Process Optimization for the Extraction of Polyphenols from Okara

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Summary

The objective of the present investigation is to examine okara, a suitable substrate for polyphenol extraction, and to develop a feasible eco-friendly process to maximize the yield of antioxidant phenolics. Box-Behnken design (BBD) based on response surface methodology (RSM) was employed to investigate the effect of temperature (°C), solvent fraction (%) and incubation time (min) on polyphenol extraction by using MINITAB 15 software. Acetone was used as solvent to extract the phenolic compounds possessing the antioxidant properties (DPPH radical scavenging activity, reducing power, and metal chelating activity). Extraction under the optimum conditions yielded total polyphenolic content of 1.16 mg/mL, DPPH radical scavenging activity of 61.07 %, metal chelating activity of 61.20 % and better reducing power. The effective model developed for antioxidant mining from okara under mild operational conditions can be a valuable technique for soybean-based food industry.

Key words: natural antioxidants, polyphenolics, soybean, okara, response surface methodology (RSM)

Introduction

Health consciousness of consumers has led to a dynamic increase in the demand for natural antioxidants, which contribute to nutritional quality of products. The food industries are meeting the demand for antioxidants by the extraction from natural sources. Plant phenolics play a major antioxidative role in the diet. They are aromatic compounds responsible for the protection against degenerative diseases (1). Dietary antioxidants quench the free radicals generated during metabolism which are responsible for disruption of membrane fluidity, lipid peroxidation, oxidative DNA damage and alteration of platelet functions because they cause quality deterioration and nutritional loss (2). Antioxidants not only have health-related effects, but they also increase the shelf life of food.

Food industries are driven to use antioxidants in the form of food additives to maintain the market value of the product by enhancing its colour, flavour and nutritional profile. Although synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are common food additives, due to their toxicity and carcinogenicity (3) their application is questioned. Therefore, the need for natural antioxidants is rapidly increasing not only in food industry but also in medicine.

Agricultural and food waste generated during the processing is becoming the ideal substrate for extraction of antioxidant polyphenolics. Several food and agro-residues such as apple, potato and onion peels, carob pods and olive tree leaves (4), raspberry waste (5), etc. have been assessed for extraction of phenolics. Among these food processing residues, okara, a waste by-product of soybean industry, can be a potential feedstock for efficient recovery of bioactive antioxidant phytochemicals. Mateos-Aparicio et al. (6) measured the carbohydrate digestion (pectin) of okara by sequential extraction and correlated it with the antioxidant activity. Okara is

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rich in indigestible complex polysaccharides and it has not been explored yet for the extraction of polyphenolics. However, total phenolic content of okara is almost half of the soybean seeds (7), but until now okara has been exploited only as nitrogen source during fermentation, animal feed or for dietary fibre extraction.

Natural antioxidants can be extracted from plant and animal sources under effective process conditions. Extraction process differs with the raw material and is also affected by several physicochemical factors. The economic viability and feasibility of the technological process of extraction is highly dependent on the availability and cost of raw material. Another important aspect apart from the cost-effective extraction and selection of substrate is the safety concern associated with the use of organic solvents. In past decades, methanol/hexane/benzene extractions were performed, but their safety issue due to potential toxic effects from the residual solvent was a point of concern. Optimization process for the reduction of use of organic solvents would be advantageous in their application in food and pharmaceutical sector. There are several reports on the extraction of polyphenols from food waste by using several solvents (2,8,9). Among them, acetone/water is the most efficient solvent for polyphenol extraction, because water in combination with acetone contributes to the creation of a moderately polar medium that ensures the efficient extraction of polyphenols and their antioxidant activities (10). The advantage of using food grade acetone is that it reduces the persistent problem of waste solvent disposal and hazards of environmental pollution. Therefore, the present article deals with the assessment of okara, a potential low-cost feedstock for extraction of antioxidant-rich phenolics with radical scavenging, reducing power, and metal chelating activities, using food grade solvent by adopting Box-Behnken design based on the response surface methodology (RSM).

Materials and Methods

Preparation of okara

Soybeans (*Glycine max*) were purchased from a local market at Kharagpur, India. Accurately weighed clean soybeans were soaked for 2 h in a ratio of soybean to water of 1:2.48 and incubated at 40 °C in a thermostatically controlled water bath (II). Soaked soybeans were subsequently dehulled and weighed to add twice the volume of water. It was blended and extracted through cheese cloth to obtain the soy milk (12). The leftover residue after cheese extraction, okara, was dried overnight at 60 °C and grinded to powder for subsequent extraction.

Extraction of antioxidant phytochemicals from okara

A mass of 1 g of powdered okara was mixed with various fractions of solvent (acetone) and incubated under different time and temperature conditions. After the incubation, the sample was centrifuged at 2000×g for 10 min to separate the insoluble fractions, and antioxidant potential was estimated in the supernatant.

Determination of the total phenolic content in the extracts

Solvent extraction of polyphenols was performed in 50-mL stoppered conical flask containing 1 g of dried okara sample. Samples were taken from the reaction mixture at specific time interval according to the experimental design. Each sample taken from the reaction mixture was centrifuged at 2000×g for 5 min. Then polyphenolic content (mg/mL) in the supernatant was estimated by Folin-Ciocalteu method (13).

Antioxidant activity

Antioxidant activity of the phytochemicals extracted from okara was assessed by measuring their radical scavenging activity, reducing power, and metal chelating activity.

Radical scavenging activity

Hydrogen atom or electron-donating ability of the corresponding extracts was measured by the bleaching of the purple-coloured methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). This spectrophotometric assay uses stable DPPH radical as a reagent (14). To 1 mL of sample, 0.5 mL of DPPH (1 mM in methanol) was added. The control sample was prepared in a similar way by adding 1 mL of acetone instead of sample. The mixtures were shaken vigorously and left to settle for 15 min at room temperature. After the incubation period, the absorbance was read against a blank at 517 nm. Inhibition of DPPH free radical (in %) was calculated as follows:

\[
\text{Inhibition} = \left( \frac{A_0 - A_S}{A_0} \right) \times 100
\]

where \(A_0\) is the absorbance of the control, and \(A_S\) is the absorbance of the sample at 517 nm.

Reducing power

Reducing power of samples was determined according to the method of Benzie and Strain (15) by measuring the coloured ferrous-triprydyltriazine complex formed due to ferric to ferrous ion reduction at pH. To 3 mL of freshly prepared ferric reducing ability of plasma (FRAP) reagent (300 mM acetate buffer, pH=3.6, 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution and 20 mM FeCl₃·6H₂O in a 10:1:1 ratio), 0.3 mL of distilled water and 0.1 mL of extract were added. The absorbance was measured after 8 min of incubation at 593 nm. Higher the absorbance, higher the reducing power of sample.

Metal chelating activity

Ferrous ion chelating activity of the extract was determined by the method of Dinis et al. (16). Into test tubes containing 1.7 mL of distilled water and 50 µL of 0.2 mM FeCl₃·4H₂O, 50 µL of sample solution were added and the mixture was left at room temperature for 1 min. To this mixture 0.2 mL of 5 mM ferrozine were added and final colour was measured at 562 nm after 10 min of incubation. The metal chelating efficiency of samples was determined by comparing with the chelating activity of ethylenediaminetetraacetic acid (EDTA). The inhibition percentage of Fe²⁺-ferrozine complex formation against blanks containing FeCl₃ and ferrozine was calculated by the formula:

\[
\text{Inhibition} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

where \(A_0\) is the absorbance of the control, and \(A_1\) is the absorbance of the sample at 562 nm.
Box-Behnken design of experiment

A three-level three-factor fractional factorial design was adopted in this study. Initially the influencing parameters were selected by preliminary experiments on the basis of one-factor-at-a-time approach. The input variables considered to be important during the extraction process were temperature (40–60 °C), solvent fraction (25–75 %) and incubation time (15–45 min). In coded terms the lowest, central and the highest levels of five variables were –1, 0 and +1, respectively. Table 1 shows the coded and actual values of the experimental variables.

The experimental data were analyzed by the response surface regression (RSREG) procedure to fit the following second-order polynomial equation:

\[ Y = \beta_{0} + \sum_{i=1}^{5} \beta_{i} X_{i} + \sum_{i=1}^{5} \beta_{ii} X_{i}^{2} + \sum_{i=1}^{5} \sum_{j=i+1}^{5} \beta_{ij} X_{i} X_{j} / 3 \]

where \( Y \) is the response (total polyphenolic content, DPPH radical scavenging activity, metal chelation and reducing power); \( \beta_{0}, \beta_{i}, \beta_{ii} \) and \( \beta_{ij} \) are constant coefficients, and \( X_{i} \) and \( X_{j} \) are the coded independent variables, which influence the response variable \( Y \). This response was preferred because a relatively few experimental combinations of the variables were adequate to estimate a potentially complex response function. Data were analyzed using MINITAB 15 software (Minitab Inc., State College, PA, USA) to find the interaction between the variables and the responses.

Results and Discussion

In the present investigation, the experimental design has been formulated in such a way to develop an empirical model to examine the interaction of different associated parameters responsible for the extraction of phenolic constituents present in okara using RSM, and also to identify the optimum conditions for a multivariable system of extraction. The predicted values were compared with the experimentally observed values to check the performance of the model as shown in Table 1. Analysis of variance for experimental set up was done to evaluate the fitness of response function. The linearity and quadratic effect of the independent variables, their interaction and regression coefficients on the response variables (Table 2) were analyzed. The goodness of fit of the model was checked by the coefficient of determination (\( R^{2} \)) for stating a good statistical model, and also to justify its robustness. The coefficient of determination (\( R^{2} \)) was calculated to be 0.9608, 0.9958, 0.9982 and 0.9999 for total polyphenolic content, DPPH radical scavenging activity, metal chelation and reducing power, respectively, which are all close to 1.

Quadratic equation for solvent extraction

Second-order polynomial equations were used to correlate the input process variables with the responses. The second-order polynomial coefficient for each term of the equation was determined through multiple regression analysis using MINITAB 15.

The mathematical expression of relationship for total polyphenolic content, DPPH radical scavenging activity, metal chelation and reducing power with variables \( A_{1} \), \( A_{2} \) and \( A_{3} \) (temperature (°C), solvent fraction (%) and incubation time (min), respectively) are given in Eqs. 4–7:
Total polyphenolic content/(mg/mL) =
= 4.049 - 0.028A_1 - 0.110A_2 - 0.004A_3 + 0.00007A_1^2 + 0.00092A_1^2 + 0.00012A_2^2 + 0.00072A_3^2 - 0.00013A_1A_2 + 0.00009A_1A_3

Metal chelation/% = + 444.02 - 13.82A_1 + 0.25A_2 - 5.57A_3 - 0.06A_1A_2 + 0.018A_3

DPPH activity/% = - 11.60 + 3.47A_1 - 1.53A_2 + 1.47A_3 - 0.033A_1^2 + 0.00252A_2^2 - 0.0036A_3^2 + 0.0166A_1A_2 - 0.0401A_1A_3 + 0.014A_2A_3

Reducing power = - 0.454 + 0.02A_1 + 0.02A_2 + 0.016A_3 - 0.000049A_1^2 + 0.00014A_2^2 - 0.00066A_3^2 - 0.00014A_1A_2 - 0.00028A_1A_3 + 0.00003A_2A_3

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Table 2. ANOVA analysis of response surface quadratic model for extraction of total polyphenols and antioxidant activity (DPPH radical scavenging activity, metal chelation and reducing power)

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<td>3665.290</td>
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^a degrees of freedom, ^b sum of squares, ^c mean squares
From the regression equations mentioned above, it can be seen that $A_2^2$, $A_3^2$ and $A_1A_2$ were significant model terms for total polyphenolic content. The surface plot in Fig. 1 shows the effect of temperature (°C) and solvent fraction (%) on the extraction of total polyphenols (mg/mL). By increasing the temperature and solvent fraction, total polyphenolic content decreased significantly. This can be due to the degradation of polyphenols and decrease in the polarity of solvent at higher temperatures (17,18). At specific temperature (40 °C) and solvent fraction (25 %), a maximum yield of polyphenols (1.16 mg/mL) was obtained.

For DPPH radical scavenging activity, $A_1$, $A_3$ and $A_1A_2$ were significant model terms. From a 3D surface plot (Fig. 2), it was observed that radical scavenging ability increased with the increase in temperature and solvent fraction up to 50 °C and 33 % respectively, but with further increase in these parameters, the percentage of inhibition of DPPH radicals started declining. Liu et al. (19) also observed the inhibition of DPPH radical scavenging activity at higher temperature.

The influencing model terms for metal chelation property were $A_2$, $A_1^2$ and $A_2A_3$. The surface plot (Fig. 3) indicates that at specific solvent fraction (25 %) and incubation time (15 min), maximum metal chelating activity can be achieved.

For reducing power, $A_1$, $A_2$ and $A_2A_3$ were significant model terms. The surface plot (Fig. 4) shows the effect of interaction between solvent fraction and incubation time on the reducing power activity of okara. It demonstrated maximum reducing power activity at 42 % solvent fraction and 20 min of incubation time.

**Validation of the model**

Validation of the optimal conditions for solvent extraction of polyphenols was done by MINITAB 15 software. The results in Table 3 present the optimal conditions for each individual response with the predicted and experimental values. The optimum conditions for radical scavenging properties were obtained at 50 °C with incubation time of 15 min using solvent fraction of 33 %. The radical scavenging activity present at 50 °C was probably due to the increasing diffusivity of the solvent in the solid matrix, which favours the extraction. The reducing power activity was obtained at 60 °C, solvent fraction of 42 % and incubation time of 20 min. Optimum condition for reducing power was noticed at higher temperature (60 °C) than that for radical scavenging proper-
ty. The stability of reducing power activity could be partly due to the formation of products of Maillard reaction. It was reported that there was an alteration in the phenolic compounds after heating, which contributed to the increase in reducing power (20).

Chelating agents are effective as secondary antioxidants because they reduce redox potential, thereby stabilizing the oxidized form of the metal ion (21). The extraction of chelating agent under optimized conditions (41 °C, 15 min, 25 % solvent fraction) showed chelation activity up to 61.20 %. Total polyphenolic content of okara (1.16 mg/mL) is similar to that reported by Gan and Latif (8). Report by Tabart et al. (22) also agreed that aqueous acetone gave better yield of total polyphenolics than methanol and ethanol. Validation of the model suggests that okara may serve as a good substrate for extraction of a significant amount of polyphenols and other antioxidants.

**Conclusion**

The obtained results provide innovativeness and eco-friendly approach for recovery of the antioxidant phenolics from a low-cost feedstock okara, which can retain the maximum bioactivity of extracted biomolecule. In the present study, maximum amount of extracted polyphenols was achieved by using a food grade solvent. It can be concluded that the optimum conditions for maximum polyphenol extraction from okara (1.16 mg/mL) can be achieved by using acetone as solvent under 40 °C, 15 min and 25 % of solvent fraction, DPPH radical scavenging activity of 61.07 % at 50 °C for 15 min and 33 % of solvent fraction, metal chelating activity of 61.20 % at 41 °C for 15 min and 25 % of solvent fraction, and better reducing power at 60 °C for 20 min and 42 % of solvent fraction.

**References**


### Table 3. Predicted and experimental values under optimum conditions for maximum total polyphenolic content and antioxidant activity (DPPH radical scavenging activity, metal chelation and reducing power)

<table>
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<tr>
<th>Responses</th>
<th>Temperature/°C</th>
<th>Solvent fraction/%</th>
<th>Incubation time/min</th>
<th>Predicted value</th>
<th>Experimental value</th>
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<tbody>
<tr>
<td>γ(total polyphenols)/(mg/mL)</td>
<td>40</td>
<td>25</td>
<td>15</td>
<td>1.156</td>
<td>1.160</td>
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<tr>
<td>DPPH radical scavenging activity/°C</td>
<td>50</td>
<td>33</td>
<td>15</td>
<td>61.230</td>
<td>61.070</td>
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<td>Metal chelation/%</td>
<td>41</td>
<td>25</td>
<td>15</td>
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<tr>
<td>Reducing power (A)</td>
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<td>42</td>
<td>20</td>
<td>0.730</td>
<td>0.732</td>
</tr>
</tbody>
</table>

