c	0.9±0.1 a
Rutin	-	-	4.7±0.4 c	-	11.1±1.0 d	1.5±0.2 a	17.2±0.7 e	2.2±0.1 b
Isoquercitrin	-	-	2.7±0.2 b	1.1±0.1 a	6.1±0.04 e	4.6±0.2 c	7.6±0.4 f	5.9±0.1 d
Vitexin	-	-	3.2±0.1 b	2.5±0.1 a	8.9±0.4 e	5.3±0.3 c	11.1±0.8 f	7.3±0.6 d
Epigallocatechin	-	-	5.9±0.2 b	5.0±0.2 a	7.2±0.1 c	7.0±0.3 c	8.9±0.3 d	7.2±0.2 c
Isovitexin	-	-	1.8±0.2 b	0.8±0.1 a	5.0±0.5 d	2.0±0.03 b	6.6±0.3 e	2.2±0.1 c
Hyperin	-	-	2.3±0.1 a	-	2.4±0.3 a	-	3.1±0.2 b	-
Afzelchin-catechin-dimethylgallate	-	-	-	-	0.5±0.04 a	-	1.2±0.1 b	-
Epicatechin-dimethylgallate	-	-	2.4±0.1 a	-	6.5±0.2 b	-	7.8±0.1 c	-
Quercetin	-	-	3.3±0.4 b	-	11.2±0.7 c	0.7±0.1 a	15.5±0.6 d	0.7±0.1 a
Sum phenolic acids	3.6±0.4 a	56.6±1.1 e	7.0±0.8 b	100.4±0.9 f	15.7±0.7 c	130.5±1.2 g	17.8±0.9 d	166.0±1.2 h
Sum flavones	77.3±0.5 h	66.4±0.5 d	76.3±0.2 g	60.8±0.2 b	68.7±0.2 e	61.8±0.5 c	69.8±0.2 f	54.3±0.6 a
Sum flavan-3-ols	-	-	13.8±0.4 b	8.1±0.2 a	34.5±0.6 e	28.7±0.3 c	47.0±0.4 f	31.7±0.4 d

Sum flavonols	-	-	13.0±0.4 d	1.1±0.1 a	30.8±0.6 e	6.7±0.2 b	43.4±0.7 f	8.8±0.4 c

BB= buckwheat enriched bread

Analyses were carried out in triplicate (n=3). Different letters in the same line indicate significantly different values ($p < 0.05$).

Table 4. Content of single bound phenolic compounds and relative classes in control (white bread) and enriched bread loaf expressed as mg/kg d.w.

Compound	Control	BB 10%	BB 20%	BB 30%
Vanillic acid	0.6±0.04 a	0.8±0.1 b	0.9±0.1 b	1.5±0.2 c
Syringic acid	0.6±0.1 a	10.0±0.3 b	18.3±0.4 c	29.7±0.7 d
p-hydroxy-benzaldehyde	4.9±0.5 a	12.8±0.4 b	21.7±0.9 c	28.1±1.3 d
p-coumaric acid	1.7±0.2 a,b	1.5±0.1 a	2.6±0.3 c	4.3±0.3 d
Sinapic acid	2.6±0.2	-	-	-
trans ferulic acid	18.9±0.2 d	17.1±0.3 c	16.5±0.2 b	15.7±0.2 a
cis ferulic acid	1.7±0.1 c	1.5±0.1 b	1.3±0.1 a	1.3±0.1 a
Sinapoyl hexose	1.3±0.1 c	1.3±0.1 c	1.0±0.1 b	0.8±0.04 a
Dihydroferulic acid	0.1±0.02 a	0.1±0.01 a	0.1±0.02 a	0.1±0.02 a
Apigenin-6-C-arabinoside-8-C-hexoside Isomer I	67.4±0.7 d	56.5±1.3 c	49.1±0.8 b	41.1±0.5 a
Apigenin-6-C-arabinoside-8-C-hexoside Isomer II	3.2±0.2 d	2.7±0.3 b,c	2.3±0.2 a,b	2.0±0.2 a
Catechin-glucoside Isomer I	-	1.2±0.1 a	1.9±0.2 b	2.7±0.4 c
Catechin-glucoside Isomer II	-	1.3±0.3 a	2.6±0.1 b	3.6±0.1 c
Catechin	-	3.2±0.2 a	5.6±0.3 b	8.7±0.2 c
Epicatechin	-	3.9±0.3 a	6.9±0.5 b	11.0±0.2 c
Swertiamacroside	-	2.3±0.2 a	5.0±0.1 b	8.0±0.4 c
Orientin	-	0.6±0.1 a	1.3±0.2 b	1.8±0.1 c
Isorientin	-	0.5±0.1 a	0.7±0.1 b	1.1±0.2 c
Rutin	-	4.2±0.1 a	4.7±0.1 b	5.7±0.3 c
Isoquercitrin	-	0.1±.03 a	0.9±0.1 b	1.0±0.1 b
Vitexin	-	1.9±0.2 a	3.9±0.1 b	7.0±0.6 c
Epigallocatechin	-	3.4±0.2 a	3.8±0.3 a	4.3±0.1 b
Isovitexin	-	0.5±0.1 a	2.4±0.3 b	3.4±0.2 c
Hyperin	-	2.1±0.3 a	2.3±0.2 a,b	3.9±0.4 c
Sum phenolic acids	32.7±0.3 a	47.3±0.2 b	67.3±0.5 c	89.4±0.4 d
Sum flavones	70.5±0.4 d	62.8±0.3 c	59.7±0.5 b	56.4±0.4 a
Sum flavan-3-ols	-	13.0±0.3 a	20.7±0.2 b	30.3±0.2 c
Sum flavonols	-	6.4±0.1 a	8.0±0.1 b	10.6±0.3 c

BB= buckwheat enriched bread

Analyses were carried out in triplicate (n=3). Different letters in the same line indicate significantly different values ($p < 0.05$).

Table 5. Content of single and bound phenolic compounds and relative classes in crust and crumb of formulated breads expressed as mg/kg d.w.

Compound	Control		BB 10%		BB 20%		BB 30%	
	Crumb	*Crust*	*Crumb*	*Crust*	*Crumb*	*Crust*	*Crumb*	*Crust*
Vanillic acid	0.6±0.1 a	0.6±0.1 a	0.8±0.1 b	0.8±0.1 b	0.8±0.1 b	0.9±0.05 c	1.0±0.1 d	2.0±0.3 e
Syringic acid	-	0.7±0.1 a	0.7±0.1 a	19.3±0.4 d	1.8±0.2 b	34.8±0.8 e	2.8±0.1 c	56.7±0.4 f
p-hydroxy-benzaldehyde	2.0±0.2 a	7.8±0.2 d	2.5±0.1 b	23.0±0.4 e	2.6±0.1 b	40.9±0.5 f	3.5±0.3 c	52.7±0.9 g
p-coumaric acid	-	1.7±0.2 d	0.1±0.01 a	3.0±0.4 e	0.2±0.03 b	4.9±0.5 f	0.6±0.05 c	8.0±0.4 g
Sinapic acid	-	2.3±0.3 a	-	3.2±0.1 b	-	3.5±0.1 c	-	4.3±0.2 d
trans ferulic acid	1.9±0.3 c,d	35.9±0.5 h	1.3±0.05 a	32.9±0.2 g	1.5±0.1 a,b	31.6±0.4 f	1.8±0.2 c	29.6±0.6 e
cis ferulic acid	-	1.6±0.1 c	-	1.3±0.03 b	-	1.2±0.1 a	-	1.1±0.1 a
Sinapoyl hexose	-	1.2±0.1 c	-	1.2±0.1 c	-	0.9±0.02 b	-	0.7±0.04 a
Dihydroferulic acid	0.03±0.005 a	-	0.03±0.004 a	-	0.03±0.005 a	-	0.04±0.003 a	
Apigenin-6-C-arabinoside-8-C-hexoside Isomer I	69.2±0.4 g	65.5±0.8 f	58.9±0.5 e	54.2±0.7 d	49.5±0.4 c	48.8±0.4 c	43.1±0.1 b	39.1±0.3 a
Apigenin-6-C-arabinoside-8-C-hexoside Isomer II	3.0±0.1 d	3.3±0.1 e	2.6± b	2.8±0.1 c	1.9±0.2 a	2.8±0.1 c	1.7±0.1 a	2.3±0.2 b
Catechin-glucoside Isomer I	-	-	1.1±0.04 a	-	1.7±0.1 b	-	2.6±0.3 c	-
Catechin-glucoside Isomer II	-	-	1.1±0.1 a	-	2.5±0.3 b	-	3.5±0.2 c	-
Catechin	-	-	3.1±0.3 a	-	5.5±0.1 b	-	8.2±0.1 c	-
Epicatechin	-	-	3.6±0.2 a	-	6.8±0.5 b	-	10.0±0.3 c	-
Swertiamacroside	-	-	2.2±0.1 a	-	4.8±0.1 b	-	7.3±0.2 c	-
Orientin	-	-	0.5±0.05 a	-	2.1±0.3 c	0.4±0.1 b	3.0±0.2 d	0.5±0.1 a
Isorientin	-	-	0.4±0.04 b	-	1.1±0.1 c	0.2±0.04 a	2.0±0.3 d	0.3±0.03 a
Rutin	-	-	4.2±0.3 c	-	6.8±0.5 d	0.3±0.04 a	9.7±0.4 e	0.7±0.1 b
Isoquercitrin	-	-	-	0.1±0.02 a	-	0.9±0.1 b	0.8±0.1 b	1.1±0.1 c
Vitexin	-	-	2.1±0.1 b	1.7±0.2 a	5.0±0.3 d	2.8±0.04 c	8.2±0.2 e	5.9±0.3 d
Epigallocatechin	-	-	3.2±0.1 a	-	3.5±0.2 a	4.0±0.2 b	4.5±0.1 c	4.2±0.04 b
Isovitexin	-	-	1.3±0.04 c	0.4±0.02 a	3.7±0.1 e	1.1±0.1 b	4.8±0.1 f	2.0±0.2 d
Hyperin	-	-	2.0±0.2 b	-	3.2±0.3 c	1.5±0.2 a	6.3±0.2 d	1.6±0.1 a
Sum phenolic acids	4.6±0.2 a	51.8± 0.8 e	7.6±0.1 b	84.6±0.4 f	11.8±0.3 c	118.7±1.2 g	16.9±0.4 d	155.2±0.8 h
Sum flavones	72.2±0.5 g	68.9±0.8 f	65.9±0.5 e	59.1±0.4 c	63.3±0.8 d	56.1±0.7 b	62.9±0.6 d	50.0±0.5 a
Sum flavan-3-ols	-	-	12.0±0.3 b	-	20.0±0.2 c	4.0±0.2 a	28.8±0.2 d	4.2±0.3 a
Sum flavonols	-	-	6.2±0.2 d	0.1±0.001 a	10.0±0.2 e	2.6±0.1 b	16.8±0.1 f	3.4±0.1 c

BB= buckwheat enriched bread. Analyses were carried out in triplicate (n=3). Different letters in the same line indicate significantly different values ($p < 0.05$).

Table 6. Total free and bound phenolic content (by HPLC-ESI-MS) and antioxidant activity of bread samples

		Total phenolic content by HPLC-ESI-MS (mg/kg d.w.)	DPPH (µmoles Trolox equivalent/100 g d.w.)	ABTS (µmoles Trolox equivalent/100 g d.w.)
		Free phenolic compounds		
Control	Loaf	109 ±1	296 ± 1	222 ± 1
	Crust	123.1 ± 0.9	443 ± 2	317 ± 2
	Crumb	81.0 ± 0.7	146.1 ± 0.9	118 ± 1
BB 10%	Loaf	172 ± 1	476 ± 3	402 ± 2
	Crust	170.3 ± 0.9	579 ± 3	498 ± 2
	Crumb	110 ± 1	368 ± 2	302 ± 2
BB 20%	Loaf	204 ± 1	679 ± 3	607 ± 3
	Crust	228 ± 1	763 ± 3	711 ± 3
	Crumb	150 ± 2	589 ± 3	503 ± 3
BB 30 %	Loaf	235 ± 1	889 ± 3	845 ± 4
	Crust	261 ± 2	949 ± 3	899 ± 4
	Crumb	178 ± 2	823 ± 2	787 ± 3
		Bound phenolic compounds		
Control	Loaf	103.2 ± 0.5	166 ± 1	115 ± 1
	Crust	120.6 ± 0.7	200 ± 1	118 ± 1
	Crumb	76.8 ± 0.5	128 ± 2	109 ± 1
BB 10%	Loaf	129.5 ± 0.8	239 ± 2	213 ± 2
	Crust	143.8 ± 0.4	382 ± 2	301 ± 2
	Crumb	91.7 ± 0.6	183 ± 1	121 ± 2
BB 20%	Loaf	155.7 ± 0.6	396 ± 2	356 ± 3
	Crust	181.3 ±0.9	452 ± 2	403 ± 2
	Crumb	105.1 ± 0.9	418 ± 3	306 ± 2
BB 30 %	Loaf	187 ± 1	485 ± 3	555 ± 3
	Crust	213 ± 1	632 ± 3	602 ± 3
	Crumb	125 ± 1	571 ± 3	501 ± 3

BB= buckwheat enriched bread. Analyses were carried out in triplicate (n=3).

Highlights

-Enrichment of bread with buckwheat flour increase its flavonols and flavan-3-ols content

-This study highlighted differences between phenolic composition of crust and crumb

-Rutin is more concentrated in crumb than crust