Process optimization of chickpea (*Cicer Arietinum* L.) seed protein isolates for functional foods

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Abstract
Chickpea protein isolates can be a good source of biologically available protein as an alternative to meat protein. Desi chickpea (Cicer arietinum L.) seeds (cultivar K850) were used in the present study for protein isolate preparation. Protein content and protein isolate yield optimization was done using central composite rotatable design (CCRD) and different combinations of process variables such as pH, meal/solvent ratio, temperature and time were selected. Response surface methodology (RSM) with a suitable experimental design was used to develop a model for optimization of process parameters to obtain protein isolates with maximum protein content and yield. A second order polynomial equation was obtained for both responses and model was fitted based on Analysis of Variance (ANOVA), coefficient of determination and lack of fit. Higher protein content in chickpea protein isolate (CPI) resulted in lower yield.

Low temperature (40˚C) and high pH (11) for longer time were used for mixing of alkali solubilization of protein and yielded chickpea protein isolates with higher protein content (93.96%). The 3D surface plots drawn between the responses and process variables indicate linear, interactive and quadratic effects which were synergistic and antagonistic. Optimized protein isolates can be good source of viable plant protein having higher protein content.

Keywords: Chickpea, optimization, protein isolates, response surface methodology (RSM).

Introduction

India is rich in agricultural diversity in terms of abundant crop diversity, soil types, climate and monsoons. Varieties of pulse crops are grown in India which are mostly consumed as 'dal' (dehusked split legumes). Legumes are the second largest consumed grains of the world after cereals. Legumes are essential component of Indian staple diet as the cheapest plant protein source for vegetarians[10].

They contain bioactive peptides which are associated with reduced risk of cancer, hypertension and antithrombotic activity and high amounts of dietary fibre in pulses enhance weight loss in obese individuals and reduce gastrointestinal diseases[3]. Chickpea (*Cicer arietinum*) also called as Bengal gram or garbanzo bean is the third most widely consumed pulse after field beans (*Vicia faba*) and dry beans (*Phaseolus vulgaris*)[14] with approximately 14.7 million tonnes[7].

Chickpea was the major pulse produced by India (7.48%) followed by pigeon pea (*Cajanus cajan*) (2.6%) in year 2015-16. Major amount of chickpea is imported in India from Australia (46%) and Russia (39%) [31].

Desi (microsperma) chickpea seed is characterized by thick brown coloured seed coat[19]. The growing demand for meat analogues or animal protein replacers as a source of functional food with high quality protein has paved interest in protein isolate production. Interest in plant protein is increasing due to its use in food and nonfood applications in various industries. Amongst the legumes, soybean protein isolates are widely used as functional ingredients with nutritionally improved quality and enhanced economic value. Chickpea seed is also a good source of protein constituting 80% of the total dry seed mass with potential health benefits. It is also a good source of dietary fibre, vitamins, polyphenols and minerals[39].

Polyphenolic compounds have health protective effect due to their ability to alleviate oxidative stress and prevention of free-radical mediated chronic diseases[11]. Polyphenols have antioxidant[35], anticancer[33], antimicrobial[22], anti-diabetic, antihypertensive[9] and antimutagenic[24] properties. The desi chickpea cultivar (K850) selected for the present study contains 255 mg/ 100 g total phenolic content (TPC) and higher antiradical activity (88%) due to polyphenols as reported by Sharma et al[28].

Effect of modification in protein solubilization method by using sodium sulphite in addition to sodium hydroxide has been studied by Sa´nchez-Vioque et al[25]. Its effect on isolate yield, chemical composition, functional properties and protein composition has also been investigated[25]. Certain physicochemical, functional and thermal properties of desi and kabuli Indian chickpea protein isolates showed significant differences as investigated by Kaur and Singh[15]. Different methods of protein isolate recovery by isoelectric precipitation and ultrafiltration consisted mainly of globulin fraction rather than albumin[21]. No work has been done on protein isolate optimization from Indian desi chickpea cultivar. Majority of the scientific studies on optimization of protein isolate preparation process have been done on watermelon[37], custard apple[34], lentil[13] and red pepper[8].

The major objective of this study is to obtain optimized conditions for chickpea protein isolate preparation having more than 90% protein content on dwb. Hence, the

optimization of protein isolate preparation from chickpea seeds is very essential for large scale production. The outcome of this study will provide optimized set of variables for protein isolate production with maximum protein content and protein isolate yield which will be desirable for further use in product development or hydrolysate production from it. Protein isolate from chickpea can be incorporated into biscuit, cakes, cookies, beverages etc. for preparation of functional foods, depending upon the physicochemical properties of protein isolates.

Material and Methods

Materials: Desi chickpea (*Cicer arietinum L.*) seeds (cultivar K850) used in the present study were procured from SVPUAT (Sardar Vallabhbhai Patel University of Agriculture and Technology), Gujarat. All the chemicals used were of analytical grade (Merck and Fischer Scientific Co., India).

Preparation of sample for protein extraction: The desi chickpea seeds (K850) were cleaned manually to remove stones and dust. Whole seeds were grinded into fine powder through mixer (Company-Havells, India) which passed through sieve with definite pore size (No. 500 IS). Fine powder was defatted with n-hexane (1:3 ratio w/v) at room temperature for 5-6 h. The defatted meal was then oven dried at 50˚C for 1 hr and passed through sieve. The defatted flour was stored at 4˚C in air tight jars until use[8].

Preparation of chickpea seed protein isolate (CPI): CPI was prepared by alkaline dissolving and acid precipitation method[16]. The protein from chickpea seed flour was extracted with selected 30 combinations of four independent variables namely pH, meal/solvent ratio, temperature and time. Chickpea flour was mixed with distilled water at different meal/ solvent ratio (MSR). pH of the suspension was made alkaline (9-11) using 0.5 N NaOH and then heated at different time-temperature (40-50 ˚C and 10-20 min) combinations in a water bath shaker (Icon Instruments Company). Alkaline pH was chosen based on earlier studies done by Wani et al[37] as low pH increased protein yield but protein content of isolate was less than 90%.

After centrifugation at 8000 x g for 10 minutes, additional extraction was carried out with half the volume of solvent. The supernatants were pooled, pH of the soluble proteins was adjusted to the isoelectric pH of 4.3 using 0.5N HCl[25]. The precipitated protein was left to stand for 3-4 hrs at 4˚C.

The precipitate formed was recovered after centrifugation at 8000 x g for 10 minutes, washed with distilled water, neutralized (pH 7.0) with 0.5 N NaOH and subsequently freeze dried. The freeze dried protein isolates were stored at 4˚C for further analysis.

Proximate analysis: Moisture, protein (Micro-Kjeldahl method), fat, crude fiber and ash of the protein isolates were determined by AOAC methods[1] namely method numbers of 925.1, 981.10, 920.85, 962.09 and 923.03 respectively. The carbohydrate content was estimated by difference method.

Experimental Design and statistical analysis: Chickpea protein isolates with high protein content were obtained using response surface methodology (RSM) with full factorial design (Central Composite Rotatable Design-CCRD). It is a common tool used for process optimization, since it varies all the variables at the same time with minimum number of assays, it shows interactive effect among independent variables and the complete effect of all the parameters on the process and the responses. It is one of the most widely used mathematical and statistical techniques despite the limitation that it assumes the existence of quadratic relationship between the process variables and response within the domain[5].

In order to determine which factor takes the response variable to a maximum or to minimum, quadratic or second order polynomial model was applied, the parabola (curvature in the plane) whose equation is mathematical for the response variable[12].

In the CCRD design, four factors involved in alkali solubilization of protein were 9-11 pH, 1/15-1/25 meal/solvent ratio (MSR, w/v), 40-50 temperature (T,˚C) and 10-20 time (t, min) have been used to generate 30 experimental runs and two responses namely, protein content (Y_1) and protein isolate yield (Y_2). Protein content was calculated by Kjeldahl method and protein yield of isolate was calculated on the basis of protein isolate weight (isolate weight x protein percent in flour)[34]. Since these are extrinsic factors affecting the protein content of chickpea isolate, hence these four factors were chosen for optimization.

Since alkaline pH and moderate temperature were used, hence functional properties of protein isolates such as solubility and yield were also maximum. During isoelectric precipitation at pH 4.5, solubility of protein is minimum, both amine and carboxyl groups along with equivalent charges are equal and hence isolates can be recovered from the solution as precipitate.

Quadratic polynomial equation (Eq.1) one for each response was used to study the effect of each variable where Y_i is the ith response (i=1-2), c_0 to c_{14} are the regression coefficients and x_1, x_2, x_3, x_4 are the dimensionless coded values for pH, MSR, T, t respectively (Table 1). In the model, real values were expressed in coded values (as in eq. 2) to determine the relative importance of every process parameter and the terms affecting the response. In eq. 1, intercept is represented by c_0, linear terms by x_1 to x_4, interaction terms[5,34,37] by $x_1 x_2$ to $x_3 x_4$ and quadratic terms by x_1^2 to x_4^2.

$$Yi = c0 + c1\,x1 + c2\,x2 + c3\,x3 + c4\,x4 + c5\,x1\,x2 + c6\,x1\,x3 + c7\,x1\,x4 + c8\,x2\,x3 + c9\,x2\,x4 + c10\,x3\,x4 + c11\,x12 + c12\,x22 + c13\,x32 + c14\,x42 \quad (1)$$

$$Xi = (xi - \dot{x}i)/\Delta xi \quad (2)$$

where X_i is the dimensionless value of independent variable (coded value), x_i is the real value of an independent variable, $\dot{x}_i$ is the real value of an independent variable at the center point and Δx_i is the step change.

The minimum and maximum limits of each process factor were based on some preliminary experiments done at pH-9, MSR-25, T-40˚C and t-25 min[34,38] which had higher isolate yield (18g/100g) but with low protein content (75-79%). Hence, from industrial facet, the amount of solvent used in isolate preparation was minimized and pH was increased to increase the protein % in the chickpea protein isolates (CPI). Triplicate analysis was performed to each replicate run (N=2x3) for estimation of protein content in CPI. RSM was performed in Design Expert 11.0.3.0 (Stat- Ease, Minneapolis, U.S.A) software. Statistical analysis was done through Analysis of Variance (ANOVA) at 95% confidence interval to test the model significance. The coefficient of variation (R^2) and adjusted coefficient of variation (Adj R^2) were also used to assess the validity of the model at $p<0.05$.

Table 1
Range and levels of the independent variables in the CCRD design

Independent variables	Symbol uncoded	Levels in the coded form				
		-2	-1	0	+1	+2
pH (X_1)	A	8	9	10	11	12
Meal/solvent ratio (MSR, w/v) (X_2)	B	1:10	1:15	1:20	1:25	1:30
Temperature (T,˚C) (X_3)	C	35	40	45	50	55
Time (t, min) (X_4)	D	5	10	15	20	25

Table 2
Central Composite Design for the effect of independent variables on response variables

Run	pH (X_1)	Meal/Solvent Ratio (X_2)	Temperature (X_3) (T,˚C)	Time (X_4) (t, min)	Protein Content (Y_1,%)	Protein Isolate Yield (Y_2, %)
1	8.00	20.00	45.00	15.00	78.31	60.97
2	9.00	15.00	40.00	10.00	81.07	62.63
3	9.00	15.00	40.00	20.00	83.56	63.15
4	9.00	25.00	40.00	10.00	76.82	63.65
5	9.00	25.00	40.00	20.00	79.69	64.92
6	9.00	15.00	50.00	10.00	75.71	57.99
7	9.00	15.00	50.00	20.00	76.78	58.57
8	9.00	25.00	50.00	10.00	73.46	59.38
9	9.00	25.00	50.00	20.00	74.17	60.55
10	10.00	20.00	35.00	10.00	80.6	64.63
11	10.00	10.00	45.00	5.00	74.91	56.72
12	10.00	10.00	45.00	15.00	81.4	58.85
13	10.00	20.00	45.00	15.00	78.29	66.93
14	10.00	20.00	45.00	15.00	77.74	68.35
15	10.00	20.00	45.00	15.00	77.11	68.81
16	10.00	20.00	45.00	15.00	78.72	65.62
17	10.00	20.00	45.00	15.00	78.97	65.18
18	10.00	20.00	45.00	15.00	78.39	66.49
19	10.00	20.00	45.00	25.00	80.56	61.47
20	10.00	20.00	55.00	15.00	71.4	63.45
21	10.00	30.00	45.00	15.00	75.19	65.91
22	11.00	15.00	40.00	10.00	84.58	59.56
23	11.00	15.00	40.00	20.00	86.21	63.45
24	11.00	25.00	40.00	10.00	77.26	71.67
25	11.00	25.00	40.00	20.00	79.79	74.68
26	11.00	15.00	50.00	10.00	73.63	57.79
27	11.00	15.00	50.00	20.00	75.54	61.85
28	11.00	25.00	50.00	10.00	70.11	67.36
29	11.00	25.00	50.00	20.00	72.15	69.82
30	12.00	20.00	45.00	15.00	76.19	73.75

Optimization and Validation: Optimization of the process parameters was done numerically to obtain conditions with maximum desirability and maximum protein content in CPI. The overall desirability function (D) was maximized by eq. 3:

$$D = (d1r1\ x\ d2r2)1/\ (r1 + r2) \quad (3)$$

where d_i is the desirability index for ith response (i=1 and 2), r_i is the relative importance whose values range from 1 to 2. Both the individual desirability and overall desirability indices ranged from 0 to 1[5]. The target was to maximize protein content in chickpea protein isolates. The optimized condition was validated by performing experiment at the nearest possible given conditions and it was found that both isolate protein content and yield were in close proximity with the variables selected by software (Table 3). The 3D surface plots were drawn to show the effect of independent variables on dependent variables (responses).

It was obtained by keeping the two independent variables at zero level and varying the other two to predict the response. The predicted protein content was 85.06 % and isolate yield was 62.2 %, while the actual protein content was 85.69% and isolate yield was 63.79%, so the desirability of this model and condition was 1 as the highest possible amount. At this condition, protein content was maximum while other components such as polysaccharides were less, hence the yield of isolate was also fair. The results of validation were similar to a study done on fenugreek by Feyzi et al.[6]

Results and Discussion

Proximate analysis of defatted chickpea protein isolate (CPI): The responses corresponding to each experimental runs performed on chickpea flour are presented in table 2. The proximate composition of the CPI is also given in table 4. Chickpea seeds have 23g/100g protein content for isolate preparation[29]. The protein content increased in chickpea protein isolates compared to whole seed flour along with decrease in fat, fiber and carbohydrate content due to alkali solubilization process used to separate soluble protein from flour. The selected combinations of independent variables resulted in chickpea protein isolates having protein content ranging from 70.11 to 86.21 % and isolate yield from 56.72 to 73.75 % (Table 3). Higher protein content in CPI had lower isolate yield and vice versa because high yield at higher temperature dissolves more starch than protein, hence low protein content [34,38].

Model fitting: The results in ANOVA table showed that (Table 5) correlation coefficient (R^2) was in the range of 0.79-0.97 for both the responses which suggests that values of the variables were in agreement with the actual values and applied model was adequate. Studies done earlier have also reported R^2 values ranging from 71.00% to 95.20% for sunflower seeds[17], germinant pumpkin seed[23], pigeonpea[32], amaranth seed[26], flax seed[18] and tomato seeds[36]. Model F value was significant ($p<0.01$) and very less chance was (<0.01%) that F value could occur due to noise.

High PRESS value (86.46) indicated that the model is good predictor of protein isolate yield and protein content. Both F values and non-significant ($p>0.05$) Lack of fit for protein content (3.13) and protein isolate yield (1.45) further validate the model and showed that model was efficient in predicting the CPI yield and protein content based on the variables selected for alkali solubilization of proteins[8,18].

Table 3
The set of constraints used for different parameters targeting CPI with high protein content

Parameters	Goal	Lower Limit	Upper Limit	Importance	Optimized value at D= 0.968
pH	In range	9	11	3	11.00
Meal/ Solvent Ratio (w/v)	In range	15	25	3	15.00
Temp (T, ˚C)	In range	40	50	3	40.00
Time (t, min)	In range	10	20	3	20.00
Protein Content (%)	Maximize	70.11	86.21	3	85.69
Protein Isolate Yield (%)	In range	56.72	73.75	4	63.79

Table 4
Proximate composition of Optimized CPI (dry wt basis, dwb)

Parameters	Whole Seed flour	Protein Isolate
Moisture %	4.3±0.12	8.25±0.14
Crude Protein (Nx6.25) %	23±0.57	93.96±0.53
Crude Fat %	5.6±0.11	0.083±0.01
Crude Fibre %	5.8±0.26	0.95±0.15
Ash %	3.2±0.15	2.99±0.22
Carbohydrate (by difference)%	58.6	2.96

Values are means ± S.D of triplicate determination

Coefficient of variation for protein content (1.36%) and protein isolate yield (2.58%) were lower than 10% which suggests reproducibility of the model[8]. Analysis of Variance (ANOVA) of independent variables for linear, interaction and quadratic terms was also significant ($p<0.05$). Positive value of coefficient of variables (Table 5) indicates synergistic effect and negative values indicate antagonistic effect. For protein content (Table 5), linear coefficients of meal/solvent ratio, temperature and time had significant effect whereas interaction coefficients of pH, temperature and quadratic coefficient of temperature were also significant ($p<0.05$). For protein isolate yield (Table 5), pH, meal/solvent ratio, temperature and time had significant effect along with the quadratic coefficient of meal/solvent ratio.

The positive coefficient of meal/solvent ratio at quadratic level indicates minimum protein content at center which increases with increase in meal/solvent ratio. The negative coefficient of pH, temperature and time at quadratic level shows maximum protein content at center which decreases with increase in variable value. Positive value of quadratic coefficient for pH indicates minimum value of isolate yield at center which increases with increase in pH values. Maximum value of isolate yield at center and lower values with increase in meal/solvent ratio, temperature and time is indicated by negative coefficient at quadratic level.

Similar results have been reported by Sethi et al[27] for the effect of quadratic coefficient of pressure and pulp on green mango mayonnaise, xanthan gum, temperature and pH on red pepper seed protein isolate by Firatligil-Durmus and Evranuz[8], alkali concentration, solvent/meal ratio and extraction time for protein recovery from watermelon protein isolates by Wani et al[37], solvent/meal ratio and pH for protein extraction from lentil flour[13].

Optimization of the process: The 3D surface plots (Figure 1and 2) were obtained by considering two independent variables constant at zero coded values and varying the other two variables. Consequently, both main and interactive effect would be studied on the responses. Linear effect of optimized independent variables on the response as shown in figure 3.

Effect of pH, meal/solvent ratio, temperature and time on protein content: Linear coefficients for pH, meal/solvent ratio and temperature were negative. Higher values of these variables yielded low protein content in CPI. Positive linear coefficient for time showed higher protein content with increase in time. Meal/solvent ratio showed antagonistic (negative) effect with pH (Figure 2a), lower meal/solvent ratios at higher pH (11) had more protein content in CPI. This was due to more water soluble (albumin), salt soluble (globulin) and acid alkali soluble (glutelin) proteins in chickpea[4].

Table 5
Estimated coefficients of fitted second order polynomial model and corresponding ANOVA values describing the effect of independent variables on responses

Source	Protein Content %			Protein Isolate Yield %		
	Coefficient	F-Value	*p*-value	Coefficient	F- Value	*p*-value
Intercept	78.20			66.90		
X_1	-0.2596	1.45	0.2473	2.54*	56.46	<0.0001
X_2	-1.92*	79.17	<0.0001	2.55*	56.94	< 0.0001
X_3	-3.16*	214.67	<0.0001	-1.37*	16.34	0.0011
X_4	1.11*	26.32	0.0001	1.10*	10.66	0.0052
$X_1 X_2$	-0.4794	3.29	0.0896	2.17*	27.52	<0.0001
$X_1 X_3$	-0.9619*	13.26	0.0024	0.3325	0.6462	0.4340
$X_1 X_4$	0.0606	0.0527	0.8215	0.6175	2.23	0.1562
$X_2 X_3$	0.6306*	5.70	0.0305	-0.3262	0.6222	0.4425
$X_2 X_4$	0.0656	0.0617	0.8071	-0.0712	0.0297	0.8655
$X_3 X_4$	-0.2369	0.8044	0.3840	-0.0262	0.0040	0.9502
X_1^2	-0.2028	1.01	0.3306	0.1733	0.3011	0.5913
X_2^2	0.0584	0.0839	0.7760	-1.07*	11.51	0.0040
X_3^2	-0.5153*	6.53	0.0220	-0.6567	4.32	0.0552
X_4^2	-0.0816	0.1635	0.6917	-1.89	35.90	<0.0001
ANOVA for the model						
F value						
Lack of Fit	25.16		<0.0001	15.59		<0.0001
R^2 Value	3.13		0.1100	1.45		0.3567
Adj R^2 Value	0.9592			0.9357		
	0.9210			0.8757		

X_1 – pH, X_2 – Meal/Solvent Ratio**,** X_3 – Temperature, X_4 – Time; *$p < 0.05$, all terms are significant

Meal/solvent ratio showed significantly higher synergistic effect with temperature than with mixing time. Increase in meal/solvent ratio alongwith increase in both temperature and time, protein content increased.

At meal/solvent ratios of 10-15 and temperature of 40-45 ˚C, isolates with higher protein content were obtained. pH showed significantly higher antagonistic with temperature on protein content than meal/solvent ratio. Positive interactive effect was seen between pH and mixing time which extracted more protein from chickpea seed meal. At lower temperature, an increase in mixing time, increased protein content (Figure 2d). The negative quadratic effect of pH, temperature and time was responsible for more concave shape of the surface plot (Figure 2).

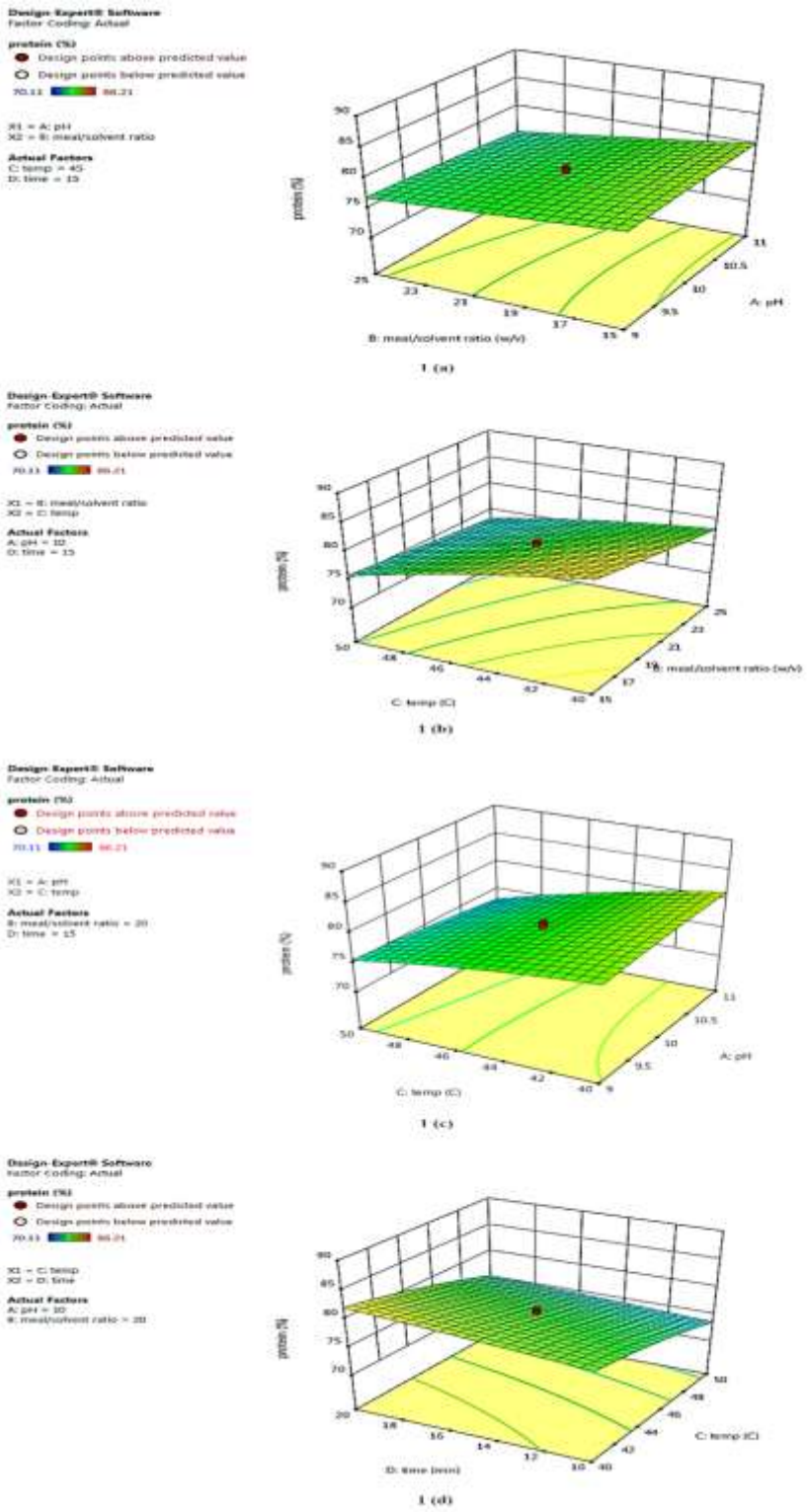

Fig. 1: Effect of independent variables on protein content %

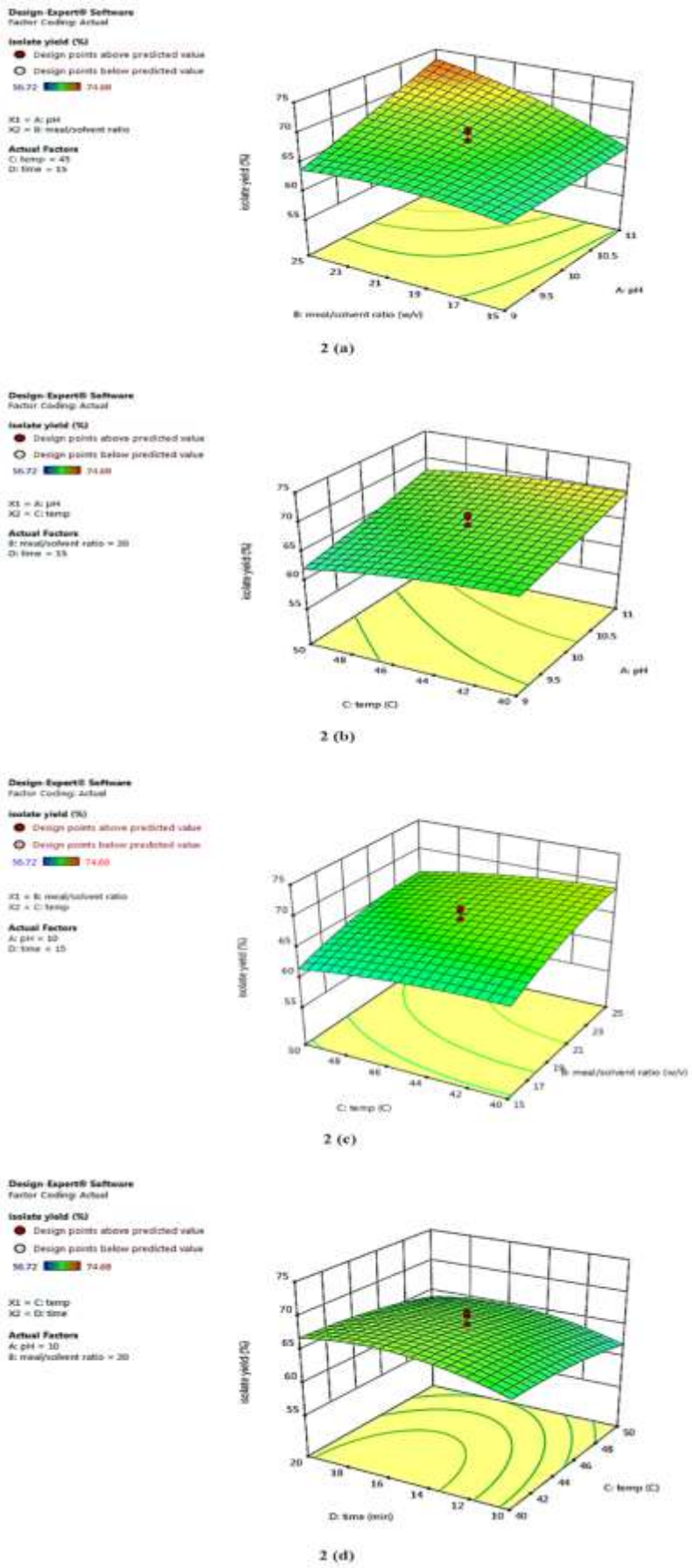

Fig. 2: Effect of independent variables on protein isolate yield %

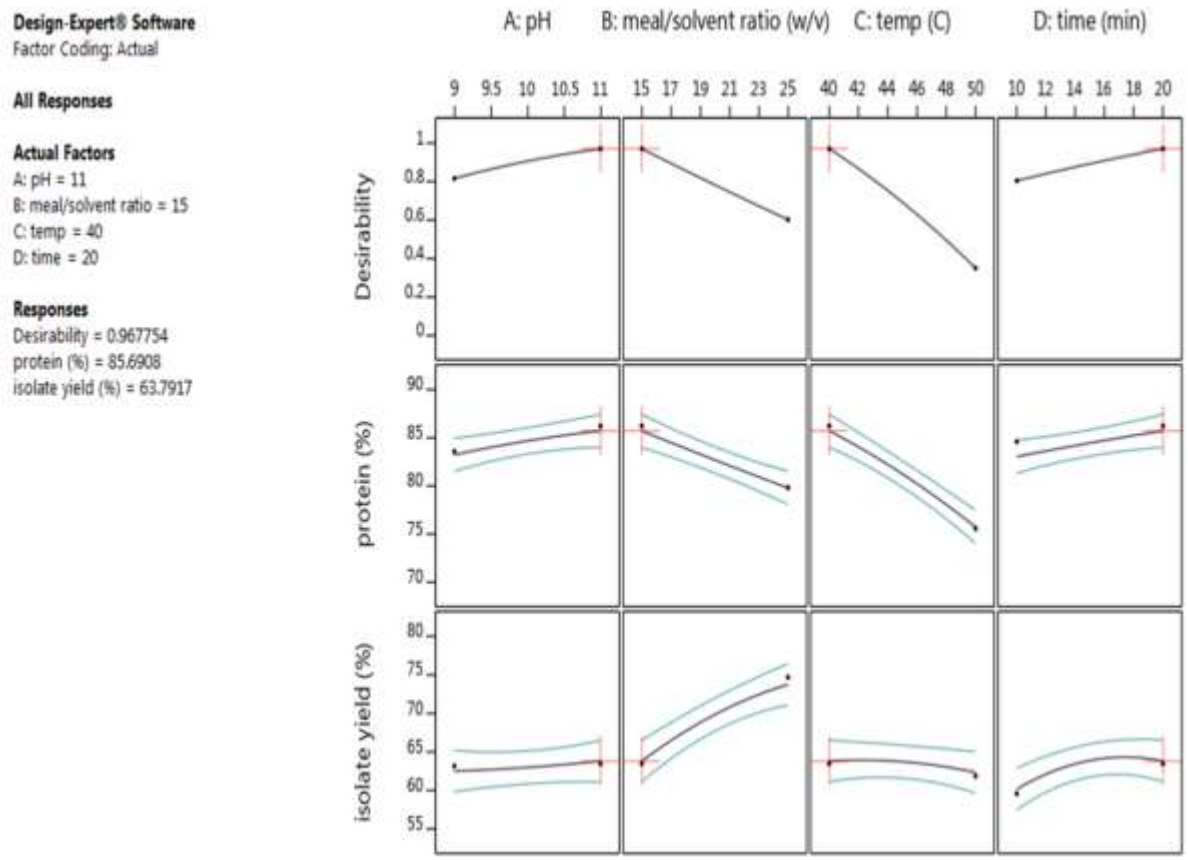

Fig. 3: Desirability and effect of independent variables on optimized respomse

The results were in agreement with Jarpa-Parra et al.[13] who optimized lentil protein extraction based on pH. Lower protein content at higher meal/solvent ratio and temperature is due to solubilization of starch which is susceptible to alkaline conditions which precipitate along with the proteins at isoelectric point. Increase in alkalinity with decrease in meal/solvent ratio and temperature, dissociates hydrogen from functional groups such as carbonyl, sulphate and breaks hydrogen bonds, leading to increase in surface charge on proteins with enhanced solubility and higher protein % in isolates[30].

Effect of pH, meal/solvent ratio, temperature and time on protein isolate yield: All the parameters showed positive linear coefficient except for temperature on protein isolate yield. With increase in temperature, isolate yield decreased whereas isolate yield increased with increase in other variables. Positive interaction coefficients between pH-meal/solvent ratio, pH-temperature and pH-time resulted in synergistic effect on isolate yield. Meal/solvent ratio showed more negative value of interaction coefficient with temperature than with mixing time on isolate yield i.e. with increase in one, the other has to be decreased to obtain higher yield. Higher temperature resulted in lower isolate yield in less mixing time used for protein extraction.

Higher pH (10-12), meal/solvent ratio (20-25) and temperature (50˚C) were accompanied with more pronounced isolate yield. Meal/solvent ratio, temperature and mixing time showed negative quadratic coefficient value which was significantly more for meal/solvent ratio and hence responsible for concave shape of the graph (Figure 3).

Other studies done on custard apple[34], red pepper seeds[8], watermelon[38], pigeon pea[18], flax seeds[20] were in agreement with the results obtained on varying the amount of solvent used, pH of alkaline condition, temperature and mixing time of alkali solubilization.

Conclusion

For optimization, protein content of chickpea isolate was important which had to be more than 90%. Preliminary study was done on 10g of flour to decide the minimum and maximum value of independent variables. Chickpea protein isolates with maximum protein content were very well achieved at higher pH (11), moderate time (20 min), lower meal/solvent ratio (1/15) and moderate temperature (40˚C). Experiments conducted at the optimized condition yielded 14.65g/100g (63.79%) protein isolate with 92.96% (dwb) protein content.

Lower meal/solvent ratio was significant from industrial point of view as higher ratio and more time for preparation will be less economical for protein isolate preparation. Yield at optimized condition was also satisfactory as any further increase in yield would result in low protein content. Chickpea protein isolates can be a source of functional food

with higher neutraceutical value based on higher protein availability and lesser carbohydrates as compared to seeds.

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