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Response surface methodology for extraction optimization of pigeon pea protein

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Abstract

Optimisation for pigeon pea protein extraction (Y) (*Cajanus cajan* (L) Millsp from the new IAPAR 43-Aratã variety) was investigated using response surface methodology. A compound central design was used with variables (X_1) NaCl concentration (0.000; 0.025; 0.050; 0.075 and 0.100M); (X_2) pH (2.5; 4.0; 5.5; 7.0 and 8.5) and (X_3) liquid:solid ratio (5:1; 10:1; 15:1; 20:1; and 25:1, v/w). A model of the second degree equation was used to create the surface responses and confirmative studies were carried out. The following equation: $\hat{Y} = -19.3733 + 8.6004x_2 - 0.508526x_2^2$ shows optimum conditions for protein extraction of about 75% yield, at pH 8.5 without NaCl regardless of the liquid:solid ratio (v/w) under the experimental conditions studied.

1. Introduction

The guandu bean or pigeon pea (*Cajanus caja*n (L.) Millsp) is a leguminous plant of the Fabbaceae family and is grown in Asia, Africa and South America (Krishna & Bhatia, 1985; Salunkhe, Kadam & Chavan, 1985). However, there is no available information on its production in different regions of the world, as it is only cultivated to meet domestics needs, especially in the developing countries (Salunkhe et al., 1985).

In Brazil, pigeon pea yield and market price have not been established, as this legume is produced only on small and medium-sized farms for subsistence. The Paraná Agronomic Institute — IAPAR, put an early dwarf variety on the market in 1990 called ''Iapar 43-aratã'' whose yield varies from 1000 to 2000 kg/ha, and reaches 4000 kg grain under some cultivation conditions.

The protein content of the pigeon pea varies from 15.5 to 28.8% (Oshodi & Ekperigin, 1989; Salunkhe, Chavan & Kadam, 1986; Vilela & El-Dash, 1985) and depends on genetic and environmental factors (Salunkhe et al., 1986).

Traditional pigeon pea products involve hydration, cooking, peeling or grinding, tinning and freezing (Salunkhe et al., 1986). The pigeon pea can also be made into good quality flour by dry grinding (Vilela & El-Dash, 1985), or maceration for 12 h at 18°C (Batistuti & Freitas, 1995). Sant'anna Filho, Vilela and Gomes (1985) obtained protein isolates from pigeon peas with possible application in food. Singh, Jambunathan and Gurtu (1981) fractioned pigeon pea proteins using water-solubility properties (albumins), salts (globulins), alcohol (prolamins) and acid/alkali (glutelins) as well as residual proteins and non-protein nitrogen. Salunkhe et al. (1986) state that, as in other legumes, the pigeon pea gobulin were the largest proteins stored and their contents varied from 60 to 70%.

A pigeon pea with 24.2% crude protein and 70% albumins and globulins was used for extraction with 0.5 M NaCl in a 0.01 M phosphate buffer, and pH 7.0 (Gopala Krishna, Mitra & Bhatia, 1977; Morton, 1976).

Various parameters, such as pH, temperature, ionic force, salt or solvent type, extraction time, solid-solvent ratio, presence of components causing linking, affect protein solubility. The solubility of a protein, as well as its functionality as a nutritional ingredient, may be affected by extraction conditions, solvent type and heat

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treatment (Liu, 1997). The extraction, isolation and fractioning procedures may differ, depending on the end-use. When isolation and fractionation is carried out for application in the food area, an extraction method in alkaline aqueous solution, followed by isoelectric precipitation at pH between 4.0 and 5.0, is used. Precipitated proteins may be separated by heat coagulation, filtration, centrifugation or ultracentrifugation (Sathe, Deshpande & Salunkhe, 1984).

Protein extraction efficiency in pigeon pea may determine the protein concentration or isolation procedure and the subsequent application of the functional ingredient in nutritional systems.

Sant'Anna et al. (1985) reported the solubility of pigeon pea protein. Maximum extraction took place at pH below 3.0 and above 7.0 and minimum extraction between pH 4.0 and 6.0. Similar observations have also been made on pigeon pea flour (Oshodi & Ekperigin, 1989). However, there are no reports in the literature on the effect of variables which may effectively influence pigeon pea protein extraction.

Response surface methodology (RSM) is a statistical-mathematical method which uses quantitative data in an experimental design to determine, and simultaneously solve, multivariate equations, to optimise processes or products (Giovanni, 1983). Thus, the optimisation of maximum pigeon pea protein extraction was investigated using RSM, with three variables: NaCl concentration, liquid:solid ratio and pH.

2. Materials and methods

2.1. Raw material and sample preparation

The Iapar 43-Aratã variety pigeon pea was used, provided by the Paraná Agronomic Institute, Londrina, Pr, Brazil. This variety has a high yield with simple management. Clean selected grains were ground in a hammer-type mill and the flour (mesh 60) was stored in polythene bags and kept at 10°C until use.

2.2. Methods

Association of Official Analytical Chemists [AOAC] (1996) descriptions were used to determine flour moisture content, flour protein content and extract protein content (Kjeldahl method, N × 6.25).

2.3. Experimental design

The effect of the variables X_1 (molar, NaCl concentration), X_2 (initial pH) and X_3 (liquid:solid ratio, v/w) at five variation levels (Table 1) in the pigeon pea protein extraction process was investigated using the central composite design for response surface methodology (Box & Draper, 1987), as shown in Table 2, with 16 experimental runs and 15 treatments in two orthogonal blocks.

The model proposed for the response (Y) was:

$$Y = b_0 + \sum_{n=1}^{3} b_n X_n + \sum_{n=1}^{3} b_{nn} X_n^2 + \sum_{n<m}^{3} b_{nm} X_n X_m$$

where b_0 is the value of the fixed response at the central point of the experiment which is the point (0,0,0); b_n, b_{nn} and b_{nm} are the linear, quadratic and cross products coefficients, respectively.

The response function investigated was $Y =$ g of soluble protein from extract/100 g flour. The data was transformed into $(Y/100)^{1/2,}$ according to Box and Draper's (1987) recommendations to assure the normality of the experimental data. Analyses of variance and regression were carried out by the SAS/STAT (Statistical Analysis System, 1989). A regression Eq. (1) was obtained from which the respective estimated values ($\hat{Y}$) were calculated to compare with the experimental data (Y).

2.3.1. Study on the response surface

The response surface enables the unique or critical points of the protein extraction from pigeon pea flour to be determined. The behaviour of the surface was investigated for the response function ($\hat{Y}$) = g soluble protein from extract/100 g flour using the regression Eq. (1).

Some criteria were established to reduce the cost and maximise extraction and yield. After fixing two of the three variables, some cuts were made on the surface to obtain a simplified equation to simulate the protein extraction.

2.3.2. Confirmative studies

The tendencies of the three variables were analysed (X_1, X_2 and X_3) using the regression Eq. (1). Later, two experiments (1 and 2) were performed, each with four treatments and three replications, in a complete randomized block design. Table 3 shows all the treatments in experiments (1 and 2) with the respective levels of the variables used.

The experimental data were used for the analyses of variance of the transformed data as $(Y/100)^{1/2}$. A further check experiment was performed, with fixed variables X_1 (at 0.0 M NaCl) and X_3 (at 5:1, liquid:solid) and the X_2 variable was varied (pH between 7.0 and 9.0 with intervals of 0.5 units). A complete randomised block design was used with five treatments and three replications per treatment. The analysis of variance of the original results was performed on these results without transformation to obtain the respective regression equation. Then a curve was established for protein grams extracted/100 g flour with the pH.

Table 1
Independent variable values of the process and their corresponding levels

Independent variables	Symbol		Levels				
	Uncodified	Codified	−2	−1	0	+1	+2
NaCl (M)	X_1	X_1	0	0.025	0.050	0.075	0.100
Initial pH	X_2	X_2	2.50	4.00	5.50	7.00	8.50
Liquid:solid ratio (v/w)	X_3	X_3	5:1	10:1	15:1	20:1	25:1

Table 2
Experimental design with the respective codified factors, variation levels and response function (Y and $\hat{Y}$)[a]

Block	Treat	Factors			Variation levels			Response function	
		x_1	x_2	x_3	X_1	X_2	X_3	Y	$\hat{Y}$
1	1	−1	−1	−1	0.025	4.0	10:1	2.51	3.32
	2	1	−1	−1	0.075	4.0	10:1	2.85	4.67
	3	−1	1	−1	0.025	7.0	10:1	12.07	9.78
	4	1	1	−1	0.075	7.0	10:1	9.88	7.66
	5	−1	−1	1	0.025	4.0	20:1	2.72	3.69
	6	1	−1	1	0.075	4.0	20:1	3.47	4.52
	7	−1	1	1	0.025	7.0	20:1	13.51	9.81
	8	1	1	1	0.075	7.0	20:1	8.97	6.96
2	9	0	0	0	0.050	5.5	15:1	3.49	3.89
	10	0	0	0	0.050	5.5	15:1	3.53	3.89
	11	−2	0	0	0.000	5.5	15:1	3.24	4.03
	12	2	0	0	0.100	5.5	15:1	3.49	3.44
	13	0	−2	0	0.050	2.5	15:1	12.86	8.90
	14	0	2	0	0.050	8.5	15:1	14.74	23.04
	15	0	0	−2	0.050	5.5	5:1	4.22	4.28
	16	0	0	2	0.050	5.5	25:1	3.41	4.15

[a] $\hat{Y}$ = estimated response function.

2.4. Pigeon pea protein extraction

The extraction experiments (Table 2) were carried in random order, beginning with block 1 (experiments 1–8). The procedure was repeated with block 2 (experiments 9–16).

All the experiments were carried out in triplicate, using 25 g of pigeon pea flour with the addition of NaCl at the established volume and molarity. The pH was adjusted according to the treatment and the extraction lasted 2 h with agitation at 250 rpm at room temperature. An 80 mesh sieve was used for filtration and the supernatant volume was measured. The soluble protein content was then determined in triplicate.

3. Results and discussion

3.1. Optimisation of the pigeon pea protein extraction conditions

Table 2 shows the response function Y (observed) and $\hat{Y}$ (estimated) expressed in g of soluble protein from the extract/100 g pigeon pea flour obtained from the triplicate means for each of the 16 treatments. The analysis of variance of the response function ($\hat{Y}$) showed that there was significant effect ($P < 0.05$). The block effect was not significant at the same level. The total determination coefficient (R^2) was 77.48%, indicating a reasonable fit of the model to the experimental data. The coefficient of variation (vc) of 18.50% indicated medium experimental accuracy (Gomes, 1978).

Table 3
Experiments and treatments with their respective protein extraction variation levels

Experiment	Treatment	Variation levels		
		x_1 (NaCl, M)	x_2 (pH)	x_3 (v/w)
1	1	0 (0.050)	2 (8.5)	0 (15:1)
	2	0 (0.050)	2 (8.5)	−1 (10:1)
	3	−1 (0.025)	2 (8.5)	0 (15:1)
	4	−1 (0.025)	2 (8.5)	−1(10:1)
2	5	−1 (0.025)	2 (8.5)	−1 (10:1)
	6	−2 (0.000)	2 (8.5)	−1 (10:1)
	7	−1 (0.025)	2 (8.5)	−2 (5:1)
	8	−2 (0.000)	2 (8.5)	−2 (5:1)

As the complete equation was significant, the mathematical model [Eq. (1)] with its respective coefficients was obtained.

$$\hat{Y} = 0.1971 - 0.0038x_1 + 0.0454x_2 - 0.0008x_3 - 0.0009x_1.x_1 - 0.0175x_2.x_1 + 0.0480x_2.x_2 - 0.0033x_3.x_1 - 0.0023x_3.x_2 + 0.0020x_3.x_3 \quad (1)$$

It was possible to compare the observed (Y) and estimated ($\hat{Y}$) values of soluble protein from extract/100 g pigeon pea flour from this regression equation, as shown in Table 2.

Experiment 14 had the highest extracted soluble protein content (14.7%) and was used to optimise and establish the criteria of the best conditions for obtaining the protein concentrate. Criteria for cost reduction and best protein extraction conditions were used, and several were, therefore, analysed and the respective equations and graphics obtained.

Criterion No. 1: $x_1 = 0$(0.05 M NaCl) and varied x_2 (pH) and x_3 (liquid:solid ratio).

$$\hat{Y}_{\text{criterion1}} = 0.1971 + 0.0454x_2 - 0.0008x_3 + 0.0480x_2.x_2 - 0.0023x_3.x_2 + 0.0020x_3.x_3 \quad (2)$$

When the response surface was analysed (Fig. 1A), maximum protein extraction ($\hat{Y}_{\text{criterion1}} = 0.45$) was obtained when x_2 (pH) was close to $+2$, that is, pH around 8.5 regardless of the x_3 variable (liquid:solid ratio).

Criterion No. 2: $x_1 = -1$(0.025 M NaCl) and varied x_2 (pH) and x_3 (liquid:solid ratio).

$$\hat{Y}_{\text{criterion2}} = 0.2000 + 0.0629x_2 + 0.0025x_3 + 0.0480x_2.x_2 - 0.0023x_3.x_2 + 0.0020x_3.x_3 \quad (3)$$

Fig. 1B shows that maximum protein extraction ($\hat{Y}_{\text{criterion2}} = 0.49$) was also obtained when x_2 (pH) was close to $+2$, that is, when the pH was close to 8.5 regardless of the x_3 variable (liquid:solid ratio), indicating a small increase of approximately 0.04 or 0.16 g soluble protein extracted/100 g flour compared with criterion No. 1.

Criteria No. 3: $x_1 = -2$ (0.00 M NaCl) and varied x_2 (pH) and x_3 (liquid:solid ratio).

$$\hat{Y}_{\text{criterion3}} = 0.2011 + 0.0804x_2 + 0.0058x_3 + 0.0480x_2.x_2 - 0.0023x_3.x_2 + 0.0020x_3.x_3 \quad (4)$$

Fig. 1C shows that maximum protein extraction ($\hat{Y}_{\text{criterion3}} = 0.56$) was obtained when the pH was around 8.5 ($x_2 = +2$) regardless of the liquid:solid ratio (x_3).

The x_1 variable was fixed at 0 (0.050 M NaCl), -1 (0.025 M NaCl) and -2 (0.00 M NaCl) using these three criteria (Fig. 1), to observe the effect NaCl addition on protein extraction. There was as increase in protein extraction as NaCl was reduced, so the addition of NaCl is not necessary for protein extraction. This indicates that the most important variable is the pH (x_2).

Criterion No. 4: $x_3 = 0$ (liquid:solid ratio; 15:1) and varied x_1 (NaCl) and x_2 (pH).

$$\hat{Y}_{\text{criterion4}} = 0.1971 - 0.0038x_1 + 0.0454x_2 - 0.0009x_1.x_1 - 0.0175x_2.x_1 + 0.0480x_2.x_2 \quad (5)$$

Fig. 2A shows that maximum protein extraction $\hat{Y}_{\text{criterion4}} = 0.56$ was obtained when x_2 was close to $+2$ (pH close to 8.5) and x_1 between -1 (0.025 M NaCl) and -2 (0.00 M NaCl).

Criterion No. 5: $x_3 = -1$ (liquid:solid; 10:1) and varied x_1 (NaCl) and x_2 (pH).

$$\hat{Y}_{\text{criterion5}} = 0.1999 - 0.0005x_1 + 0.0477x_2 - 0.0009x_1.x_1 - 0.0175x_2.x_1 + 0.0480x_2.x_2 \quad (6)$$

Variables	Levels				
	-2	-1	0	+1	+2
x1 NaCl concentration(M)	0.000	0.025	0.050	0.075	0.100
x2 initial pH	2.500	4.000	5.500	7.000	8.500
x3 Liquid:solid ratio(v/w)	5:1	10:1	15:1	20:1	25:1

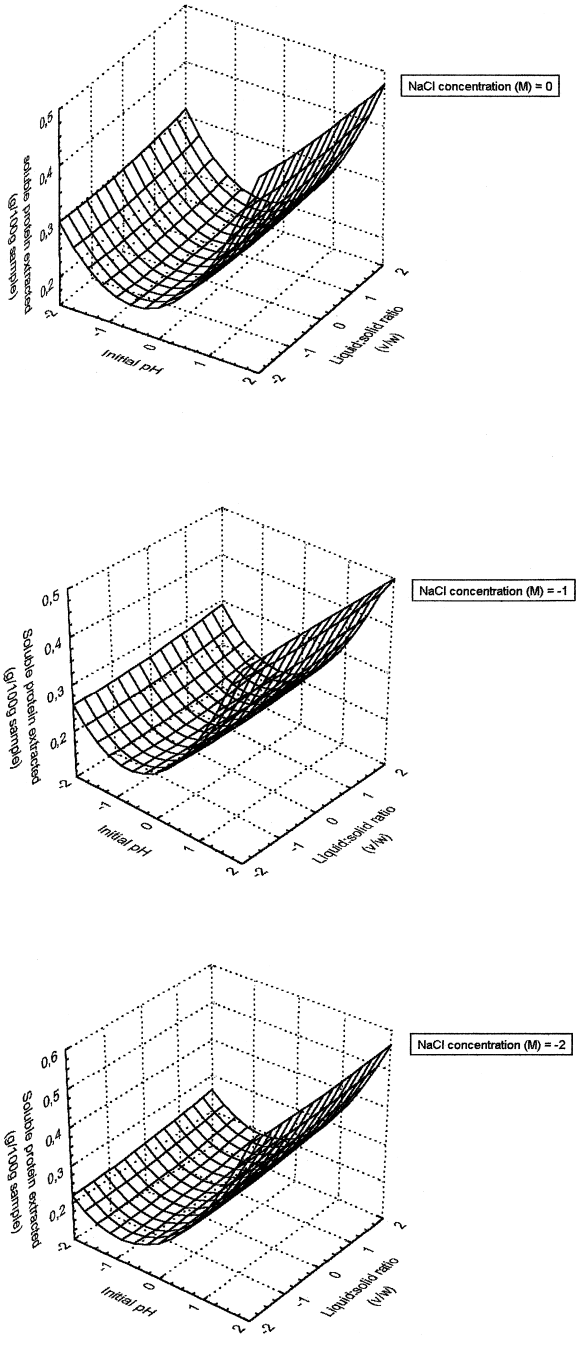

Fig. 1. Soluble protein extraction (g/100 g sample) by the initial pH and the liquid:solid (v/w) ratio with the variable $x_1 = 0$ (A), $x_1 = -1$ (B) and $x_1 = -2$ (C).

Variables	Levels				
	-2	-1	0	+1	+2
x1 NaCl concentration(M)	0.000	0.025	0.050	0.075	0.100
x2 initial pH	2.500	4.000	5.500	7.000	8.500
x3 Liquid:solid ratio(v/w)	5:1	10:1	15:1	20:1	25:1

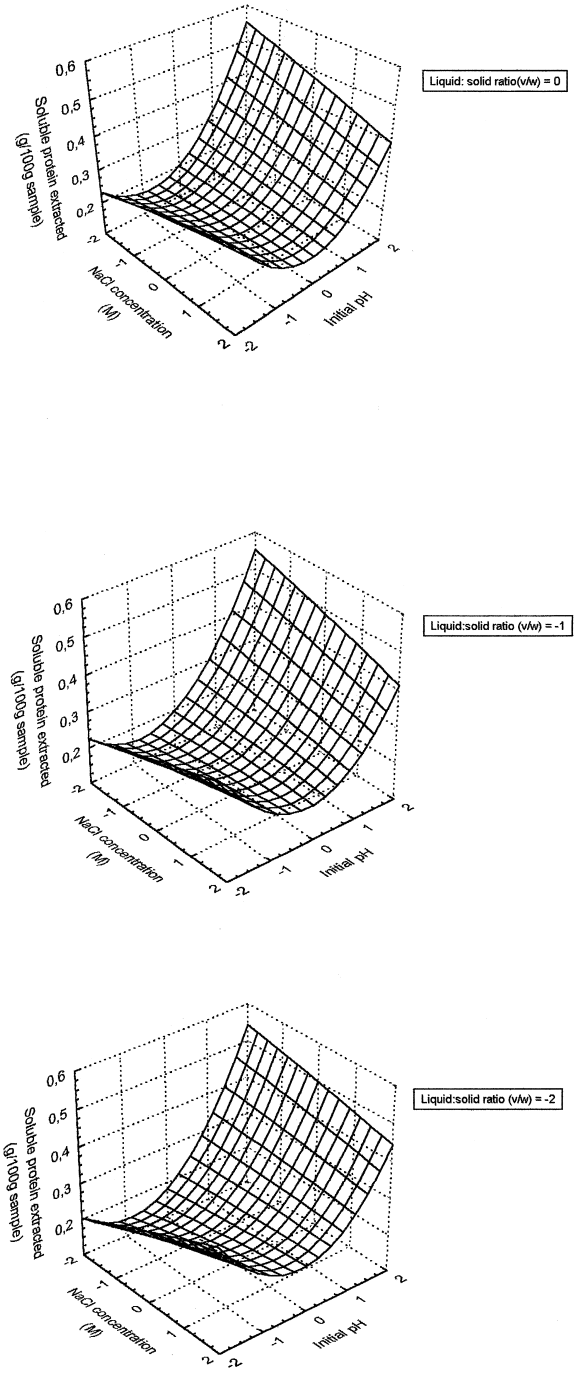

Fig. 2. Soluble protein extraction (g/100 g sample) by the NaCl concentration and initial pH with the variable $x_3 = 0$ (A), $x_3 = -1$ (B) and $x_3 = -2$ (C).

Fig. 2B shows that maximum protein extraction ($\hat{Y}_{\text{criterion5}} = 0.49$) occurred when x_1 was close to or lower than 0 (0.050 M NaCl) and x_2 close to $+2$ (pH close to 8.5).

Criterion No. 6: $x_3 = -2$ (liquid:solid; 5:1) varied x_1 (NaCl) and x_2 (pH).

$$\hat{Y}_{\text{criterion6}} = 0.2067 + 0.0104x_1 + 0.0500x_2 - 0.0009x_1.x_1 - 0.0175x_2.x_1 + 0.0480x_2.x_2 \quad (7)$$

Fig. 2C shows that the maximum protein extraction ($\hat{Y}_{\text{criterion6}} = 0.56$) was observed when x_2 was close to 2 (pH close to 8.5) and x_1 was close to -1 to -2 (from 0.025 to 0.00 M NaCl).

Criteria 4, 5 and 6 (Fig. 2) show, again, that the maximum protein extraction occurred when the pH was close to 8.5 and the saline concentration was close to 0.00 M. All these indicators suggest that the maximum protein extraction would be obtained when the pH was close to 8.5 and the saline concentration close to 0.0 M, regardless of the liquid:solid ratio. Thus, it was possible to simplify regression Eq. (1), to $\hat{Y}$ simplified equation considering only the x_2 variable (pH):

$$\hat{Y}_{\text{simplified}} = 0.1985 + 0.0454(x_2) + 0.0477(x_2)^2 \quad (R^2 = 74.93\%)$$

where $x_2 = \text{pH}$; $(x_2)^2 = (\text{pH})^2$.

The maximum protein extraction point was estimated from the $\hat{Y}_{\text{simplified}}$ equation as being $\hat{Y}^*_{\text{simplified}} = 4.031$, that is, 16.3 g protein extracted from 100 g flour at 8.5 pH.

3.2. Confirmative tests

The regression Eq. (1) was obtained using the response surface methodology, which indicated some tendencies of the three variables investigated [$X_1 =$ NaCl concentration (M); $X_2 =$ initial pH and $X_3 =$ liquid:solid ratio (v/w)]. Two experiments were carried out with fixed X_2 variable (pH) to confirm the data obtained by Eq. (1). Table 4 shows the results from experiment 1.

The analysis of variance showed a significant difference ($P < 0.01$) among the treatments with a 0.41% variation coefficient . As treatments 1, 3 and 4 do not differ significantly and treatment 4 used the lowest NaCl concentration (0.025 M) and the lowest liquid:solid ratio (10:1), it was decided to carry out experiment 2 for lower costs.

Table 5 shows the results of the soluble protein content-extracted (g/100 g flour) (Y) from experiment 2.

The analysis of variance indicated a significant difference ($P < 0.01$) among the treatments with a 0.31% variation coefficient. As treatment 7 had the greatest protein extraction content (g/100 g flour) without the addition of NaCl, and the lowest liquid:solid ratio, a further check test was made. The NaCl was kept constant at 0.00 M and the liquid:solid ratio was kept at

Table 4
Extracted soluble protein content (Y) from experiment 1 of the confirmative studies

	Variables			Y (g/100 g sample)	
Treatment	X_1 = NaCl (M)	X_2 = pH	X_3 = liq:sol (v/p)	Original data	Data in $(Y/100)^{1/2}$
1	0.050	8.5	15:1	15.8	0.40
2	0.050	8.5	10:1	15.0	0.398
3	0.025	8.5	15:1	16.2	0.414
4	0.025	8.5	10:1	17.8	0.407

Table 5
Extracted soluble protein content (Y) from experiment 2 of the confirmative studies

	Variables			Soluble protein (g/100 g sample)	
Treatments	X_1 = NaCl (M)	X_2 = pH	X_3 = liq:sol (v/w)	Original data	Data in $(Y/100)^{1/2}$
4	0.025	8.5	10:1	15.4	0.404
5	0.000	8.5	10:1	16.1	0.412
6	0.025	8.5	5:1	15.7	0.408
7	0.000	8.5	5:1	16.4	0.417

5:1, and the pH was varied between 7.0 and 9.0, at intervals of 0.5 pH units to confirm treatment 7. Table 6 shows the results of this experiment.

The analysis of variance of the original data, without transformation to $(Y/100)^{1/2}$ indicated significance ($P < 0.01$) with a 87.87% determination coefficient (R^2). The following regression equation was obtained:

$$\hat{Y} = -19.3733 + 8.6004X_2 - 0.508526X_2^2,$$

where X_2 = pH.

This equation confirmed the classic phenomenon of protein extraction in function of the pH, in the 7.0–9.0 range (Fig. 3).

When the equation $\hat{Y} = -19.3733 + 8.6004X_2 - 0.508526X_2^2$ was derived, a maximum point was obtained, that is $\hat{Y} = 17.0$ g extracted soluble protein from 100 g flour at pH = 8.5. Thus, it was confirmed that these experimental results were analogous to those estimated by the simplified regression equation because, when using $\hat{Y}_{\text{simplified}} = 0.1985 + 0.0454(x_2) + 0.0474(x_2)^2$, where (x_2) = pH and $(x_2)^2 = \text{pH}^2$, $\hat{Y} = 16.3$ g extracted soluble protein/100 g flour, a value similar to 16.99 g/100 g flour obtained by the quadratic equation.

The response surface methodology, with some adopted criteria, indicated that, in this study, maximum protein extraction was obtained when the pH was close to 8.5 and the saline concentration close to 0.0 M, regardless of the liquid:solid ratio.

Therefore, some confirmative experiments were carried out and the following optimum condition for protein extraction was reached: no NaCl; pH = 8.5 and liquid:solid ratio = 5:1. Under these conditions protein extraction was 16.9 g protein extracted/100 g sample and 74.8% yield. These results differ, in part, from other researchers who optimised protein extraction from other grains.

Table 6
Mean soluble protein content extracted (g/100 g sample) in the check test, pH varied from 7.0 to 9

Treatment	pH	Soluble protein (g/100 g sample)
1	7.0	15.8
2	7.5	16.7
3	8.0	16.8
4	8.5	16.9
5	9.0	16.9

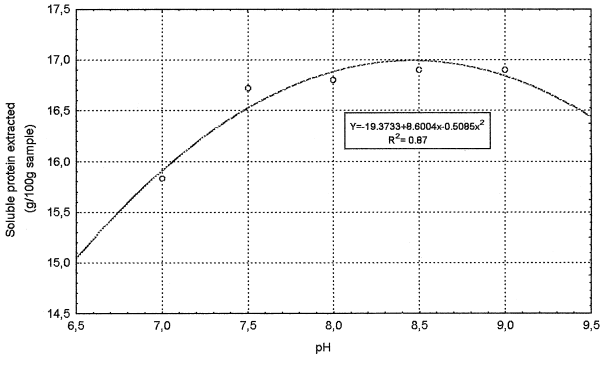

Fig. 3. Soluble protein extraction (g/100 g sample) by the pH.

Rustom, López-Leiva and Nair (1991) optimised protein extraction from peanuts (*Arachis hypogaea* L.) with water using response surface methodology and found significant time, temperature, pH and liquid:solid ratio effects on protein extraction, and concluded that

optimum extraction conditions were: pH = 8.0; time = 30 min; temperature = 50°C and liquid solid ratio = 8:1.

In another study, Bello and Okezie (1989) optimised protein extraction conditions from winged bean flour (*Psophocarpus tetragonolobus* (L.) DC) and determined the effects of several factors on protein extraction, such as pH, temperature, time and liquid:solid ratio, and found optimum conditions when time = 30 min, pH = 12 and liquid:solid ratio 20:1. Temperature did not have a significant effect.

Kadam and Salunkhe (1984) state that, among the solvents used to extract winged bean protein (*Psophocarpus tetragonolobus* (L.) DC) in a liquid:solid ratio of 5:1, NaOH at 0.1 M concentration was the most effective. They further report that different legume proteins have a common minimum dispersion point at an acid pH of 4.0 and great quantities of nitrogenised constituents may be extracted by dilution either in NaOH or HCl for a maximum dispersion pH. They also observed that, without salts, only small quantities of proteins are dissolved at pH values below 5.0. Solubility increases rapidly to pH 7.0 but then increases gradually to pH 10.0. Preparation of legume protein concentrates and isolates is advantageous for nutritional applications, as each process used to obtain these concentrates and isolates has advantages and limitations. Protein extraction in an alkaline medium, especially at high pH values, may destroy and racemize amino acids and also cause the formation of new compounds, such as lisinoalanine, which may be toxic, and the aggregation of proteins which may reduce the protein solubility. On the other hand, preparation of concentrates and isolates may significantly reduce the anti-nutritional factors, such as phytohaemaglutins, tannins, phytates and protein inhibitors, and oligosaccharides, such as stachyose, verbascose and raffinose, which cause flatulence, and therefore offers nutritional advantages (Desphande, Sathe & Salunkhe, 1984; Kadam & Salunkhe, 1985).

4. Conclusions

The optimum pigeon pea protein extraction (*Cajanus cajan* (L) Millsp) condition with about 75% yield was obtained from the $\hat{Y} = -19.3733 + 8.6004X_2 - 0.508526X_2^2$, equation, when the pH was approximately 8.5 without the addition of NaCl regardless of the liquid:solid ratio (v/w) under the experimental conditions investigated, which varied from 5:1 to 25:1.

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