CHEMICAL AND MICROBIAL EVALUATION OF BISCUITS MADE FROM WHEAT FLOUR SUBSTITUTED WITH WHEAT SPROUTS

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ABSTRACT

The aim of this work was to produce biscuits from wheat flour substituted with different amounts of wheat sprout powder (2.5–7.5%). The biscuits were subjected to chemical, phytochemical and microbial evaluations. The crude protein, fat and ash contents and the energy value of the biscuits increased with increasing percentage of wheat sprout powder. Adding sprouts resulted in higher values of phenolics, alpha-tocopherol and antioxidant activity. There was no statistically significant difference in the contents of total phenolics and alpha-tocopherol between biscuits supplemented with 5% sprouts and biscuits substituted with 7.5% sprouts. The phenolic content in biscuits containing 7.5% sprouts was 245 mg GAE/100 g dw compared with 110 mg GAE/100 g dw in control biscuits. Antioxidant activity was the highest in biscuits substituted with 7.5% sprouts. All levels of substitution of wheat flour with wheat sprouts had an effect on the nutritional properties of biscuits, but the substitution level of 2.5–5% is recommended for the improvement of their sensorial properties. The biscuits produced had a low microbial load and were microbiologically safe. *Escherichia coli, Salmonella* spp., and sulfite-reduction clostridia were not detected in any sample during the period of investigation from 2 to 60 days of storage.

KEYWORDS:

Biscuits, wheat sprouts, antioxidants, phenolics, alpha-tocopherols, microbial evaluation

INTRODUCTION

Wheat is one of the "three major" cereal crops, with an annual production of more than 700 million tons. In 2018, the total world harvest was about 730 million tons compared with 516 million tons of rice and 1482 million tons of corn (http://www.fao.org/statistics). Wheat is the most popular energy crop for the production of confectionery products because its protein (gluten) has unique properties, combining strength and elasticity required to produce bread, cookies, cakes (Akhtar et al., 2008; Adeoye et al., 2017).

Germination is a simple, inexpensive and environmentally friendly method of producing plant foods with functional properties. Germination reduces the level of antinutrients, increases the nutritional and medicinal properties of food and induces the accumulation of secondary metabolites, some of which have been characterized as antioxidants (Aguilera et al., 2013; Tarasevičienėa et al., 2019). Germination promotes the accumulation of phenolic acids, flavonoids

and vitamins, which are health promoters and therefore increase grain value (Oh and Rajashekar, 2009). An increase of simple sugars and amino acids in germinated seeds of wheat (Yang et al., 2001), barley (Rimsten et al., 2003) and rice (Saman et al., 2008) is the result of the activation of hydrolytic enzymes that break down the complex molecules of starches, proteins and polysaccharides. Žilić et al. (2014) found that germination increases the nutritional value of the grain and intensifies the biosynthesis of tocopherols, niacin and riboflavin in the embryonic axis. Also, germination generates and increases the amount of bioactive components such as ascorbic acid, tocopherols, tocotrienols and phenolic compounds, leading to an increase in antioxidant activity (Frias et al., 2005; Fernandez-Orozco et al., 2008; Sytar et al., 2018; Tarasevičienė et al., 2019).

Among bakery products, biscuits/cookies and crackers are the most popular snack foods (Nagi et al., 2012). Biscuits are the most commonly consumed cereal food items along with bread. They are inexpensive food products, with varied taste, are readily available and have a long shelf life (Kulkarni, 1997; Nagi et al., 2012). Biscuits contain flour, saccharides and fats in their basic formulation. During storage, they can undergo oxidation, causing deterioration of their organoleptic properties (Reddy et al., 2005). Lipid oxidation can be prevented by the addition of antioxidants. In recent years, there has been a great interest in natural antioxidants because of their role in preventing the auto-oxidation of fats and oils. Some of them such as α-tocopherol, β-carotene, and ascorbic acid are used in bakery products. These compounds are effective in enhancing the shelf life of bakery products, but they have lower efficiency than synthetic antioxidants (Nanditha and Prabhasankar, 2009).

Today, there is a great interest in functional foods, commonly known as “superfoods”. There is no clear definition of superfoods – the term is used for foods that combine nutritional and health benefits and disease prevention (Pasias et al., 2018). Nowadays, functional food studies on cereals and pseudocereals have been particularly interested in cereal products with high antioxidant properties (Chlopicka et al., 2012; Havrlentova et al., 2014; Lao et al., 2017; Zykin et al., 2018), and breeders have developed colored wheat with high anthocyanin content in seeds and sprouts to improve mill quality (Sytar et al., 2018). There has been an increase in the use of sprouted grains in the human diet, and an increase in scientific research dealing with their nutritional traits and

phytochemical contents (Benincasa et al., 2019). Sprouting has been suggested as an efficient and effective method to enhance functional value (Abderrahim et al., 2012). Sytar et al. (2018), also suggested that sprouts are a rich source of total phenolics, flavonoids and anthocyanins. Alvarez-Jubete et al. (2010) documented the influence of sprouting of amaranth, quinoa and wheat on polyphenol profile and antioxidant capacity. Khattak et al. (2007) described that germinated seeds are nutritionally and functionally enhanced materials compared with non-germinated seeds. Zilic et al. (2014) reported that interest in incorporating bioactive ingredients such as phenolic antioxidants and vitamins from sprouts into popular foods has grown rapidly, the main reason being increased consumer health awareness. Bakery products such as bread or biscuits in the form of functional foods can deliver a high concentration of antioxidants which can have a role in the protection from diseases, such as cardiovascular and degenerative diseases, and cancer (Arts and Hollman, 2005). The use of wheat sprouts may be significant in the development of functional foods (Cevallos-Casals and Cisneros-Zevallos, 2010; Benincasa et al., 2015). Wheat flour is the main ingredient of biscuits but it is nutritionally very poor. Therefore, the addition of wheat sprouts can improve functional and nutraceutical properties of products. The aim of the present study was to produce biscuits from wheat flour substituted with different amounts of wheat sprout powder, and to study its effect on the chemical, phytochemical and microbial properties of biscuits.

MATERIAL AND METHODS

Preparation of wheat sprouts

Seeds of winter wheat (*Triticum aestivum* L.) cultivar 'Simonida' supplied by the Institute of Field and Vegetable Crops, Novi Sad (Serbia) were used for germination. Wheat sprouts were obtained through germination of whole wheat grains. Germination was performed according to the method described by Vale et al. (2014) with small modifications. Grains were washed with distilled water, and sterilized with hydrogen peroxide (5%, v/v) for 5 minutes, and then rinsed many times with distilled water. The treated wheat grains were steeped in distilled water, at room temperature at 25°C for 24 h. The steeped wheat kernels were spread on double layers of filter paper in Petri dishes and placed in a plant growth chamber (FOC, Cooled Incubator, Velp scientifica). Every day the Petri dishes were sprayed with distilled water. The germination process proceeded in the growth chamber under controlled conditions, which were: temperature 20 °C, in the dark, 6 days. Sprouts reached 2–4 cm in length. They were mechanically separated and shade dried for 14 h, at

50 °C. The dried sprouts were light brownish in color. The material was ground by a flint mill to fine powder and stored at -20 °C and used for further processing (for determination of crude protein, crude fat, ash, alpha-tocopherol and total phenolic content) (Figure 1).

Figure 1. Wheat sprouts (left) and Wheat sprouts powder (right)

Preparation of biscuit dough and biscuits

The main ingredients used were purchased at a local market in Serbia and included type - 500 white wheat flour (producer – Fidelinka), sugar and baking powder (producer – Centroproizvod) and butter (producer – Imlek). The ingredients used for making wheat flour biscuits substituted with wheat sprout powder are given in Table 1.

Table 1. Ingredients used for making wheat flour biscuits substituted with wheat sprout powder

Ingredients (g)	**control**	**2.5%**	**5.0%**	**7.5%**
Wheat flour	100	97.5	95	92.5
Wheat sprout powder	-	2.5	5	7.5
Powdered sugar	50	50	50	50
Butter	38	38	38	38
Baking soda	0.5	0.5	0.5	0.5
Baking powder	0.5	0.5	0.5	0.5
Water	20	20	20	20

Dough for all biscuits including control was prepared by the manual technique. The basic formulation used was 100g type - 500 wheat flour, 50g sugar, 38g fat (butter), 0.5g baking powder, 0.5g baking soda and 20ml water (control). Biscuits with wheat sprouts were prepared in the same way, with part of wheat flour substituted with 2.5%, 5% and 7.5% wheat sprout powder. Flour, baking powder and baking soda were sieved and mixed together. Fat and sugar were creamed together and then flour and baking powder were added and mixed. Water was added and the dough

was kneaded to reach proper consistency. The prepared dough was subjected to sheeting of 4 mm thickness. Finally, the sheets were cut to 4.5 diameter by using a die and were subjected to baking at 180 ± 5 °C for 20 ± 2 min. The baked biscuits were cooled to room temperature and sealed in polyethylene packs. Biscuit samples for determination of alpha-tocopherol were stored at -20 °C. Total phenolics and antioxidant activity were determined on the second day after baking, and the proximate analysis of biscuits (fat, protein, ash, starch) was obtained in the first week after baking. Biscuit samples were stored at room temperature to assess shelf-life. For this purpose, microbiological analysis was performed at 2, 30 and 60 days of storage.

Proximate Analysis

Moisture, crude fat, crude proteins and ash were determined in wheat sprouts and wheat flour type - 500. Biscuits were evaluated for crude protein, crude fat, total starch and carbohydrate content. Moisture content was measured by the gravimetric method using an oven at 105°C for 18 h. Crude proteins were determined by the micro-Kjeldahl method. The nitrogen conversion factor used for crude protein calculation was 6.25. Crude fat was determined by the Soxhlet extraction method with petrol-ether as solvent. The carbohydrate content (%) was calculated by subtracting crude ash, fat and protein content from 100% dry matter. Total starch content was determined using the Ewers method. The total energy was determined by the Atwater method (Osborne and Voogt, 1978). The total energy or the caloric value was estimated by calculation using the water quantification factors of 4, 9 and 4 kcal, respectively, for protein, fat and carbohydrate. 1Kcal/100 g = [(4 × carbohydrate) + (4 × protein) + (9 × fat)].

Extraction of Antioxidant Compounds and Determination of Antioxidant Activities

The extraction process

Samples (milled sprouts and biscuits) were extracted with 50% acetone (50% acetone and 50% water) at room temperature in an ultrasonic bath for 30 min. The weight ratio of sample to solvent was 1:10 in favor of solvent. The extracts were filtered and then centrifuged at 5000 rpm for 10 min. The clear supernatants obtained by extractions were kept in the dark in the fridge and used for the determination of the total phenolic content and antioxidant activity.

Determination of total phenolic content (**TPC)**

The total phenolic content was determined using a modified Folin-Ciocalteu colorimetric method (Singleton et al., 1999; Liu et al., 2002). An aliquot of 40 µL of extracts or Gallic acid standard solution was mixed with 3.16 mL of distilled water, whereupon 200 µL of Folin-Ciocalteu reagent was added. After 8 min. 600 µL of 20% Na_2CO_3 solution was added. The solution was well mixed and incubated for 2 h at room temperature. Thereafter, absorbance was measured at 760 nm. The results were expressed as milligrams of Gallic acid equivalents per 100 g dry matter (mg GAE/100g dm).

Antioxidant capacity

Antioxidant properties were determined by the ABTS assay, according to Re at al. (1996). The ABTS stock solution in distilled water was prepared from 7 mM ABTS and 2.45 mM potassium persulphate, and then incubated in the dark for 12–16 h at room temperature. The ABTS working solution was prepared by diluting the stock solution with ethanol to an absorbance of 0.70 ± 0.05 at 734 nm. An aliquot of 10 µL of extracts was mixed with 1 mL of prepared ABTS solution and mixed for 6 min. Absorbance was measured at 734 nm, with distilled water as a reference. Results were expressed as Trolox equivalent antioxidant capacity (µmol TE/100 g dm) and as percentage of inhibition of the ABTS radical.

DPPH methods

The antioxidant capacity of the extracts was also studied through the evaluation of the free radical-scavenging effect on the 1.1-diphenyl-2-picrylhydrazyl (DPPH) radical. An aliquot of 0.1 ml of extract was mixed with 3.9 ml DPPH solution ($6 \cdot 10^{-5}$M). The mixture was thoroughly vortex-mixed and kept in the dark for 30 min. Then, the absorbance was measured at 515 nm, with distilled water as a reference. Results were expressed as percentage of inhibition of the DPPH radical and also as Trolox equivalent antioxidant capacity (µmol TE/100 g dm). Percentage of inhibition of the DPPH radical was calculated according to the following equation:

$$\mathrm{I}\,(\%) = \frac{AB-AA}{AB} \cdot 100$$

where: AB is the absorbance of DPPH solution without extracts and AA is absorbance of DPPH solution with extracts.

Analysis of alpha-tocopherols in wheat sprouts and biscuits

The wheat sprouts and biscuits were milled to a fine powder with particle size < 500 µm. Sample preparation for the determination of alpha-tocopherol was performed according to the method of Žilic et al. (2014), with some modifications. The sample (0.5 g) was mixed with 5 mL of n-hexane. The mixture was rigorously shaken at 2500 rpm (IKA, Germany) at 5°C, 30 min. After centrifugation at 6000 rpm for 15 min, the upper layer was separated and evaporated under N_2 (Nitrogen generator, MICRO, Italy) keeping temperature constant at 70±2°C (water bath) in the dark. The dried sample was then redissolved in 2.5 mL of methanol, vortexed and centrifuged at 5000 rpm for 10 min. After being vortex-mixed and centrifuged, the samples were filtered through a 0.45 µm pore size nylon filter and directly injected into the chromatograph.

The content of alpha-tocopherols was determined by the HPLC method (Gimeno et al., 2000).

Stock standard solution of alpha-tocopherol was prepared in methanol at a concentration of 500 μgmL^{-1} and stored at -20°C in dark vial. Separation by HPLC was carried out using a Waters liquid chromatographic system (USA) with a 1525 binary pump, a 717 autosampler, a TCR3 column heater and a 2420 dual absorbance detector. The column was ODS2 Spherisorb (250x 4 mm, i.d., 5 µm). The analytical column was kept at 45°C. The mobile phase was methanol–water (96:4, v/v) and the elution was performed at a flow-rate of 2 ml min^{-1} in isocratic mode. The injection volume was 50 µl. Detection was performed at 292 nm and each run lasted 5 min. The data were stored and processed by the Empower software. Based on the calibration curve (Y =1.03e + 004x-2.75e + 0.004, R^2 = 0.999095), the content of alpha-tocopherol was determined.

Microbial Evaluation of biscuits

The microbiological analysis of the biscuits was carried out 48 hours after baking, and then at 30 and 60 days of storage. The total viable count of produced cookies was determined using the standard microbiological plating method (Radulović and Petrušić, 2011; Giwa et al., 2012; Đukić et al., 2017). Samples of biscuits from each treatment group were milled to powder under aseptic conditions. Five g of the sample was dissolved in 45 ml of sterile water and vortexed for homogenization at 3000 rpm for 5 min. The supernatant was decanted (basic dilution). Thereafter, a 10-fold dilution of each sample homogenate was made. An aliquot of 1 ml from selected dilutions of each sample was inoculated aseptically into labeled agar plates of the media in three repetitions (agars: Nutrient Agar was used for the total number of aerobic mesophyll bacteria; Endo agar for

Escherichia coli, SS agar for *Salmonella;* and Sabouraud Dextrose Agar for the number of molds). For the determination of the presence of sulphite-reducing clostridia, test tubes with 1 ml of basic dilution were heated in a water bath for 10 min at 80 °C, and then the Sulphite agar was poured into the tubes. The height of the agar in the tubes was > 14 cm and the agar distance from the closure <1 cm. Petri dishes were incubated at 37°C ± 2°C for 24 to 48 hours, except for molds where incubation period was 3 to 5 days, at 28°C ± 2°C. At the end of the incubation period, colonies were numerated. All media (agars) were prepared according to the manufacturer's instructions and autoclaved at 121 °C for 20 minutes.

Statistical Analysis

Experimental data in this research were statistically analyzed by a one-way ANOVA, followed by the least significant test, which was used to detect significant differences among the means. The level of significance was assigned at $p< 0.05$ for chemical composition, and $p< 0.01$ for the content of phenolics and antioxidant activity. The statistical analyses were performed using the program STATISTICA 12 (StatSoft, Inc. 2014).

RESULTS AND DISCUSSION

Chemical characteristics

The chemical analysis of wheat flour and wheat sprouts is given in Table 2. Wheat sprouts had higher level of crude protein, ash and fat compared with type - 500 wheat flour. Also, the results on total phenolics (1440 mg GAE/100g dm) and alpha-tocopherol (60.05 mg/kg) showed that wheat sprouts were a good source of these compounds. This is in agreement with previous studies (Cevallos-Casals end Cisneros-Zavallos, 2010; Benincasa et al., 2015; Sytar et al., 2018). The increasing content of phenolic compounds during the germination of cereals is explained by enzyme activity during germination (Sharma et al., 2016). Germination induces the synthesis or activation of hydrolytic enzymes in germinating grain, resulting in structural modifications and synthesis of compounds of high biological and nutritional value (Kaukovirta-Norja et al., 2004; Wilhelmsson, 2004; Bondia-Pons et al., 2009). Germination results in a higher nutritional and functional value associated with the quality and quantity of nutrients, biologically active compounds and antioxidant potential (Cornejo et al., 2015). Tarasevičienė et al. (2019) found that

the level of alpha-tocopherol in germinated wheat grains was 5.3 times higher than in non-germinated grains.

Table 2. Chemical analyses of wheat flour and wheat sprouts

Flour	Moisture %	Crude protein %	Crude fat %	Ash %	Total phenolic mg GAE/100 g dm	alpha-tocopherol mg/kg
Wheat	8.58	9.50	1.30	0.50	nd	nd
Sprouts	10.82	32.90	1.44	3.79	1440	60.05

nd-not derermined

Biscuits without wheat sprouts (control sample) contained lower level of ash, crude protein, fat and energy value. Results on crude protein, ash, fat, carbohydrates and energy value in biscuits are shown in Table 3.

Table 3. Chemical composition of biscuit

	Crude protein %	Crude fat %	Total Carbohydrates %	Starch %	Ash %	Energy value kcal/100 g
Control	6.76±0.07d	17.74±0.04c	74.85±0.12a	36.64±0.40c	0.65±0.00d	486.08±0.20d
2.5% sprouts	6.947±0.08c	18.27±0.04b	74.07±0.12b	38.37±0.37a	0.71±027c	488.53±0.24c
5% sprouts	7.14±0.06b	19.09±0.05a	72.91±0.01c	37.29±0.43b	0.86±0.00b	492.01±0.24a
7.5% sprouts	7.54±0.07a	18.97±0.07a	72.59±0.01d	34.72±0.42d	0.90±0.00a	491.21±0.32b

a - d Means of duplicate determination. Means with the same superscripts within the column are not significantly different ($p>0.05$). Means without the same superscripts within each column are significantly different ($p<0.05$)

The crude protein content of the biscuits increased from 6.76% to 7.54% (Table 3) with increasing percentage of sprout flour ($p<0.05$). This was due to the addition of sprout flour, which is a good source of supplemental protein. The chemical analysis showed that the content of crude proteins was 9.5% in wheat flour and 32.90% in wheat sprouts (Table 2). In cereals, the protein content

increased after sprouting, as the result of enzymatic and phytohormonal changes or a compositional change following the degradation of other constituents (Kim et al., 2012).

The fat content increased from 17.74% (control) to 19% in biscuit samples with 5 and 7.5% sprouts, where it was the highest. This increase may be due to the higher content of fat in sprout flour compared with wheat flour. The fat in food can affect its shelf stability. Fat can cause oxidative deterioration and food spoilage. These results are in accordance with the findings for barley sprouts (Agu and Okoli, 2014), soybean (Hegstad, 2008) and sprouted brown rice (Kim et al., 2012).

Ash content is an overall estimate of the presence of mineral elements in food (Obeta et al., 2019). The ash content ranged from 0.65% to 0.90%, and was significantly higher in biscuits substituted with sprouts than in control biscuits ($p<0.05$). Sprouts are a good source of mineral elements. The ash content in sprouts was 3.79%, compared with 0.5% in wheat flour (Table 2). The slight increase in the ash content of biscuits substituted with sprouts compared with control biscuits might be due to phytase enzyme activity during sprouting (Inyang and Zakari, 2008; Yaqoob et al., 2017).

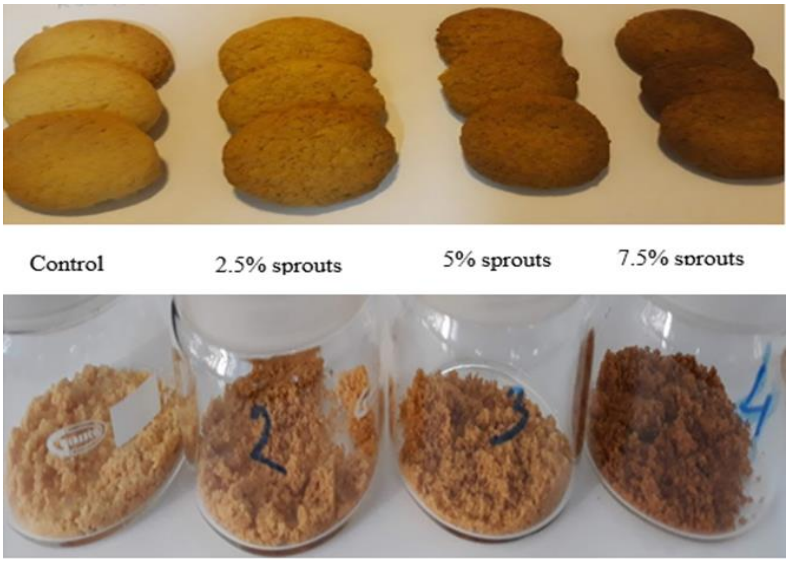
The carbohydrate content decreased, but the energy content increased with increasing amount of substituted sprouts, which was due to the increase in the contents of protein and fat. This is in accordance to investigation of Agu and Okoli (2014). The increase in the energy value of biscuits with substitution of sprouts in comparison with control biscuits could be the result of increased fat content since fat has almost twice the energy content of protein and carbohydrates (Roday, 2007).

A reduction in the content of carbohydrates after sprouting can be attributed to an increase in α- and β-amylases during sprouting and the consequent hydrolysis of starch (Anuchita and Nattawat, 2010). These results on the carbohydrate, protein and fat contents are in accordance with Delgado-Andrade et al. (2009).

The baking conditions: high temperature (up to 200 °C) and short baking times (less than 20 min) reduced the water content (< 10%) and promoted a brown surface (Ait-Ameur et al., 2006). The moisture content in the biscuits was low (less than 2.5%). The low moisture content in the biscuits in this study proved that biscuits belong to bakery products with a long shelf-life. The most visible transformation is connected with the color of biscuits. This is due to the chemical reactions occurring during baking, essentially the Maillard reaction and caramelization (Xu and Chang,

2008). All of this has an effect on the flavor of products and their acceptability by consumers. The color of control biscuits was light but turned brown with increasing amount of wheat sprouts, reaching dark brown in biscuit samples containing 7.5% sprouts (Figure 2).

Figure 2. Baked biscuit and milled biscuit



For consumers, biscuits with 2.5% and 5% sprouts had a pleasant taste and aroma, while those containing 7.5% sprouts had a somewhat harsher taste and were less acceptable. Earlier research reported that the development of color during baking, which is due to both the Maillard reaction and caramelization, is strongly correlated with the contents of compounds possessing antioxidant capacity (Manzocco et al., 2001).

The total phenolic content (TPC)

The phenolic content in biscuits increased with increasing percentage of sprouts. Biscuits with 7.5% sprouts had 245 mg GAE/100 g dw compared with control (biscuits without wheat sprouts), which contained 110 mg GAE/100 g dw, i.e. the increase was 2.2 times higher. The addition of the lowest amount of wheat sprouts (2.5%) significantly ($p<0.01$) increased the phenolic content. This was most likely attributed to the rich source of phenolic content in wheat sprouts used for biscuit preparation. As explained by Zhou et al. (2011) and Xu et al. (2014) during seed germination, oxidative stress forces cereal sprouts to develop self-defensive strategies i.e. to

enhance the activity of antioxidant enzymes and the rate of synthesis of active compounds such as phenolics and flavonoids.

In this paper, we did not examine the content of total phenolics in the biscuit dough before baking. Therefore, we could not argue that the process of baking had an effect or not, i.e. if the content of phenolics increased or decreased. But when biscuits with and without sprouts were compared, a significantly higher total phenolic content was found in biscuits with sprouts than in biscuits without sprouts (control biscuit). This increase can be the result of using supplemental sprouts as a good source of total phenolics. Moreover, some processes inside the matrix, such as the Maillard reaction, can also increase the content of total phenols. Maillard-type reaction products are involved to some extent in the estimation of phenolic compounds (Samaras et al., 2005). This is due to the heat-induced products (reductones and melanoidins) from the Maillard reaction, but also polyphenolic oxidation products and caramelization products can influence/affect the TPC estimation. The formation of compounds resulting from the Maillard reaction after heat treatment is reported as a possible contribution to the increased TPC (Holtekjølen et al., 2008).
(Table 4).

Table 4. The content of phenolic, alpha-tocopherol and antioxidative activity of biscuit

	Total phenolic mg GAE/100 g dm	DPPH % inhibition	DPPH µmol TE/100 g dm	ABTS %inhibition	ABTS µmol TE/100 g dm	alpha-tocopherol mg/kg
Control	110±11.88c	9.97±0.59d	59.1±4.92d	16.05±0.30d	411±9.4d	32.46±0.21b
2.5 % sprouts	180±11.44b	16.28±1.65c	117.8±16.25c	24.40±1.37c	648±39.02c	33.74±0.5a
5% sprouts	235.9±5.73a	25.86±1.21b	214.8±11.96b	37.50±2.50b	1035±72.22b	34.56±0.16a
7.5% sprouts	245.5±18.14a	39.60±1.92a	342.5±18.45a	47.50±2.11a	1325±65.28a	34.07±0.1a

a - d Means of triplicate determination, except for alpha-tocopherol (duplicate determination). Means with the same superscripts within the column are not significantly different ($p>0.01$). Means without the same superscripts within each column are significantly different ($p<0.01$)

Antioxidant activity of biscuits

The biscuits produced following the formulation given in Table 1 – control biscuits and those made with 2.5%, 5% and 7.5% of the T-500 wheat flour substituted with wheat sprouts – were tested for their radical scavenging activity using the DPPH test. The results of DPPH• radical scavenging activity, expressed as % inhibition, or µmol TE, obtained after the storage of biscuits (48 h), are shown in Table 4. The substitution of type -500 wheat flour with sprouts resulted in increased antioxidant activity of biscuits i.e. increased % inhibition. The obtained values depended on the amount of substitution sprouts; as their amount increased, % inhibition also increased. The increase in the antioxidant activity of biscuits with wheat sprouts could be attributed to the significantly higher total phenolic content in wheat sprouts in comparison with wheat flour. Biscuits prepared with apple, wheat fibers and wheat bran as substitutes for wheat flour also showed significant increases in antioxidant capacity (Bilgiçli et al., 2007).

DPPH radical scavenging activity was higher in biscuits with sprouts than in control biscuits (Table 4), as the result of a higher polyphenol content in wheat sprouts. There was a high coefficient of correlation ($R^2 = 0.87$) between TPC and DPPH (% inhibition) radical scavenging activity of samples. Reduction of DPPH radicals revealed that the examined extracts possess radical inhibitors or scavengers with the potential to act as primary antioxidants. They might react with free radicals, in the first place with peroxy radicals, which are the major propagators of the auto-oxidation chain of fat (Sharma and Gujral, 2014). Also, there was a high correlation ($R^2 = 0.915$) between TPC and ABTS (% inhibition), and the results showed that supplemental/substitutional sprouts can affect the antioxidant activity (ABTS assay). The antioxidant activity of bakery products can be modified by active oxidative enzymes present in ingredients of compounds used in bakery production, or oxidized by ambient oxygen (Chlopicka at al., 2012). Germination of cereals/pseudocereals has been suggested as an effective method to increase antioxidant compounds (Zhou et al., 2011; Xu et al., 2014). However, this process can also affect the Maillard reaction in products (Abderrahim et al., 2012). The total antioxidant potential is a benefit not only from polyphenolic compounds, but is also attributed to the Maillard reaction products. The polyphenols in biscuits come from natural ingredients (especially sprouts) used for their preparation. Maillard compounds are formed during the baking process. Maillard compounds, particularly melanoidins, have been shown to have antioxidant effects in vitro. Various studies demonstrated the high antioxidant capacity of melanoidins, which act through a

chain breaking, oxygen scavenging, free radical direct scavenging, metal chelating mechanism (Borelli et al., 2003). Products of the Maillard reaction are strongly correlated with the content of compounds which possess antioxidant capacity (Manzocco et al., 2001; El-Massry et al., 2003; Jing and Kitts, 2000). The creation and accumulation of melanoidins, the Maillard reaction products, having various degrees of antioxidant activity, may contribute to the increase in the antioxidant capacity (Que et al., 2008; Miranda et al., 2009). There are studies reporting increasing levels of correlation between antioxidant activity and total phenolic content during dehydration (Deepa et al., 2007). The Maillard reaction often occurs along with other reactions and processes. The increasing of antioxidant activity could be the result of synergistic interactions between phenols and compounds such as carbohydrates and proteins in samples (López-Perea et al., 2019; Xu et al., 2019). The presence of a large amount of sugar also increases antioxidant activity, since soluble carbohydrates can act synergistically with phenolic compounds and increase antioxidant activity (Xu et al., 2019). Earlier research showed that antioxidant activity in bakery products depends on manufacturing conditions such as baking temperatures, the formation of indigestible complexes with bread proteins or starch and recipes (Dziki et al., 2014). Baking affected the antioxidant capacity of the samples due to water soluble antioxidants (Horszwald et al., 2010). Processing such as baking (Somoza, 2005; Sharma and Gujral, 2014) and microwave roasting (Baba et al., 2014) increase antioxidant activity. This can be explained by the formation of brown pigments, melanoidins, the products of the Maillard reaction, which occurs during baking (Manzocco et al., 2000; Xu and Chang, 2008; Chauhan et al., 2015; Hussain et al., 2019). The products of the Maillard reaction have higher antioxidant activity compared with their precursors (Nicoli et al., 1997; Sharma and Gujral, 2014; Jan et al., 2015). The Maillard reaction products can act as antioxidants and scavenge free radicals (Jing and Kitts, 2000; El-Massry et al., 2003), which contribute to better antioxidant activity of the bakery product. This can explain the results of this study: biscuits with the maximum level of wheat flour substitution (7.5%) were dark in color, as the result of the formation of brown pigments, melanoidins, and had the highest antioxidant potential, as measured by DPPH and ABTS assay (Table 4). Melanoidins are able to scavenge peroxyl, hydroxyl, ABTS+ (2,2’-azobis-3-ethylbenzothiazoline-6-sulphonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals, to chelate metal ions (Yoshimura et al., 1997; Morales and

Jimenez-Perez, 2001; Bersuder et al., 2001; Del Castillo et al., 2007; Michalska et al., 2008), and have the ability to prevent oxidative stress in cellular cell lines (Horszwald et al., 2010).

The content of alpha-tocopherol

The content of alpha-tocopherol in wheat sprouts was 60.05 mg/kg (Table 2), which was significantly higher than the content in wheat flour – 10 mg/kg (Hejtmánková et al., 2010). The percent substitution of wheat flour with wheat sprout powder led to an increase in the level of alpha-tocopherol in biscuits. There was no statistically significant difference between biscuits with 2.5, 5 and 7.5% wheat sprouts ($p<0.01$). Biscuits with 5% sprouts had 34.56 mg/kg compared with control biscuits, which contained 32.46 mg/kg. Pasias et al. (2018) found that the addition of different ingredients (oats, nuts, chocolate, whole grains with fruits, vanilla, sesame etc.) in bread, biscuits, breadsticks and sweet buns can affect the content of alpha-tocopherol in bakery products. Leenhardt et al. (2006) and Siro et al. (2006) reported that temperature during biscuit making did not induce a great loss of vitamin E. Conversely, there are some investigations which suggest that tocol losses during bread making could be attributed to direct oxygenation during dough making and heat destruction during baking (Leenhardt et al., 2006). Dough making resulted in 20 to 60% reduction in the content of tocopherols and tocotrienols (Wennermark and Jagerstad, 1992). This can be due to the incorporation of oxygen in the dough, which makes oxidation of unesterified polyunsaturated fatty acids easier. The end results are lipid oxidation and destruction of vitamin E (Nanditha et al., 2009). This paper did not examine the content of alpha-tocopherol in the biscuit dough before baking. Therefore, we cannot argue if baking had an effect or not, i.e. if the content of alpha-tocopherol increased or decreased. But, the comparison between biscuits with and without sprouts showed a significantly higher content of alpha-tocopherol in biscuits with sprouts than in biscuits without sprouts (control biscuits).

According to the nutrient reference intake, vitamin E can be declared on foodstuffs. This is defined by the Regulation (European Union, EU) No. 1169/2011 of the European Parliament and of the Council on the provision of food information to consumers. For vitamin E, this Regulation considers foodstuffs containing 15% of the reference daily intake. As specified by the Commission Regulation (EU) No. 432/2012, the claim: “Vitamin E contributes to the protection of cells from oxidative stress” can be used only for foodstuffs containing at least a significant amount of Vitamin

E –18 mg/kg (which is 15% of the reference daily intake)**.** In all sampled biscuits, the content of alpha-tocopherol higher than 18 mg/kg (ranging from 32.46 to 34.56), which is nearly 28% of the daily value.

Microbiological evaluation of biscuits

The total counts of bacteria and molds did not change much during the periods of investigation (from 2 to 60 days). At 60 days of storage, the bacterial count was low, and the highest count was in samples with 7.5% sprouts, i.e. below 4·10 cfu/g. In all samples, the mold count was lower than 10 cfu/g (Table 5).

Table 5. Microbial evaluation (the number of total count of bacteria and molds) in biscuit

	Bacteria (cfu/g)			Molds (cfu/g)		
Biscuit	2days	30 days	60 days	2 days	30 days	60 days
Control	<10	10	10	<10	<10	<10
2.5% sprouts	<10	10	10	<10	<10	<10
5% sprouts	<10	<10	<10	<10	<10	<10
7.5% sprouts	<10	<10	<40	<10	<10	<10

This range is acceptable compared to the standard (1,000 cfu/g for molds, and 10,000 cfu/g for bacteria), and all samples in this study were within the microbial limit standard. *Escherichia coli*, *Salmonella* spp. and sulfite-reduction clostridia were not detected in any sample. Microorganisms play a significant role in the determination of the shelf life of food products, and they are responsible for food spoilage. These results indicate that biscuits were prepared under good hygienic conditions, using good manufacturing practices. Improper preparation, handling and storage can cause a significant increase in microbial numbers. There was no contamination after production and during storage.

CONCLUSION

The prepared biscuits were rich in protein, carbohydrates, mineral elements and energy. The results obtained indicated that the substitution of wheat flour with wheat sprout powder in biscuits increased their antioxidant activity, i.e. functional characteristics. The substitution of wheat flour with wheat sprout powder is a good way to obtain products with higher nutritional value and good antioxidant properties. The analysis of variance showed that the total contents of bioactive components (phenolics, alpha-tocopherol) and antioxidant activity were significantly different between control biscuits and biscuits with sprouts. There was no statistically significant difference ($p<0.01$) in the content of total phenolics and alpha-tocopherol between biscuits supplemented with 5% sprouts and those supplemented with 7.5% sprouts. Antioxidant activity was the highest in biscuits with 7.5% sprouts. The total antioxidant potential is a benefit not only from polyphenolic compounds, but is also attributed to the products of the Maillard reaction. Biscuits with 2.5% and 5% sprouts had a pleasant taste and aroma, although the taste of biscuits with 7.5% sprouts was less acceptable for consumers. Microbial counts of control biscuits were the same as those of biscuits substituted with sprouts; therefore, their shelf-life was exactly the same. Biscuits produced with and without wheat sprouts had a low microbial load, were microbiologically safe and therefore had a long shelf life under proper storage conditions. The results of the microbiological analysis can be attributed to the good quality of the raw materials used for the preparation of biscuits, the effect of the heat treatment, as well as good manufacturing practices applied during making, handling and storage of products.

The Authors declare that there is no conflict of interest.

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