INTERNATIONAL JOURNAL OF FOOD AND NUTRITIONAL SCIENCES

IMPACT FACTOR ~ 1.021

Official Journal of IIFANS

Research Paper   *Open Access*

OPTIMISATION OF EXTRACTION OF PROTEIN ISOLATE FROM RAPESEED MEAL USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Optimisation of protein extraction from defatted rapeseed meal was done by response surface methodology. A central composite design including independent variables of pH (8, 9, 10, 11, 12), solvent to meal ratio (5:1, 10:1, 15:1, 20:1, 25:1 v/w), NaCl concentration (0, 0.5, 1, 1.5, 2 M) and time (40, 50, 60, 70, 80 min) were used. The selected responses (dependent variables) which evaluate the extraction process were protein isolate yield (g), protein concentration (%) and whiteness index. The four factors had positive effect on protein yield isolate and protein concentration. But pH and solvent to meal ratio had negative effect on whiteness index while the other two had positive effect. The optimized conditions for pH, solvent to meal, NaCl concentration and time are 10.5, 14:1 (v/w), 0.9 M and 53 min., respectively. The suitability of the model was verified by extracting the protein under optimized conditions as determined by the model.

Keywords: Response surface methodology, optimization, rapeseed, protein extraction, whiteness index

INTRODUCTION

Rapeseed is one of the major cultivated crops in India. India produces about six million tons of rapeseed. India ranks third after China and Canada in production contributing 12 percent of total world production. With second position in area and third position in production, India holds a premier position in world economy. It is a key edible oilseed crop in India which accounts for one third of the oil produced in India (Kumar et. al., 1990). Rapeseed has a high protein content of 15-20 % which further increases to around 40 % in the defatted meal. Defatted meal is mainly used as animal feed, to improve sugarcane yield, as fertilizer to increase yield of tea plant, green leafy vegetables, papaya, orchids, tobacco, etc. Because of the high protein content of the defatted meal it has immense potential to be a rich source of low cost protein. Rapeseed protein contains a well balanced amino acid profile but the use of the meal is limited in food applications due to the presence of antinutritional factors such as glucosinolates which interferes with thyroid function reducing growth, erucic acid that has potential to produce toxic effects in heart, phytates which strongly bind polyvalent metal ions such as zinc and iron making them unavailable for metabolism and phenolics that have bitter flavour and make the protein products darker in colour (Diosady, Rubin, and Chen, 1990).

Proteins are the building blocks of the human body. But apart from being structural component, protein as such can perform other functional properties which have a wide array of uses in the commercial food industry. The amino acid composition mainly determines the physicochemical properties of the proteins in terms of both electrostatic and hydrophilicity properties. The electrically charged groups of protein molecules play an important role in determining the nature of interaction of protein with other substances (Phillips, Kinsella and Whitehead, 1994) in foods and for industrial purposes. The amino acid composition compares favourably with that of FAO/WHO reference protein (Sarwar, et. al., 1984). Due to the antinutritional factors in the protein rich meal, the protein has to be extracted for any food use. The extraction, solubility and functional properties of protein are affected by parameters like pH of the solvent, solvent to meal ratio, temperature, ionic force, salt or solvent type, extraction time and presence of compounds that causes linking (Quanhong and Caili, 2005).

Response surface methodology (RSM) is a very effective tool for optimization of processes where factors and their interactions affect the desired response. The methodology encompasses use of various types of experimental designs, generation of polynomial equation and mapping of the response over the experimental domain to determine the optimum response (Chauhan, Bhardwaz and Chakrabarti, 2013). The main advantage of RSM is the reduced number of experimental trials required to evaluate the multiple parameters and their interactions. It uses the design as a central technique. The technique requires minimum of experiments saving time and thus is more effective than conventional methods used for optimization. The 3D plots are used to study response surfaces and determine the optimum (Silpradit et. al., 2010). The central composite design (CCD) is the most popularly used design

of RSM. The CCD has three types of design points, (i) 2-level factorial or fractional factorial design points, (ii) axial (or α) points and (iii) centre points (Chang et. al., 2002, Beg, Sahai, Gupta, 2003; Chauhan and Chakrabarti, 2011; Box and Draper, 1987, Singh B, Kaur S and Ahuja N, 2006) . CCD is used to estimate the co-efficient of quadratic model. All point descriptions are in terms of coded values of factors. CCD requires 5 level of each factor viz, -α, -1, 0, 1, +α. One of the major attributes of the CCD is that its structure lends itself to sequential experimentation. RSM technique can therefore, be used to determine the optimized levels of the different factors that will influence extraction of protein isolate, the protein content and whiteness index of the protein isolate.

MATERIALS AND METHODS

MATERIALS

Commercially defatted and dehulled meal of rapeseed after oil pressing procedure was obtained from a local factory. The meal was further defatted with hexane and used in this study as starting material. All the other chemicals were of analytical grade. The pH was adjusted with 1M HCl or 1M NaOH with a pH meter (Eutech).

METHODS

EXTRACTION OF PROTEIN

Protein was extracted by a modified method of Vioque et. al., (2000) and Sadeghi & Bhagya, (2009). Rapeseed defatted flour (10 g) was suspended in solvent in the required ratio. The solvent contained 0.05 M Tris buffer, 0.25% sodium sulfite (Na_2SO_3) and sodium chloride (NaCl) of given concentration. Na_2SO_3 was used to prevent oxidation of polyphenols and to avoid the darkening of the final product while NaCl was used to prevent the binding of phenolic acids to the protein (Sadeghi and Bhagya, 2009). The suspension was extracted by stirring continuosly at room temperature. After centrifugation at 8,000 × g, two more extractions were carried out with half of the volume of alkaline solution. The supernatants were pooled and adjusted to the isoelectric point (pH 3.8) for precipitation of the soluble proteins which was recovered by centrifugation at 8,000 × g. The precipitate was washed with distilled water, adjusted to pH 3.8 and freeze-dried. The moisture content of the isolates was determined.

PROTEIN ISOLATE YIELD

The weight of the protein isolate was taken as protein isolate yield (Digital balance, Denver instruments).

PROTEIN CONTENT

The protein content of the rapeseed meal was determined by micro-kjehdahl method (AOAC, 1998).

MOISTURE CONTENT

For moisture content determination by vacuum drying method was used (AOAC, 1980).

COLOUR MEASUREMENT AND WHITENESS INDEX

The *L, a, b* parameters of the extracts were taken with Color Measurement Spectrophotometer (Hunter Color-Lab Ultrascan Vis). *'L'* denotes lightness of the sample, *'a'* denotes the redness or greenness and *'b'* denotes the blueness or yellowness. The whiteness index was calculated as WI= L-3b (Salcedo-Chaävez B., et. al., 2002).

EXPERIMENTAL DESIGN

To study the response pattern and determine optimum combination of the variables, a central composite experimental design with four variables was used. Experimental range and levels of the independent variables, viz. X_1 (pH, P), X_2 (solvent:meal ratio, S), X_3 (NaCl concentration, C), and X_4 (time, T) at five levels used to design the process are given in Table 1. The effect of the independent variables (Table 1) in the extraction process is shown in Table 2. Six centre points of the design were used to allow for estimation of a pure error sum of squares. All the experiments were carried out in random order so as to maximize the effects of unexplained variability in the observed responses due to extraneous factors. Low and high level of factors were coded as -1 and +1, and midpoint was coded as 0. The factor level of trials that ran along axes drawn from the middle of the cube through the centers of each face of the cube were coded as −α and +α.

The variables were coded according to the following equation

$x_i = (Xi - \overline{X}i) / \Delta Xi$

where, x_i is the dimensionless value of an independent variable, Xi is the real value of an independent variable, Xi is the real value of an independent variable at the center point, ΔXi is the step change. The specific codes are:

x_1 (pH) = (P- 10)/1,

x_2 (solvent: meal) = (S-15)/5,

x_3 (concentration) = (C-0.25)/0.13, and

x_4 (time) = (T-60)/10

Table1. Experimental range and levels of the independent variables used to design the process

Independent variables	Symbol		Levels				
	Uncodified	Codified	-α	-1	0	+1	+α
pH	X_1	x_1	8	9	10	11	12
Solvent:Meal (v/w)	X_2	x_2	5:1	10:1	15:1	20:1	25:1
NaCl (M)	X_3	x_3	0	0.5	1	1.5	2
Time (min)	X_4	x_4	40	50	60	70	80

Table 2. Central composite design (CCD) for the preparation of protein isolate and its responses

Run	Factors				Response 1	Response 2	Response 3
	pH	Solvent:Meal (v/w)	NaCl (M)	Time (min)	Protein isolate yield (g)	Protein Content (%)	Whiteness index
1	9	10:1	0.5	50	1.17	67.25	61.03
2	11	10:1	0.5	50	2.1	71.15	53.03
3	9	20:1	0.5	50	0.72	69.97	47.29
4	11	20:1	0.5	50	1.52	73.2	45.44
5	9	10:1	1.5	50	1.68	68.15	65.3
6	11	10:1	1.5	50	2.14	72.5	55.91
7	9	20:1	1.5	50	1.08	72.45	56.8
8	11	20:1	1.5	50	2.1	74.21	53.56
9	9	10:1	0.5	70	1.27	76.55	55.67
10	11	10:1	0.5	70	2.28	80.35	49.34
11	9	20:1	0.5	70	0.88	79.35	54.7
12	11	20:1	0.5	70	1.89	82.85	54.53
13	9	10:1	1.5	70	1.58	77.15	60.79
14	11	10:1	1.5	70	2.88	80.15	53.08
15	9	20:1	1.5	70	1.11	79.75	65.07
16	11	20:1	1.5	70	1.8	84.45	63.51
17	8	15:1	1	60	0.86	69.95	58.79
18	12	15:1	1	60	2.45	76.85	49.22
19	10	5:1	1	60	1.35	70.21	63.39
20	10	25:1	1	60	1.48	76.37	60.08
21	10	15:1	0	60	1.68	73.55	53.22
22	10	15:1	2	60	1.54	74.31	66.47
23	10	15:1	1	40	1.77	65.24	55.87
24	10	15:1	1	80	1.86	83	60.46
25	10	15:1	1	60	1.68	75.15	56.72
26	10	15:1	1	60	1.54	77.25	56.73
27	10	15:1	1	60	1.37	74.85	56.72
28	10	15:1	1	60	1.87	76	56.72
29	10	15:1	1	60	1.63	74.15	59.22
30	10	15:1	1	60	1.58	73.15	59.21

STATISTICAL ANALYSIS

Design-Expert Version 6.0.11 (Stat-Ease Inc., Minneapolis, USA) was used to conduct the experimental design. The protein isolate yield, percentage of protein content and whiteness index obtained were taken as dependent variables or responses. The model proposed for the response is given by the following equation.

$$Y_i = b_0 + \sum_{n=1}^{4} b_n x_n + \sum_{n=1}^{4} b_{nn} x_n^2 + \sum_{n \neq m=1}^{4} b_{nm} x_n x_m$$

where, Y_i (i =1, 2, 3) is predicted response for protein isolate yield, protein content and whiteness index; b_0 is the value of the fitted response at the center point of the design that is point (0,0,0,0). b_n, b_{nn} and b_{nm} are the linear, quadratic and interaction regression terms, respectively. The quadratic model was used for the analysis.

The 'p' value of the regression co-efficient explains the pattern of mutual interaction between variables, the smaller the value of 'p', the corresponding co-efficient is more significant. The optimum level of pH, NaCl concentration, solvent to meal ratio and time was obtained by maximizing the protein isolate yield, protein content and whiteness index through numerical optimization. The quality of fit of second order equation was determined by co-efficient of determination R^2 and its statistical significance was determined by F test. The individual and interactive effects of the independent variables were evident from the model graphs.

RESULTS AND DISCUSSION

ANOVA FOR PROTEIN ISOLATE YIELD

Proposed model (2nd order polynomial regression) equation for response is

Meal yield = +1.61 + 0.21*A + 0.090*B + 0.14*C + 0.45*D + 0.093*A^2 - 0.026*B^2 + 0.19* C^2 + 0.20* D^2 + 0.032*A* B - 0.012*A*C + 0.022*A*D - 0.003*B*C + 0.013*B*D - 0.086*C*D

Here, A is pH, B is solvent to meal ratio, C is NaCl concentration and D is time. The equation in terms of

coded factors can be used to make predictions about the response for the level of each factor. The coded equation is useful for identifying the impact of the factors by comparing the factor coefficient. The ANOVA for response surface quadratic model is given in Table 3. The model F value of 14.49 implies that the model is significant. There is only 0.01 % chance that an F-value this large could be due to noise. Values of "Prob>F" less than 0.05 indicate model terms are significant. In this case, A, C, D, A^2, C^2, D^2 are significant model terms. The "lack of fit" is not significant relative to pure error which further indicates the validity of the model. The "lack of fit" value of 1.89 indicates that there is 25.03 % chance that the value this large could be due to noise. The prediction equation showed a good fit with the experimental design since the R^2 value of 0.9312 indicated that 93.12% of the variability within the range values studied could be explained by the model (Fig. 1). 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 10.55%. It means that the model was quite reproducible. "Adeq precision" measures the signal to noise ratio. A value greater than 4 is desirable. The ratio of 14.082 indicates that the model can be used to navigate the design space. 3D contour plots were drawn to demonstrate the main and interactive effects of the independent variables on the dependent variables. These graphs (Fig. 2) were obtained by fixing two variables at coded zero level while the other two variables

varied to predict the response variable (protein isolate yield).

Table 3. Coefficient of the fitted model for the determined response

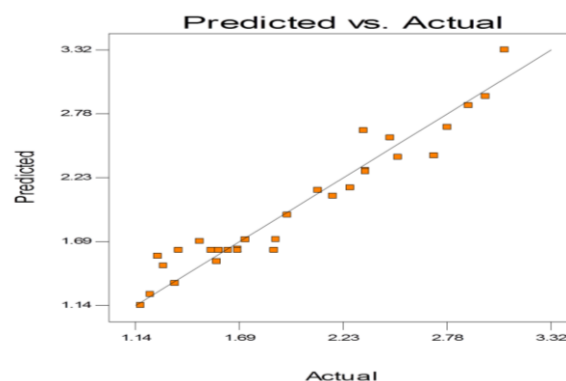| Coefficient	Protein isolate yield
Model F-value	14.49
Prob>F	<0.0001
Lack of fit	1.89
R^2 value	0.9312
CV value	10.55 %
Adeq precision value	14.082

Figure1: Comparative predicted and actual values for protein isolate yield at R^2= 0.93
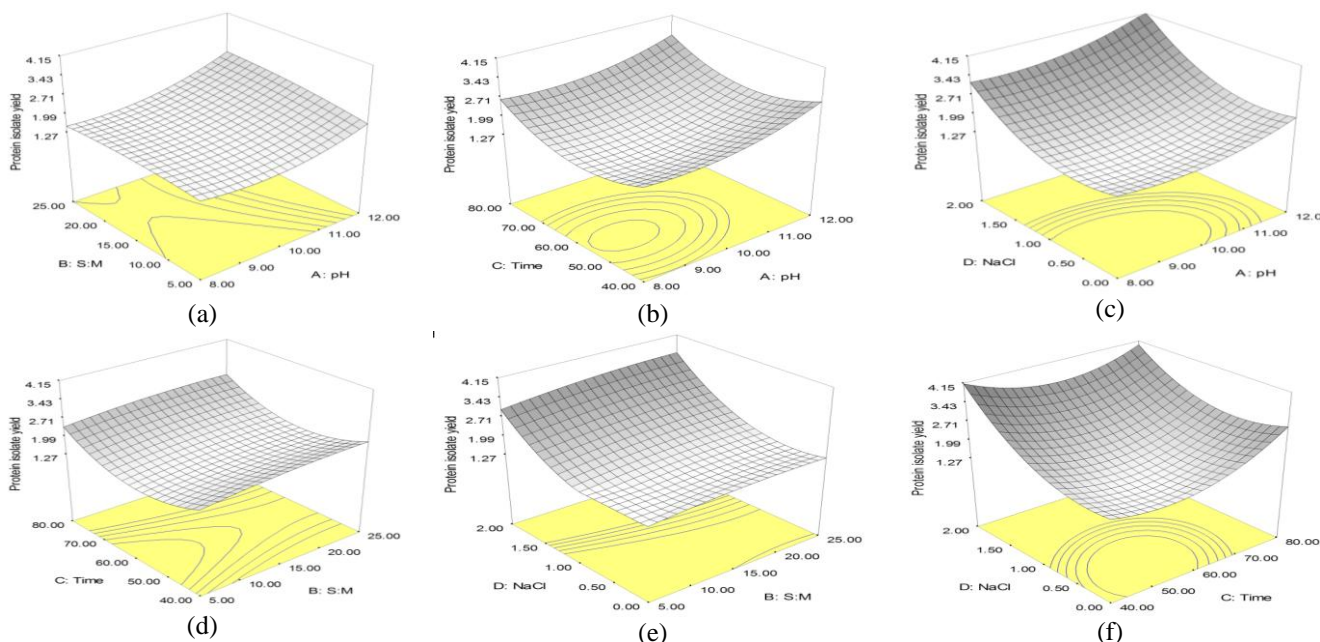

Figure 2: Effect of interaction of the factors on Protein isolate yield

ANOVA FOR PROTEIN CONTENT

Proposed second order polynomial regression equation for response is
Protein content = + 75.09 + 1.75*A + 1.47*B +0.40*C + 4.47*D - 0.11*A^2 - 0.13*B^2 + 0.027*C^2 + 0.075*D^2 - 0.12*A* B - 0.039*A*C + 0.11*A*D + 0.18*B*C + 0.089*B*D - 0.21*C*D

The factors are coded as stated earlier. The equation in terms of coded factors can be used to make predictions about the response for the level of each factor. The coded equation is useful for identifying the impact of the factors by comparing the factor coefficient. The ANOVA for response surface quadratic model is given in Table 4. The model F value of 20.05 implies that the

model is significant. There is only 0.01 % that an F-value this large could be due to noise. Values of "Prob>F" less than 0.05 indicate model terms are significant. In this case, A, B and D are significant model terms. The "Lack of fit" is not significant relative to pure error which is good as we want the model to fit. The "Lack of fit" value of 1.10 indicates that there is 48.71 % chance that the value this large could be due to noise. The prediction equation showed a good fit with the experimental design since the R^2 value of 0.9493 indicated that 94.93% of the variability within the range values studied could be explained by the model (Fig. 3). The ANOVA for response surface quadratic model is given in Table 4. The CV of the model was 1.97%. As a general rule, a model can be considered reasonably reproducible if its CV value is not greater than 10% (Firatligil-Durmus and Evranuz, 2010). "Adeq precision" measures the signal to noise ratio. A value greater than 4 is desirable. The ratio of 17.125 indicates that the model can be used to navigate the design space. 3D contour plots were drawn to demonstrate the main and interactive effects of the independent variables on the dependent variables. These graphs (Fig. 4) were obtained by fixing two variables at coded zero level while the other two variables varied to predict the response variable (protein content).

Table 4. Coefficient of the fitted model for the determined response

Coefficient	Protein content
Model F-value	20.05
Prob>F	<0.0001
Lack of fit	1.10
R^2 value	0.9493
CV value	1.97 %
Adeq precision value	17.125
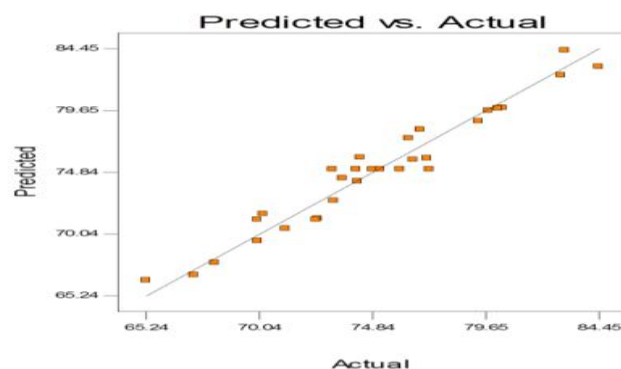
Figure 3: Comparative predicted and actual values for protein content at R^2= 0.94
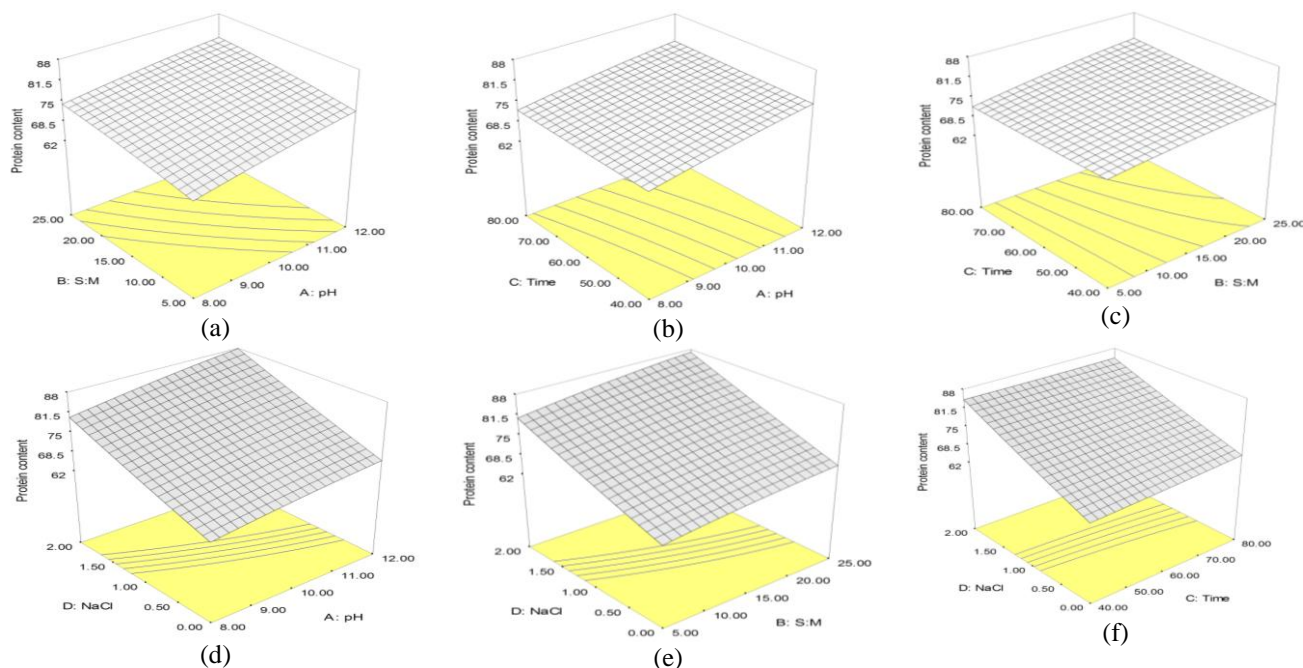

(a) (b) (c)

(d) (e) (f)

Figure 4. Effect of interaction of the factors on protein content

ANOVA FOR WHITENESS INDEX

Proposed 2nd order polynomial regression equation for response is

Whiteness Index $= + 57.56 - 2.39*A - 0.83*B + 3.31*C + 1.14*D - 1.30*A^2 + 0.63*B^2 + 0.16*C^2 - 0.26*D^2 + 1.54*A*B - 0.34*A*C + 0.42*A*D + 1.31*B*C + 3.19*B*D + 0.21*C*D$

Similarly as earlier, the coded equation can be used to make predictions about the response for the level of each factor and is also useful for identifying the impact of the factors by comparing the factor coefficient. The

ANOVA for response surface quadratic model is given in Table 5. The model F value of 19.35 implies that the model is significant. There is only 0.01 % that an F-value this large could be due to noise. Values of "Prob>F" less than 0.05 indicate model terms are significant. In this case, A, B, C, D, AB, BC, BD and A^2 are significant model terms. The "Lack of fit" value of 2.00 is not significant relative to pure error which is good as we want the model to fit. There is only 23.00 % chance that the value this large could be due to noise. The prediction equation showed a good fit with the experimental design since the

R^2 value of 0.9478 indicated that 94.78% of the variability within the range values studied could be explained by the model (Fig. 5). The CV of the model was 2.92%. As a general rule, a model can be considered reasonably reproducible if its CV value is not greater than 10% (Firatligil-Durmus and Evranuz, 2010). "Adeq precision" measures the signal to noise ratio. A value greater than 4 is desirable. The ratio of 17.125 indicates that the model can be used to navigate the design space. 3D contour plots were drawn to demonstrate the main and interactive effects of the independent variables on the dependent variables. These (Fig. 6) graphs were obtained by fixing two variables at coded zero level while the other two variables varied to predict the response variable (Whiteness index).

Table 5. Coefficient of the fitted model for the determined response

Coefficient	Whiteness Index
Model F-value	19.35
Prob>F	<0.0001
Lack of fit	2.00
R^2 value	0.9478
CV value	2.97%
Adeq precision value	17.125

Table 6. Optimum of condition (based on graphical optimisation), predicted and experimental value of the response at that condition

Factors	Optimum conditions	
pH	10.47	
Solvent:Meal (v/w)	14.15:1	
NaCl (M)	0.9	
Time (min)	53.06	
Responses	**Predicted**	**Experimental**
Meal Yield (g)	1.61	1.67±0.12[a]
Protein Content (%)	75.09	74.21±0.03[a]
Whiteness Index	57.56	57.42±0.03[a]

[a]Mean value of five determinations
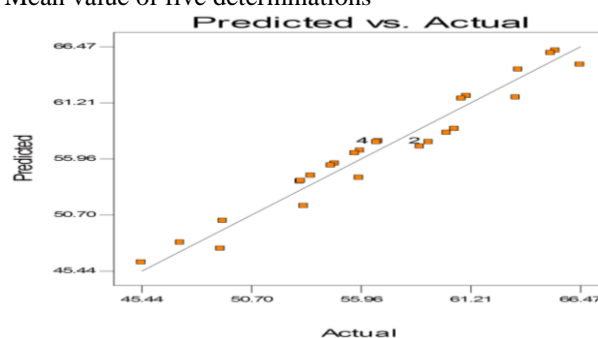
Figure 5: Comparative predicted and actual values for whiteness index at R^2= 0.94

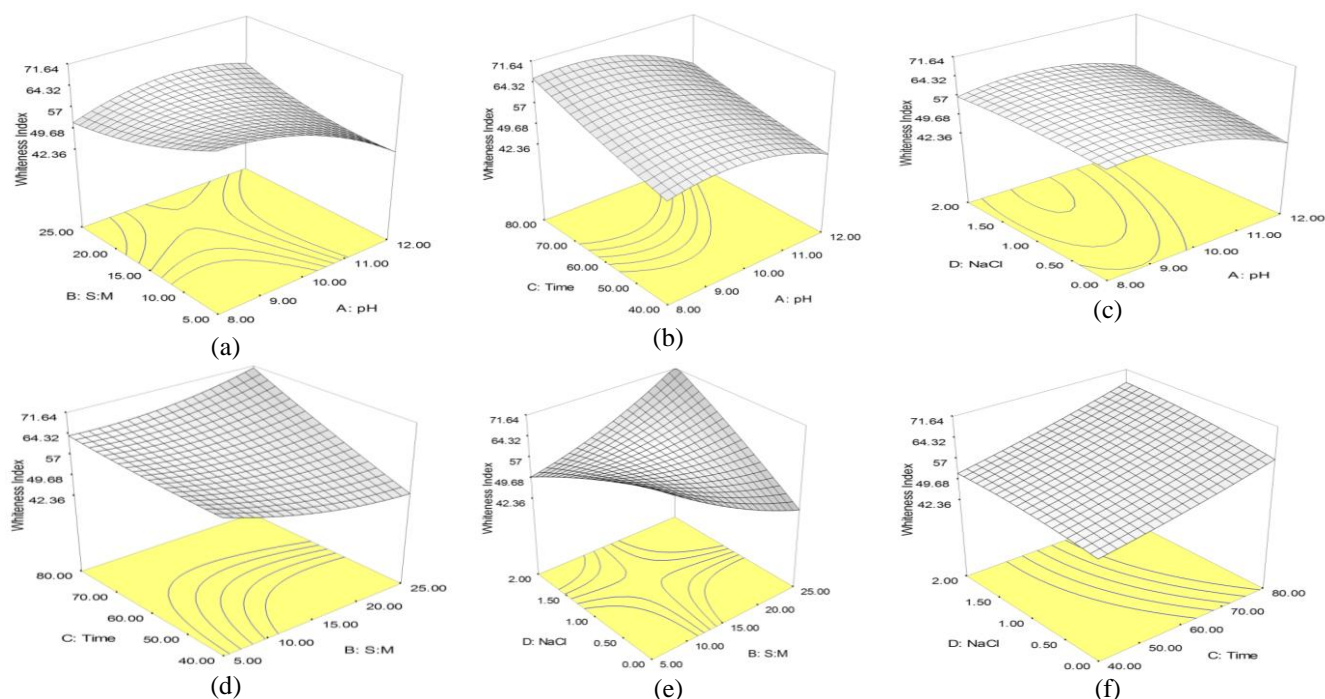(a)   (b)   (c)

(d)   (e)   (f)

Figure 6. Effect of interaction of the factors on whiteness index

EFFECT OF THE DIFFERENT FACTORS AND THEIR INTERACTION ON THE RESPONSES

From review of several published literature on protein extraction the four parameters viz, pH, time, solvent to meal ratio and NaCl concentration (Firatligil-Durmus and Evranuz, 2010; Wani et. al.; Kanu et. al., 2007; Mune, Minka, Mbome, 2008) were selected to study their effects on the responses. Therefore, the central

composite design was done using the four factors. The minimum and maximum levels were chosen from related published literature.

Nitrogen extractability increases at high pH. It was found to be maximum at pH 12 (Ghodsvali et. al.,2005). Extraction at higher pH was not done as it had undesirable effect on protein isolate. Wang et.al., 1999, reported that high pH caused protein hydrolysis and denaturation resulting in an unacceptable odour and flavour. Increased pH also increased Maillard reaction that darkened the product, decreased nutritive value of protein, especially essential amino acid such as lysine, while increased the extraction of non-protein component, which coprecipitated with protein leading to lower protein purity. The effects of the four parameters seem to be similar for both protein isolate yield and protein content. With increase in the pH both protein isolate yield and protein content increased. The extraction was more beyond pH 10 but it was quite linear from pH 11 to 12 (Manamperi et. al., 2007). A further increase in pH could not be attained as the meal turned dirty green in colour which is an undesirable characteristic and the solvent to meal ratio could not be maintained.

The meal to solvent ratio had a positive effect on both protein isolate yield and protein content i.e. with increase in meal to solvent ratio the extraction of protein increased. The extraction of protein increased with increase in solvent to meal ratio in the beginning but the rate of increase slowed down later. The increase of extraction in the beginning was because of availability of more solvent that allowed more extraction. But the slow increase in extraction with further increase in ratio may be due to solvent which was not enough to disperse and extract protein (Loomis & Battaile, 1996).

NaCl concentration also had a positive effect on extraction of protein. With the increase of NaCl the effect on extraction increases. The phenolic compounds present in rapeseed are bound to the protein by various mechanisms in aqueous medium such as hydrogen bonding (Mason, 1955), covalent bonding (Loomis and Battaile, 1996), hydrophobic interaction (Hagerman & Butler, 1978) and ionic bonding (Rubino et. al., 1996). Extraction of protein in aqueous medium in presence of NaCl prevents the formation of the complexes thus aids in the process of extraction of protein. But excess of the salt interferes with the functional properties such as foaming capacity, foaming stability, emulsion capacity and emulsion stability of the protein. NaCl has a negative effect on both the emulsion capacity and stability of protein concentrates (Adubiaro et. al. 2012, Ogungbenle, 2008). NaCl have a positive effect on the foaming capacity at lower concentrations only (Andualem and Gessesse, 2013).

Extraction of protein increased with time i.e. the factor also had a positive effect. Increase in time increases the interaction between the solvent and meal which increases the extraction of protein. However, increasing the time beyond one hour causes frothing of the solution which is due to denaturation of the protein and coagulation of protein matrix (Kanu et. al., 2007).

Whiteness index (WI) of the extracted protein was also affected by the four factors. The interaction of the factors and their effect on WI index is shown in Fig. 6. pH and solvent meal ratio had negative effects while NaCl concentration and time had positive effects. The values of whiteness index ranged between 45 and 66. Decrease in phenolic compounds increases the WI (Salcedo-chaävez, B., et. al., 2002). Negative effect on WI because of increase in pH may be due to the fact that high pH accelerates the protein and phenolic reaction (Marcone and Kakuda, 1999; Bejosano and Corke, 1998). Positive effect of NaCl is explained by the fact that NaCl prevents the binding of phenols to protein as explained earlier. The longer interaction time helped in breaking protein phenol bonds. Xu and Diosady, 2002 found that a 72.5% decrease in phenolic compound would cause an increase of WI by 28.4%.

VERIFICATION OF THE RESULTS

The capability of the mathematical model obtained to predict the optimization of the response values using the recommended levels of the factors was tested. The values of the responses obtained experimentally were in agreement with the predicted ones (Table 6). Thus, it can be said that the model can be successfully used for prediction of optimized responses.

Quanhang and Caili (2005) optimized the extraction of protein germinated pumpkin seed and found significant effects of solvent/meal ratio and found that optimum conditions were: solvent/meal ratio of 30.2:1 (v/w), NaCl concentration of 4.26% and a extraction time of 18.1 min. Wani et al. (2006) studied the extraction of protein from watermelon seed and concluded that maximum protein yield was obtained by extracting seed meal with a 1.2% NaOH concentration, solvent/meal ratio of 70:1, extraction time of 15 min and temperature of $40^{o}C$. Liadakis et al. (1995) worked on optimization of protein extraction from tomato seed and found that optimum extraction was achieved by extracting at pH 11.5 at $50^{o}C$ for 20 min at ratio of 1:30 (w/v). Rustom et al. (1991) reported that time, temperature, pH and solvent/meal ratio had significant effects found the optimum extraction conditions were: pH of 8.0, time of 30 min; temperature of $50^{o}C$ and solvent/meal ratio of 8.1. Mizubuti et al. (2000) found the optimum conditions for pigeon pea protein extraction were pH at 8.5 and solvent/meal ratio at 5.1. Oomah et al. (1994) determined for extracting protein from flaxseed meal optimum conditions that were solvent to meal ratio of 10 l/kg, 0.8 mol/L NaCl and pH 8.0.

CONCLUSION

In conclusion, response surface methodology technique was found to be very useful in determining the optimization of conditions for extraction of protein isolate. Protein isolate was extracted from defatted protein meal that remained after oil extraction. The quadratic model developed exhibited a non-significant value for lack of fit and high value for the coefficient of determination. The optimum extraction was achieved by extracting the meal in

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meal: solvent ratio of 14:1 (v/w) at pH 10.5 with NaCl concentration of 0.9 M in 53 min.

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