Response Surface Methodology for Optimization of Protein Isolate from Defatted Custard Apple Seed (Annona Squamosa)

Raj Kumar, Renu Sharma
Research Scholar, Assistant Professor
IKGPTU, India; BGIET,Sangrur

Tanuja Srivastava
Director & Professor
BGIET, Sangrur

DC Saxena
Professor
SLIET, Longowal, Punjab.

ABSTRACT
Defatted custard apple (Annona squamosa) seed flour, after extraction of oil was used as a source of protein to meet the nutritional requirements of most of the people of developing countries. The poisonous nature and presence of antinutrients in these oil seeds limits their use as food for livestock and man. However, to utilize the seeds for food, either removal of the antinutrients and/or isolate the proteins contents is necessarily required. In the present study, custard apple seed protein isolate (CASPI) was prepared by using alkali method. A three-factor five-level, central composite rotatable design (CCRD) of Response surface methodology (RSM) was adopted to study the effect of three independent variable namely pH (7-11%), NaOH concentration (0.6-2%) and Flour to solvent ratio (20% - 60%) on the dependent variables like protein content (%) and protein yield (%). The numerical optimization technique gives the different optimized conditions for the custard apple seed protein isolate were pH (11), NaOH concentration (0.67M) and flour to solvent ratio (1:60 w/v). The experimental samples under the optimum process conditions resulted protein content 68.07% and protein yield 18.69% which were in close proximity to the predicted values. The closeness of actual values 68.07% and 18.69% and predicted values 66.89% and 18.07% for protein content and protein yield confirms the validation of RSM model.

Keywords
Defatted custard apple seed flour, protein isolate, RSM, Numerical optimization, CCRD.

1. INTRODUCTION
Global food production has greatly increased but food consumption per head in developing countries has not increased proportionately because of high birth rates. Under-nutrition is often a major problem in most of the countries of the world. In order to provide good nutrition for human existence and to fulfill the protein shortage, the Interest in newer sources of protein has grown in developing countries. As part of the quest for newer sources, some lesser-known oil seeds have been evaluated for their nutritional qualities in India [1-7]. Protein isolates from vegetal sources have gained importance in food industry because of their high protein contents and due to the versatility of their functional properties that can also improve the nutritive quality of foods [8-9].

The Annonaceae family contains a considerable number of plants of economic significance because of their edible fruits. These crops represent the fruits of tropical America, Australia, Africa, Malaysia and India (In India the custard apple is one of them) with a very sharp and short season, lasting for about three months a year. Custard apple (Annona squamosa) is popularly called as sitaphal in South India and sharifa in North India. It is heart shaped fruit weighing about 150gm with a very bumpy skin. When ripe, pulp is creamy, very sweet and pleasantly flavoured. It is usually eaten as dessert. The seeds of custard apple are so hard that they may be swallowed whole with no ill effects but the kernels are very toxic. The seeds contain 25.5% oil used in soap and paint industries. The seed cake can be used as green manure for agriculture. The seeds, leaves and young fruits are insecticidal [10]. The seeds contain 7.7% moisture, 8.5% crude protein, 9.7% ash, 5.2% crude fiber, 40% fat and 34.1% carbohydrate along with some minerals like potassium, calcium, phosphorous, sodium and magnesium [11].

Traditionally, oils and protein isolates are obtained from oilseed. In the oil extraction process, a by-product obtained is protein rich cake. Recently, there is major interest in the defatted cake because they possesses a high quantity of protein. It has been established that custard apple seed contain ample amount of protein but due to the poisonous nature of seeds prevented its use as food. According to Usman, L. A. 1 , Ameen O. M. , Ibiyemi S. A. and Muhammad, [12] the presence of antinutrients in these oil seeds often causes their inferior nutritional qualities, and hence limits their use as food for livestock and man. However, it is possible to utilize the seeds for food by either removing the antinutrients and/or isolate the proteins contents and subsequently use them in food processing industries. Currently, study is focused on the waste products generated by the food industry indicated they are an alternative source of oils and protein. The main objective of the present work is to isolate the protein from

Table 1 Values of independent variables at five levels of the CCRD design

Independent variables	Uncoded	Levels in coded form				
		-1.68	-1	0	+1	+1.68
pH	X_1	5.64	7	9	11	12.36
NaOH Concentration	X_2	0.12	0.6	1.3	2	2.48
Flour to solvent ratio	X_3	6.36	20	40	60	73.64

defatted custard apple seed flour and to study the effects of pH, NaOH concentration and flour to solvent ratio on protein content and protein yield of custard apple seed protein isolate by using Response Surface Methodology (RSM).

2. MATERIALS AND METHOD

2.1 Raw material:

Custard apple seed were collected from local market of Sailu, Distt. Parbhani (Maharashtra). Well matured, fully eye opened fruit, slightly yellow and green in colour was selected which was free from blemishes and mechanical injuries. Then the seed of custard apple were separated from fruit pulp manually by splitting the fruit.

2.2 Preparation of custard apple seed flour:

Whole fruit (matured) were procured from market with then removing seed from fruit. Seeds were then properly cleaned and dried. Then cleaned seeds were soaked in water for overnight (12hrs) at room temperature to facilitate manual dehulling of seeds. After dehulling, seeds were splited and dried at 65^0 c until constant moisture content was attained. Drying was followed by grinding in grinder, sieving through 60 mesh size sieve. The groud endosperm part of the seed was extracted with soxhlet extractor to remove most to remove most of the fat. The resulting defatted custard apples seed flour was packed in air tight polythene bag and stored at refrigerated condition until it was used for further processing i.e. for protein isolation preparation.

2.3 Preparation of custard apple seed protein isolate (CASPI)

Custard apple seed protein isolate (CASPI) was prepared by using alkali method [13]. For the preparation of CASPI 15 g defatted custard apple seed flour weighed and transferred in to clean and dry conical flask. To this 100 ml water was added and mixed. The pH was set at 9.0, with 1N NaOH, and kept in water bath-cum shaker (200 rpm, 60º C) for 30 min. The slurry was centrifuged at3000 rpm for 15 min. The supernatant was separated and precipitated at pH 4.5 by using 1N HCl, and again centrifuged at 5000 rpm for 10 min. Protein curd and whey was obtained, whey was discarded. Protein curd was washed (water), freezed and then dried at 60^0c for 12 hrs. The final product CASPI was stored in glass bottles for further use.

2.4 Selection of optimum pH for protein isolate

Custard apple seed flour (constant weight i.e 15g) was extracted with 1M NaOH solution at constant flour to solvent ratio (i.e. 1:20 w/v) with continuous stirring at different pH for 30 minutes. Results obtained in alkaline condition, was increased extraction rate with the increase in pH. Whereas, at acidic pH (pH 6) protein yield was observed to be low and at high alkaline pH, protein yield was high. Hence, it was concluded 7-11 pH was optimum for extraction of protein.

2.5 Selection of optimum proportion of flour to solvent ratio

According to Mizubuti et al. [14] both pH and solid/solvent ratio were important factors impacting protein content and/or yield. Custard apple seed flour (constant weight i.e. 15g) was extracted with 1M NaOH solution at a different solvent-to-flour ratio with constant stirring at constant pH for 30 minutes. The results were shown that, at very low flour to solvent ratio i.e. 1:10 (w/v), yield was low and flour swelled due to water adsorption, extracting solution concentration became dense, and at solvent ratio 1:70 (w/v), no significant difference was shown. Hence, it was concluded 1:20 to 1:60 solvent to solid ratio was optimum for extract of protein

2.6 Selection of optimum level of NaOH concentration

Similarly preliminary trials were conducted to obtain the optimum limit of NaOH concentration for extraction of protein. Protein isolate was prepared at different concentration of NaOH (0.0, 0.6, 1.5, and 2.5). Among these four combinations, flour containing 0.0 M, there was no yield of protein because of ionic strength and concentration. Whereas flour containing 0.6 M and 2.0 M NaOH, protein was solubilized and gave proper yield of protein. Hence, it was concluded 2.0 M and 2.5 M NaOH concentration was optimum for extract of protein.

3. EXPERIMENTAL DESIGN

A three-factor five-level, central composite rotatable design of Response surface methodology (RSM) was adopted in the experimental design [15]. The main advantage of RSM lies in reduction of experimental runs needed to provide sufficient information for statistically acceptable result. Table 1 shows independent variables selected for the experiments. The variables and their levels were chosen by taking trials of samples as literature concerning the preparation of protein isolate from custard apple seed is scanty. The independent variables were percentage of pH (7-11%), NaOH concentration (0.6-2%) and Flour to solvent ratio (20% - 60%).The five levels of the process variables were coded as -1.68, -1, 0, 1 and +1.68 (Montgomery, 2001). The dependant variables for quality parameters were protein content and

protein yield. Design in coded (x) form and at the actual levels (X) is given in Table 2.

Table 2 Experimental design in coded and uncoded levels for custard apple seed protein isolate

Sr. No.	Coded variables			Uncoded variables		
	X_1	X_2	X_3	X_1 pH	X_2 NaOH Concentration	X_3 Flour to solvent ratio
1	-1	-1	-1	7	0.6	20
2	1	-1	-1	11	0.6	20
3	-1	1	-1	7	2	20
4	1	1	-1	11	2	20
5	-1	-1	1	7	0.6	60
6	1	-1	1	11	0.6	60
7	-1	1	1	7	2	60
8	1	1	1	11	2	60
9	-1.68	0	0	5.63	1.3	40
10	1.68	0	0	12.36	1.3	40
11	0	-1.68	0	9	0.12	40
12	0	1.68	0	9	2.47	40
13	0	0	-1.68	9	1.3	6.36
14	0	0	1.68	9	1.3	73.63
1	0	0	0	9	1.3	40

5						
16	0	0	0	9	1.3	40
17	0	0	0	9	1.3	40
18	0	0	0	9	1.3	40
19	0	0	0	9	1.3	40
20	0	0	0	9	1.3	40

4. ANALYSIS OF DATA

A complete second order quadratic model employed to fit the data and adequacy of the model was tested considering R^2 (the coefficient of multiple determination, a measure of the amount of variation around the mean explained by the model), Adjusted R^2 (a measure of the amount of variation around the mean explained by the model, adjusted for the number of terms in the model), predicted R^2 (a measure of how good the model predicts a response value) and Fischer's F-test. Coefficient of determination R^2 , is defined as the ratio of the explained variation to the total variation and is measure of the degree of fit [16]. It is also the proportion of the variability in the response variables, which is accounted for the regression analysis [17]. When R^2 approaches unity, the better the empirical model fits the actual data. The smaller the value of R^2, the less relevance the dependent variables in the model have in explaining the behavior variation. The models were then used to interpret the effect of various predictors on the response. The analysis of variance tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were jugged statistically by computing the F-value at probability (p) of 0.01 or 0.05. The regression coefficients were then used to make statistical calculation to generate contour maps from the regression model. Optimization of press parameters was done by partially differentiating the model with repeat to each parameter, equating zero and simultaneously solving the resulting functions. Design expert 6.0 (version 6.0, by STAT-EASE inc., USA) was used for optimization of selected parameters.

5. STATISTICAL ANALYSIS OF RESPONSES

The responses such as protein content and protein yield for different experimental combinations were related to the coded variables (xi, i=1,2 and 3) by a second degree polynomial (Equation 1) as given below:

$$Y = \beta 0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_{12} + \beta_{22} x_{22} + \beta_{33} x_{23} + \beta_{12} x_1.x_2 + \beta_{13} x_1.x_3 + \beta_{23} x_2.x_3 + \varepsilon \ldots .(1)$$

Where x_1, x_2, and x_3 are the coded values of pH, NaOH concentration and flour to solvent ratio. The Coefficients of the polynomial were represented by β_0 (constant), β_1, β_2, β_3 (linear effects); β_{12} , β_{13},β_{23} (interaction effects) ; β_{11} , β_{22}, β_{33} (quadratic effects) ; ε (random error). Multiple regression analysis was used for data modeling and statistical significance of the terms was examined by analysis of variance. Design expert 6.0 (version 6.0, by STAT-EASE inc., USA) was used for statistical analysis of the data.

6. RESULTS AND DISCUSSION

Variation of responses (Protein content and Protein yield) with independent variables (pH, NaOH concentration and flour to solvent ratio) are shown in Table 3. A complete second order model (Equation 1) was tested for its adequacy and series of three dimensional response surfaces were drawn using design expert software version 6.0 to visualize the variation of response with independent variables

6.1 Effect of Process variables on Protein Content (%)

Protein content (%) was calculated by difference between crude protein (NX6.25) and non protein nitrogen. Crude protein was determined by Kjeldahl peocedure [18]. Table 3 and Table 4 showed the coefficients of the model and other statistical attributes of response (Protein content). Regression model fitted to experimental results of protein content showed that model F value of 27.27 was significant. The chance of large model F-value due to noise was only 0.01%. In the case, X_1, X_2, X_3, X_1^2, X_1X_2, X_1X_3, X_2X_3 are significant model terms. The fitted model was also expressed by coefficient of determination R^2, which was found to be 0.9608.

Table 3: Analysis of variance for Protein Content (%)

Source	Coefficient of Model terms	Sum of squares	Mean square	DF	F Value	Prob>F
Model	62.709	170.268	18.918	9	27.266	<0.0001
X_1	0.504	3.470	3.470	1	5.002	0.0493
X_2	-1.397	26.676	26.676	1	38.446	0.0001
X_3	1.104	16.651	16.651	1	23.998	0.0006
X_1^2	-0.923	12.291	12.291	1	17.714	0.0018
X_2^2	-0.014	0.003	0.003	1	0.004	0.9472*
X_3^2	0.281	1.139	1.139	1	1.642	<0.2289*
X_1 X_2	-2.498	49.920	49.920	1	71.946	<0.0001
X_1 X_3	2.261	40.924	40.924	1	58.981	<0.0001
X_2 X_3	1.511	18.283	18.283	1	26.350	0.0004

***Non-significant at 5% level of significance, df: degrees of freedom**

Table 4: Analysis of variance results of equation 1

Response	Source	Sum of squares	Df	Mean squares	F-value	P-value
Protein Content	Regression	170.268	9	1.891	27.266	<0.0001
	Lack of Fit	2.980	5	5.951	0.751	0.6193
	Pure error	3.960	5	7.920		
	Residual	6.940	10	6.940		
	Total	177.206	19			
	R^2-value	0.9608				

Considering all the above criteria, the model (Eq. 2) was selected for representing the variation of protein content and for further analysis.

Protein Content = $62.70 + 0.50X_1 – 1.39X_2 + 1.10X_3 - 0.92X_1^2 - 0.01X_2^2 + 0.28X_3^2 - 2.49X_1X_2 + 2.26X_1X_3 +1.51X_2X_3$ …………….. **(2)**

Where X_1, X_2, X_3 coded values of pH, NaOH concentration and flour to solvent ratio respectively. Above equation showed that linear terms X_1 and X_3 possesses highly significant positive effect with F-value 5.00 and 24.00 respectively while NaOH concentration (X_2) had significant negative effect with F-value 38.45 on the protein content. P-values of X_1, X_2 and X_3 were found to be 0.0493, 0.0001 and 0.0006 respectively. Quadratic term of pH (X_1^2) was having significant negative effect with F-value 17.71 and P-value 0.0018. The interaction term X_1X_3 and X_2X_3 possesses significant positive effect with P-values P<0.0001 and 0.0004 while X_1X_2 showed negative significant effect with P-value less than 0.0001. As the linear terms pH and flour to solvent ratio have significant positive effect on the proteincontent of protein concentrate. So increase in pH and flour to solvent ratio leads to increase in protein content while increase in NaOH concentration leads to decrease in protein content of the protein concentrate as the concentration of sodium hydroxide has negative significant effect on the protein content. In **Fig.1.** Protein content (%) was found to be increasing with increase in pH at low NaOH, whereas it was found to be decreasing with increase in pH at high NaOH concentration. This may be due to the fact that when samples were extracted at high pH and NaOH concentration, higher amount of starch was introduced into the concentrate. Starch granules, already damaged by milling process, are susceptible to alkali conditions, which increases their solubility at high pH values. Then, when the pH of the extract solution is decreased to the isoelectric point of proteins, some of the solubilized starch is precipitated along with the protein [19-20]. Similar effect of NaOH concentration was depicted from the Fig.1. on the protein content. It was found to be increasing with increase in NaOH concentration at low pH whereas it was found to be decreasing with increase in NaOH concentration at high pH.

The effect of pH and flour to solvent ratio on the protein content (%) of the custard apple seed protein isolate is shown in Fig.2. It was depicted from figure that when NaOH concentration was kept constant, protein content slightly decreases with increase in pH and flour to solvent ratio. This may be due to the fact that at high pH, extraction of some non-protein compounds such as lipids and carbohydrates occurs which results in decrease in protein content [20] and at low flour to solvent ratio, flour swelled due to water absorption, extracting solution concentration became dense causing the viscosity of the solution increased which hampered molecular diffusion results in velocity of extraction decrease [21].

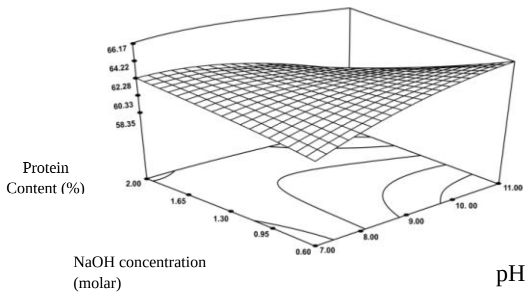

Fig .1. Response Surface plot for the effects of pH and NaOH concentration on protein content (%) of custard apple seed protein isolate

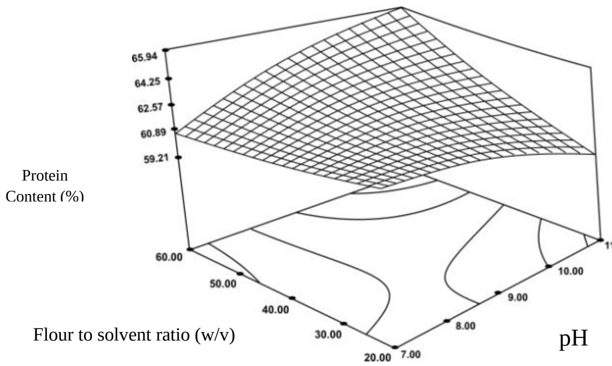

Fig .2. Response Surface plot for the effects of pH and flour to solvent ratio on protein content (%) of custard apple seed protein isolate

Fig.3. showed the effects of NaOH concentration and flour to solvent ratio on the protein content (%) of custard apple seed protein isolate. It was seen that protein content decreased with increase in NaOH concentration and increased with increasing flour to solvent ratio. This may be due to the increase in surface area for solubilization of protein molecules at higher flour to solvent ratio. Velocity of extraction also increases by increase in flour to solvent ratio at particular level [21].

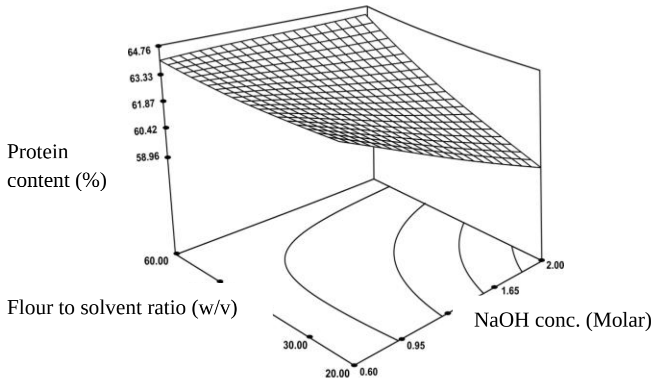

Fig .3. Response Surface plot for the effects of NaOH concentration and flour to solvent ratio on protein content (%) of custard apple seed protein isolate

6.2 Effect of Process variables on Protein Yield (%)

Protein yield (%) was estimated as the percentage of protein mass of the concentrate obtained with respect to the initial flour protein mass [22]. All the masses were estimated on a dry weight basis and moisture content was determined according to AOAC methods (1990). The Protein yield was in range of 3.166 to 22.980% for protein concentrate. **Table 5 and Table 6** showed the coefficients of the model and other statistical attributes of response (Protein yield). Regression model fitted to experimental results of protein yield showed that model F value of 170.88 was significant. The chance of large model F-value due to noise was only 0.01%. In the case, X_1, X_2, X_3, X_1^2, X_2^2,X_3^2, X_1X_2, X_1X_3 are significant model terms. The fitted model was also expressed by coefficient of determination R^2, which was found to be 0.9935.

Table 5: Analysis of variance for Protein Yield (%)

Response	Source	Sum of square	Df	Mean square	F-value	P-value
Protein Content	Regression	438.95	9	4.88	170.88	<0.0001
	Lack of Fit	2.29	5	4.58	4.06	0.0749
	Pure error	5.63	5	1.13		
	Residual	2.85	10	2.90		
	Total	441.81	19			
	R^2-value	0.9935				

Table 6: Analysis of variance results of equation 1

Source	Coefficient of Model terms	Sum of squares	Mean square	DF	F Value	Prob> F
Model	14.612	438.951	48.772	9	170.881	<0.0001
X_1	4.7508	308.243	308.243	1	1079.97	<0.0001
X_2	1.111	16.873	16.873	1	59.119	<0.0001
X_3	2.325	73.886	73.886	1	258.872	<0.0001
X_1^2	-1.052	15.960	15.960	1	55.919	<0.0001
X_2^2	-0.335	1.624	1.624	1	5.690	0.0382
X_3^2	-0.689	6.846	6.846	1	23.988	0.0006
X_1X_2	1.217	11.858	11.858	1	41.547	<0.0001
X_1X_3	0.872	6.090	6.090	1	21.337	0.0010
X_2X_3	-0.272	0.594	0.594	1	2.081	0.1797*

***Non-significant at 5% level of significance,df: degrees of freedom**

Considering all the above criteria, the model (Eq. 3) was selected for representing the variation of protein content and for further analysis.

Protein yield = $14.61 + 4.5X_1 – 1.11X_2 + 2.33X_3 – 1.05X_1^2 - 0.34X_2^2 - 0.69X_3^2 + 1.22X_1X_2 + 0.87X_1X_3 – 0.27X_2X_3$ …………….. **(3)**

Where X_1, X_2, X_3 coded values of pH, NaOH concentration and flour to solvent ratio respectively. Above equation showed that linear terms X_1 , X_2 and X_3 possesses highly significant positive effect with F-value 1079.97, 59.12 and 258.87 respectively on the protein yield. P-values of X_1, X_2 and X_3 were found to be <0.0001, <0.0001 and <0.0001respectively. Quadratic term of pH (X_1^2), NaOH concentration (X_2) and flour to solvent ratio (X_3) was having significant negative effect with F-value 55.92, 5.69 and 23.99 respectively at P<0.05. P values of pH (X_1^2), NaOH concentration (X_2) and flour to solvent ratio (X_3) were found to be <0.0001, 0.0382 and 0.0006. The interaction term X_1X_2 and X_1X_3 possesses significant positive effect with P-values 0.0001 and 0.0010 respectively. While X_2X_3 showed non-significant negative significant effect with P-value 0.1797. As the linear terms pH, NaOH concentration and flour to solvent ratio have significant positive effect on the protein yield of protein concentrate. So increase in pH, concentration of sodium hydroxide and flour to solvent ratio leads to increase in protein yield of the protein concentrate of custard apple seed protein isolate. The relative effect of different variables on protein yield of protein concentrate can also be seen from the three dimensional plots. In **Fig.4.** Protein yield (%) was found to be increasing with increase in pH and NaOH concentration. It can be due to the higher solubility of protein and some non-protein compounds at high pH. It was reported that the high alkali concentration helps to break down the hydrogen bonds and to dissociate hydrogen from carbonyl and sulphate groups [23]. The increased surface charge on protein molecules leads to an enhanced solubility in the solvent system and ultimately increase in protein yield [24].

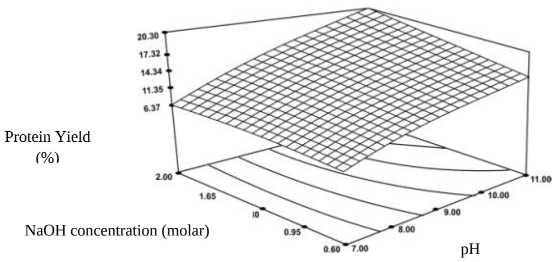

Fig .4. Response Surface plot for the effects of pH and NaOH concentration on protein Yield (%) of custard apple seed protein isolate

The combined effect of pH and flour to solvent ratio on protein yield is represented by **Fig. 5.** In this Fig. protein yield (%) increased with increase in pH and flour to solvent ratio. Similar trend was observed in interactive effect of pH and flour to solvent ratio reported by Mizubuti et al.,[14].

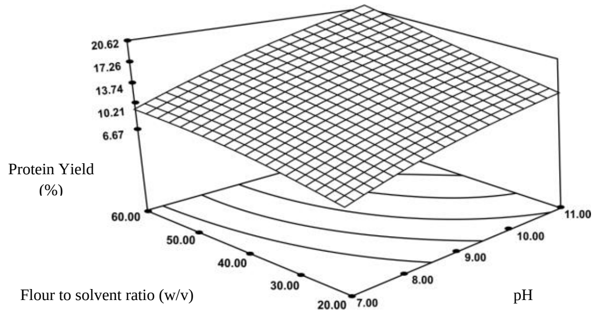

Fig .5. Response Surface plot for the effects of pH and flour to solvent ratio on protein Yield (%) of custard apple seed protein isolate

The interactive effect of NaOH concentration and flour to solvent ratio on protein yield is shown in **Fig.6.** where protein yield (%) increases linearly with increase in concentration of sodium hydroxide and flour to solvent ratio.

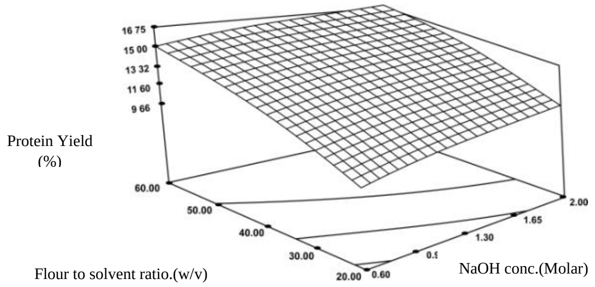

Fig .6. Response Surface plot for the effects of NaOH and flour to solvent ratio on protein Yield (%) of custard apple seed protein isolate

7. OPTIMIZATION

A numerical multi-response optimization technique was applied to determine the optimum combination of pH, NaOH concentration and flour to solvent ratio for the production of protein concentrate with respect to its yield and protein content. The assumptions were to develop a product which would have maximum protein content and protein yield. Therefore, among responses, these parameters were attempted to be maintained whereas other parameters were kept within range. Under these criteria, the uncoded optimum operating conditions for the development of protein concentrate were pH (11), NaOH concentration (0.67) and flour to solvent ratio (1:60) respectively. The responses predicted by the Design expert -6 software for these optimum process conditions resulted protein content 68.07% and protein yield 18.69%.

8. VERIFICATION OF RESULTS

The suitability of the model developed for predicting the optimum response values was tested using the recommended optimum conditions of the variables to validate experimental and predicted value of the responses. The experimental samples under the optimum process conditions resulted protein content 68.07% and protein yield 18.69% which were in close proximity to the predicted results as shown in **Table 7.**

Table 7: Predicted and Actual values of the responses at the optimized conditions of experiment

Parameters	Uncoded	Responses	Actual Value	Predicted Value	Variation
pH	11	Protein Content (%)	68.07	66.89	1.72
NaOH Concentration	0.67	Protein Yield (%)	18.69	18.07	3.31
Flour to solvent ratio (w/v)	1:60				

9. CONCLUSION

The present study was conducted to develop protein concentrate and to study the effects of pH, NaOH concentration and flour to solvent ratio on protein content and protein yield of custard apple seed protein concentrate by using Response Surface Methodology (RSM). The results of different experiments showed that higher pH resulted in maximum protein content and protein yield while higher NaOH concentration and flour to solvent ratio resulted in minimum protein content and maximum protein yield whereas higher flour to solvent ratio resulted in maximum protein content and protein yield. The different optimized conditions obtained by numerical optimization for the custard apple seed protein concentrate were pH (11), NaOH concentration (0.67M) and flour to solvent ratio (1:60 w/v). The findings of the present work demonstrates the feasibility of developing protein concentrate by alkali method for custard apple seed flour. The information obtained in this study will allow food scientists to search for optimal preparation strategies for using the various products of protein isolates of custard apple seed.

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