J Food Sci Technol (January 2016) 53(1):621–630
DOI 10.1007/s13197-015-2074-y

ORIGINAL ARTICLE

Antioxidant capacity and bioaccessibility of buckwheat-enhanced wheat bread phenolics

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Revised: 15 September 2015 / Accepted: 22 October 2015 / Published online: 31 October 2015

Abstract The impact of an in vitro procedure that mimics the physiochemical changes occurring in gastric and small intestinal digestion on the antioxidant capacity and bioaccessibility of phenolic compounds from 16 types of buckwheat-enhanced wheat breads was assessed. The methodology was based on the Global Antioxidant Response (GAR) which combined bioaccessible antioxidant capacity of the soluble fraction from digestible portion measured by the standard Trolox Equivalent Antioxidant Capacity (TEAC) assay and antioxidant capacity of the insoluble fraction from the undigested portion by the QUENCHER method. The bioaccessibility of the phenolics was measured in the soluble fraction with Folin-Cicalteu reagent and in the insoluble fraction by modified QUENCHER method. The studies showed almost 20-fold higher GAR values as compared to the antioxidant capacity of the respective undigested reference breads. The bioaccessible antioxidant capacity of soluble fraction from digestible portion increased significantly whereas the undigested residue displayed antioxidant capacity that accounted for up to 15 % of the GAR. The bioaccessible phenolics accounted for up to 90 % of the total phenolics after digestion and were highly correlated with GAR results of buckwheat-enriched wheat breads. Our results indicate that in vitro digestion is the crucial step that releases of high amount of phenolic antioxidants. The combination of QUENCHER assay with Total Antioxidant Capacity (TAC) and Total Phenolic Content (TPC) assay estimated on Folin-Ciocalteu reagent has been useful for the determination of the bioaccessible antioxidant activity and phenolics of the soluble and insoluble fraction of buckwheat-enhanced wheat breads.

Keywords Buckwheat-enhanced wheat bread · Gastrointestinal digestion · Bioaccessibility model · Global Antioxidant Response (GAR) · Bioaccessible phenolic compounds

Introduction

Efforts are increasingly numerous and more common actions to the variety of conventional diet to protect the consumer against diseases such as diabetes, hypertension, heart disease and cancer (Sun and Ho 2005; Dewettinck et al. 2008; Pedersen et al. 2011). Currently, wheat flour is usually used in bread making (Giménez-Bastida et al. 2015). Recently, scientists have demonstrated that the usage of buckwheat flour as an ingredient in food recipe can provide beneficial health effects (Lin et al. 2009; Gawlik-Dziki et al. 2009; Wronkowska et al. 2010; Zhang et al. 2012).

Buckwheat has been a commonly-eaten food, it is grown in many countries, but Russia is the biggest producer in the world. Buckwheat contains rutin (quercetin-3-rutinoside), and therefore is the preferred source of this flavonoid. Rutin as a metabolite has antioxidant, anti-inflammatory, and anti-cancer properties. Moreover, it thickens the walls of blood vesses, thus reducing their permeability and preventing arteriosclerosis. Buckwheat is most commonly used for producing flour and groats (Holasova et al. 2002; Sun and Ho 2005; Kreft et al. 2006; Gawlik-Dziki et al. 2009).

Determination of the antioxidant capacity of cereals is not easy and depends on the polarity of the solvents and type of bonds, which are connected to the cell wall. Serpen et al.

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(2007) showed that antioxidants bound to a cell wall have high antioxidant capacity but they are difficult to extract and the solvents of different polarity have to be used. The techniques usually used to extract bioactive compounds from food, which consist of extraction and hydrolysis steps, present some restriction: 1) low extraction efficiency depending on the polarity of the solvent (Pellegrini et al. 2007), 2) the alkaline hydrolysis does not release all linked antioxidants (Serpen et al. 2007), 3) the combined effect between antioxidants may be slightly degraded during the extraction of antioxidants and due to the antioxidant capacity measurements in various extracts (Serpen et al. 2008). Therefore, an attractive approach is developing a system without extraction step. On the other hand, several common techniques have been used to evaluate the TAC of bread (Michalska et al. 2008; Martinez-Villaluenga et al. 2009), however these methods are limited due to the solubility of the antioxidant compounds in environment of reaction. Nonetheless, beside the extraction technique, there is still an insoluble fraction existing in the foodstuff. To surmount this trouble, Gökmen et al. (2009) elaborated a straight method called QUENCHER to assess the TAC of foods without the extraction. This allows the investigation of the antioxidant capacity of whole antioxidant stuff.

One of the major topics about the positive effects of polyphenols is their bioavailability and metabolism. The bioavailability of nutritional compounds is based on their gastrointestinal stability, and liberation from the food matrix (called bioaccessibility), and the efficacy of their trans-epithelial transition. The bioavailability of the ingredients used orally in a pure form is typically much greater than the bioavailability of the same component contained in the matrix of the food product. In many cases, the individual nutrients are chemically linked with the other components, which greatly limit their release in the intestine. *In vitro* digestion models are generally used for investigation of the structural changes, digestibility, and deliverance of food ingredients according to simulated digestive environment. Studies based on model systems that simulate human physiological system are common, cheap and helpful in understanding the metabolic pathway of food (Anson et al. 2009; Li et al. 2014). When food is exposed to in vitro intestinal digestion, generally the activity of the bioaccessible fraction obtained only is analysed, while the undigested fraction is discarded (Rufián-Henares and Delgado-Andrade 2009). The QUENCHER procedure enables to evaluate the antioxidant activity in the residue portion.

The goal of the present study was to assess the antioxidant capacity and bioaccessibility of 16 types of buckwheat-enhanced wheat bread phenolic compounds after an *in vitro* digestion. In the first part of this work, gastrointestinal digestion was conducted in order to release the *in vivo* available antioxidant compounds. Afterwards, the antioxidant ability of this fraction was evaluated using a standard procedure. In the second part, the undigested portion was also studied used the QUENCHER method. Determination of the bioaccessible phenolic compounds in buckwheat-enhanced wheat breads was addressed for the first time.

Material and methods

Chemicals and reagents

Alpha-amylase, pepsin, pancreatin, bile salts, rutin, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulphate and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PCL-ACW kits were purchased from Analytik Jena (Leipzig, Germany). All other reagents were from POCh, (Gliwice, Poland). Water was purified with a Mili-Q-system (Milipore, Bedford, USA).

Buckwheat enhanced wheat breads making

Dark wheat flour type 2000, white wheat flour type 500 and white buckwheat flour were purchased from local market. Roasted buckwheat groat was obtained from a local industry and it was milled into a flour. The white buckwheat flour or flour from milling roasted buckwheat were used for the replacement of dark or white wheat flour of 10, 20, 30 and 50 % (*w*/*w*). Buckwheat enhanced white and dark wheat breads and reference wheat breads were prepared. In general, 16 types of buckwheat-enhanced wheat bread was prepared as follows: 4 types of white wheat bread enhanced with white buckwheat flour, 4 types of white wheat bread enhanced with flour from milled roasted buckwheat groats, 4 types of dark wheat bread enhanced with white buckwheat flour and 4 types of dark wheat bread enhanced with flour from milled roasted buckwheat groats. The ingredients and the baking condition were shown in Table 1. At least three loaves of each type of bread baked. The samples of bread were freeze-dried, milled and stored in the freezer until analysis.

In vitro digestion of breads

An *in vitro* digestion model system was performed according to the method of (Delgado-Andrade et al. 2010). It comprised three stages: oral, gastric and intestinal digestion. Oral digestion was performed using 0.125 mL of an alpha-amylase solution (final concentration: 1.3 mg/mL of 1 mM $CaCl_2$ pH 7.0) per 0.5 g of each sample was brought to a final volume of 5 mL of milli-Q water. The mixture was then incubated at 37 °C for 30 min. After that pH was adjusted to 2 with HCl 6 N, a pepsin solution (final concentration: 0.16 g/mL of 0.1 mL) was added in an amount of 0.025 g of pepsin/0.5 g of sample, then incubated at 37 °C for 2 h with the shaking, for

Table 1 Buckwheat-enhanced wheat breads formulation and baking conditions

Ingredient and conditions	Substitution level (%)				
	0	10	20	30	50
Buckwheat enhanced white wheat breads					
White wheat flour (g)	350	315	280	245	175
White buckwheat flour (g)	–	35	70	105	175
Flour from roasted buckwheat groats (g)	–	35	70	105	175
Buckwheat enhanced dark wheat breads					
Dark wheat flour (g)	350	315	280	245	175
White buckwheat flour (g)	–	35	70	105	175
Flour from roasted buckwheat groats (g)	–	35	70	105	175
Water (mL)	228*	228*	228*	228*	228*
	250**	250**	250**	250**	250**
Salt (g)	3.5	3.5	3.5	3.5	3.5
Yeast (g)	10.5	10.5	10.5	10.5	10.5
Fermentation:					
Temperature (°C)	37	37	37	37	37
Time (min)	90	90	90	90	90
Pieces of dough (g)	250	250	250	250	250
Proofing (75 % rh)					
Temperature (°C)	37	37	37	37	37
Time (min)	25	25	25	25	25
Baking:					
Temperature (°C)	250	250	250	250	250
Time (min)	30	30	30	30	30

*Buckwheat enhanced white wheat breads

**Buckwheat enhanced dark wheat breads

the gastric digestion. Next, the pH was adjusted to pH 6 with 1 M $NaHCO_3$ dropwise, and 1.25 mL of pancreatin + bile salts mixture was added (final concentration: 0.004 g/mL of pancreatin and 0.025 g/mL of bile salts of 0.1 M $NaHCO_3$) for the intestinal digestion. The pH was then adjusted to pH 7.5 with 1 M $NaHCO_3$, and samples were incubated at 37 °C for 2 h with the shaking. After that, the digestive enzymes were deactivated by heat treatment for 4 min at 100 °C. The samples were cooled and centrifuged at 3200 x g for 60 min at 4 °C in a Beckman GS-15 R centrifuge (Fullerton, CA, USA) to separate soluble and insoluble fractions. Insoluble fraction was frozen, lyophilized and used for the QUENCHER assays.

Determination of total phenolic content in breads before digestion and bioaccessible phenolics of the soluble fraction after digestion.

Bread samples (0.25 g) were extracted with 67 % aqueous methanol (5 mL) for 40 min at room temperature. Then samples were centrifuged at 5000 x g for 10 min in a Beckman GS-15 R centrifuge. All extractions were carried out in triplicate.

A total phenolic content (TPC) estimated based on Folin-Ciocalteu reagent was determined according to Shahidi and Naczk (1995). Appropriate extracts (0.25 mL) were mixed with 0.25 mL 50 % aqueous Folin-Ciocalteu reagent and 0.5 mL saturated sodium carbonate solution and 4 mL of water. After that the mixture was incubated at room temperature (25 min.) and centrifuged at 5000 x g for 10 min in a Beckman GS-15 R centrifuge. Absorbance was measured using a spectrophotometer Shimadzu UV-160 1PC at $\lambda = 725$ nm. The data were calculated on rutin equivalents.

Determination of antioxidant capacity in breads before digestion and bioaccessible antioxidant capacity of the soluble fraction after digestion by ABTS and photochemiluminescence (PCL) assays.

ABTS assay

The ABTS assay was determined following the procedure described by Re et al. (1999) with a slight change. Extracts from bread samples were prepared according to the same procedure as above. The $ABTS^{+\bullet}$ was prepared the day before the measurements. Aqueous solution of ABTS (7 mM) was mixed with 51.4 mM solution of potassium persulfate. The mixture was allowed to stand at room temperature (in dark) for 12–16 h. The $ABTS^{+\bullet}$ stock solution was diluted with a 67 % methanol so that the absorbance was in the range of

0.70 ± 0.02 at λ = 734 nm. The $ABTS^{+\bullet}$ working solution (1.48 mL) was mixed with 0.020 mL of the extracts of bread or Trolox standards and measured at 30 °C after 6 min at λ = 734 nm using a spectrophotometer Shimadzu UV-160 1PC (Kyoto, Japan). Trolox was used for standard calibration (0.1–2.0 mM) and results were expressed as μmol Trolox/g D.M. (dry matter).

PCL assay

0.1 g of bread samples were extracted with 1 mL of miliQ water for 3 min with a vortex. Next, the samples were centrifuged for 5 min. (16,100 x g, 4 °C) in an Eppendorf 5415 R centrifuge (Hamburg, Germany). All extractions were carried out in triplicate.

The photochemiluminescence (PCL) assay was used to measure the antioxidant activity of buckwheat-enhanced wheat bread extracts against superoxide anion radicals ($O_2^{-\bullet}$) with a Photochem® apparatus (Analytik Jena. Leipzig, Germany) (Zieliński et al. 2012). The free radicals are visualized with the chemiluminescent detection reagent luminol which works as photosensitizer as well as oxygen radical detection reagent. These reactions take place in the Photochem®. Briefly the antioxidant activity of extracts of buckwheat-enhanced wheat bread was determined using an ACW kit.

Exactly 1.5 mL reagent 1 (sample diluent), 1 mL reagent 2 (buffer solution), 0.025 mL reagent 3 (photosensitizer) and 0.01 mL extracts of bread was mixed and measured. The amount of the added extract was such that the intensity of luminescence falls within the scope of the standard curve. Therefore, the antioxidant capacity was calculated by comparison delay time PCL-ACW assay of sample with a Trolox standard curve.

Determination of the bioaccessible total phenolic content and bioaccessible antioxidant capacity of the insoluble fraction after bread digestion.

TPC-QUENCHER method

This method used the ability of the functional groups of antioxidants to reduce the Folin-Ciocalteu reagent at the solid/liquid interface. It was a compilation of classical methods for the determination of phenolic compounds and the modified method by Serpen et al. (2008).

To 0.3 g of freeze-dried insoluble fraction from buckwheat-enhanced wheat breads was added 0.25 mL 50 % ethanol solution of Folin-Ciocalteu reagent and shaken for 30 s using a laboratory vortex type Genie 2 (Bohemia, NY, USA). Then was added 0.25 mL of saturated sodium carbonate, and 4 ml of deionized water. The mixture was left in the dark for 25 min at room temperature, after that the sample was centrifuged at 13 200 x g at 4 °C for 5 min. The absorbance was measured at a wavelength of λ = 725 nm using a spectrophotometer Shimadzu UV-1601PC. The total phenolic content was calculated on rutin equivalents.

ABTS-QUENCHER method

This procedure was applied to the freeze-dried insoluble fraction from buckwheat-enhanced wheat breads, and it was conducted as described by Serpen et al. (2008).

To 0.1 g of samples added 6 mL of 50 % ethanol solution of $ABTS^{+\bullet}$ stock solution prepared as in point. 2.4.1. The mixture was mixed using a magnetic stirrer at room temperature for 24.5 min. Then, the samples were centrifuged at room temperature (14 000 x g, 5 min). The absorbance was measured at a wavelength λ = 734 nm using a spectrophotometer Shimadzu UV-160 1PC. When the absorbance would be out of the range of the standard curve, samples were diluted of cellulose (microcrystalline cellulose, 50 μm). Antioxidant capacity of samples was calculated from a standard curve of Trolox (0.5 mM to 3 mM).

Statistical analysis

Results were expressed as mean ± SD of three different extractions. Data were evaluated by analysis of variance (ANOVA), followed by the Fischer test with the significant difference $P < 0.05$. Analyses were performed using the STATISTICA 7.1 (Tulsa, OK, USA). Pearson test for correlation analysis was used.

Results and discussion

The in vitro digestion model to simulate the physiological processes (transit time, pH and enzymatic conditions) occurring in the gastrointestinal tract of the human digestive system and has been widely used to study the complex multistage process of human digestion (Li et al. 2014). These models deliver a beneficial choice to animal and human models by quickly screening food components. The best in vitro digestion technique would offer precise results in a short time and would therefore help supply systems for different components and structure (Hur et al. 2011). Polyphenols and other antioxidants are susceptible to degradation during digestion due to the effects of pH and enzymes and in the present study, the antioxidant activity of the buckwheat-enhanced wheat bread samples was determined before and after an *in vitro* digestion.

Total phenolic content (TPC) before and after digestion

The total phenolic content of white and dark buckwheat-enhanced wheat breads before and after gastrointestinal digestions *in vitro* was summarised in Fig. 1. Our study showed that

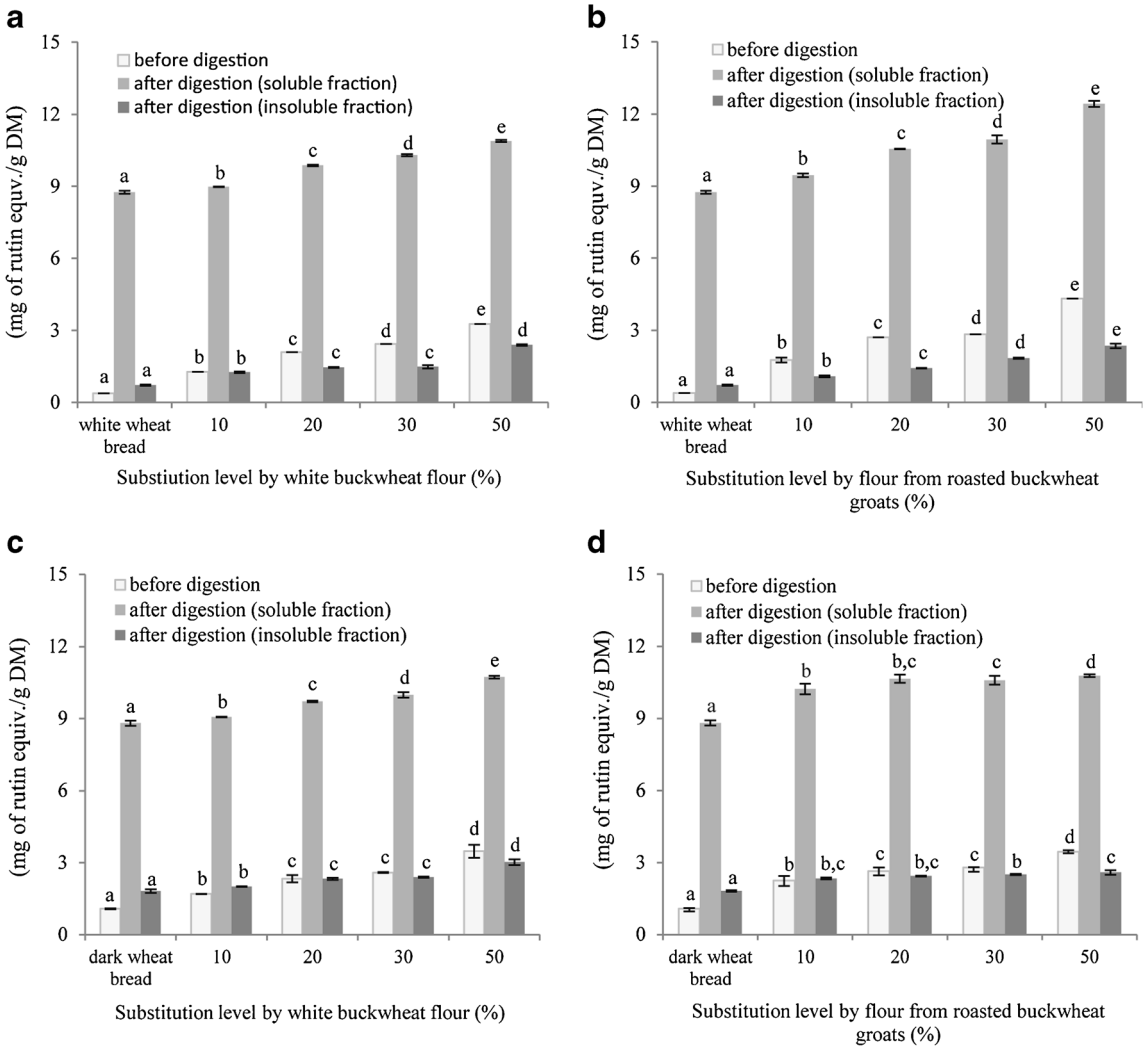

Fig. 1 The total phenolic content of buckwheat enriched wheat breads before and after gastrointestinal digestions in vitro. **a**) buckwheat-enhanced white wheat breads; white wheat flour was substituted by white buckwheat flour; **b**) buckwheat-enhanced white wheat breads; white wheat flour was substituted by flour from roasted buckwheat groats; **c**) buckwheat-enhanced dark wheat breads; dark wheat flour was substituted by white buckwheat flour; **d**) buckwheat-enhanced dark wheat breads; dark wheat flour was substituted by flour from roasted buckwheat groats. Different lowercase letters marked on the columns for each fraction of buckwheat enriched wheat breads indicate significant differences amongst the means ($p < 0.05$)

the content of TPC measured before digestion was lowest in the samples of the reference bread formed on the basis of white and dark wheat flour. Furthermore, the total phenolic content in the dark wheat flour was about three times higher than in white flour (1.8 mg rutin equiv./g DM and 0.38 mg rutin equiv./g DM respectively). These differences could be due to the evidences that most of the phenolic compounds from the whole grains are present in bran fraction (Zieliński and Kozłowska 2000; Horszwald et al. 2010; Gani et al. 2012). Moreover, the increasing addition of buckwheat flour (white and roasted) caused an increase of TPC in non-digested buckwheat-enhanced wheat breads. The highest increase was found in buckwheat-enhanced white wheat breads. Eight-fold increase was found in 50 % addition of white buckwheat flour as compared to white wheat bread and eleven-fold increase in 50 % addition of flour from roasted buckwheat groats as compared to white wheat bread. In buckwheat-enhanced dark wheat breads this increase ranged from 1.5 to 3 times as compared to dark wheat bread.

It is known that both pH and the digestion process result in starch hydrolysis, proteolysis and releasing phenolics from their conjugation forms as well as cell wall matrices. Hence the nutrients and non-nutrient are in their simplest forms (amino acids, fatty acids and simple sugars) before absorption through the intestinal walls (Gião et al. 2012). In this study, the total phenolic content was increased after *in vitro* gastrointestinal digestion. In soluble fraction this increase was significantly higher than in insoluble fraction (QUENCHER). It was interesting that the total phenolic content in the

soluble fraction of the reference white wheat bread was comparable to the content of these compounds in the dark wheat bread (with high difference of these compounds before digestion). This is in accordance with the results achieved by other scientists (Gawlik-Dziki et al. 2009; Chandrasekara and Shahidi 2012). Liyana-Pathirana and Shahidi (2005) also demonstrated that simulated gastrointestinal conditions significantly increased the TPC of extracts obtained from wheat whole grains and their flour, germ and bran fractions. In contrast, the TPC of the insoluble fraction was more than twice higher in the dark wheat bread. Addition in an amount of 10, 20, 30 and 50 % of white buckwheat flour or flour from buckwheat roasted groats caused an increase of the TPC in the soluble fraction, which ranged from 8.98 mg rutin equiv./g DM to 12.43 mg rutin equiv./g DM. While in the insoluble fraction the TPC was ranged from 1.26 mg rutin equiv./g DM to 3.03 mg rutin equiv./g DM.

Antioxidant capacity before and after digestion

The antioxidant capacity of the bread samples determined against $ABTS^{+\bullet}$ radicals before and after gastrointestinal digestion *in vitro* are shown in Fig. 2. It was found that replacement of white or dark wheat flour by white or roasted buckwheat flour at amounts 10, 20, 30 and 50 % increases the antioxidant capacity in the extracts of buckwheat-enhanced wheat bread. The highest antioxidant activity was found in breads with addition of 50 % of both buckwheat flours: more than three times in the case of white buckwheat flour, and about four times in the case of roasted buckwheat groats flour as compared to the reference wheat breads (before digestion).

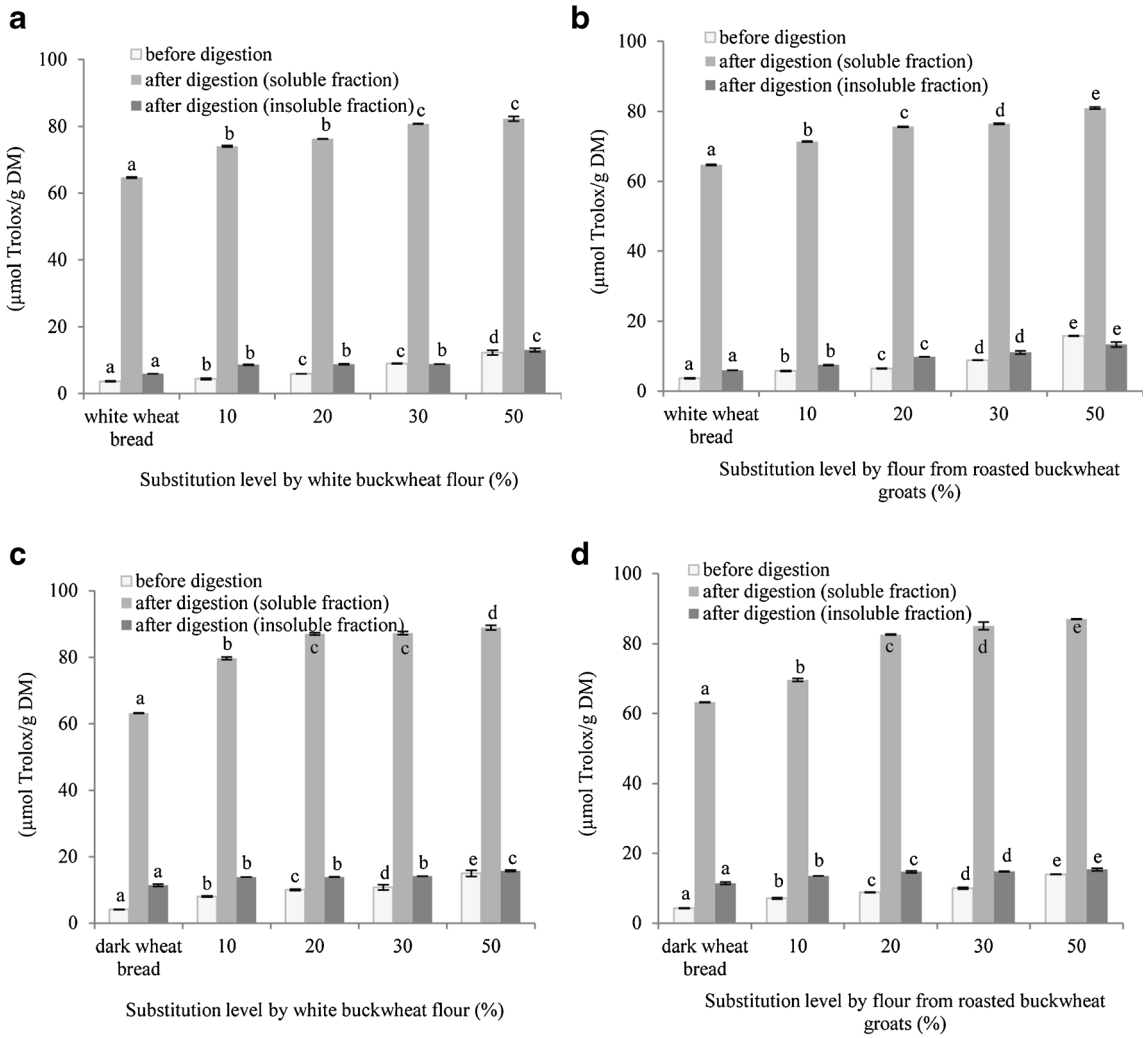

Fig. 2 The antioxidant capacity determined against $ABTS^{+\bullet}$ radicals of buckwheat enriched wheat breads before and after gastrointestinal digestions in vitro. Description as under Fig. 1

After the gastrointestinal digestion the antioxidant activity was significantly increased in all types of bread. It has been found that the buckwheat-enhanced dark wheat breads showed higher ability to scavenge ABTS radicals when compared to buckwheat-enhanced white wheat breads. The antioxidant activity soluble fraction ranged from 71.36 to 88.98 μmol Trolox/g DM. In contrast, antioxidant activity insoluble fraction determined by ABTS-QUENCHER method, was on the level of from 7.43 to 15.77 μmol Trolox/g DM. These results are similar to those obtained by Delgado-Andrade et al. (2010). However, increasing substitution (10, 20, 30, and 50 %) of both buckwheat flours in white and dark buckwheat-enhanced wheat breads was statistically ($p < 0.05$) significant on the antioxidant capacity after digestion. It was interesting that the antioxidant capacity of the insoluble fraction remaining after digestion constituted approximately 15 % of the total antioxidant capacity. According to Baublis et al. (2000) phenolic acids are mainly in bran layer of grains and mostly exist as a covalently linked form with insoluble polymers. They found that digestive pH conditions affected a considerable intensification in antioxidant activity of high fibre cereal foods. This suggests that acid environments cause changes in the activity, structure and/or absorption of water soluble antioxidants. These authors also indicate that acid hydrolysis impact soluble phenolic accumulation by leading to liberate of free phenolic acids from fibre. Consequently, the antioxidant capacity of grains, flour or bread could be additionally higher as a result of digestive processes (Rufián-Henares and Delgado-Andrade 2009).

The antioxidant capacity of white and dark buckwheat-enhanced wheat breads against superoxide anion radicals ($O_2^{-\bullet}$), measured by PCL assay before and after gastrointestinal digestion *in vitro* are shown in Fig. 3. The highest scavenging effect was noted in dark wheat flour bread extracts,

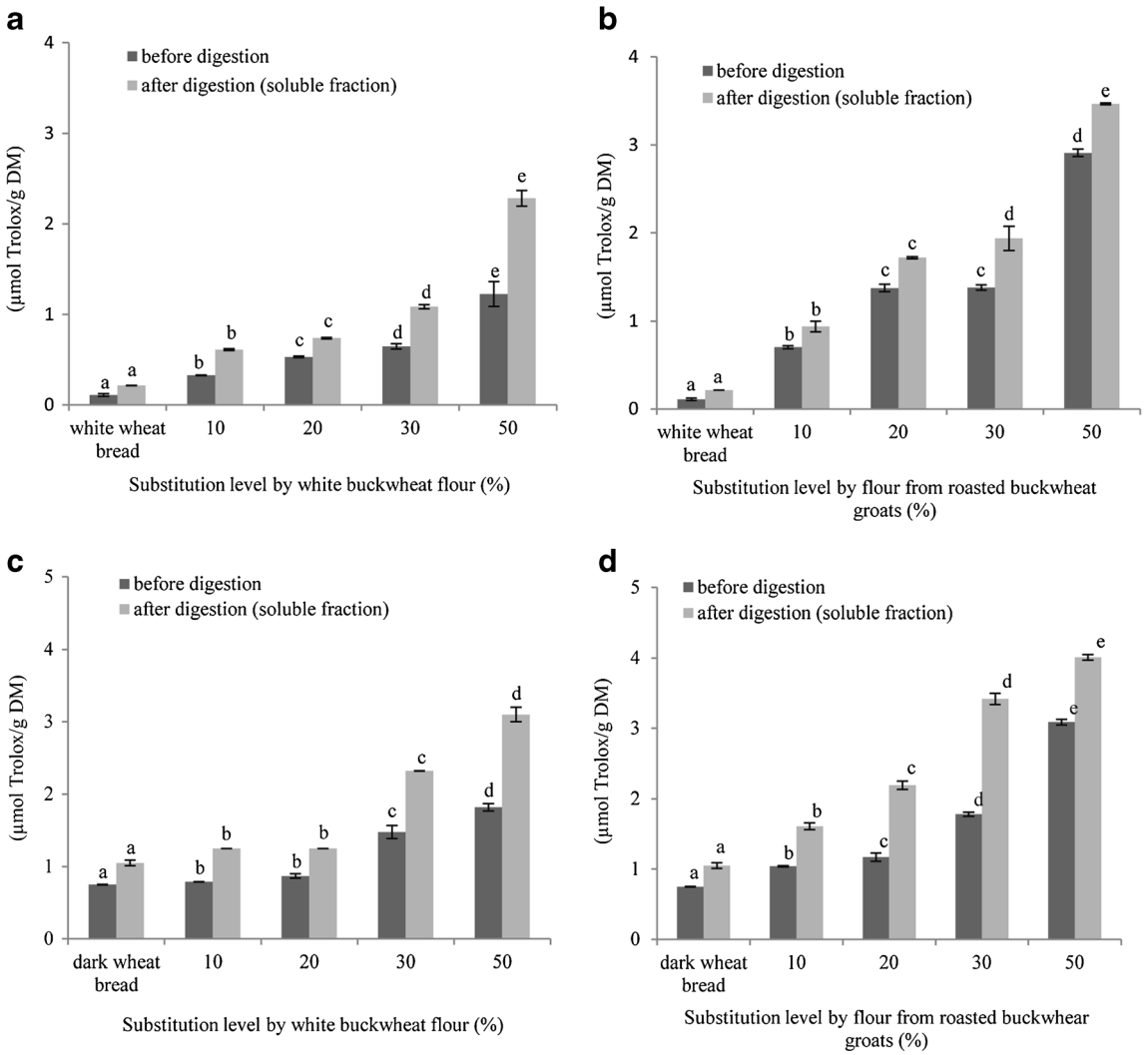

Fig. 3 The antioxidant capacity determined by PCL assay of buckwheat enriched wheat breads before and after gastrointestinal digestions in vitro. Description as under Fig. 1

whereas the white wheat flour bread extracts showed the lowest ability to scavenge superoxide anion radicals. The same relation was reported by Horszwald et al. (2010). Antioxidant activity measured by PCL method, similarly as ABTS method, was statistically ($p < 0.05$) higher as percent of buckwheat flours was increasing in buckwheat enhanced wheat breads. This increase may be due to the participation of buckwheat, which is rich in antioxidant compounds (Zieliński et al. 2006; Zieliński et al. 2012; Zhang et al. 2012). We also observed that the scavenging effect obtained by the PCL assay was much lower compared to the ABTS assay. This relation was also observed by other researchers (Zieliński et al. 2012; Horszwald et al. 2010).

After digestion, antioxidant activity was increased average 1.5-fold in all types of bread. However, it was much lower compared to that determined by the ABTS assay. In addition, it was found a progressive increase of the PCL values due the increasing substitution level (10, 20, 30, and 50 %) of white buckwheat flour or flour from roasted buckwheat groats. Due to the limitations by the methodology, the ability to scavenge superoxide anion radicals has not been determined in the insoluble fraction.

Effect of soluble and insoluble fraction after gastrointestinal digestion on global antioxidant response (GAR) of buckwheat-enhanced wheat breads

There are not many reports of applying the approach of global antioxidant response (GAR) to the total antioxidant activity (soluble + insoluble after gastrointestinal digestion). In this study, the GAR of a soluble fraction from a digestible portion was measured using a conventional procedure and insoluble fraction from the non-digestible portion was measured by the QUENCHER method. It was found a higher level of GAR for dark wheat bread as compared to white wheat bread (75.70 μmol Trolox/g DM and 70.81 μmol Trolox/g DM, respectively) (Fig. 4). Also, the substitution of white buckwheat flour showed higher GAR than substitution of flour from roasted buckwheat groats. However, these differences are not statistically significant. This result is similar to that obtained by Delgado-Andrade et al. (2010). Thus, the ability to neutralize free radicals depends on the stage of digestion and the type of bread. The antioxidant capacity was raised with the increasing level of buckwheat flours. However, Vallejo et al. (2004) showed that after *in vitro* pepsin digestion the content of flavonoid was not reduced. This may suggest that flavonoids are not digested in the small intestine, whereas the pancreatin digestion significant decreases in concentration of flavonoids. Consequently, the high increase in the antioxidant capacity after the digestion was due to the degradation of high molecular weight phenolic compounds to the low molecular phenolic acids.

Antioxidants constitute important defence against free radicals. Therefore, the evaluation of the antioxidant capacity is a valuable tool for the selection of foods rich in antioxidants. To this objective, various databases have been available allowing to estimating the total antioxidant activity of food (Baublis et al. 2000; Fardet et al. 2008; Gökmen et al. 2009). Nonetheless, food products have difficult structures where single antioxidant compounds can exist in different form: soluble and insoluble. Consequently, it is significant to investigate antioxidant activity of food after *in vitro* digestion process (Gawlik-Dziki et al. 2009; Chandrasekara and Shahidi 2012; Li et al. 2014). However, linked polyphenols are most likely disengaged later in the colon after fermentation. Oral bioavailability of the bioactive compounds present in the pure form is typically much greater than the bioavailability of the same components contained in the matrix of the food product, because they need to be released from the matrix before absorption. In many cases, individual nutrients are chemically bound to other components, which greatly limit their release in the intestine (Delgado-Andrade et al. 2010; Hur et al. 2011; Chandrasekara and Shahidi 2012). Majority of food polyphenols occur in the form of esters or glycosides which are not absorbed in the native form. Therefore, these compounds must be hydrolysed prior to absorption (Gawlik-Dziki et al. 2009; Li et al. 2014).

Correlation studies

Correlations between the total phenolic content (TPC) and the global antioxidant response (GAR) in buckwheat-enhanced wheat breads are illustrated in Table 2. The bioaccessible phenolics were highly correlated with GAR values in all types of bread. The highest correlation was found in dark wheat bread enhanced with white buckwheat flour ($r = 0.99$). In addition, a high positive correlation was found in the soluble ($r = 0.82$) and insoluble fraction ($r = 0.99$) after bread digestion.

Conclusion

In this study we showed that substitution of wheat flours by white or roasted buckwheat flour allowed for enrichment of wheat breads in phenolic compounds. The substitution of wheat flours by buckwheat flours at the level of 10, 20, 30 and 50 % enhanced the antioxidant

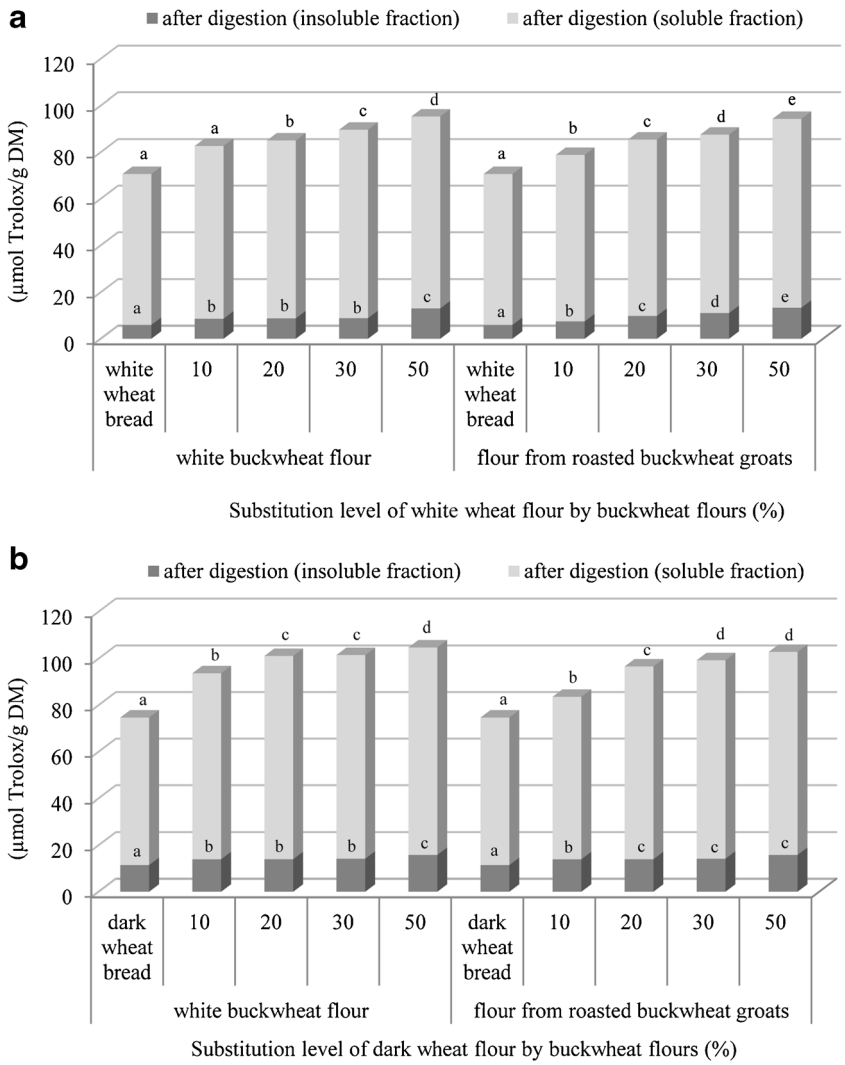

Fig. 4 Effect of soluble and insoluble fraction after gastrointestinal digestion in vitro on global antioxidant response (GAR) of **a** white and **b** dark buckwheat-enhanced wheat breads. Description as under Fig. 1

properties and increased the total phenolic content in buckwheat-enhanced white and dark wheat breads. The results provided in this study indicated that *in vitro* digestion was the crucial step in formation of the antioxidant capacity due to the releasing the high amount of phenolic compounds. On the other hand, it is obvious that after digestion insoluble fraction is not usually analysed by conventional methods. In conclusion, the determination of total phenolic content and antioxidant capacity in this faction by QUENCHER assay is highly recommend. The combination of QUENCHER assay with Total Antioxidant Capacity (TAC) and Total Phenolic Content (TPC) assay estimated on Folin-Ciocalteu reagent has been useful for the determination of the bioaccessible antioxidant activity and phenolics of the soluble and insoluble fraction of buckwheat-enhanced wheat breads.

Table 2 Correlation between the content of total phenolic compounds and the antioxidant capacity of buckwheat- enhanced wheat breads

Type of buckwheat enhanced wheat breads	Type of buckwheat flour	Before digestion	After digestion	
			Soluble fraction	Insoluble fraction
Buckwheat-enhanced white wheat breads	White flour	$r = 0.95$	$r = 0.91$	$r = 0.99$
	Flour from roasted groats	$r = 0.77$	$r = 0.93$	$r = 0.99$
Buckwheat-enhanced dark wheat breads	White flour	$r = 0.99$	$r = 0.82$	$r = 0.90$
	Flour from roasted groats	$r = 0.95$	$r = 0.88$	$r = 0.99$

Acknowledgments This work was supported by grant No. 1346/B/P01/2010/38 from the National Science Centre. This article is a part of the Ph.D. thesis of D. Szawara-Nowak.

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