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Optimization of Malted Sorghum Protein Extraction by Response Surface Methodology

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Four independent variables viz. ultrasonic power, pH, extraction time and solvent/meal ratio were selected. The extraction process was evaluated by a selected response like protein yield and the second-order model obtained revealed 96.7% of coefficient of determination. Selected response, which evaluated the extraction process, was protein yield and the second-order model obtained for protein yield revealed a coefficient of determination of 96.7%. The optimal extraction conditions for protein were determined as follows; Ultrasonic power, pH, extraction time and solvent/meal ratio were 400W, 8.0, 40min, and 20:1 (v/w) respectively. Protein yield was primarily affected by Ultrasonic power, pH and solvent/meal ratio. These conditions resulted in protein yield of 5.43g of soluble protein from extract/100 g malted sorghum flour, which was agreed closely with the predicted value 5.36%. The adequacy of the model was confirmed by extracting the protein under optimum values using the model. These results may help in designing the process of optimal protein extraction from malted sorghum flour.

Keywords: Response surface methodology, Malted sorghum, Optimization, Protein extraction

Introduction

Sorghum is an extremely important crop in Asia, Africa and other semi-arid regions of the world due to it's relatively drought tolerance compared to other cereals (Anglani, 1998). It can grow under very harsh conditions such as infertile soils and excessive heat, conditions that are unsuitable for maize or wheat production. Sorghum is nutritionally equivalent to most cereals, and its protein content is quite variable. However, for human nutrition sorghum protein is deficient in critical amino acids, most importantly lysine (FAO, 1995; FAO, 2002). Most literatures report several instances of levels ranging from 6 to 16 (Hoseney *et al*., 1994).

Recently, there has been increased interest in sorghum as a gluten-free cereal to substitute the gluten-rich cereals in the diet of people suffering from celiac disease (Elkhalifa *et al*., 2005). The functional properties of sorghum proteins can be used to define how flour proteins can be used to supplement or replace more toxic protein sources. Extraction of proteins is desirable for the utilization of any new protein material and can be added in small amounts to food products for a specific aspect (Ghavidel and Prakash, 2006).

Malting has been identified as a traditional processing technology that could possibly be used to improve the nutritional quality of the protein (Wang and Fields, 1978). The process of malting comprises three unit operations, viz. steeping, germination and drying. A number of factors are known to have an effect on the development of enzymes synthesized during germination and thus on the quality of the malt produced. Some authors studied the increase of efficiency of sorghum water extract (Abdul *et al*., 1999; Randhawa *et al.*, 2002; Muhammad *et al.*, 2005).

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Various parameters such as pH, temperature, ultrasonic power, ionic strength, solvent type, extraction time, solvent/meal ratio, presence of components causing linking, affect protein extractability (Wani *et al.,* 2006). The extraction, isolation and fractionation procedures differ depending on the end use. Generally, protein concentrates or isolates are prepared by the extraction of protein-rich material in alkaline solution which is then precipitated at isoelectric pH between 4.0 and 5.0 for food application (Mwasaru *et al.,* 2000; Aluko and McIntosh, 2001; Chavan *et al.*, 2001; Chove *et al.*, 2001; Lqari *et al*., 2002; Bilgi and Celik, 2004; Aluko *et al*., 2005).

Response Surface Methodology (RSM) is an affective statistical technique for optimizing complex processes. It is wide used in optimizing the extraction process variables, such as protein, polysaccharides, anthocyanins, Vitamin-E and phenolic compounds from varied materials (Qiao *et al*., 2009).

In this study, the main objective was to optimize the extraction of protein from Chinese malted sorghum. RSM was designed to systemic analyze the effects of extraction parameters on the yields of protein from Chinese malted sorghum.

Materials and Methods

Materials: Sorghum (*Sorghum bicolor* L. Moench) was purchased from the local market. All the chemicals used were of analytical grade and purchased from Sinopharm Chemicals Reagent Company (SCRC), Shanghai, China.

Soaking, malting and preparation of sorghum flour: After removing chaff and unviable grain, sorghum grains (1000 g) were thoroughly cleaned by washing with tap water and then soaked in wooden ash extract. The grains were soaked for 24 h at 30°C with the soaking water being changed at 6 h interval. After soaking, the grains were spread on jute bags and covered

with the same material in a secluded and dark area. Malting was allowed to proceed for different time intervals (3, 5 and 6 days) and the temperature of malting kernels was 25°C. The growth was terminated by kilning in a forced air oven at 40°C for 24hr. The withered rootless were gently brushed off and dried grain were milled using a bench-top attrition mill (Dade, DFT-600, 25000 rpm, Zhejiang Linda Mechanic Co., Ltd., China). The resultant flour was sieved into a particle size of 70-mesh. The flour was then packaged in a low density polyethylene bag and was stored using plastic containers with lids in a refrigerator at 4°C for later analysis.

Protein extraction: Malted sorghum flour meal was extracted with selected 29 combinations of independent variables such as pH (7.0-9.0), solvent/meal ratio (10:1 to 30:1 v/w) and extraction time (mixing) (20-60 min) (Table I). Malted sorghum flour (10 g) was extracted with deionized water in a stirred glass vessel at constant temperature. The pH of suspension was kept constant during extraction time by adjusting with 0.5 mol equiv/L NaOH or 0.5 mol equiv/L HCl. Ultrasonic treatment was performed in an ultrasonic cell disintegrator (JBT/C-YCL400T, Xinzhi Biotechnology and Science Inc., Lingbo, Henan Province, China) with 50 ml of Chinese malted sorghum slurry exposed to extract for 20min time at varied ultrasonic power. Ice bathing was used to ensure the temperature of solution was below 50°C in the whole extraction processing.

The slurry was centrifuged (ZOPR-52D refrigerating centrifuge) at 10,000 rpm for 20 min, the supernatant was collected and the soluble protein content was determined according to the method of Lowry, Rosebrough *et al*., (1951). All the experiments were carried out in triplicate.

Proximate analysis: Moisture, crude fat, crude protein and ash content were determined according to the methods of AOAC, (1997).

Experimental design and statistical analysis: Response surface methodology was used to determine the influence of four independent variables and the optimum conditions of protein isolation. The process variables and the responses were defined from published data (Rustom *et al*., 1991; Oomah *et al.*, 1994; Liadakis *et al*., 1995; Liadakis *et al.,* 1998; Mizubuti *et al*., 2000; Moure *et al*., 2002; Quanhang and Caili, 2005). The effect of the variables ultrasonic power (X_1), pH (X_2), extraction time (mixing) (X_3) and solvent to meal ratio (X_4) in malted sorghum protein isolation process was investigated. Each variable was coded at five levels: -2, -1, 0, 1, 2 (Table 1).

Table 1. Variables and levels for central composite design

Variable	Symbol	Coded variables levels[a]				
		-2	-1	0	1	2
Ultrasonic power (W)	X1	300	350	400	450	500
pH	X2	7	7.5	8	8.5	9
Extraction time (mixing)	X3	20	30	40	50	60
Solvent to meal ratio (v/w)	X4	10	15	20	25	30

[a]Passage from coded variable (X_i) level to natural variable (x_i) level is given by the following equations: $x_1 = 5X_1+40$; $x_2=0.5X_2+ 8.0$; $x_3=10x_3+40$; $x_4= (5X_4+20)$

The variables were coded according to the following equation;

$$X_i = (x_i - \bar{x}_i)/x_i \quad \text{.....1}$$

Where, X_i is the dimensionless value of an independent variable, x_i is the real value of an independent variable, $_i$ is the real value of an independent variable at the center point, "x_i is the step change.

The response function investigated was $Y_=$ g of soluble protein from extract/100 g flour. A Central Composite Design (CCD) was arranged to allow for fitting of second-order model (Nakai and Dou, 2006) (Table 2). The CCD combined the vertices of hybercube whose coordinates were given by 2n factorial design (runs 1-16) with the star points (runs 17-24). The star points were added to the factorial design to provide for estimation of curvature of the model. Five replicates at the center point of the design (runs 25-29) were used to allow for the estimation of the pure error sum of squares. All experiments were carried out in a randomized order to minimize any effect of extraneous factors on the observed responses.
The model proposed for response (Y) was;

$$Y = b_0 + \sum_{n=1}^{4} b_n X_n + \sum_{n=1}^{4} b_m \; X_n^2 \sum_{n\neq m=1}^{4} b_{nm} X_n X_m \quad \text{.....2}$$

Where, b_0 is the value of the fitted response at the center point of the design, which is point (0, 0, 0, 0). b_n, b_{nn} and b_{nm} are the linear, quadratic and interaction regression terms respectively. The objective in the optimization process was to find a common value for the dependent variables; thus we used the desirability method. The Statistical Analysis System (28) software Version 6.03 was used in all statistical analysis and evaluation.

Predicted values (Y) is transformed into a value of d, which falls in the range [0, 1] by using Design-Expert 7.1 and measures the desirability degree of the response in reference to the optimum value intended to be reached. In our case, we wanted dependent variable to be in range. The ideal optimum value is d=1; an acceptable value for d can be between 0.6 and 0.8 ($0.6 < d < 0.8$). The optimum condition was verified by conducting experiments under these conditions. Surface plots

were generated by assigning constant (zero) values to two of the four variables and solving the fitted equations as a quadratic equation in the remaining two variables.

Table2. Central composite design arrangement, responses for protein yield

Run	Actual values				Protein yield (Y),%	
	X1	X2	X3	X4	Experimental	Predicted
1	350	7.5	30	15:1	4.38±0.36	4.35
2	450	7.5	30	15:1	4.45±0.23	4.35
3	350	8.5	30	15:1	5.31±0.31	5.21
4	450	8.5	30	15:1	5.26±0.09	5.32
5	350	7.5	50	15:1	4.80±0.31	4.73
6	450	7.5	50	15:1	4.81±0.63	4.78
7	350	8.5	50	15:1	4.90±0.24	5.06
8	450	8.5	50	15:1	5.29±0.16	5.21
9	350	7.5	30	25:1	4.42±0.20	4.40
10	450	7.5	30	25:1	4.41±0.20	4.37
11	350	8.5	30	25:1	5.30±0.01	5.44
12	450	8.5	30	25:1	5.53±0.08	5.51
13	350	7.5	50	25:1	4.97±0.40	5.02
14	450	7.5	50	25:1	5.05±0.19	5.04
15	350	8.5	50	25:1	5.53±0.09	5.53
16	450	8.5	50	25:1	5.51±0.04	5.65
17	300	8	40	20:1	5.32±0.15	5.26
18	500	8	40	20:1	5.32±0.12	5.38
19	400	7	40	20:1	3.74±0.18	3.88
20	400	9	40	20:1	5.49±0.16	5.35
21	400	8	20	20:1	5.07±0.13	5.13
22	400	8	60	20:1	5.72±0.13	5.65
23	400	8	40	10:1	4.33±0.29	4.43
24	400	8	40	30:1	5.02±0.51	4.91
25	400	8	40	20:1	5.43±0.21	5.36
26	400	8	40	20:1	5.37±0.28	5.36
27	400	8	40	20:1	5.29±0.23	5.36
28	400	8	40	20:1	5.26±0.19	5.36
29	400	8	40	20:1	5.40±0.02	5.36

X_1: ultrasonic power, X_2: pH, X_3: Extraction time, X_4: solvent/meal ratio.

Results and Discussion

Proximate analysis of malted sorghum flour: The proximate analysis of malted sorghum flour is presented in Table 3. The sorghum had a protein content of 11.41 g/100 g dry solids considered adequate for protein recovery. The majority of sorghum proteins are found within the endosperm of the kernel, distributed within both protein bodies and the endosperm's protein matrix. The major sorghum protein fractions are prolamins and glutelins, with prolamins predominating. The prolamins in sorghum is kafirin-a protein similar to maize zein in molecular weight, structure, solubility, and amino acid composition (Da and Taylor, 2005). Kafirin is found to predominate in three forms: α-, β-, and γ-kafirin, with α-kafirin being the major form (Musigakun and Thongngam, 2007). These sorghum prolamins are located within the protein bodies in the starchy endosperm (Taylor *et al.*, 1984). Like many cereals, the limiting amino acid in sorghum is lysine (Waniska and Rooney, 2000).

Table 3. Proximate composition of malted sorghum flour

Parameters	Composition, g/100g dry solids
Moisture	9.70±0.04
Crude fat	3.60±0.11
Crude protein (Nx6.25)	11.41±0.08
Ash	2.69±0.06
Carbohydrate	59.58±0.12

Fitting the models: Malted sorghum flour was extracted for its protein following 29 combinations of four independent variables (ultrasonic power, pH, extraction time, solvent/meal ratio) (Table 1). Results revealed that the experimental protein yield varied from 3.74 to 5.53 g soluble protein/100 g malted sorghum flour. Quanhang and Caili (2005) found that the protein yield was 2.77-7.86 g protein/100 g germinant pumpkin seeds. Liadakis *et al.*, (1995) extracted 43.9-57.3% of the proteins contained in tomato seed meal.

The application of RSM yields the following regression equation, which is an empirical relationship between protein yield and the test variable in coded units, as given in the following equation.

Final equation in terms of actual factors

Protein extraction

$$Y = 5.37 + 0.030X_1 + 0.37X_2 + 0.13X_3 + 0.12X_4 - 0.011X_1^2 - 0.19X_2^2 + 0.0074X_3^2 - 0.17X_4^2 + 0.024X_1X_2 + 0.012X_1X_3 - 0.009X_1X_4 - 0.13X_2X_3 + 0.044X_2X_4 + 0.061X_3X_4$$

The predicted values of protein yields were calculated using the regression model and compared with experimental values in Figure 1. The total determination coefficient (R^2) was 96.7%, indicating a reasonable fit of the model to the experimental data. Earlier studies have reported values for R^2 ranging from 58.5 to 99.03% for watermelon seed (Wani *et al.*, 2006), peanut (Rustom *et al.*, 1991), germinant pumpkin seed (Quanhang and Caili, 2005), amaranth seed (Saloedo *et al.*, 2002), flaxseed (Oomah *et al.*, 1994) and tomato seed (Liadakis *et al.*, 1995).

The significance of each coefficient was determined using the Student t-test and p-value. Solvent/meal ratio, pH and extraction time were the most significant factors ($p<0.001$). Especially, pH and solvent/meal ratio showed highly significant ($p<0.001$) at linear and quadratic terms. An interaction effect of pH and solvent/meal ratio was significant ($p<0.05$) and ultrasonic power was not significant factor. Liadakis *et al.*, (1995) and Wani *et al.*, (2006) reported a similar effect.

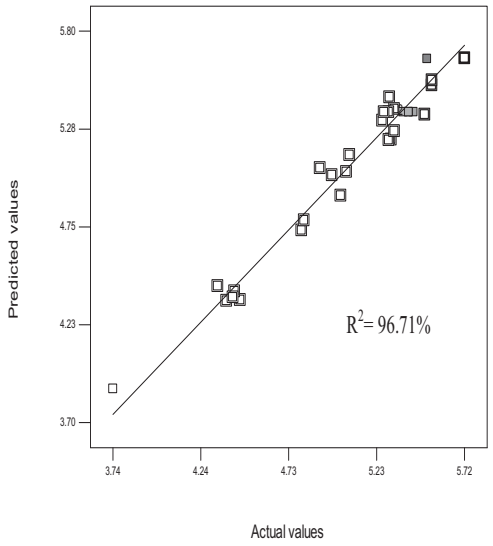

Figure 1. Comparison between predicted and observed protein yield (g soluble protein/100 g flour)

Analysis of variance (ANOVA) of independent variables was performed. The statistical analysis data revealed that linear, quadratic and interaction terms were significant ($p< 0.05$). The lack of fit test measures the failure of the model to represent data in experimental domain at points which are not included in the regression. There was a non-significant lack of fit that further validates the model ($p>0.05$) (Table 4). The coefficient of variation (CV) is the ratio of the standard error of estimate to the mean value of observed response expressed as a percentage. It is a measure of reproducibility of the models. The CV of the model was calculated as 2.31%. As a general rule, a model can be considered reasonably reproducible if its CV is not greater than 10%.

Table 4. Analysis of variance (ANOVA) on the effect of protein extraction condition

Source	Sum of Squares	df	Mean Square	F-Value	p-value prob > F
Model	6.0719	14	0.4337	31.4458	0.0001*
A-Temp.	0.0210	1	0.0210	1.5271	0.2355
B-pH	3.2479	1	3.2479	235.4918	0.0001*
C-Et(mixing)	0.4040	1	0.4040	29.2947	0.0001*
D-RMS	0.3542	1	0.3542	25.6878	0.0001*
AB	0.0093	1	0.0093	0.6786	0.4229
AC	0.0024	1	0.0024	0.1776	0.6794
AD	0.0012	1	0.0012	0.0939	0.7634
BC	0.2819	1	0.2819	20.4434	0.0004**
BD	0.0308	1	0.0308	2.2331	0.1558
CD	0.0601	1	0.0601	4.3609	0.0542
A^2	0.0030	1	0.0030	0.2231	0.6434
B^2	0.9613	1	0.9613	69.7053	0.0001*
C^2	0.0015	1	0.0015	0.10908	0.7458
D^2	0.8170	1	0.8170	59.2403	0.0001*
Residual	0.2068	15	0.0137		
Lack of Fit	0.1840	10	0.0184	4.0225	0.0689
Pure Error	0.0228	5	0.0045		
Cor Total	6.2788	29			

*Significant at 1% level; ** Significant at 5% level

B, C, D, BC, B^2, D^2 are significant model terms.

Optimization of the process: The 3D surface plots were drawn to illustrate the main and interactive effects of the independent variables on the dependent one. These graphs were obtained by fixing two variables at coded zero level (Table 2) while varying the remaining two variables and predicting the response variable (protein yield).

Figure 2a shows the effect of ultrasonic power and pH; pH exerted a quadratic effect on protein production, whereas ultrasonic power had a linear effect. Ultrasonic power did not seem to affect protein extraction in the selected range whereas extraction time exerted a linear effect, as shown in Figure 2b Figure 2c shows the effect of the ultrasonic power and of solvent/meal ratio (SMR) on protein production. A quadratic effect of solvent/meal ratio and linear effect of ultrasonic power on the response were observed.

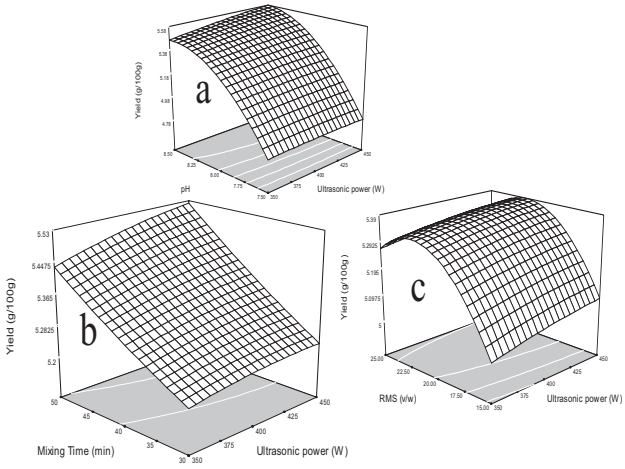

Figure 2. Surface plots for protein yield of malted sorghum flour; (a) Effect of ultrasonic power power and pH on protein yield with extraction time 40 min, solvent (water)/meal ratio 20:1 (v/w); (b) Effect of ultrasonic and extraction time on protein yield with pH 8.0, solvent water)/meal ratio 20:1 (v/w); (c) Effect of ultrasonic power and solvent (water)/meal ratio on protein yield with extraction time 40 min, pH 8.0.

The graph shown in Figure 3a indicates that both pH and extraction time had a quadratic effect on protein extraction. Figure 3b depicts the influence of pH and solvent/meal ratio; it can be seen as a quadratic effect for both pH and solvent/meal ratio. The results indicated that the increase in pH and solvent/meal ratio extracted higher amount of protein from malted sorghum flour. Effect of extraction time and solvent/meal ratio on protein yield is presented in Figure 3c.

The results revealed that extraction time did not have any significant effect on the protein extraction while an increase in solvent/meal ratio showed an increasing trend for protein extraction. Considering all the response, it is evident that solvent/meal ratio, extraction time and pH had a significant effect on protein yield while the effect of ultrasonic power was more limited.

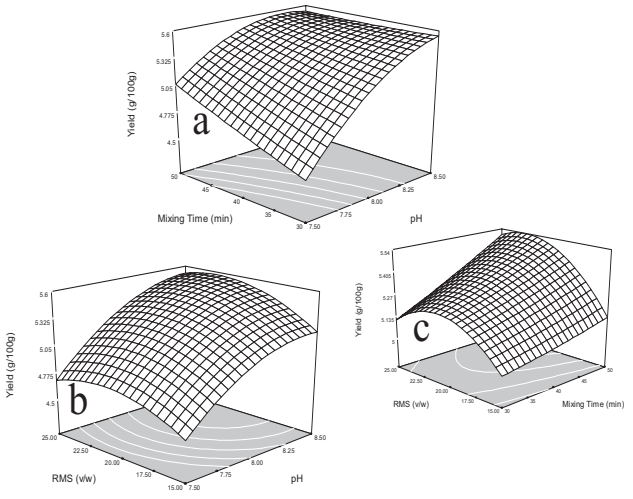

Figure 3. Surface plots for protein yield of malted sorghum flour; (a) Effect of pH and extraction time on protein yield with ultrasonic power 400W, solvent (water)/meal ratio 20:1 (v/w); (b) Effect of pH and solvent (water)/meal ratio on protein yield with ultrasonic power 400W, extraction time 40 min; (c) Effect of extraction time and solvent/ meal ratio on protein yield with ultrasonic power 400W, pH 8.0.

Optimum extraction conditions were estimated by the desirability method using a Design-Expert 7.1 Software. A solvent/meal ratio of 20:1 (v/w), extraction time of 40 min, pH of 8 and ultrasonic of 400W were found to be optimal in range for protein extraction from malted sorghum flour. Ghavidel and Prakash (2006) reported that flours obtained from ungerminated green gram, cowpea and *Bengal* gram had a broad apparent isoelectric pH range 3–5 and the highest solubility of the protein for germinated samples occurred at pH 6. Normally the protein solubility characteristics are influenced by factors such as origin, processing conditions, pH, ionic strength and the presence of other ingredients (Kinsella, 1976). The proteins of the germinated samples are more soluble and this might be due to the high proteolysis activity during germination which leads to an increase in the protein solubility resulting from hydrolysis of the storage proteins.

Studies of the extraction of malted sorghum flour protein could not be traced; however, similar studies using other plant material have been reported. Quanhang and Caili (2005) worked on protein extraction from germinated pumpkin seed and found significant effects of solvent/meal ratio and concluded that optimum conditions were: solvent/meal ratio of 30.2:1 (v/w), NaCl concentration of 4.26% and a reaction time of 18.1 min. Wani *et al*., (2006) studied the extraction of watermelon seed protein and concluded that maximum protein yield was obtained by extracting seed meal with a NaOH concentration of 1.2%, solvent/meal ratio of 70:1, extraction time of 15 min and ultrasonic of 400W. Liadakis *et al.,* (1995) worked on protein extraction from tomato seed and found that optimum extraction could be achieved by extracting one part of tomato seed meal with 30 parts of water (w/v) at pH 11.5 at 50°C for 20 min. Rustom *et al.,* (1991) reported that significant effects of time, temperature, pH and solvent/meal ratio were found and concluded that optimum extraction were: pH of 8.0, time of 30 min; temperature of 50°C and solvent/meal ratio of 8.1. Mizubuti *et al.,* (2000) state that optimum conditions for protein extraction from pigeon pea were no NaCl, pH 8.5 and solvent/meal ratio 5.1. Oomah *et al*., (1994) determined that solvent to meal ratio of 10:l per kg, 0.8 mol/L NaCl and pH 8.0 were optimum conditions for extracting protein from flaxseed meal.

Confirmative tests: The suitability of the model equation for predicting the optimum response value was tested using the recommended optimum conditions. When optimum values of independent variables (ultrasonic power 400W, solvent/meal ratio 20:1 v/w, extraction time 40min, pH 8.0) were incorporated into the regression equation, 5.36 g/100 g protein yield was obtained whereas experiments at optimum conditions gave a protein yield of 5.43 g/100 g. Thus, predicted values from fitted equations and observed values were in very good agreement.

Conclusion

Protein was extracted from malted sorghum flour using response surface methodology with 29 selected combinations of ultrasonic power, pH, solvent/meal ratio and extraction time. The experimental value of protein yield varied from 3.74 to 5.53g soluble protein/100 g malted sorghum flour. The second order polynomial model developed for protein yield exhibited a non-significant value for lack of fit and high value for the coefficient of determination. The variables which showed greatest effect on the extraction yield of protein were the pH and solvent/meal ratio. Optimum extraction of malted sorghum protein with water could be achieved by extracting one part of malted sorghum meal with 20 parts of water (w/v ratio) at pH 8 at 400W for 40 min. These conditions resulted in protein yield of 5.43 g of soluble protein from extract/100 g malted sorghum flour. The response surface methodology technique proved to be a useful tool in establishing optimum conditions for extracting of protein from malted sorghum flour.

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