EXTRACTION OF PHENOLIC COMPOUNDS FROM AGRO-INDUSTRIAL WASTES AND EVALUATION OF THEIR ANTIOXIDATIVE POTENTIAL

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Abstract

Constant world population growth imposes the need for the production of more food, and as a consequence, has an increase in the organic-rich wastes. These agro-industrial wastes represent a problem from the environmental viewpoint, and their utilization as the raw materials for the production of value-added products has become one of the main topics of the biorefinery. The aim of this research was to use soybean meal, co-product of dietary oil production, as a model agro-industrial by-product for optimization of phenolic compounds extraction. In this paper, ethanol influenced microwave-assisted extraction is chosen since it is recognized as eco-friendly and food-safe.

Optimization of the extraction included finding the optimal liquid/solid ratio, extraction duration, and microwave power. The same method was applied for the extraction of phenolics from apple pomace, apple pomace mixed with soybean meal, ultrasound-treated soybean meal, and wheat bran. Antioxidative potential of all extracts was evaluated using a fixed reaction time 2,2-diphenyl-1-picrylhydrazyl (DPPH) method as well as a steady-state measurement DPPH method. 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) i.e., ABTS and ferric reducing antioxidant power (FRAP) methods were applied as well.

Under optimized conditions (liquid/solid ratio of 15 mL/g for 70 s at a microwave power of 300 W) 3.87 mg gallic acid equivalent (GAE)/g was extracted from soybean meal, which represented a substantial increase compared to 2.46 mg GAE/g obtained before optimization. Results showed that extracts had different behavior towards different radicals depending on the type of the extracted phenolics. Apple pomace extract was the most potent towards DPPH• radical, while the most effective extract towards ABTS•+ radical was obtained from apple pomace and soybean meal mixture. The soybean meal extract showed the highest reducing power assessed via the FRAP method.

Results obtained in this study show that optimization of the extraction enabled a substantial increase in the extracted phenolics and that the method could be successfully applied with other agro-industrial by-products providing extracts with antioxidative potential.

Key words: Microwave extraction, Eco-friendly, Agro-industrial by-products, Phenolic compounds, Radical-scavenging activity, Ferric reducing antioxidant power.

1. Introduction

Secondary metabolites such as alkaloids, mycotoxins, pigments, plant growth factors and phenolic acids are produced in little amounts and are systematized as the nutritive constituents in the plants. These bioactive compounds have been found to reveal a multiplicity of beneficial effects on the human health. The anti-mutagenic, antioxidant, anti-microbial, anti-inflammatory, anti-allergic effect of these compounds has been confirmed in past few years [1]. Among plants secondary metabolites, bioactive phenolic compounds possess the most abundant interest due to their different functions in the plants, including assimilation of nutrients, protein synthesis, enzyme activity, photosynthesis, cell signaling and protection against adverse environmental conditions. How phenolic compounds are present in all plant organs, they are inevitable in human nutrition [2].
Consequently, the researchers strive for finding plants, fruits, vegetables and agro-industrial residues as rich sources of the bioactive phenolic compounds.

Solid-liquid extraction of the bioactive compounds with organic solvents along with water extraction is an important step in the production of phytochemicals as additives for formulations of dietary supplements, food or feed ingredients and pharmaceutical products, and a growing trend today towards the extraction was observed. The main propose is to find much more efficient method, more economically accepted, and environment safest in terms of the yield of these bioactive compounds [1]. Extraction yield depends on the type of solvents (polarity), extraction time and temperature, liquid/solid ratio, as well as the chemical composition and physical characteristics of the origin plant samples. The plant material may contain the phenolic compounds ranging from simple (e.g., phenolic acids, anthocyanins) to highly polymerized substances (e.g., tannins) in different quantities. Thus, there is no uniform procedure convenient for phenolic extraction from plant materials [3], and the scientific research is still focused on finding the most suitable extraction methods. There are strives to find and apply the innovative eco-friendly technologies for processing plant materials such as supercritical fluid extraction, subcritical water treatment, enzyme-assisted subcritical water treatment, ultrasound-assisted extraction, microwave-assisted extraction and others. Microwave-assisted extraction (MAE) technology is a green extraction technique that provides numerous benefits such as the reduction of the extraction time and solvent consumption, the possibility of simultaneously extracting multiple samples, drastically improving sample throughput. MAE technology downsizes the time required for the extraction of polyphenols from plant material due to microwave energy is efficiently transmitted to the origin material through molecular interaction with the electromagnetic field. Namely, MAE merges high temperature and pressure for optimum release of the phenolic compounds while simultaneously destroying the cell wall and releasing of the components into a solvent [5]. Thus, MAE technology efficiently extracts compounds of interest associated to plant materials and is one of the preferred methods.

On the other hand, in the world the growing knowledge of consumers to issues concerning food additive safety culminates in an augmented attempt in finding alternative additives and preservatives from natural and probably safer sources. Food manufacturers are encouraged to use natural antioxidants instead of synthetic compounds to maintain the nutritional values of their products. So, the commercial antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) can be replaced by plant extracts particularly polyphenols obtained from agro-industrial by-products. Application of bioactive extract from agro-industrial by-products as a constituent of food supplements and drugs is a new, perspective and modern approach [6]. The potential application of soybean meal, apple pomace and wheat bran as sources of bioactive compounds, particular antioxidant polyphenols, continue to attract a lot of scientific attention and offers up new possibilities. With reference to data, the soybean meal represents 70% of the residue after the extraction of oil from soybeans, while the production of apple juice yields 25% of waste, apple pomace posing a great risk if disposed of directly in the environment [7, 8 and 9].

Also, the soybean meal, apple pomace and wheat bran have a diverse nutritional profile [7, 8 and 9], which enable easy shifting from waste into the edible and value-added products. Thus, the valorization of these agro-industrial wastes by extraction of the valuable compounds is still justified and offers possibilities for transforming by-products into industrial commodities. In details, the overall global apple pomace, generated residues of apples, production is exceeded 3,600 tons per year. It is mostly consisted of skin and flesh (95%), seeds (2 to 4%), and stems (1%). Apple pomace is an abundant source of digestible fiber, pectin, and phenolic compounds, and researchers reported that the composition of apple pomace is: 9.0% moisture, 2.27% fat, 2.37% protein, 1.6% ash, 84.7% carbohydrate, 5.6% starch, 54.2% total sugar, and high quantities of calcium, potassium, and magnesium [7]. Another agro-culture product, wheat bran, is cheap lignocellulosic biomass that represents the main by-product of wheat flour production and hence it is abundant in the world. Exactly, a by-product stream accounted for about 150 million tons of bran per year. Wheat bran comprises approximately 12% of water, 13-18% of protein, 3.5% of fat and 56% of carbohydrates, and contained large amount of phenolic compounds [8]. Production of soybean meal is by far the most important due to its reached > 60% of the total global production of meals (more than 200 million metric ton per year). Soybean meal is a co-product after oil extraction from dehulled soybean seeds, and the defatted meal consists of 10.7% moisture, 47.5% protein, 0.5% fat, 3.5% fibre, and 6.0% ash [9, 10]. Literature data reported that soybeans may contain isoflavones, anthocyanins, phytic acid, saponins, phenolic acids, hydroxybenzoic and hydroxycinnamic acids, isoflavonoids and isoflavones [11].

According to the mentioned nutritional value and abundant content of phenolic acid, in this research the extraction process was optimized, and the presence of the bioactive compounds and its biological activities such as antioxidant were evaluated. Evaluation of the efficiency of several extraction parameters to extract phenolic compounds from soybean meal,
apple pomace, wheat bran and blend composed with soybean meal and apple pomace have been done. Thereafter, the quality of the extracts was monitored through determination of total polyphenol content, as well as evaluating the DPPH• and ABTS•+ radical-scavenging activities and ferric reducing antioxidant power.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Gallic acid (trihydroxybenzoic acid), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-s-triazine (TPTZ) and Folin-Ciocalteu's reagent were all purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents were of analytical grade.

2.1.2 Sample materials

Soybean meal was purchased from Soja Protein, Bečej, Serbia. Ultrasound treatment of 10 g soybean meal, soaked in 50 mL of distilled water, was performed using ultrasound probe (Bandelin sonoplus ultrasonic homogenizer HD 2200, SG 213G horn, TT 19 probe tip) at 35% of amplitude for 5 min. The raw wheat bran was obtained from local manufacturer Vega (Čenta, Serbia) and the apple pomace was prepared after homemade pressing apples. The all substrates were dried to constant weight in the vacuum oven (Vacuum oven VD23, Binder, Tuttingen, Germany) at 60 °C, grinded utilizing a ball mill (Mixer Mill MM 400, Retsch, Haan, Germany) and sieved to obtain substrates with particle sizes between 200 and 800 µm (200 mm stainless steel sieve with 200 and 800 µm openings; Gilson Company, INC, United States).

2.2 Extraction process of phenolic compounds

The extraction process of phenolic compounds with previously reported solvent 20% aqueous ethanol [12] was performed by the fast microwave-assisted extraction using a household microwave oven (LG MC7849HS). The first step was the optimization of microwave-assisted extraction process utilizing a soybean meal, as the most potent agro-industrial waste in polyphenol content, so three different parameters were examined in order to optimize extraction: 1) liquid/solid ratio (9 to 20 mL/g), 2) extraction duration (35 to 70 s), and 3) microwave power (160 to 400 W). Batch experiments of extraction were performed in 100 mL Erlenmeyer flasks and the liquid and solid phase were separated using a centrifuge (Sigma 2-16P, Sigma, Osterode am Harz, Germany) at 8,000 rpm for 3 min. Phenolic compounds in supernatant labelled as extracts are present in the soluble form i.e. free phenols. The percentage of dry matter in the extracts was measured on a moisture analyzer (Kern MLS-A, Balingen, Germany). All extracts were prepared in triplicate.

2.3 Analytical methods

2.3.1 Determination of total polyphenol content

The determination of the total polyphenol content in prepared extracts was analyzed spectrophotometrically according to Folin-Ciocalteu's method with small changes [12]. Briefly, 0.050 mL of the extract sample was oxidized with 0.25 mL Folin–Ciocalteu’s reagent, and the reaction was neutralized with 1.0 mL of 15% Na2CO3 and 3.7 mL of distilled water. This mixture was kept in dark at room temperature for 2 h and afterwards absorbance was read at 750 nm with blank which was prepared in the same way only with distilled water instead extract sample. The results were expressed as gallic acid equivalents (GAE) per gram of extract dry matter through the calibration curve of gallic acid (0 to 1.6 mg/mL).
2.3.2 Determination of DPPH radical scavenging activity

The estimation of antiradical properties of the extract samples was conducted using a DPPH assay through two different methods: conventional, measured at a fixed reaction time [13], and steady-state measurement as described by Mishra et al. [14], both with minor changes. Namely, 0.18 mL of 0.1 mM DPPH methanolic solution was mixed with 0.02 mL of appropriately diluted extract (1–5 mg/mL) and decrease in absorbance was monitored at 517 nm using a microplate spectrophotometer (Multiskan™ GO, Thermo Scientific™, Waltham, USA). The all tested extracts reached at steady state after 60 to 80 min. Analysis conducted using fixed reaction time was done according to the same experimental procedure, but inhibition of DPPH radical was measured only at one time, 30 min. A blank was prepared by using the pure methanol and 0.02 mL of diluted extract. A control sample was prepared in the same way with except that 0.02 mL of pure solvent was taken instead of sample. The results were expressed as Trolox equivalents (TE) per gram of extract dry matter (μmol TE/g d.m.) through the standard curve of Trolox reagent, water-soluble analogue of vitamin E (0 to 0.2 mg/mL). Besides, collected results are calculated as an effective concentration, \( EC_{50} \) as well as an inhibition concentration, \( IC_{50} \).

2.3.3 Determination of ABTS⁺ radical scavenging activity

The second method which was used to estimate the antiradical properties of extract samples was ABTS⁺ radical scavenging assay. Determination of ABTS⁺ radical scavenging activity was performed following the method of Jovanović et al. [13], by utilizing the appropriately diluted extracts (1–5 mg/mL). The results were expressed as Trolox equivalents (TE) per gram of extract dry matter (μmol TE/g d.m.) through the standard curve of Trolox reagent, water-soluble analogue of vitamin E (0 to 0.2 mg/mL), as well as an inhibition concentration, \( IC_{50} \), of extract needed to achieve inhibition of 50% of the radical.

2.3.4 Determination of ferric reducing antioxidant power

The reducing capacity of extract sample was determined by ferric reducing antioxidant power i.e. FRAP method described earlier with slight modifications [13]. Analysis was conducted following the next procedure: 0.03 mL of extract (1–5 mg/mL) was mixed with 0.9 mL of FRAP solution which consists of 10 volumes acetate buffer (0.3 M, pH 3.6), 1 volume TPTZ reagent (10 mM solution dissolved in 40 mM HCl) and 1 volume ferric chloride (20 mM solution). The results were expressed as the Trolox equivalents (TE) per g of dry matter of extract.

2.4 Statistical analysis

The experimental results included three replications. The results were presented as mean ± standard deviation. Statistical differences were determined by one-way analysis of variance (ANOVA). Tukey test was applied as a test of posterior with a level of significance of 95%. All the tests were considered statistically significantly at p<0.05. Statistical analyses including calculations were performed using OriginPro 9.0 software package (Origin Lab Corp., Mass. USA).

3. Results and Discussion

3.1 Optimization of phenolic compounds extraction from agro-industrial wastes

The phenolic compounds exhibit various biological activities such as antioxidant, antinutagenic and anti-inflammatory. Soybean and its by-products are a well-known source of isoflavones, the main compounds from the group of flavonoids, which are most often mentioned for their numerous positive effects in the prevention and treatment of cardiovascular diseases, obesity, osteoporosis and cancer [15]. It is proved that agricultural by-product, such as soybean meal, what bran and apple pomace, represent the abundant sources of valuable food molecules: fermentable sugars, proteins and phenolic compounds [11, 15, 16, and 17]. Extraction of these bioactive phenolic compounds from agricultural by-products represents an alternative origin for obtaining natural antioxidants, which are considered completely safe in comparison with synthetic ones. In addition, the extraction of polyphenols enables the improvement of its availability and consequently the disclosure of biological activity [12]. Due to low toxicity, ethanol has been proved as most efficient solvent for the extraction of bioactive phenolic compounds from different plants material. In order to improve the extraction efficiency of polyphenols from soybeans, ethanol is mainly used with water in various concentrations due to its high dielectric properties and an aqueous ethanol evinces a better effect compared to mono-component solvents. Also, the extraction with aqueous ethanol is recognized as eco-friendly and safe method [2, 18]. The extraction process can be accelerated utilizing of microwaves or ultrasound waves, such as demonstrated in our previously study during the extraction of natural antioxidants from yellow soybean [2].

According mentioned facts, the first purpose of this research was to optimize the extraction process...
of phenolic compounds by used eco-friendly and safety aqueous ethanol method. Optimization was performed by empirical method and the relationship of the total polyphenol content and that of liquid/solid ratio, extraction time and microwave power, are graphically presented in Figure 1. The total phenolic content of soybean meal before optimization was found to be 2.46±0.05 mg GAE/g d.m., which was in a good agreement with the results obtained in several studies [2, 9, and 18]. On the other hand, Malenčić et al., [19], reported much higher values for varieties of black and brown soybean seeds, ranging from 4.94 to 6.22 mg GAE/g d.m. The discrepancies in the obtained results can be attributed to the solid sample varieties and different extraction solvent.

It's known that the liquid/solid ratio is relevant parameter in the extraction process, since the diffusion mechanism relies on the difference in concentrations between solid sample and the solvent, as well as that the amount of collected substrates depends on the amount of solvent [18]. The ratio of ethanol and soybean meal sample was varied from 9:1 to 20:1 (Figure 1a), so that by increasing the volume of ethanol up to 15 mL, the phenolic content significantly increases reaching the peak of 2.63±0.05 mg GAE/g d.m., (p<0.05). The observed release of phenolic compounds after increasing liquid/solid ratio is associated with the difference of concentration gradient and establishing the diffusion before equilibrium was reached. After establishing equilibrium state, the addition of ethanol (20:1 ratio) didn't promote polyphenols extraction, and can be headlined that the amount of extracted compounds was depleted. Accordingly, the liquid/solid ratio of 15:1 is more abundant and economical and is chosen for subsequent optimization step. Figure 1b presents the effect of extraction duration on the efficiency of polyphenols extraction and it is clearly that the examined time was positively correlated with the total polyphenol content. Namely, with prolonging the time from 35 to 70 s, the total polyphenol content notes by a steady rise from 2.46±0.05 to 3.41±0.09 mg GAE/g d.m. In this case, the equilibrium state between soybean meal and ethanol was not achieved, but the prolonging the time period past a 70 s had a higher thermal effect on the extract sample, which was undesirable in this research because the ethanol was evaporated. Therefore, 70 s is selected as the most suitable extraction time. In the last step, the effect of microwave on the extraction was analyzed through the examining the influence of oven microwave power from 160 to 450 W (Figure 1c). The results from Figure 1c indicate that total polyphenol content from soybean meal at 160 W of microwave power was 3.41±0.09 mg GAE/g d.m. Further increase of microwave power to 300 W enhanced the yield of polyphenol recovery and the yield peaked at 3.87±0.10 mg GAE/g d.m. However, raising the power past 300 to 450 W decreased the total polyphenol content to 3.24±0.08 mg GAE/g d.m. This decrease may be due to the thermal destruction of polyphenols at high temperature caused by the higher microwave power 450 W. Previous studies found that the interaction between microwave power and extraction time significantly affected extraction efficiency of flavonoids, but didn't have positive effect on the extraction of proanthocyanidins from plants [20, 21]. In general, it can be noted that by varying the extraction conditions the total polyphenol content was increased by almost for 60%, indicating that the examined process parameters exerted great influence on the extraction of phenolic compounds from soybean meal. So, the maximum extractability of total polyphenol content may be obtained by applying of 20% ethanol, a liquid/solid ratio of 15 mL/g, an extraction time of 70 s and a microwave power of 300 W.

Validation of MAE method under the optimized parameters was conducted using the several others agro-industrial wastes wheat bran, apple pomace and blend of apple pomace and soybean meal (Figure 2). The total polyphenol content of wheat bran was found to be 1.82±0.27 mg GAE/g d.m., which is in excellent concurrence with the literature data [16, 22]. Contrary, Zhao et al., [23], reported much greater content of phenolic compounds, so noted disagreements only could be attributed to the wheat varieties and various extraction parameters. Zhou and Yu, [24], reported that among the solvents tested, 50% acetone extracts contained a higher level of total phenolics from wheat, whereas ethanol was the least effective solvent. By using fast microwave extraction, from apple pomace was extracted 2.25±0.08 mg GAE/g d.m. of phenolic components. In comparison with other studies, certain polyphenol content was identical to the content obtained after aqueous extraction of apple pomace conducted by Reis et al., [25]. The release of phenolic compounds from apple pomace during extraction with 50% acetone was great, and the achieved value of total polyphenols was found to be 3.31 mg GAE/g [17]. In the study carried out by Ajila et al., [26], 80% ethanol and 80% methanol were found to be more efficient than water for extracting total phenolics from apple pomace and the polyphenol content of the apple pomace extracts was found to be in the range of 5.78–16.12 mg GAE/g d.m. of samples, depending on the solvent, extraction time and temperature, much greater than results presented in Figure 2. The polyphenol content in the blend of apple pomace and soybean meal also was determined and it was found that the polyphenol content increased from 2.25±0.08 to 2.55±0.12 mg GAE/g d.m. and significantly depended solely on the addition of soybean meal (p<0.05). Comprehensively, the results obtained in this section of research show that the optimized MAE could be successfully applied with all analyzed agro-industrial wastes, providing extracts with an abundant amount of polyphenols.
3.2 Antioxidant capacity of extracts from different agro-industrial wastes

Antioxidants are responsible in preventing oxidative damages to the cellular components as a consequence of biochemical reactions. Some phenolics and flavonoids appeared to be more active than vitamins for this purpose and their activities depend on the structure and total number of hydroxyl groups [6]. Thus, the antioxidant capacity of extracts from different agro-industrial wastes was evaluated applying three assays, scavenging activity DPPH• and ABTS++ radicals and ferric reducing antioxidant power. Although the DPPH• method is one of the most commonly used methods for determining the antioxidant activity of phenolic compounds, it is generally performed by measurement after 30 min, which for some compounds is not enough time to reach their maximum antioxidant potential. Therefore, two DPPH methods were used in this research, namely the steady state method, where the result is expressed as the EC50 value, i.e. the concentration of extract that is necessary to achieve
50% of the total antioxidant effect of a compound, and the fixed time method where the result is expressed as IC$_{50}$ value, i.e. the concentration of the extract that after 30 min leads to inhibition of 50% DPPH$_{•}$ radicals. In the case that compounds react rapidly with the DPPH$_{•}$ radical, these values are equal. The compounds with fast kinetics reach a steady state in less than 30 min, a medium between 30 min and 1 hour, while those with slow kinetics need more than 1 h to reach steady [14].

The present results (Figure 3) revealed that there is a significant difference in the kinetic behavior of disappearance of DPPH$_{•}$ radical in presence of different extracts of the same concentration, obtained from four various agro-industrial wastes varied significantly. It’s evident that the time required achieving maximum scavenging of DPPH$_{•}$ radical differs depending on the concentration and type of antioxidant extracts. Specifically, these antioxidant extracts take more than 30 min, as suggested in the assay with a fixed time, to achieve their maximum potential in scavenging DPPH$_{•}$ radical. The steady state of all obtained extracts at used concentrations was established only after 60 min. So, based on the mentioned classification, all extracts can be classified as the ones with slow kinetics as shown in Figure 3. This is to some extent expected because most of the phenolic compounds found in agro-industrial by-products are classified as intermediate or slow kinetic antioxidants. To compare the kinetics, we determined the EC$_{50}$ and IC$_{50}$ values for Trolox and found that it follows the fast kinetics in reaction with the DPPH$_{•}$ radical, and therefore the EC$_{50}$ and IC$_{50}$ are equal. Namely, it was found to be 0.05 mg/mL for EC$_{50}$ and IC$_{50}$ values (Table 1). In comparison with Trolox, the all extracts do not show enviable potential. The EC$_{50}$ and IC$_{50}$ values for wheat bran extract were 6.42±0.08 and 7.02±0.13 mg/mL respectively, which was in concordance with the results from literature data which showed that wheat bran has an IC$_{50}$ value of 5.25 mg/mL [27]. The IC$_{50}$ value (466.3 μg/mL) of methanol extract from soybean meal in DPPH scavenging activity [6] was even four times smaller than values reported in our study (17.90 ± 0.14 mg/mL). Antioxidant activity of polyphenol extracts from apple pomace also was tested using DPPH method.

![Figure 3. Kinetic behaviour of the extracts obtained from: a) soybean meal, b) apple pomace, c) apple pomace and soybean meal, and d) wheat bran, after applying MAE technology.](image-url)
and the IC_{50} were ranged from 12.24 to 23.42 μg/g d.w. depending on the extraction conditions [26], and antioxidant potential is in disagreement with results from Table 1. These disagreements can be attributed to the different solvents used for extraction and dissimilar process parameters, as well as the species and varieties of soybean meal, apple pomace and wheat bran. The lowest IC_{50} value (13.33 mg/mL) was detected in the extract of raw soybean meal [9], which is also in disagreement with the results from this study.

Besides, the DPPH scavenging activity pattern coincides completely with the phenol content in extracts after applying extraction process, but there are significant differences among the results of antioxidant activity (p<0.05), whereby the best activities are achieved by extracting phenolic from soybean meal (Table 1). The soybean meal extract obtained by MAE showed the highest value of both antioxidant activity (0.044±0.008 μmol TE/g d.m.) and total polyphenol content (3.87±0.10 mg GAE/g d.m.), which is due to the higher content of phenolic acids in this extract, whereas the extract obtained by extracting from blend of apple pomace and soybean meal showed significantly higher values of antioxidant activity (0.049±0.006 μmol TE/g d.m.) and total polyphenol content (2.55±0.12 mg GAE/g d.m.) as compared to extracts prepared only from apple pomace (p<0.05). It’s noticeable that apple pomace extract has much lower antioxidant potential compared to other three extracts.

The antioxidant activity of all prepared extracts was assessed also by their potential to scavenge ABTS⁺ radical cation and the results expressed through IC_{50} values are summarized in the Table 2. On first sight, the results are very similar to the ones obtained by the previously discussed DPPH scavenging activity, but differences were noted. The most abundant ABTS scavenging activity is evident for extract from blend of apple pomace and soybean meal, with IC_{50} value 4.08±0.12 mg/mL and 0.511±0.010 μmol TE/g d.m., and extract from soybean meal with IC_{50} value 5.13±0.17 mg/mL and 0.429±0.009 μmol TE/g d.m. Considering to the results from Table 1 and Table 2, it’s possible to see the higher values of Trolox equivalents in blend extract compared to apple pomace extract, while the situation is reversed in ABTS⁺ scavenging activity. From this it can be concluded that in the apple pomace there are polyphenols better soluble in methanol, which is used as a solvent for DPPH radical activity. Also, for all extracts a much higher antioxidant activity of extracts assayed by the ABTS⁺ method compared to the DPPH method was observed. These discrepancies in the extract could be ascribed to the differences in solubility of the obtained extracted polyphenols since different solvents are used. In addition, various mechanisms are attributed to the radical scavenging of these radicals. Namely, although the quenching of both DPPH and ABTS⁺ radicals takes place via electron or hydrogen transfer mechanisms, electron transfer is dominant in the reactions between phenol and ABTS⁺. On the other hand, various factors like (a) stereo-selectivity of the radicals, (b) solubility of the extract in different testing systems, (c) polarity of the solvent, (d) functional groups have been reported to affect the capacity of extracts to react and quench different radicals [28]. The IC_{50} value obtained for the wheat bran

<table>
<thead>
<tr>
<th>Extracts from agro-industrial wastes</th>
<th>(EC_{50}) of DPPH (mg/mL)</th>
<th>(IC_{50}) of DPPH (mg/mL)</th>
<th>μmol TE/g d.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean meal</td>
<td>12.18±0.08*</td>
<td>17.90±0.14*</td>
<td>0.044±0.008*</td>
</tr>
<tr>
<td>Apple pomace</td>
<td>5.83±0.12d</td>
<td>7.08±0.21*</td>
<td>0.108±0.009*</td>
</tr>
<tr>
<td>Apple pomace and soybean meal</td>
<td>10.33±0.10e</td>
<td>10.48±0.26e</td>
<td>0.049±0.006e</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>6.42±0.08e</td>
<td>7.02±0.13e</td>
<td>0.057±0.005e</td>
</tr>
<tr>
<td>Control - solution of Trolox</td>
<td>0.05±0.04*</td>
<td>0.05±0.04*</td>
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<tr>
<th>Extracts from agro-industrial wastes</th>
<th>(IC_{50}) of ABTS⁺ (mg/mL)</th>
<th>ABTS⁺ (μmol TE/) g d.m.</th>
<th>FRAP (μmol TE/) g d.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean meal</td>
<td>5.13±0.17c</td>
<td>0.429±0.009e</td>
<td>0.060±0.009e</td>
</tr>
<tr>
<td>Apple pomace</td>
<td>16.47±0.15c</td>
<td>0.033±0.008e</td>
<td>0.038±0.008e</td>
</tr>
<tr>
<td>Apple pomace and soybean meal</td>
<td>4.08±0.12d</td>
<td>0.511±0.010e</td>
<td>0.056±0.009e</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>6.60±0.14b</td>
<td>0.160±0.009e</td>
<td>0.053±0.011e</td>
</tr>
<tr>
<td>Control - solution of Trolox</td>
<td>0.20±0.03c</td>
<td>/</td>
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</tr>
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</table>

Legend: * TE represents Trolox equivalent; Results are expressed as means ± standard deviation (n=3); Means with different letters in the same column are significantly different (p<0.05).
extract was found to be 6.60 ± 0.14 mg/mL. According to Zhou and Yu [24], TEAC was found to be ranged from 3.09 to 15.26 μmol TE/g for Akron wheat bran and from 2.74 to 12.04 μmol TE/g for Trego wheat bran, since another group of researchers [29] explained the lower values (0.81–12.81 μmol TE/g of defatted bran) by the fact of wheat varieties, locations, extraction methods, and the way used to prepare ABTS⁺.

In the reducing power assay, the presence of antioxidants in the extract samples would result in the reduction of Fe³⁺ to Fe²⁺ by donating an electron. The amount of complex was monitored by measuring the formation of Perl’s blue, and increasing absorbance indicates an increase the ability to reduce TPRZ-Fe (III) complex to TPTZ-Fe (II). The obtained results are given in Table 2 and it’s noticeable that the values of Trolox equivalent are in accordance with the results obtained by DPPH and ABTS methods. Besides, it was important that reducing power was also in a correlation with total polyphenol content. The ferric reducing power of soybean meal extracts was found to be 0.060±0.009 μmol TE/g d.m., and in comparison with the extracts of bend composed with apple pomace and soybean meal, and wheat bran, was not observed a statistically significant difference (p>0.05). Reducing power obtained for apple pomace extract was found to be 0.038±0.008 μmol TE/g d.m., much lower than other three extracts indicating that extracted polyphenols only have greater activity in solvent like as methanol.

Generally, it can be concluded that the use of non-conventional procedure, like as MAE with 20% ethanol can significantly improve phenolic compounds extraction from the soybean meal, apple pomace and wheat bran, concurrently to short the time of extraction (only 70 s) and reduce the energy consumption (power 300 W). The obtained extracts, in addition to the high content of polyphenols, also exhibit high antioxidant activity, both the ability to scavenge DPPH⁺ and ABTS⁺ radicals and reducing power.

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4. Conclusions

- This research has studied the utilization of soybean meal, by-products of dietary oil process, for optimization of polyphenol extraction. It was encouraging to find that MAE with ethanol was found to be rapid, effective and cheap method for obtaining natural antioxidants from soybean meal, apple pomace, blend of apple pomace and soybean meal, and wheat bran.

- Namely, the extraction of polyphenol content varied depending on the liquid/solid ratio, extraction time and microwave power, so consequently the temperature of extraction. The polyphenol content of the soybean extract was found to be in the range of 2.46–3.87 mg GAE/g d.m. of samples, depending on the varied extraction parameters. As the most appropriated conditions for efficient polyphenol extraction were adopted liquid/solid ratio of 15 mL/g, extraction time of 70 s and microwave irradiation power of 300 W. Validation experiments showed that these conditions yielded the total polyphenol contents of 3.87, 2.25, 2.55 and 1.82 mg GAE/g d.m. of soybean meal, apple pomace, blend of apple pomace and wheat bran, respectively. The results obtained in this study showed that soybean meal as an agro-industrial waste exhibited notable antioxidant activity when compared to apple pomace and wheat bran. Performed experimental analyses showed that extracted polyphenols were highly correlated to antioxidant activity. The obtained dried extract of soybean meal, apple pomace and blend of apple pomace and soybean meal exhibited most satisfactory antioxidant activity in terms of scavenging DPPH⁺ and ABTS⁺ radicals, even as ferric reducing power. Among them, apple pomace extract was the most potent towards DPPH⁺ radical, while the most effective extract towards ABTS⁺-radical was obtained from blend of apple pomace and soybean meal. By analyzing the ability to reduce ferric ion, a soybean meal extract proved to be the most efficient.

- Finally, based on obtained total phenolic content, soybean meal may be an exploitable resource for natural antioxidant production by an eco-friendly extraction procedure. Likewise, the wheat bran and apple pomace, along with the blend composed of apple pomace and soybean, have a great potential to be the substrates for extraction polyphenols. Extracts from agro-industrial by-products, abundant in the polyphenols content, may be labelled as bioactive compounds, more specifically as the antioxidants, which can be implemented as components of food and/or feed in order to increase the nutritional values or product stability, as well as to develop the new healthy products.

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