Phenolics composition and their biochemical stability confirmation by IN VITRO gastrointestinal conditions simulation, for a new functional fermented beverage based on sprouted buckwheat

CATERINA BRAJDES (DUMITRU)1*, GABRIELA BAHRIM1, RODICA DINICA2, CAMELIA VIZIREANU3

1Dunarea de Jos University of Galati, Dunarea de Jos University, Faculty of Food Science and Engineering, 111 Domnească Street, 800201, Galati, Romania
2Dunarea de Jos University, Faculty of Science111 Domnească Street, 800201, Galati, Romania
*Corresponding author: dumitrukati@yahoo.com

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Abstract

The sprouted buckwheat contains a large amount of polyphenols, especially flavonoids. Based on soluble antioxidants and other bioactive compounds the functional beverage derived from sprouted buckwheat can exert beneficial effects on human health. The nutritive assimilation and the in vivo effect are very important to assure human health. The aim of this study is to examine through by using classical and modern methods the content of polyphenols from a new functional beverage based on the lactic fermentation of the sprouted buckwheat and their biochemical stability by in vitro simulation of gastrointestinal conditions. The results show that polyphenols from functional beverage present a quantitative content decreasing of 39.19% and also a reduction of 27.41% of antioxidant activity in gastrointestinal tract.

Keywords: Fagopyrum esculentum Moench, sprouted buckwheat, functional beverage, polyphenols, flavonoids, antioxidant activity, in vitro gastrointestinal (GI) conditions simulation

1. Introduction

The essential element of life, the oxygen, is necessary for the human body to breathe and to produce energy. As a consequence of such activities, free radicals are generated, reactive oxygen species (ROS), which can cause damages, particularly of proteins, lipids, nucleic acids (L. PHAM-HUY [1]). The oxidative damages represent the cause of cardiovascular diseases, obesity, cancer, dyslipidemia or Alzheimer's disease. The oxidative stress is countered by antioxidants. They are designed to neutralize the free radicals by donating an electron or hydrogen atom (M.P. KAHKONEN & al.[2]), thereby inhibiting the oxidation mechanisms that lead to chronic and degenerative diseases.

The polyphenols are secondary metabolites synthesized in plants, and are involved in the response to the ultraviolet rays, or the aggression of some pathogens (C.H. BECKMAN [3]). They are known as phytochemicals, due to their potential beneficial effects on human health, through preventive action, as well as by reducing the risk factors of chronic diseases (J. GRY & al. [4]).

The abundance of flavonoids differs in layout mode and the number of hydroxyl groups, methoxy, and glycosides groups attached to the C-O bonds and C-C, and to the blackbone (E. MIDDLETON & al.[5]). Nowadays, more than 7,000 flavonoidic compounds are known (T. FOSSEN & al. [6], T. FATIMA &al. [7]).

In nature, most of the phenolic compounds occur in glycosylated form. The flavonoids are the most abundant polyphenols in human diet (A. SCALBERT [8]). The main flavonoids
include the following subclasses: flavones, flavonols, flavanones, flavanols, flavanones, isoflavones, chalcones and anthocyanidines (R. TSAO [9]).

The flavons occur as aglicons in food. These are compounds that possess a double bond between C2 and C3 and no -OH group at C3: luteolin, apigenin, orientin, vitexin, isoorientin, isovitexin. Than flavones, flavonols are in addition to the -OH group in position 3: quercetin, quercitrin, rutin, isorhamnetin.

The biosynthesis of flavonols and flavones directly depends on the presence of light, therefore, they are found mainly in the leaves (K.M. HERRMANN [10]). Flavonons and flavononols are characterized by the presence of a bond between saturated C2-C3 and one oxygen atom (carbonyl group) in position 4: naringin, hesperidin taxifolin. Flavanols represent a class of flavonoids that use the skeleton of 2-phenyl-3, 4-dihydro-2 h-chromen-3-ol. These compounds include catechins, epicatechins and their derivatives. The catechins are the epicatechins epimers, both + and – epicatechins, and are the most widely found optical isomers in nature. Epigallocatechins and gallocatechins contain in addition a hydroxyl phenolic group, and by heating release pyrogallol. Proanthocyanidins which have catechins as monomers are called procyanidins, while proanthocyanidins which have gallocatechins as monomers are called prodelphinidins (D. FERREIRA & al.[11]). Medical researchers showed that catechins are involved in neutralizing free radicals, chelating metals in redox reactions, inhibiting the transcription factors related to cancer, and inhibiting the oxidative enzymes, having effects on the slowing down the progress of aging and in Alzheimer's disease prevention, cardiovascular diseases, and very effective in diabetic nephropathy (T. YOKOZAWA & al.[12]). These phenolic compounds showed potential for treatment of diseases related to lifestyle, such as type 2 diabetes, obesity, and metabolic syndrome (D.S. BARBOSA [13], R. COOPER & al. [14]). EGC and EGCG have higher antioxidant activity because of large number of groups –OH groups (J. MUNOZ-MUNOZ & al.[15]). Structure of chalcone contains an aromatic ketone that forms the central core for a variety of biologically important compounds: phlorizin. Isoflavones differ from flavons by arranging the B core into position 3: genistein, daidzein, coumestrol.

Common buckwheat (Fagopyrum esculentum Moench) is considered a major source of flavonoids; it contains important quantities of orientin, vitexin, isoorientin, quercitin, especially rutin (quercetin 3-rutinoside) (Z. LUTHAR [16]). Some studies showed that phenolic compounds are bonded to cell wall components (K. ADOM & H. LIU [17]), while other studied found that they are distributed in both full seeds (V. HUNG & N. MORITA [18]) and especially the leaves and inflorescences. After 7 days of germination, the content of flavonoids increases nearly 20 times (C. BRAJDES & C. VIZIREANU [19]). Functional beverage based on sprouted buckwheat presents high flavonoid content. The acid environment of the stomach and the alkaline environment of the intestine lead to structural changes of flavonoids.

The aim of this study is to analyses the qualitative and quantitative changes and antioxidant activity evaluation of the pholyphenols, especially of the flavonoids, from a functional beverage obtained by lactic acid fermentation of mixture based on sprouted buckwheat, honey and inulin, by in vitro simulated gastrointestinal (GI) conditions, in order to predict the functional role of this product in vivo.

2. Materials and methods

Fermented beverage and chemicals

The fermented beverage based on sprouted buckwheat extract, buckwheat honey and inulin was obtained in laboratory by lactic acid fermentation using a Lactobacillus plantarum strain as starter.
Starter culture *Lactobacillus plantarum*, Vege Start 60 (lyophilised) provided by Christian Hansen Company, Denmark, had a concentration of 4.8×10^10 CFU/g.

The lyophilized inulin was provided from the Company SC Enzymes and Derivates, Romania.

Buckwheat honey was purchased from Smitty Bee Honey Inc. Company in Iowa, USA.

Buckwheat seeds were germinated in the automatic sprouted EasyGreen (Seed & Grain Tech Inc, China) which keeps the constant humidity by water-spraying at regular intervals. Seeds were germinated for seven days. After germination the seeds were dried at 40°C, and then ground in a ball mill. The finely chopped buckwheat sprout was mixed with sterile water to get a final concentration of 6 g /100 mL. The suspension was maintained at 45 ºC for 30 minutes on a water bath, and afterwards the temperature was increased up to 90 ºC and was maintained for 10 minutes to inactivate the spoilage microbiota prior to controlled fermentation. The suspension was then filtered through a vacuum pump.

**In vitro gastrointestinal conditions simulation**

The simulation of *in vitro* gastrointestinal (GI) conditions was obtained by simulating gastric and intestinal juices according to the method of KOS [20]. Simulated gastric juice was obtained by suspending pepsin (3 g/L) into a sterile solution of 0.5% NaCl. The pH was established at value of 2.0 using concentrated solution of HCl.

Simulated intestinal juice was obtained by pancreatin suspension (1 g/L) and bile salts (0.45 g/L oxigall) into a sterile solution of 0.5% NaCl. The pH was established at value of 8.0 with a concentrated solution of 0.1 M NaOH.

Functional beverage (0.2 mL) was mixed with 0.3 mL 0.5% NaCl solution and with gastric juice or intestinal juice, respectively, at volume of 1 mL, and incubated at 37 ºC at 50 rpm (S. KOS [20]).

Because the gastric chyme is eliminated in a rate of 2-3 kcal / min (H. BRENER [21]), it is considered that 100 mL of liquid (energy of functional beverage based on sprouted buckwheat is 39.6 kcal/100 mL) will reach the duodenum within 20-30 minutes, and the bowel will last 90 minutes. Samples were collected at baseline, 15 and 30 minutes after the simulated gastric conditions, and at 30, 60 and 90 minutes of intestinal conditions simulation.

**Total polyphenols analysis**

The total polyphenol content (TPC) was determined by spectrophotometric analysis, using T80 UV/VIS spectrophotometer and gallic acid as standard, according to the method described by the International Organization for Standardization (ISO, 14502-1). Briefly, 1.0 mL of supernatant of centrifuged beverage was transferred in duplicate to separate tubes containing 5.0 mL of a 1/10 dilution of Folin-Ciocalteu’s reagent in water. Then, 4.0 mL of a sodium carbonate solution (7.5% w/v) was added. The tubes were then allowed to stand at room temperature for 60 min and then the absorbance was measured at wavelength of 765 nm. 1 cm quartz cells were used for absorbance measurement. The total polyphenols contents are expressed as mg of gallic acid equivalents per 100 mL of beverage (C. ANESINI [22]).

**Total flavonoids contents determination**

The total flavonoid content in the extracts was determined by a spectrophotometric method based on the formation of complex flavonoid-aluminium with a maximum absorbance at wavelength of 420-430 nm (Bozin et al., 2008) [23]. Briefly, 1 mL of supernatant of centrifuged beverage was mixed with 1 mL of 2.5% AlCl₃, 2 mL of 10% sodium acetate
solution, and 70% ethanol to a total volume of 10 mL. The experiments were run in duplicate, and after incubation at room temperature for 30 minutes, the absorbance of the reaction mixtures was measured at wavelength of 420 nm comparing with control sample. The flavonoid content was determined from a standard curve prepared with rutin, quercetin (ranging from 10 to 50 μg/mL final volume) and expressed as mg quercetin or rutin equivalents.

**DPPH scavenging assay**

The free radical scavenging activity (RSA) was determined using the protocol established by Brand – Williams (1995) [24] based on 2,2 – diphenyl – 1 – picrylhydrazyl (DPPH) assay. A volume of 3.975 mL of DPPH (6x10⁻⁵ M) working solution was added to a cuvette of 1 cm and the absorbance at wavelength of 515 nm was measured (A₀) using a T80+ UV/VIS spectrophotometer. Subsequently, 25 μL of supernatant of centrifuged beverage was added into the cuvette with DPPH, and the absorbance was measured after 10 min (A). The antioxidants present in the methanol extract of the samples reduced the DPPH and faded the colour of the solution in direct correlation with the antioxidant concentration. The free radical scavenging activity was measured according to the following equation:

\[
RSA, \% = \frac{(A₀ – A)}{A₀} \times 100
\]

**HPLC-MS analysis of flavonoids**

For HPLC analysis it has been use the HPLC Agilent 1200 system (Agilent, USA) with DAD detector hooked up with MS single quadrupole Agilent 6110 (Agilent, USA). The equipment has positive ionization ESI mode under the following conditions: capillary voltage: 3000 V; temperature: 350 °C; nitrogen flow rate: 8 l/min; m/z: 100-1000. The flow rate of mobile phase was 0.5 mL/min, temperature of column was 25°C and the wavelengths of 280 nm to 340 nm were selected. The analyses were conducted using C18 chromatographic column Eclipse XDB (ZORBAX, USA), sizes 4.6 x 150 mm, 5 μm. The supernatant of centrifuged beverage for the determination of total polyphenols was priori prepared by filtering through 0.45 μm filter and injected into the HPLC. The mobile phase consisted of water and 0.1 % acetic acid/acetoniitre (solvent A) and acetoniitre and 0.1% acetic acid (solvent B). Identification of polyphenolic compounds was done in correlation with specific standards.

**FTIR analysis**

The FTIR analysis of the supernatant of centrifuged beverage was using the Magna-IR Spectrometer equipment 350 (Nicolet Instrument Corporation, USA), by recording the spectra from frequencies of 4000 to 600 cm⁻¹, with a spectral resolution of 1 cm⁻¹, using total reflection attenuation technique (ATR).

**Statistical analysis**

All experiments were carried out in triplicates. Statistical analysis was done using Microsoft Excel 2007. The average values standard deviation is reported.

**3. Results and discussion**

To produce a biological effect *in vivo*, it is essential that polyphenolic compounds from functional lactic acid fermented beverages based on sprouted buckwheat, to arrive in sufficient quantities in the target tissues. The bioavailability of these compounds is conditioned by their absorption from the intestine, which in its turn is determined by the chemical and molecular structure.
The bioactive compounds content of functional beverage were identified with reference compounds and literature data on the basis of their HPLC retention times and mass spectra (Figure 1 and Table 1).

**Fig. 1.** The HPLC chromatogram of functional beverage (detection at wavelength of 280 nm)

**Table 1.** Mass spectrometry data of phenolic content of fermented sprouted buckwheat beverage

<table>
<thead>
<tr>
<th>Peak</th>
<th>R_t (min)</th>
<th>[M + H]+(m/z)</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.2</td>
<td>379</td>
<td>Hidroxisecolariciresinol</td>
</tr>
<tr>
<td>2</td>
<td>4.7</td>
<td>307</td>
<td>Epigallocatechin (EGC)</td>
</tr>
<tr>
<td>4</td>
<td>7.0</td>
<td>459</td>
<td>Gallocatechingallate (GCG)</td>
</tr>
<tr>
<td>5</td>
<td>9.4</td>
<td>269</td>
<td>Coumestrol</td>
</tr>
<tr>
<td>6</td>
<td>12.1</td>
<td>291</td>
<td>Catechin</td>
</tr>
<tr>
<td>8</td>
<td>14.0</td>
<td>449</td>
<td>Orientin</td>
</tr>
<tr>
<td>9</td>
<td>14.5</td>
<td>449</td>
<td>Isoorientin</td>
</tr>
<tr>
<td>10</td>
<td>15.4</td>
<td>433</td>
<td>Vitexin</td>
</tr>
<tr>
<td>11</td>
<td>16.1</td>
<td>611</td>
<td>Rutin</td>
</tr>
<tr>
<td>12</td>
<td>16.4</td>
<td>493</td>
<td>Isorhamnetin glucuronid</td>
</tr>
<tr>
<td>16</td>
<td>23.4</td>
<td>317</td>
<td>Isorhamnetin glucuronide arabinosid</td>
</tr>
</tbody>
</table>

As it can be seen in Figure 1 and Table 1, the dominant peak is vitexin. This is 8-β-D-glucopyranosyl-apigenin, found in many medicinal herbs. Its presence in food is required, taking into account the effects of antiinflammatory and antioxidant (M. BORGHII [25]). Also, other three flavones, orientin, isoorientin and luteolin obviously show peaks. The flavonols from the functional beverage are represented by rutin, isorhamnetin, quercetin, isorhamnetin glucuronide arabinosid. Flavan-3-ols are been represented by epigallocatechins, gallocatechin gallate, catechin gallate. As lignin, it was identified the hidroxisecolariciresinol and as isoflavons, the coumestrol.
The main flavonoids identified in the functional beverage include flavonols, flavones, isoflavones, catechins and lignans. In plants, the flavonoids are ubiquitous, generally found in glycosylated form, most often, carbohydrates and glucose or rhamnose (K. PANDEY [26]). Similar results have been recorded by Luthar (1992) [16] and Zielinska et al (2012) [27].

The polyphenolic content and antioxidant activity variation during GI passage are presented in Figure 2.

In Figure 2, it can be seen that both the total polyphenols and flavonoids are influenced by the acid environment of the stomach, and the alkaline environment of the intestine. In the gastric passage, the concentration of total polyphenols decrease by 12%, while in the duodenal passage decrease by 18.6%. Therefore the quantity of polyphenols absorbed will be 41.9% lower compared to the amounts of the oral intake. In the gastric segment, the total flavonoids show a 13.46% decrease followed by other 25.73% in the intestinal segment. Therefore a total drop of 39.19% of the polyphenols quantity should be expected. The antioxidant activity that follows polyphenolic profile shows a decrease of 11.95% in gastric area, and a decrease of 27.41% in the intestinal area, so there is a total drop of 27.41%.

The HPLC chromatograms after gastric and intestinal simulations are presented in Figure 3 and Figure 4.
During *in vitro* the gastric simulation, the flavonoids have suffered some changes. The flavonols concentration decreased, due to hydrolysis of the glycosides bond. Thus, the rutin was hydrolyzed to quercetin and isoquercitrin. Similar results were obtained by Wang [28]. The flavon concentration decreased as well.

The coumestrol, a member of the isoflavonoids family, is considered a phytoestrogen. The quantity present in the beverage comes both from buckwheat extract and added buckwheat honey. It was noted that the passage through the stomach segment does not affect its structure. The secoisolariciresinol and the hidroxisecoisolariciresinol are the main present lignans in the roots, stems, cereals, oleaginous plants, nuts, vegetables and fruits (S. WILLO [29], T. SICILIA [30]). During the gastric digestion, the hidroxisecoisolariciresinol is converted to enterodiol and enterolactone (D. INGRAM [31]). This phytoestrogen is recognized for its preventive effects against breast cancer, colon cancer, atherosclerosis and diabetes.

The catechins show very pronounced increases. This is due to the acidic hydrolysis of procyanidines and prodelphinidines from the pericarp of both buckwheat and buckwheat honey, which released the specific monomers. Similar results were obtained by Martinez-Ortega (2001) [32] and Spencer, J (2003) [33].
In Figure 4 it can be seen the increase of peak-5 corresponding to the coumestrol, due to the fact that at alkaline pH, it is released from the fragments of the existing pericarp in the beverage. In addition to its estrogenic activity, the coumestrol acts as an antioxidant because of its ability to donate electrons from hydroxyl groups. Georgetti et al. (2003) [34] and Lee et al. (2006) [35] have suggested that the coumestrol would have antiradicals activity which is 20 times larger than of genistein and daidzein. Wong (1988) [36] has suggested that the coumestrol might have antihepatotoxic and antiinflammatory activity.

Hidroxiseoisolariciresinol is still converted at enterodiol and enterolactone. It has been noticed an increasing of peak corresponding to the luteolin, as a result of glycoside (orientin and isoorientin) hydrolysis. Other studies showed that the high consumption of food containing luteolin is associated with a reduced risk of chronic diseases (M. LÓPEZ-LÁZARO [37]). Numerous experimental data showed that luteolin possesses a wide range of pharmacological effect including antioxidant, antimicrobial, anti-inflammatory, and anticarcinogenic activities (G. SEELINGER [38]).

The content of flavonols is reduced further to the alkaline pH of the small intestine, due to the hydrolysis of glycosidic bond, but in a softer manner. The effect of the alkaline pH of the environment on flavonols was found by Sroka and Belz [39]. It was noticed that the area of the rutin peak is lower than the decrease observed in acidic medium. The same observation was found by Luo [40].

The instability of catechins and epicatechins in the intestinal juice and buffer with pH over than 7.4 it has been previously shown by Yoshino (1999) [41], Zhu (1997) [42] and Su (2003) [43]. At the increasing pH, the catechins suffer oxidative degradation and polymerisation.

Thus the epimerisation at pH 8.0 is 50 times faster than at pH 5.0. Ishino (2010) [44] showed that the epimerisation process is influenced by the fact that these catechins with 3 hydroxyl groups on the B ring were faster epimerized than the ones with 2 hydroxyl groups. Also, the catechins with galloyl -ester groups in position 3 are slower epimerized than the ones without this group. In this way there could be an interaction between the B ring and the galloyl group from the 3 position, reducing the stability of the catechins. They also found that during the epimerization, there is a variable degree of hydrolysis of the galloyl group.

Henning (2008) [45] showed that the bioavailability of flavan-3-ols (EGCG and EGC) was degraded at a rate of 50% in 2-3 hours, in an alkaline medium at pH 7.0. EGCG and EGC were much more susceptible to deterioration compared to EC (epicatechins) and ECG that remained stable [40].

In accordance with the fundamentals of IR spectroscopy (B. STUART [46]), three absorption fields can be identified: 4000-2500 cm⁻¹ (I), 2000-1500 cm⁻¹ (II) and the region of fingerprint (1500 - 400 cm⁻¹). FTIR spectra of fermented beverage based on buckwheat, presents in the first region (3700 -3000 cm⁻¹), a broadband absorption, corresponding to the –OH group, respectively the polyphenols. In the same region, the area of 2980 and 2850 cm⁻¹ corresponds to the vibrations of stretching of the – C-H bonds inside the CH₃ or CH₂ groups. The second region (2000 - 1500 cm⁻¹) corresponds to the absorption of the carbonyl groups. It was noticed that the major peak in this area is the one close to the frequency of 1639.1 cm⁻¹, this peak corresponding both to the asymmetric stretches of C = O bonds, and to the esteric groups (J. ČOPÍKOVÁ [47]). It can be seen that in the vibrational spectrum of intestinal simulation has been produced a growing band characteristic to carbonilic groups, due to oxidation and polymerisation reactions of the catechins with the releasing of galloyl - ester groups. The third region, 2000-400 cm-1, meets with the region of fingerprint of the beverage. Glucose, fructose, and sucrose present characteristic peaks in this region: 776, 816.8, 864.8
and 922.4. Also, in this area there are the peaks with following frequencies: 1412.6, 1342.2 and 1248.1 cm$^{-1}$. The first two peaks are attributed to vibrations of the C-H bonds of pyranose cycles from carbohydrates structure. It highlights the presence in this area of intense peak at 1026 cm$^{-1}$, characteristic of the sugar fragment (glycosylation) (H. SCHULTZ [48]).

![FTIR fingerprint of fermented beverage](image)

**Figure 5.** FTIR fingerprint of fermented beverage

- solid line: fermented beverage;
- dashed line: fermented beverage after gastric simulation;
- dotted line: fermented beverage after intestinal simulation

### 4. Conclusions

The proposed new lactic fermented beverage containing sprouted buckwheat, honey buckwheat, has functional properties based on their content of polyphenols, particularly flavonoids.

The tests of biochemical stability of flavonoids gastric and intestinal passages were realized by GI conditions simulation.

It was demonstrate by HPLC and FTIR analysis that flavonoids from fermented beverage arriving at enterocytes level in sufficient amount for being absorbed and exercising their actions. Also, the antioxidant activity of the beverage remains quite strong after the intestinal passage.

Promote of the production of the fermented food with high bioactive activity is according modern concepts for consumer’s health and food safety assurance and also to increase the life quality.

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