ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

ARCHIVIO ISTITUZIONALE
DELLA RICERCA

Alma Mater Studiorum Università di Bologna
Archivio istituzionale della ricerca

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

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*
Verardo, V., Glicerina, V., Cocci, E., Frenich, A.G., Romani, S., Caboni, M.F. (2018). Determination of free and bound phenolic compounds and their antioxidant activity in buckwheat bread loaf, crust and crumb. LEBENSMITTEL-WISSENSCHAFT + TECHNOLOGIE, 87, 217-224 [10.1016/j.lwt.2017.08.063].

*Availability:*
This version is available at: https://hdl.handle.net/11585/620822 since: 2018-02-08

*Published:*
DOI: http://doi.org/10.1016/j.lwt.2017.08.063

*Terms of use:*

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (https://cris.unibo.it/).
When citing, please refer to the published version.

(Article begins on next page)

12 October 2024

This is the final peer-reviewed accepted manuscript of:

Vito Verardo, Virginia Glicerina, Emiliano Cocci, Antonia Garrido Frenich, Santina Romani, Maria Fiorenza Caboni, *Determination of free and bound phenolic compounds and their antioxidant activity in buckwheat bread loaf, crust and crumb*, LWT, Volume 87, 2018, Pages 217-224, ISSN 0023-6438,

https://www.sciencedirect.com/science/article/pii/S0023643817306333

The final published version is available online at:

https://doi.org/10.1016/j.lwt.2017.08.063

Rights / License:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

*This item was downloaded from IRIS Università di Bologna (https://cris.unibo.it/)*

***When citing, please refer to the published version.***

# Accepted Manuscript

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

Vito Verardo, Virginia Glicerina, Emiliano Cocci, Antonia Garrido Frenich, Santina Romani, Maria Fiorenza Caboni

PII: S0023-6438(17)30633-3

DOI: 10.1016/j.lwt.2017.08.063

Reference: YFSTL 6485

To appear in: *LWT - Food Science and Technology*

Received Date: 7 April 2017

Revised Date: 18 August 2017

Accepted Date: 20 August 2017

Please cite this article as: Verardo, V., Glicerina, V., Cocci, E., Frenich, A.G., Romani, S., Caboni, M.F., Determination of free and bound phenolic compounds and their antioxidant activity in buckwheat bread loaf, crust and crumb, *LWT - Food Science and Technology* (2017), doi: 10.1016/j.lwt.2017.08.063.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

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

Vito Verardo [1*], Virginia Glicerina [2], Emiliano Cocci [3], Antonia Garrido Frenich [4], Santina Romani [2,5], Maria Fiorenza Caboni [2,5]

[1] *Department of Nutrition and Food Science, University of Granada, Campus of Cartuja, 18071 Granada, Spain.*

[2] *Interdepartmental Centre of Agri-food Industrial Research (CIRI Agroalimentare), Alma Mater Studiorum - University of Bologna, Quinto Bucci 336, 47521 Cesena (FC), Italy*

[3] *Agribologna Consortium, via Canali 1, 40127 Bologna, Italy*

[4] *Department of Chemistry and Physics (Analytical Chemistry Area) and Research Centre for Agricultural and Food Biotechnology (BITAL), Agrifood Campus of International Excellence, ceiA3, University of Almería, Carretera de Sacramento s/n, 04120 Almería, Spain.*

[5] *Department of Agricultural and Food Sciences, Alma Mater Studiorum - University of Bologna, Piazza Goidanich 60, 47521 Cesena (FC), Italy*

* Corresponding author: Vito Verardo, Department of Nutrition and Food Science, University of Granada, Campus of Cartuja, 18071 Granada, Spain. E-mail: vitoverardo@ugr.es

ACCEPTED MANUSCRIPT

**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

ACCEPTED MANUSCRIPT

## 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 ($\lambda$ = 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

derivatives that contributed to this improvement were syringic acid, *p*-hydroxybenzaldehyde and swertiamacroside. Vanillic, syringic, *p*-coumaric, sinapic, dehydroferulic and sinapoyl-hexose acid derivatives were detected in free form in formulated breads, but they are present only in bound form in refined wheat and buckwheat flours. This confirmed the hypothesis of other authors that mixing, sourdough fermentation and baking process facilitate the release of bound phenolic compounds to its free form (Angeloni & Collar, 2011; Yu & Beta, 2015)

To better evaluate the distribution of phenolic compounds in control and buckwheat enriched breads, the crumb was separated from the crust in each loaf samples; free phenolic fraction of the two section of loaf is reported in **Table 3**.

Control and enriched samples showed that crust has the higher phenolic content compared to the crumb. These data totally fitted with the results reported in other works (Balestra et al., 2011; Lu et al. 2014; Yu & Beta, 2015). According to Vitali, Dragojevic, and Sebecic (2009) the high content of phenolic compounds in crust should be due to the effect of baking temperature that probably hydrolyzes some complex phenols resulting in an increase of extractable phenolic content. Anyway, Gélinas and McKinnon (2006) hypothesized that also Maillard reactions are involved to some extent in the content of phenolic compounds in bread crust.

Only four phenolic compounds were determined in control crumb; basically, apigenin-6-C-arabinoside-8-C-hexoside isomers represented more than 95 % of its total phenolic content; minor phenolics were *trans* ferulic acid and *p*-hydroxybenzaldehyde. Twelve phenolic compounds were quantified in control crust. Apigenin-6-C-arabinoside-8-C-hexoside was the principal phenolic compound followed by the *trans* ferulic acid and their sum corresponds to 81.9% of total phenolic compounds.

To our knowledge, no studies on the distribution of phenolic compounds in buckwheat crumb 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.

*Trans* ferulic acid was the second phenolic compound in bread crust; its content in crumb was from 16 to 25 times lower than in crust. Moreover, the ferulic content in buckwheat breads was lower than control bread. Similar trend has been showed by *p*-hydroxybenzaldehyde.

Crumbs of breads formulated with buckwheat flours contained increasing amounts of flavan-3-ols (from 12 to 29 mg/kg bread d.w.) according to the buckwheat flour ratio added during bread formulation.

Rutin was also detected in bound form and its content was higher in buckwheat bread crumb than crusts confirming the low thermal stability of this compound as previously reported for its free form.

*3.3. Antioxidant activity of bread samples*

The results of antioxidant activity measured by DPPH and ABTS radicals, and total phenolic content, expressed as sum of each phenolic compound determined by HPLC-ESI-MS, are reported in **Table 6**.

Total free phenolic content in bread loaf varied between 109 and 235 mg/kg bread d.w., buckwheat bread samples showed higher amounts of these compounds compared to control.

These data confirm the results showed in previous works (Angioloni, & Collar, 2011; Szawara-Nowak, Bączek & Zieliński, 2016) that demonstrate as the multigrain bread exhibited increased polyphenol content, higher polyphenol bioaccessibility and higher antioxidant power than bread obtained with single grains.

Significant correlations have been found between total phenolic content and DPPH and ABTS assay results. Briefly, DPPH assay showed a positive correlation ($r = 0.9354$, $p < 0.001$) with total phenolic amounts, and according to Yu et al. (2013) and Yu and Beta (2015), crusts showed the highest scavenging activity due to the high phenolic content and probably to the high presence of Maillard reaction products that could react with DPPH radical.

Significant differences ($p<0.05$) were found among the buckwheat bread samples; in fact high ratio of buckwheat flour correspond to high phenolic content and radical scavenging activity. Similarly, ABTS assay reported a positive correlation ($r = 0.9338$, $p < 0.001$) with free total phenolic content in breads. ABTS scavenging capacity was comprised between 118 and 899 µmoles TEAC/100 g bread d.w.; according to results obtained by Yu et al. (2013) and Yu and Beta (2015), crust samples showed the highest antioxidant capacity, and buckwheat breads scavenging capacity increased when high buckwheat ratio was used during the bread formulation.

High correlation was found between DPPH and ABTS assay results ($r = 0.9950$, $p< 0.001$).

Total bound phenolic compounds content ranged between 77 and 213 mg/kg bread d.w. These values are apparently in contrast with the data of other authors (Abdel-Aal & Rabalski, 2013; Yu & Beta, 2015) which showed that bound phenolic content was higher than free phenolic content in bread obtained with wheat flour; according to Yu et al. (2013), the bound phenolic content in refined flour could be more than ten times lower than in whole wheat flours.

Antioxidant activity, measured by DPPH and ABTS assays, decreased with the diminution of buckwheat flour quantity. As noticed for free phenolic fraction, in each kind of bread crust samples showed higher antioxidant activity than in loaf, and the last one presented higher antioxidant activity than crumb. These results could be justified by a strong Maillard reaction development in crust than in crumb.

Positive correlations were found between total bound phenolic content and DPPH ($r = 0.7765$, $p < 0.05$) and between total bound phenolic content and ABTS ($r = 0.8361$, $p < 0.05$).

## 4. Conclusions

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

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

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

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

ACCEPTED MANUSCRIPT

**Acknowledgments**

Vito Verardo thanks the Spanish Ministry of Economy and Competitiveness (MINECO) for "Juan de la Cierva" and "Ramon y Cajal" contracts.

**References**

Abdel-Aal, E.S.M., & Rabalski, I. (2013). Effect of baking on free and bound phenolic acids in wholegrain bakery products. *Journal of Cereal Science*, *57*, 312-318.

Angioloni, A., & Collar, C. (2011). Polyphenol composition and "in vitro" antiradical activity of single and multigrain breads. *Journal of Cereal Science*, *53*, 90-96.

Balestra, F., Cocci, E., Pinnavaia, G. & Romani, S. (2011). Evaluation of antioxidant, rheological and sensorial properties of wheat flour dough and bread containing ginger powder. *LWT - Food Science and Technology*, *44*, 700–705.

Blandino, M., Sovrani, V. Marinaccio, F., Reyneri, A., Rolle, L., Giacosa, S., ... & Arlorio, M. (2013). Nutritional and technological quality of bread enriched with an intermediated pearled wheat fraction. *Food Chemistry*, *141*, 2549–2557.

Buchner, N., Krumbein, A., Rohn, S., & Kroh, L.W. (2006). Effect of thermal processing on the flavonols rutin and quercetin. *Rapid Communications in Mass Spectrometry*, *20*, 3229–3235.

Cho, Y.J., & Lee, S. (2015). Extraction of rutin from Tartary buckwheat milling fractions and evaluation of its thermal stability in an instant fried noodle system. *Food Chemistry*, *176*, 40–44

Dziki, D., Rozyło, R., Gawlik-Dziki, U., & Swieca, M. (2014). Current trends in the enhancement of antioxidant activity of wheat bread by the addition of plant materials rich in phenolic compounds. *Trends in Food Science & Technology*, *40*, 48-61.

Gélinas, P., & McKinnon, C.M. (2006). Effect of wheat variety, farming site, and bread-baking on total phenolics. *International Journal of Food Science and Technology*, *41*, 329–332.

Gil, A., Ortega, R. M., & Maldonado, J. (2011). Wholegrain cereals and bread: a duet of the Mediterranean diet for the prevention of chronic diseases. *Public Health Nutrition, 14(12A),* 2316–2322.

Gianotti, A., Danesi, F., Verardo, V., Serrazanetti, D.I., Valli, V., Russo, ... & Bordoni, A. (2011). Role of cereal type and processing in whole grain *in vivo* protection from oxidative stress. *Frontiers in Bioscience, 16,* 1609-1618.

Giménez-Bastida, J. A., & Zieliński, H. (2015). Buckwheat as a functional food and its effects on health. *Journal of Agricultural and Food Chemistry, 63,* 7896−7913.

Giménez-Bastida, J.A., Piskuła, M., & Zieliński, H. (2015). Recent advances in development of gluten-free buckwheat products. *Trends in Food Science & Technology, 44,* 58-65.

Giménez-Bastida, J.A., Zieliński, H., Piskuła, M., Zielinska, D., & Szawara-Nowak, D. (2017). Buckwheat bioactive compounds, their derived phenolic metabolites and their health benefits. *Molecular Nutrition & Food Research, 61,* 1600475.

Gómez-Caravaca, A.M., Verardo, V., Berardinelli, A., Marconi, E., & Caboni, M. F. (2014). A chemometric approach to determine the phenolic compounds indifferent barley samples by two different stationary phases: A comparison between C18 and pentafluorophenyl core shell columns. *Journal of Chromatography A, 1355,* 134–142.

Inglett, G.E., Chen, D., Berhow, M., & Lee, S. (2011). Antioxidant activity of commercial buckwheat flours and their free and bound phenolic compositions. *Food Chemistry, 125*, 923–929.

Lin, L.Y., Liu, H.M., Yu, Y.W., Lin, S.D., & Mau, J.L. (2009). Quality and antioxidant property of buckwheat enhanced wheat bread. *Food Chemistry, 112,* 987–991.

Lu, Y., Luthria, D., Fuerst, E.P., Koszonas, A.M., Yu, L., & Morris, C.F. (2014). Effect of processing on phenolic composition of dough and bread fractions made from refined and whole wheat flour of three wheat varieties. *Journal of Agricultural and Food Chemistry 62*, 10431−10436.

Parejo, I., Codina, C., Petrakis, C., & Kefalas, P. (2000). Evaluation of scavenging activity assessed by Co(II)/EDTA-induced luminol chemiluminescence and DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical assay. *Journal of Pharmacological and Toxicological Methods*, *44*, 507-512.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine, 26,* 1231–1237.

Stokic, E., Mandic, A., Sakac, M., Misan, A., Pestoric, M., Simurina, O., … & Sedej, I. (2015). Quality of buckwheat-enriched wheat bread and its antihyperlipidemic effect in statin treated patients. *LWT - Food Science and Technology, 63*, 556-561.

Szawara-Nowak, D., Bączek, N., & Zieliński, H. (2016). Antioxidant capacity and bioaccessibility of buckwheat-enhanced wheat bread phenolics. *Journal of Food Science and Technology, 53(1),* 621–630.

Szawara-Nowak, D., Koutsidis, G., Wiczkowski, W., & Zielinski, H. (2014). Evaluation of the in vitro inhibitory effects of buckwheat enhanced wheat bread extracts on the formation of advanced glycation end-products (AGEs). *LWT - Food Science and Technology, 58*, 327-334.

Verardo, V., Arráez-Román, D., Segura-Carretero, A., Marconi, E., Fernández-Gutiérrez, A., & Caboni, M.F. (2010). Identification of buckwheat phenolic compounds by reverse phase high performance liquid chromatography-electrospray ionization-time of flight-mass spectrometry (RP-HPLC-ESI-TOF-MS). *Journal of Cereal Science, 52,* 170-176.

Verardo, V., Gomez-Caravaca, A.M., Segura-Carretero, A., Caboni, M.F., & Fernandez-Gutierrez, A. (2011). Development of a CE-ESI-microTOF-MS method for a rapid identification of phenolic compounds in buckwheat. *Electrophoresis, 32, (6-7),* 669-673.

Verardo, V., Arráez-Román, D., Segura-Carretero, A., Marconi, E., Fernández-Gutiérrez, A., & Caboni, M.F. (2011). Determination of free and bound phenolic compounds in buckwheat spaghetti by RP-HPLC-ESI-TOF-MS: effect of thermal processing from farm to fork. *Journal of Agricultural and Food Chemistry, 59,* 7700–7707.

Vitali, D., Dragojevic, B., & Sebecic, B. (2009). Effects of incorporation of integral raw materials and dietary fibre on the selected nutritional and functional properties of biscuits. *Food Chemistry, 114,* 1462-1469.

Yu, L., Nanguet, A.L., & Beta, T. (2013). Comparison of antioxidant properties of refined and whole wheat flour and bread. *Antioxidants 2,* 370-383.

Yu, L., & Beta, T. (2015). Identification and antioxidant properties of phenolic compounds during production of bread from purple wheat grains. *Molecules, 20,* 15525-15549.

Zilic, S., Sukalovic, V. H. T., Dodig, D., Maksimovi, V., Maksimovi, M., & Basic, Z. (2011). Antioxidant activity of small grain cereals caused by phenolics and lipid soluble antioxidants. *Journal of Cereal Science, 54,* 417-424.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

**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 |

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

**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 ($p < 0.05$).

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

**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 |

ACCEPTED MANUSCRIPT

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 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 ($p < 0.05$).

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

**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 ($p < 0.05$).

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

**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 ($p < 0.05$).

ACCEPTED MANUSCRIPT

**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).

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

**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

ACCEPTED MANUSCRIPT