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The extraction of antioxidative compounds from rusks enriched with millet flour (*Panicum miliaceum* L.)

DAJANA T. POLEKSIĆ[1,2], MILICA Ž. PAVLIĆEVIĆ[1], JELENA M. RAKOVIĆ-SIMIĆ[3], VLADISLAV A. RAC[1], BILJANA V. VUCELIĆ-RADOVIĆ[1] and VESNA M. RAKIĆ[1]*

[1]*Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11080 Zemun, Serbia,* [2]*Aleksandrija Fruška Gora D.O.O., Železnička bb, 21238 Čurug, Serbia and* [3]*Faculty of Physical Chemistry, University of Belgrade, Studentski trg 16, Belgrade, Serbia*

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Abstract: Two different ways of extracting antioxidative compounds (including soluble polyphenols) from rusks made from wheat flour with added millet (*Panicum miliaceum* L.) were compared, *i.e.*, solvent extraction and *in vitro* digestion. Wheat flour was replaced by millet flour in amounts of 10, 20 or 30 wt. % (per dry mass). Solvent extraction was realized using a mixture of ethanol and water in different percentages, with or without the addition of formic acid. The total content of phenolic compounds (TPC) was determined using Folin–Ciocalteu reagent, while the antioxidative capacity was measured by the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. The efficiency of solvent extraction was enhanced by the addition of formic acid. The addition of millet flour in amounts up to 20 % enhanced the antioxidative properties. It was shown that *in vitro* digestion was more efficient in the extraction of antioxidative compounds, in comparison with solvent extraction.

Keywords: *in vitro* digestion; antioxidative activity; Folin–Ciocalteu method; DPPH test.

INTRODUCTION

In recent decades, the interest in polyphenols – plant secondary metabolites is increasing steadily. These ubiquitous plant compounds comprised of phenol structural units are involved in plant growth, photosynthesis, reproduction and defense against ultraviolet radiation or aggressive pathogens.[1] Interest in these compounds contained in fruits and vegetables is growing, especially because they could act as free radicals scavengers, *i.e.*, agents that can play a role in preventing or minimizing the effects of oxidative stress.[2] Therefore, food containing plant polyphenols have become an integral part of human diet, since their long term

* Corresponding author. E-mail: vesna.rakic@agrif.bg.ac.rs
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consumption is supposed to protect against development of many diseases, such as cardiovascular disorder, diabetes, cancer, osteoporosis or neurodegenerative diseases.[3]

For these reasons, there is an emerging demand for assimilating qualitative and quantitative data about phenolic compounds (PC) or certain categories of them both in plants and in final food products available on the market. These data are important since they could elicit proper advice on the recommended amounts for consumption. Moreover, in addition to the components naturally present in the raw materials, bioactive substances (such as polyphenols) extracted from plant material are often added into the final products in the food industry.[4] Hence, there is a necessity to find suitable ways of extraction that would allow maximum yields of these compounds. Generally, polyphenols are polar molecules, thus being soluble in polar solvents or in their mixtures with water.[5] Numerous literature sources offer data on the total extraction of phenolic compounds (TPC) by methanol,[6] ethanol,[7] acetone,[8] *etc*. However, only the use of green chemicals is recommendable; hence, from the above mentioned, the best choice is the use of ethanol.[5] Importantly, insight into literature data indicates that, in the case of extraction from the same vegetable material, the use of different solvents gives different TPC contents.[5] In addition, an important aspect is the bioavailability of these compounds, so the data obtained by chemical extraction are not necessarily useful when considering the biological activity of some foods.[9] From this viewpoint, the convenient way of PC extraction is the application of *in vitro* digestion of the plant material and/or final industrial products. *In vitro* methods that mimic the physicochemical processes involved in the digestion processes that occur in the upper gastrointestinal tract of humans are economical and non-invasive and are particularly recommended during food product development.[10]

Bread has been the basis of human diet since ancient times. Cereal-based food products, such as bread, are the source of all macronutrients (proteins, fats and carbohydrates) and additionally provide minerals, vitamins and other micronutrients, including polyphenols.[11] However, being widely consumed staple food, bread could be seen as a matrix to which bioactive components could be added.[12] Since the composition of cereals varies depending on their type, one of the strategies to obtain functionally enriched bread is to produce it from more than one type of cereal.

In this work, rusks, bakery products characterized by long shelf life, good digestibility and low fat content, produced from wheat flour (type 400) and flour obtained from the whole grains of millet (*Panicum miliaceum* L.) were investigated. Millets are considered to be the first cereal grains cultivated by early human civilization while today, they are understudied and underutilized in comparison to other major cereals, although in recent years, this cereal has become an

attractive subject of research.[13] It was shown that millets are rich sources of phenolic compounds with proven *in vitro* antioxidant activity.[14] Here, the extraction of TPC was realized either by solvent (mixtures of ethanol and water with or without the addition of formic acid) or by *in vitro* enzymatic methods simulating the gastrointestinal conditions of humans. The total contents of phenols and antioxidative activities were estimated using spectrophotometric methods.

EXPERIMENTAL

In this work, rusks were prepared from wheat (type 400) and millet flour: in the rusk dough, wheat flour was replaced by 10, 20 or 30 wt. % of millet flour and denoted as R-*, where * stands for millet flour content percent. Rusk made just from wheat flour type 400 was taken as a control sample (R-0). The samples were ground using a Brabender rotary mill. The final diameters of obtained particles were in the range 7–9 μm. The moisture content in the samples was determined by drying weighed samples for 2 h at 105 °C. Then, the samples were cooled in a desiccator before reweighing. The moisture content was calculated as the difference in wet and dry mass.

As a pilot study, extraction of polyphenols from R-0 was performed using different mixtures of ethanol and water, as already reported in the literature,[7,8,15] in order to select the most efficient one. Mixtures used for extractions were ethanol (80 %)–water (20 %), ethanol (70 %)–water (30 %) and ethanol (70 %)–water (29.5 %)–formic acid (0.5 %). In all extractions, 6 g of ground rusk was extracted with 25 mL of solvent at 25 °C. The best extraction time was determined in a separate set of experiments using the same rusk that was treated by the chosen extraction mixture for 3, 6, 10, 16 and 24 h. After all extractions, the samples (suspension of rusk in the solvent) were centrifuged at 11000 rpm for 30 min and stored at –20 °C, prior to further experiments. The contents of soluble TPC in the liquid phases were determined and taken as the extraction efficiency criterion.

For *in vitro* digestion, rusks were digested at 37 °C in an orbital shaker (Lab Companion SI-600R Benchtop Shaker) at 120 rpm, following a previously described procedure.[16] Briefly, about 1 g of the ground sample was weighed on an analytical balance, placed in a 200 mL screw capped conical flask and after addition of 15 mL of distilled water and 10 mL 0.85 % NaCl shaken for 10 min, followed by the addition of 1 mL of porcine α-amylase (50 units mL^{-1}, EC 232-565-6, diluted in 20 mM sodium phosphate buffer pH 6.9, containing 1 mM $CaCl_2$). After 5 min, a sufficient amount of 0.15 M HCl was added to obtain pH < 2.5, what was followed by the addition of porcine pepsin (1 mL of 20 mg mL^{-1}, EC 232-629-3, dissolved in 20 mM HCl). Afterwards, the slurry was incubated for 2 h under the same conditions. At the end of this stage (which imitates gastric phase of digestion), bile salts (4 mL of solution 150 mg mL^{-1} dissolved in 0.15 M $NaHCO_3$), 4 mL of porcine pancreatin (18.75 mg mL^{-1}, EC 232--468-9 diluted in 0.15 M $NaHCO_3$) and 1 mL of porcine mucin (75 mg ml^{-1}, EC 282-010-7, dissolved in distilled water) were added and the incubation was prolonged for a further 3 h. The supernatants recovered afterwards were centrifuged at 11000 rpm for 30 min and stored immediately at –20° C for at least 48 h before the analysis of TPC were performed. These results were compared with the ones obtained using the mixture ethanol (70 %), water (29.5 %) and formic acid (0.5 %), which was found to be the most efficient in the pilot study. In order to be comparable, those extractions were performed using the same conditions as in the case of *in vitro* digestion (5 h, 37 °C, ratio of solid to liquid (g mL^{-1}) 1:40).

Just before the determinations of the soluble TPC contents and antioxidative capacities, the supernatants from *in vitro* digestion, and the liquid phases obtained after extraction by ethanol were centrifuged additionally at 14000 rpm for 10 min.

The quantification of soluble TPC in the samples is based on their reaction with a colorimetric reagent, which allows measurement in the visible region of the spectrum. The Folin–Ciocalteu (FC) method is the best known, being based on the transfer of electrons from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes in alkaline medium, which results in the formation of chromogens in which the metal is in its lower valence state. The concentration of the complex can be determined spectroscopically at 765 nm. In order to avoid methanol interferences, the modifications proposed by Cicco *et al.*[17] were used herein. Aliquots of 100-μL sample were mixed with 100 μL of FC reagent and 200 μL of distilled water. The mixtures were kept in the dark for 2 min and then, 1600 μL of Na_2CO_3 (5 % in water) was added, in order to create basic conditions (pH ≈ 10) for the redox reaction between phenolic compounds and FC reagent. After incubation for 20 min at room temperature in the dark, the absorbance was determined at 765 nm against the reagent blank. Standard curve was constructed using concentrations of 0, 0.25, 0.50, 0.75 and 1 mg mL^{-1} of gallic acid. The total phenolic content is expressed as gallic acid equivalents (mg of GAE per 100 g).

Antioxidative capacities of the investigated samples were estimated using the DPPH test. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical which serves to monitor chemical reactions involving radicals. After accepting a hydrogen from a corresponding donor, DPPH loses its characteristic deep purple color (λ_{max} 515–517 nm) and turns colorless or pale yellow. The assay is performed according to the protocol of Sanchez Moreno *et al.*[18] Thus, 130 μL of sample was mixed with 1870 μL of DPPH solution in methanol, the absorbance (at 516 nm) was determined after the samples had been kept for 30 min in the dark, at room temperature. The antiradical activity of tested samples is expressed as equivalent of reference standard compound (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid – trolox). The results are expressed as μmol g^{-1} trolox equivalents.

The used chemicals were of appropriate quality, all enzymes and bile salts were Sigma–Aldrich products, while all other chemicals were of analytical grade quality and produced by Merck.

All experimental procedures – the extractions or digestions and chemical analysis were performed in triplicate. The data were analyzed using XLSTAT 2014 software. The obtained results are given as mean values ± *SD* (standard deviation) and further subjected to one-way analysis of variances (ANOVA) in order to determine the differences between multiple means in continuous variables. Post hoc analysis to determine extent of influence was realized using the Tukey test. Statistical significance was calculated at the level of significance $p < 0.05$.

RESULTS AND DISCUSSION

The time of solvent extraction was determined as described in the experimental section and the results are shown in Fig. 1. All three extraction mixtures were applied to the control rusk (R-0). It is evident that the extraction was almost completed after approximately 10 h. However, for the purpose of convenient organization of experiments, the extraction was performed for 16 h, at 25 ° C. It is also evident that the extraction was faster and more effective in the presence of formic acid.

The amounts of total polyphenols, extracted after 16 h and determined using FC reagent are presented in Table I. It is evident that the method of extraction and the composition of rusk both had significant influences on the amount of extracted polyphenols from rusks at the $p < 0.05$ level (F (2, 6) = 11.20; $p = 0.0094$). Two way ANOVA for the independent factors was performed at a level of significance 0.05 and degrees of freedom: 2 and 6. The extraction was more effective when performed with lower amount of ethanol and with the addition of formic acid. In accordance with the literature data, these findings could be explained by the fact that higher polarity of solvent medium allows higher degree of extraction of bound polyphenols, enabling the simultaneous extraction of polyphenols of different molecular weights. Besides, the presence of formic acid enables acidic hydrolysis of polyphenols, thus making their extraction easier.[6,15,19] These results were further confirmed by those found for the antioxidative activities of extracts obtained using three different extraction solutions (presented in Table II).

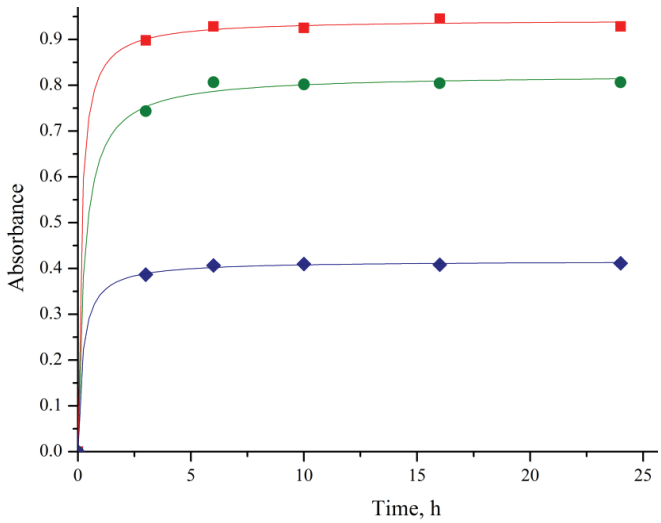

Fig. 1. Extraction from the control rusk (R-0) using the mixtures: ethanol (80 %)–water (20 %) (♦); ethanol (70 %)–water (30 %) (●) and ethanol (70 %)–water (29.5 %)–formic acid (0.5 %) (■). The lines on the graph do not have physical meaning, they are presented to guide the eye.

TABLE I. Content of soluble polyphenols from the control rusk (R-0) extracted using different mixtures of solvents; means with different superscript roman letter are significantly different (calculated by Tukey test at $p < 0.05$)

Extraction mixture	Ethanol (80 %)–water (20 %)	Ethanol (70 %)–water (30 %)	Ethanol (70 %)–water (29.5 %)–formic acid (0. 5 %)
Total polyphenols (mg 100 g^{-1})	38.11±0.72^{a}	46.42±2.49^{b}	53.66±1.74^{c}

It is evident from the results presented in Table II that the method of extraction had significant influence on antioxidative activity of R-0 sample at the $p < 0.05$ level (F (2, 6) = 54.26; p = 0.00014). As with the TPC contents, the highest antioxidative activity was found for the extract obtained using the mixture ethanol (70 %)–water (29.5 %)–formic acid (0.5 %).

TABLE II. Concentration of antioxidants from control rusk R-0 determined by the DPPH method (expressed as μmol g^{-1} trolox equivalents) extracted by different extraction methods; means with different superscript roman letter are significantly different (calculated by the Tukey test at $p < 0.05$)

Extraction mixture	Ethanol (80 %)–water (20 %)	Ethanol (70 %)–water (30 %)	Ethanol (70 %)–water (29.5 %)–formic acid (0.5 %)
μmol/g (trolox equivalents)	1.11±0.02[a]	1.25±0.05[b]	1.46±0.06[c]

Since the extraction performed using the mixture ethanol (70 %)–water (29.5 %)–formic acid (0.5 %) proved was the most efficient one, the effect of millet addition in rusks was examined using just this method of extraction. To express the results per dry mass, the moisture content in the rusks was determined and values in between 7 and 8 wt. % were found (7.66, 7.56, 7.39 and 7.19 % for R-0, R-10, R-20 and R-30, respectively.

The amounts of total polyphenols extracted from rusks seemed to be influenced by the addition of millet flour (Table III). However, a statistically significant influence was seen just in the case of the R-20 sample, with 20 wt. % of millet flour. Evidently, increasing percent of millet flour further does not change the extracted amount of TPC.

TABLE III. Content of soluble polyphenols from rusks containing different percentage of millet flour released by extraction of mixture containing ethanol (70 %)–water (29.5 %)–formic acid (0.5 %), expressed as equivalents of gallic acid; means with different superscript roman letter are significantly different (calculated by Tukey test at $p < 0.05$)

Type of rusk	R-0	R-10	R-20	R-30
Total polyphenols (mg/100 g)	53.66±1.74[a]	56.11±0.72[a]	48.36±0.38[b]	48.90±0.13[b]

On the contrary, significantly higher antioxidative activities of extracts obtained from rusks with higher contents of millet flour were found (at the $p < 0.05$ level, Table IV). It is important to notice that the difference in TPC content in the rusks made with or without addition of millet flour most probably stems not from difference in their polyphenol composition, since in both millet and wheat, *p*-coumaric, hydroxybenzoic and ferulic acid are major components,[6,14] but rather from the fact that wheat contains around 3.5 times more total polyphenols when compared to millet.[6,14]

The lack of correlation between total phenolic content and antioxidative activity could be explained by the presence of compounds, such as tannins and phy-

tic acid,[20] that act as antioxidants and are present in higher amount in millet compared to wheat.[21] However, similarly to the TPC content, the addition of millet in an amount greater than 20 % resulted in a decrease in the antioxidative activity – the insight into the results presented in Table IV shows that the antioxidative activity of rusk containing 30 wt. % of millet is similar to antioxidative activity of control rusk obtained just from wheat flour. These results could be explained by the fact that rusks enriched with millet contain a higher percentage of dietary fibers[22] and amylopectin (results not presented here) in comparison with that one produced from wheat flour alone. In fact, the addition of millet to the rusks enabled an increase in the percentage of polysaccharides with highly branched molecules. The formation of bonds between phenolic compounds and polysaccharide molecules has already been reported in the literature.[23] Thus, the previously mentioned branched moieties could contribute to the formation of hydrogen and van der Waals bonds with phenolic compounds, which could explain the decrease in the antioxidative activity found for rusk R-30.

TABLE IV. Antioxidant activities of the extracts obtained using a mixture containing ethanol (70 %)–water (29.5 %)–formic acid (0.5 %) as determined by the DPPH method (expressed as μmol g^{-1} trolox equivalents); means with different superscript roman letter are significantly different (calculated by the Tukey test at $p < 0.05$)

Type of rusk	R-0	R-10	R-20	R-30
μmol g (trolox equivalents)	1.46 ± 0.06^{a}	1.71 ± 0.08^{b}	1.86 ± 0.06^{c}	1.47 ± 0.09^{a}

Furthermore, the rusks were also subjected to *in vitro* enzymatic digestion. In order to compare the results, the extraction with the mixture ethanol (70%)––water (29.5 %)–formic acid (0.5 %) was additionally performed using the same conditions of temperature, duration and solid/liquid ratio as in the case of *in vitro* digestion. The results are presented in Table V. For all the investigated samples, the contents of soluble PC were importantly higher in the extracts obtained by *in vitro* digestion, than in those obtained using the mixture of solvents. It should be emphasized here, however, that the aromatic amino acid residues that remain in solution after enzymatic digestion also react with the Folin–Ciocalteu reagent.[24–26] Since this drawback of the FC method is commonly known, it was of particular

TABLE V. Content of soluble polyphenols released by two different methods (expressed as equivalents of gallic acid (in mg 100 g^{-1} dry mass)); means in the same column with different superscript roman letter are significantly different (calculated by the Tukey test at $p < 0.05$)

Sample (rusks)	Method of extraction	
	Solvent extraction	*In vitro* digestion
0 % millet	24.6 ± 0.2^{a}	91.1 ± 0.2^{a}
10 % millet	22.7 ± 0.2^{b}	87.2 ± 0.4^{b}
20 % millet	20.7 ± 0.3^{c}	83.2 ± 0.3^{c}
30 % millet	19.9 ± 0.2^{d}	80.1 ± 0.4^{d}

importance to determine the antioxidant activity of the obtained extracts. The results are given in Table VI. It could be seen that the antioxidative activities of the extracts obtained by *in vitro* digestion are just 1.06 to 1.16 times higher than the activities of the extracts obtained by solvent extraction. It is, however, important to emphasize that these differences are statistically significant.

TABLE VI. Antioxidant activities of extracts determined by the DPPH method (expressed as μmol g^{-1} trolox equivalents) extracted by different methods; means in the same column with different superscript roman letter are significantly different (calculated by the Tukey test at $p < 0.05$)

Sample (rusks)	Method of extraction	
	Solvent extraction	*In vitro* digestion
0 % millet	0.93±0.03[a]	1.08±0.02[a]
10 % millet	1.07±0.03[b]	1.18± 0.04[b]
20 % millet	1.14±0.02[c]	1.21±0.03[c]
30 % millet	1.01±0.01[d]	1.09±0.04[a]

Hence, it could be noticed that both the results of TPC contents (Table V) and antioxidative activities (Table VI) show that *in vitro* digestion leads to a slightly higher efficiency in extracting the bioactive compounds, in comparison with the solvent extraction. These results are in agreement with results obtained by McCarthy *et al.*,[27] which might stem from the fact that during *in vitro* digestion, hydrolysis compounds are released that contribute to antioxidant activity of the extract. Finally, it could be noticed that the DPPH test provides comparable results on the antioxidative activities of the extracts obtained by solvent extraction and by *in vitro* digestion. In contrast, the FC method yields very different results, which must be interpreted in the light of the aforementioned drawbacks of this method. Thus, it should be commented here that the DPPH method could be considered as more reliable, while the results obtained by the FC method must be taken with caution when evaluating the antioxidative potential of foods and food supplements.

CONCLUSIONS

The results obtained in this work clearly show that the efficiency of extraction of soluble polyphenols and other antioxidative compounds from the investigated material is strongly influenced by the composition of the extraction medium. Specifically, it was found that the efficiency of extraction was improved by lowering the amount of ethanol in the extraction mixture and by the addition of formic acid.

The results obtained in this work suggest that researchers should be advised to use *in vitro* digestion as the appropriate method for the extraction of bioactive components and the evaluation of antioxidative potential. This high-yielding ext-

raction method is always applied in the same way and the obtained results can be validly compared.

The results obtained using *in vitro* digestion indicate that in the specific material analyzed in this work, *i.e*., rusks made with different contents of millet flour, the addition of millet flour enhanced their antioxidative properties. However, this effect was noticed only if millet was added in amount up to 20 wt. % Addition of millet in higher amounts led to a drop in antioxidative properties, probably due to interaction of antioxidants with branched polysaccharide molecules.

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ИЗВОД

ЕКСТРАКЦИЈА АНТИОКСИДАТИВНИХ ЈЕДИЊЕЊА ИЗ ПШЕНИЧНИХ ДВОПЕКА СА ДОДАТКОМ ПРОСА (*Panicum miliaceum* L.)

ДАЈАНА Т. ПОЛЕКСИЋ[1,2], МИЛИЦА Ж. ПАВЛИЋЕВИЋ[1], ЈЕЛЕНА М. РАКОВИЋ-СИМИЋ[3], ВЛАДИСЛАВ А. РАЦ[1], БИЉАНА В. ВУЦЕЛИЋ-РАДОВИЋ[1] и ВЕСНА М. РАКИЋ[1]

[1]Пољопривредни факултет Универзитета у Београду, Немањина 6, 11080 Земун, [2]Александрија Фрушка Гора Д.О.О., Железничка бб, 21238 Чуруг и [3]Факултет за физичку хемију Универзитета у Београду, Студентски трг 16, Београд

У овом раду су поређена два начина (екстракција растварачима и *in vitro* дигестија) за екстракцију антоксидативних једињења (укључујући и растворне полифеноле) из пшеничних двопека са додатком проса (*Panicum milliaceum* L.) У двопецима је пшенично брашно замењено брашном од проса у износу од 10, 20 или 30 теж. % (по сувој маси). Екстракција растварачима је вршена смешом етанола и воде у различитом односу, са додатком и без додатка мравље киселине. Садржај укупних фенолних једињења (TPC) је одређен помоћу Folin–Ciocalteu-овог реагенса, док је антиоксидативни капацитет одређен применом DPPH (2,2-дифенил-1-пикрилхидразил) теста. Ефикасност екстракције растварачима је повећана додатком мравље киселине. Додатак брашна од проса у количини од 20 % побољшао је антиоксидативна својства двопека. *In vitro* дигестија се показала као ефикаснији метод за екстракцију антиоксидативних једињења у поређењу са екстракцијом растварачима.

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