APPLICATION OF ENZYMATIC TREATMENT TO IMPROVE THE CONCENTRATION OF BIOACTIVE COMPOUNDS AND ANTIOXIDANT POTENTIAL OF WHEAT AND RYE BRAN

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Abstract

The present study was undertaken to establish the effect of enzymatic treatment on the content of total phenolic compounds and antioxidant activity in enzymatically treated bran. Enzymatic hydrolysis of bran was carried out by α-amylase from *Bacillus amyloliquefaciens* (Sigma Aldrich) for breakdown the bonds between glucose monomers in starch. Multi enzyme complex (Viscozyme L) containing a wide range of carbohydrases were used for depolymerisation of cellulose and hemicelluloses molecules. The 80% ethanol was used to extract the antioxidant compounds from bran. Free radical scavenging activity of samples was measured using 2.2-diphenyl-1-picrylhydrazyl (DPPH). Assay and the data were expressed in Trolox equivalents (TE) per 100 g^{-1} of sample, as well the reducing power was determined using ferric reducing antioxidant power (FRAP) assay and the data were expressed in the same indices. The obtained results showed that the enzymatically treated bran samples had the highest concentration of total phenolic compounds, on the other hand the enzymatically treated bran showed higher antioxidant potential than non-enzymatically treated bran samples. Extract from enzymatically treated rye bran had the highest concentration of phenolic compounds, 1230±42.57 mg GAE 100 g^{-1} DW. The lowest concentration of phenolic compounds was found in untreated wheat bran samples and this amount was equal to 377±9.78 mg GAE 100 g^{-1} DW. Two different methods of evaluation of the bran antioxidant activity showed potential usefulness of enzymatic treatment.

***Keywords*:** phenolic compounds, antioxidant activity, bran, enzymatic hydrolysis.

Introduction

Bran of wheat and rye is a by-product from the milling process of flour and is a composite material formed from different histological layers, and three different strips. The outer strip corresponds to outer pericarp (epidermis and hypodermis), the inner one corresponds to the aleurone layers, and the intermediate one remains a composite of several tissues (inner pericarp, testa, and nuclear tissue (Hemery et al., 2010). Phenolic compounds derived from whole grain as well as from grain fractions have health-promoting effect. In the plant kingdom, phenolic compounds are essential molecules against oxidative damage, as they have UV-absorption properties and radical-scavenging activities. Therefore, the majority of the phenolic compounds are located in the most external tissues of the plant (Liu et al., 1995). In wheat grain, most of the phenolic compounds are located in the bran, which constitutes the outermost parts of the grain. One of the milling functions is cereal gain dehulling and debranning with the aim to obtain the white flour without any impurities.

Cereal grains and especially outer parts of the cereal are good source of phenolic compounds. In cereal grains located considerable amount of bioactive compounds e.g. phenolic acids, saponins, while flavonoids and phytoestrogens are presented in small quantities (Dordević et al., 2010). Scientific works imply that phenolic compounds have relatively high antioxidant activity, which may promote to their health benefits. The most predominant phenolic compound in cereals is ferulic acid, which forms up to 90% of total polyphenols. Other phenolic acids like p-coumaric, m-coumaric, syringic and vanilic acid have also been reported in cereals (Hosseinian, Mazza 2009).

Grain as well as bran chemical composition including phenolic compounds mostly depends from grain genetic / agricultural backgrounds, growing conditions and storage. On the other hand strong effect on chemical composition renders the milling procedure (Adom et al., 2005).

Liyana-Pathirana and Shahidi (2006) reported that the contribution of bound phenolics to the total phenolic content in wheat was significantly higher than free and esterified fractions, and the bound phenolic fraction demonstrated a significantly higher antioxidant capacity than free and esterified phenolics. Li et al. (2010) have reported that the phenolic compounds in cereals were mostly found in three forms: insoluble (66–80%), soluble conjugated (17–30%) and free phenolics (6%). The covalently bound ferulic acids during the fermentation of wheat bran fiber in a human model colon were released (Kroon et al., 1997). Although the solvent extraction is the major method to extract bioactive compounds from plant materials, or to obtain plant extracts rich in bioactive compounds. In the world science there have been several contentious moments e.g. after the extraction of bioactive compounds by using different types of solvents the components have low recovery and strict regulations for the use of these kind of products in the food industry. Enzymatic hydrolysis is one of the extraction techniques without any organic solvents and toxic chemicals that gave positive result and advantages among other conventional procedures. Main mechanisms of enzymatic hydrolysis are convert water-insoluble components into water soluble materials (Athukorala et al., 2006). For example, Heo et al. (2005) reported that enzymatic hydrolysis of brown seaweeds gained high bioactive compound yield and showed enhanced biological activity compared

with water and organic extract counterparts. Alrahmany et al. (2013) was reported that the enzymatic hydrolysis of oat bran give the possibility to increase the concentration of total phenolic acids upon treatment with carbohydrases. The purpose of this study was to investigate the content of total phenolic compounds and antioxidant activity in enzymatically treated bran in order to evaluate the effect of enzymatic treatment on these properties.

Materials and Methods

Experiments were done at the Latvia State Institute of Fruit – Growing collaboration with Riga Technical University.

Chemicals

Ethanol (96%) was received from SIA Jaunpagasts Plus (Company Jaunpagasts Plus Ltd., Latvia). Methanol, ethanol, ethyl acetate, asodium chloride (NaCl), hydrochloric acid (HCl), Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (Steinheim, Germany).

Bran samples

Summer wheat (*Triticum aestivum*) and rye *(Secale cereale)* bran samples were collected from industrial mills in Latvia:

1) SC Dobeles dzirnavnieks – small particle size wheat bran (WSSD);
2) SC Dobeles dzirnavnieks – wheat bran with large particle size bran (WLSD);
3) SC Rigas dzirnavnieks – large particle size wheat bran (WLSR);
4) SC Jelgavas dzirnavnieks – small particle size rye bran (RSSJ).

Enzymes

Industrial enzymes preparations produced by "Novozyme Corporation" (Bagsvaerd, Denmark) and purchased from Sigma-Aldrich. Two commercial preparations of enzymes: α-amylase from *Bacillus amyloliquefaciens* and Viscozyme L from *Aspergillus spp.*, were used to hydrolyze carbohydrates. α-amylase has a declared activity ≥250 units g^{-1}, optimum conditions of enzymatic pretreatment is pH 5.0–8.0, temperature 55±1 °C and incubation time 0.5 h (Demirkan et al., 2004) form Viscozyme L declared activity is 100 FBG g^{-1}, optimum conditions are pH 4.6, temperature 44±1 °C and incubation time 3.2 h. In this scientific work enzymes were tested both independently and in combination for establishing the synergetic interaction

Enzymatic Hydrolysis

For α-amylases treatments, wheat bran (10 g) was mixed with 90 mL of distillated water in 1000 mL Reagent bottle with screw cap with dilutions 1 : 9, and then 500 µL of α-amylase was added. Hydrolysis was carried out in a water bath at temperature 55±1 °C, incubation time 0.5 h and shaking intensity 60 rpm. After starch hydrolysis and enzyme inactivation (10 min temperature 100±1 °C) wheat bran mash was 3 minutes homogenized, the pH of the suspension was adjusted to pH 4.6 with 0.2 mL 50% citric acid in each dilutes and Viscozyme L 400 µL was added. Incubation time is 3.2 h, temperature 44±1 °C, and shaking intensity 60 min^{-1}.

Extraction of Phenolic acids

The free phenolic acids were isolated using the procedure explained by Wang et al. (2006) with slight modification, and soluble conjugated phenolic compounds were isolated using procedure described by Robbins (2003) and is depicted Figure 1.

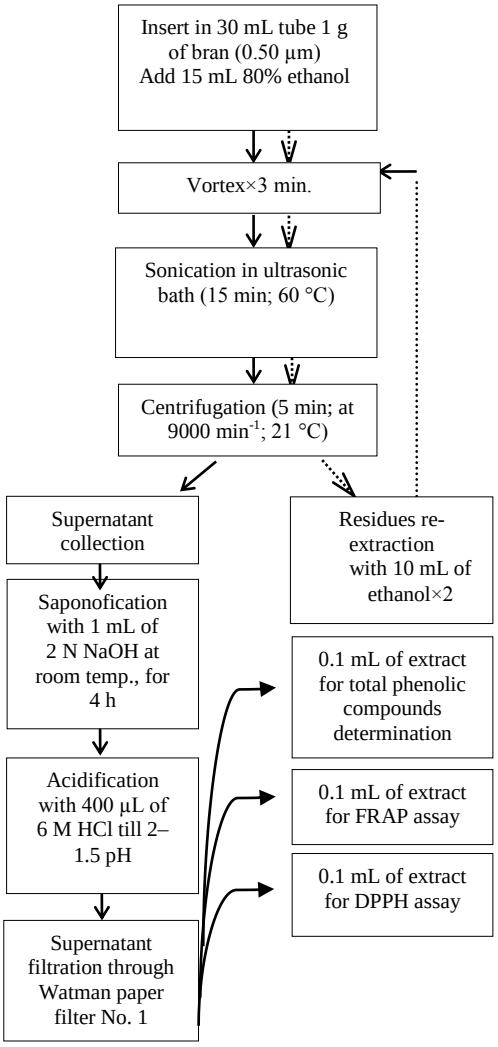

Figure 1. Flow diagram of extraction procedure of phenolic compounds

After free phenolic compounds extraction (×3 times) with 80% ethanol, after supernatant collection and the alkaline hydrolysis with 2 N NaOH was done. Alkaline hydrolysis was carried out at room temperature for 4 h, and after the hydrolysis the alkaline extract was neutralised with 6 M HCl. For elimination of precipitation the filtration through Watman paper filter No. 1 was done. The antioxidant activity and total phenolic compounds was immediately analyzed.

Determination of total phenolic compounds

Determination of total antioxidants reducing capacity by using Folin-Ciocalteu reagent was carried out as described by Sacchetti et al. (2009) with minor modifications. To volumetric flasks (10 mL) were pipette 100 μL of analyzed sample, 5 mL of deionized water and 0.5 mL of Folin reagent. After 3 minutes was added 1 mL of saturated sodium carbonate solution and supplemented with dionized water to 10 mL. The mixture was incubated for one hour at 23±2 °C, under dark, and then measured the absorption on wavelength λ=765 nm. The results obtained were expressed as mg gallic acid equivalent (GAE) per g dry weight (DW).

Free Radical Scavenging Activity

Free radical scavenging activity of samples was measured using the 2.2-difenyl-1-picrylhydrazyl (DPPH) according to the procedures described by Yen, Chen (1995) with slight modification. The extracts (100 μL) were reacted with 2.9 mL of DPPH solution (0.0039 g DPPH in 100 mL methanol). Absorbance of the cereal extracts was determined using UV – Visible Spectrophotometer SHIMADZU at 515 nm. Free radical scavenging activity of the samples was expressed as mg Trolox equivalent antioxidant capacity per 100 g^{-1} dry weight (mg TEAC g DW).

Free reducing antioxidant power (FRAP)

Free reducing antioxidant power (FRAP) was determined by its ability to reduce ferric to ferrous ions. When iron is complexed with 2, 4, 6-tripyridyl-s-trizine (TPTZ) in sodium acetate solution at an acidic pH, its reduction results in a color change of the solution, from pale rust to blue. The absorbance of the solution at 593 nm reflects the extent of reduction. The reduction power was expressed as mg Trolox equivalent. The extracts (100 μL) were reacted with 3.6 mL FRAP reagent and after vortex the absorption was spectrophotometrically detected.

Statistical analysis

Data was processed by SPSS software version 17.0. Data was analysed using descriptive statistics and processed by one-way analysis of variance ANOVA (one way ANOVA), as well as for comparing all bran samples depending from pre-treatment were used (two way ANOVA). Microsoft office software version 2007 was used to determine significant differences between the samples.

Results and Discussion

The plant cell wall is a complex design of polysaccharides. For the complete hydrolysis of these polysaccharides, a battery of enzymes is needed. By specific enzymatic treatments, cell wall polymer properties can be altered which can be utilised in food processing. Many plant cell walls contain phenolic acids residues that are ester-linked to the polysaccharide network. In grasses and cereals, these phenolic compounds (hydroxycinnamic acids) are mainly found esterified to arabinoxylans (5-*O*-feruloyl group). In decotyledons, such as spinach and sugar beet, ferulic acid is esterified to *O*-2 or *O*-3 position of arabinose and to *O*-6 position of galactose residues in pectin (Ralet et al., 1994; Fry, 1982).

The main activity of the Viscozyme L enzyme preparation is endo- (EK 3.2.1.4), exo-xylanases (EK 3.2.1.74), endo- (EC 3.2.1.4), exo- glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Endo-glucanase and exo- glucanase are known to act synergistically in cellulose hydrolysis (Wood, McRae, 1978) while β-glucosidase is needed for hydrolysing cellobiose (Woodward, Wiseman, 1982). Feruloyl esterase (FAE; E.C. 3.1.1.72), are sometimes called hemicellulase accessory enzymes, subclass of the carboxylic acid esterases (E.C. 3.1.1.1). They splits the ester bond between the glycosides and hydroxycinnamic acids, which are predented in the plant cell wall (Williamson et al., 1998). The esterases act to enable and facilitate the access of glycosyl hydrolases to the backbone wall polymers. Most feruloyl esterases act synergistically with xylanases, cellulases or pectinases to breakdown complex plant cell wall carbohydrates (Faulds, Williamson, 1995; Kroon, Williamson, 1996). Investigation into the effect of enzymatic treatments, on the content of bioactive compounds of the wheat and rye bran, revealed that treatments had a significant effect on the content of phenolic compounds, anthocyanins, as well as antioxidant potential. The influence of enzymatic treatments on the chemical compositions of the wheat and rye bran products indicated that the use of enzymes yielded a higher concentration of bioactive compound, than the untreated bran (Figure 2).

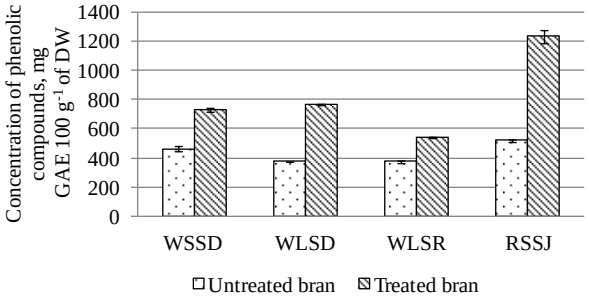

Figure 2. Concentration of total phenolic compounds in wheat and rye bran

The highest concentration of total phenolic compounds were found in enzymatically treated rye bran sample (RSSJ), and this amount was equal to 1230±42.57 mg GAE 100 g^{-1} DW, followed by (WLSD) – 765.2±1.65 mg GAE 100 g^{-1} DW, (WSSD) – 730.8±13.05 mg GAE 100 g^{-1} DW, and (WLSR) – 541.9±3.41 mg GAE 100 g^{-1} DW respectively. While the concentration of total phenolic compounds in untreated rye bran sample (RSSJ) were 520.7±7.17 mg GAE 100 g^{-1} DW, followed by (WSSD), (WLSD), (WLSR), 461.3±17.16, 377.4±0.41, 377.0±9.78 mg GAE 100 g^{-1} DW, respectively. The similar data was reported by Sungsopha (2009), which implies that after enzymatic treatments of rice bran the total phenolic compounds was increased by

476%, from 223.16 mg GAE 100 g^{-1} DW to 836.21 mg GAE 100 g^{-1} DW, and after author was made some conclusions, that it's due to the effect that of enzymatic hydrolysis, liberates and frees phenolic components and increases the level of total phenolic compounds. Khoddami et al. (2013) was reported that bran treated with carbohydrases is significantly higher compared to untreated bran. Highest increase in vanillic and caffeic acids relative to the untreated bran was achieved by cellulase (3.7-fold) and Viscozyme (4.4-fold). The total content of free and bound phenolic acids was 668.5 μg g^{-1} for untreated oat bran and after enzymatic treatments with Viscozyme L this amount has increased to 1116.0 μg g^{-1}.

Microstructure of bran

Wheat and rye bran is a multilayered composite, comprising a range of tissues, including the pericarp (epidermis, hypodermis, cross and tube cells) with the attached seed coat, the nucellar epidermis, the aleurone layer, and remnants of the starchy endosperm. All of these tissues are dietary fiber with very low bioactive compounds and antioxidant bioaccessibility and bioavailability. Research concerning the bioaccessibility of phenolic compounds and other antioxidants from solid matrices are important, since only the compounds released from the food matrix and/or absorbed in the small intestine are potentially bioavailable and in a condition to exert their beneficial effects (Tagliazucchi et al., 2009). Phenolic compounds bound to dietary fiber need to be hydrolyzed by specific enzymes in the upper area of the intestine; otherwise, these compounds will not be bioaccessible for absorption in the human intestine but will be susceptible to degradation by the colonic microflora in the large intestine (Perez et al., 2009). Our study imply that the using of enzymes gives the possibilities release the bound form of phenolic compounds which increase the bioavailability of these material.

Microscopy of the bran samples showed that degradation of cell walls was initiated in the pericarp layer (Figure 3B), as well in the starch/protein matrix (Figure 3C).

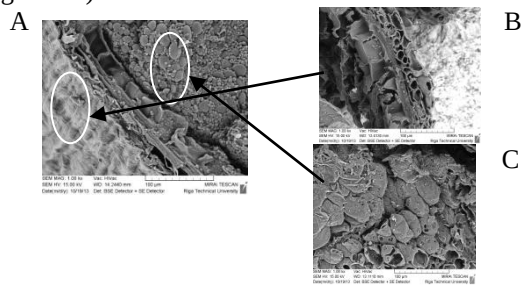

Figure 3. Microstruture of bran obtained by SEM
A– untreated wheat bran; B–enzymatically treated wheat bran (pericarp degradation), C–enzymatically treated wheat bran (starch/protein matrix degradation)

During the enzymatic hydrolysis the degradation of cell walls were observed by scanning electron microscope (Figure 3). Enzyme aided hydrolysation had large effects on wheat and rye bran characteristics, and partial hydrolysis of cell wall components was reflected in altered bran microstructure.

Free Radical Scavenging Activity

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reductions capability of DPPH is determined by the decrease in its absorbance induced by antioxidant. In this study, the total antioxidant capacity of wheat and rye bran treated by using enzymes significantly increased ($p<0.05$) the number of equivalents of all the antioxidant standards. Analyzing data of free radical scavenging activity was detected only one exception for WLSR bran sample. After enzymatic treatment antioxidant capacity of this sample decreased from 18.35±0.70 to 15.84±0.59 mmol Trolox 100 g^{-1} (Figure 4). It was useful to know that Folin-Ciocalteau assay give the possibility estimate not only phenolic compounds but also amino acids, carbohydrates, ascorbic acid, and other components which may increase the antioxidant activity of the samples. How it was reported in Kim and Wampler work that determination of total phenolic compounds by chemical method Folin-Ciocalteau assay gives the higher value than using instrumental HPLC method. There was reported that two methods are different, with different advantages and disadvantages (Kim, Wampler, 2011). Another author was reported that Folin-Ciocalteau assay gives a crude estimate of the total phenolic compounds present in an extract, whereas the free radical scavenging assay is not only specific to polyphenols (Prior et al., 2005). Our other work which at the moment not published suggest that during the extraction using different types of solvents as well as different techniques give opportunity extract from plant materials not only phenolic compounds but also some another components, which can interact with Folin-Ciocalteau reagent and simultaneously providing incorrect results. The highest scavenging effect of bran extracts on DPPH radical was observed in enzymatically treated bran (Figure 4).

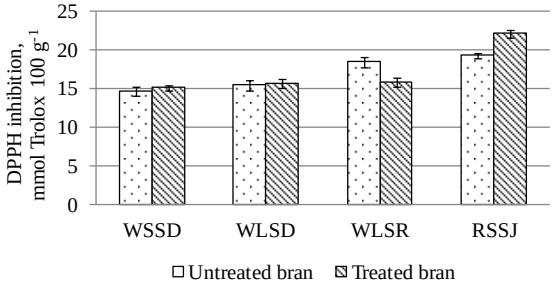

Figure 4. Free radical scavenging activity of bran extracts expressed as mg Trolox equivalent antioxidant capacity per 100 g^{-1} of dry weight

The highest scavenging effect was recorded in RSSJ bran sample (22.03±0.49 mmol Trolox 100 g^{-1}) followed by WLSR (15.84±0.59 mmol Trolox 100 g^{-1}), WLSD (15.63±0.53 mmol Trolox 100 g^{-1}), and WSSD

(15.06±0.32 mmol Trolox 100 g^{-1}). The Sungsopha was reported similar results obtained during enzymatic hydrolysis of rice bran. After enzymatic treatments the antioxidant capacity was significantly increased (Sungsopha et al., 2009).

The antioxidant power of bran extracts was evaluated by FRAP assay in Figure 5.

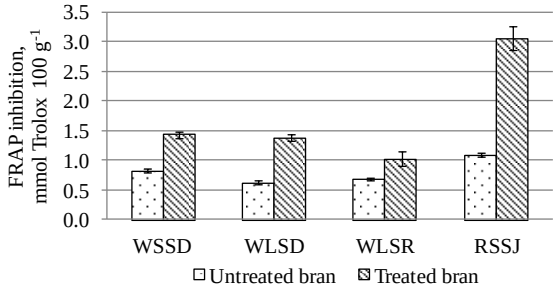

Figure 5. Free reducing antioxidant power of bran extracts expressed as mg Trolox equivalent antioxidant capacity per 100 g^{-1} of dry weight (mmol Trolox 100 g^{-1} DW)

Our obtained results showed that enzymatically treated rye bran has a greater ability of antioxidant properties than other bran samples RSSJ (3.05±0.20 mmol Trolox 100 g^{-1} DW), followed by WSSD (1.43±0.06 mmol Trolox 100 g^{-1} DW), WLSR (1.03±0.12 mmol Trolox 100 g^{-1} DW). Comparing the data it's possible to conclude that the antioxidant power of enzymatically treated bran compared to untreated was increased by 2.8 fold for RSSJ, 2.2 fold for WLSD, 1.7 folds for WSSD and 1.5 fold for WLSR respectively. On the other hand the significantly positive correlation was observed using the FRAP assay (R^2=0.970). Literature data from McCarthy and other authors shows that they got similar significantly strong correlation between total phenolic compounds and FRAP activity (McCarthy et al., 2012).

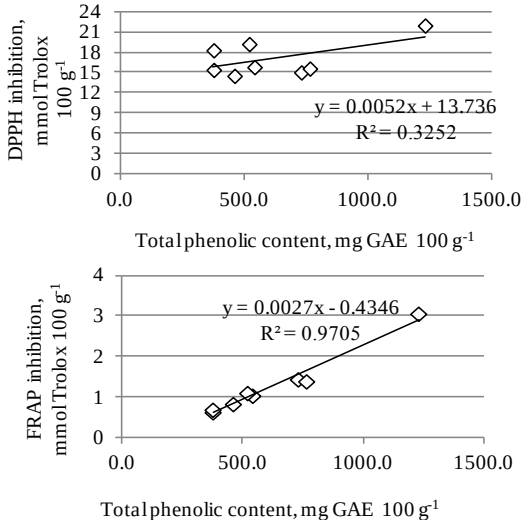

Figure 6. Correlation graphs for DPPH (A) and FRAP (B) mmol Trolox 100 g^{-1} values and total phenolic contents

In the present study, correlation graphs were plotted between IC50 values (including of DPPH, FRAP) and total phenolic contents. Two typical correlation graphs (*i.e.*, DPPH *vs* total phenolic, FRAP *vs* total phenolic) are shown in Figure 6.

Several studies have attempted to correlate the DPPH scavenging activity of molecules to total phenolic content and to individual phenolic acids. In that respect Li, Wu, and Huang (2009) found that there was no direct correlation between DPPH inhibitory activity and total phenols, ferulic or caffeic acid contents of Radix angelicae sinensis, although they reported a correlation with 1/IC50 values (Li et al., 2009).

Another authors Gamel, Abdel-Aal (2012) were reported that total phenolic contents of barley samples correlated with DPPH inhibitory capacity. Verardo et al. (2011) found a strong correlation (R^2=0.93) of DPPH result of five oat cultivars to total free phenolic compounds. In this study the correlation (R^2=0.325) between DPPH and total phenolic compounds for the enzymatically treated wheat and rye bran is much weaker. The obtained results are incomparable due to fact that during the extraction of phenolic compounds was used different extraction techniques, which can effect on the extraction capacity.

Conclusions

This study suggest, that enzymatic treatment of wheat and rye bran are effective methods to improve the concentration of bioactive compounds and antioxidant activity. Therefore, these bran products may be exploited as a potent source of bioactive compounds and antioxidants, for nutraceutical and functional food products.

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