Cowpea Proteins. 1. Use of Response Surface Methodology in Predicting Cowpea (*Vigna unguiculata*) Protein Extractability

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Response surface methodology was used to establish optimum conditions for extracting the protein nitrogen of cowpea meal (*Vigna unguiculata*). Water, 0.01 M NaOH, 0.5 M NaCl, 70% ethanol, and phosphate buffer (pH 7.0) were used as solvents. Parameters examined included meal–solvent ratio, extraction time, and temperature. The extracts obtained were analyzed for total and nonprotein nitrogen. Maximum extraction (83%) was predicted with 0.01 M sodium hydroxide. The regression equations obtained gave good predictions of cowpea protein extractability in all solvents. Defatting the cowpea flour resulted in a slight increase in the predicted nitrogen extractable. Preliminary experiments indicated that the pH and ionic strength of the extracting buffer influenced nitrogen extractability. The highest yields occurred at pH 12.0 (96%) and an ionic strength of 1.0 (76%).

Unlike soybean and allied oilseed proteins, the proteins from other legume seeds have not been studied in great detail. However, these legume seeds play a significant role in the diets of a majority of the world's population (Aykroyd and Doughty, 1964). There is thus the need to understand details of the biosynthesis, physicochemical and functional properties, and changes in processing and storage of these proteins. This necessitates an accurate method for protein isolation which is the purpose of this work.

Information on the extraction and characterization of cowpea proteins could not be found in the literature. There are, however, a number of reports on the isolation and fractionation of other legume proteins. For most of the initial reports (Osborne and Campbell, 1898; Bailey et al., 1935; Starker and Gortner, 1931) on seed proteins, the main objective was to isolate protein fractions, giving little consideration to the physical factors which might affect the extraction. Later work showed the importance of factors such as temperature and time of extraction, meal particle size, meal-to-solvent ratio, pH, and age of the meal on nitrogen extractability (O'Hara and Saunders, 1937; Nagel et al., 1938; Smith et al., 1952; Djang et al., 1953; Smith et al., 1959; Powrie, 1961; Patel and Johnson, 1974; Grant et al., 1976). Most of these experiments aimed at optimizing extraction conditions to give the best yield of nitrogenous components. Although many separate factors were investigated, the effect of a combination of factors on the nitrogen extractability was neglected. Thus, we have attempted to establish optimal conditions for extracting cowpea proteins using response surface methodology (RSM).

MATERIALS AND METHODS

Materials. Cowpea (*Vigna unguiculata*) variety "Adua Ayera" grown in Ghana was used throughout this work. The seeds were milled in a laboratory pulverizing mill (Weber Bros. and White Metal Works, Chicago, IL) to pass through a No. 008 screen. The resulting flour was used for the extractions. Defatted flour was prepared by extracting with petroleum ether for 6 h in a Soxhlet extractor.

Experimental Methods. (*a*) *Effect of Extraction Time on Nitrogen Extractability in 0.01 M Sodium Hydroxide.* A 5.0-g sample of cowpea flour was mixed with 50 mL of 0.01 M sodium hydroxide and shaken in a water bath (Eberbach Corp., Ann Arbor, MI) at 25 °C for times ranging from 30 min to 24 h. The material was centrifuged at 20000*g* for 20 min at 4 °C, the supernatant decanted, and the pH recorded. The residue was redissolved and the extraction repeated twice. Each of the supernatants obtained was made up to 100 mL and aliquots taken for nitrogen determination by the Kjeldahl method (AACC, 1969).

(*b*) *Effect of pH and Ionic Strength on Nitrogen Extractability.* The ionic strengths of solutions of different pH (1.0–13.6) were adjusted to 1.0 by adding sodium chloride. A 5.0-g sample of cowpea flour was mixed with 100 mL of solution. These were then extracted at 25 °C for 2 h and the dispersions centrifuged at 20000*g* for 20 min; total nitrogen was then estimated by the Kjeldahl method.

The effect of ionic strength on nitrogen extractability was similarly investigated by extracting 5.0 g of flour with a series of 100 mL of phosphate buffers (pH 7.0) with ionic strengths ranging from 0.005 to 4.0.

(*c*) *Establishment of Optimum Conditions for Extracting Cowpea Proteins in Different Solvents Using Response Surface Methodology.* (i) Experimental Design. The design used was a central composite rotatable design for $K = 3$ (Cochran and Cox, 1957). The variables were dependent variable (response): Y = percent nitrogen extracted; independent variables: X_1 = time of extraction (min), X_2 = temperature of extraction (°C), and X_3 = meal-to-solvent ratio.

The design was as given by Cochran and Cox (1957; Table 8A.8). The upper and lower levels of the three factors, time of extraction (X_1), temperature of extraction (X_2), and meal-to-solvent ratio (X_3), were set considering previous experimental data. The midpoints and the remaining two levels were calculated. The level of factors used in this design corresponding to the coded values are given in Table I.

(ii) Nitrogen Extraction. Using a combination of time, temperature, and meal-to-solvent ratio, 20 duplicate extractions were performed for each solvent (0.01 M sodium hydroxide, 0.5 M sodium chloride, water, phosphate buffer pH 7.0, and 70% ethanol). The mixture was centrifuged

Table I. Levels of Factors Used in Experimental Design

	actual levels of factors		
coded level	time (X_1), min	temp (X_2), °C	meal/solvent ratio (X_3), g/mL
−1.682	30.0	15	0.05
−1	72.5	19	0.08
0	135.0	25	0.125
1	197.5	31	0.17
1.682	240	35	0.20

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Table II. Regression Equations for Predicting Nitrogen Extractability from Cowpea Flour

solvent	equation[a]	R^2
water	$Y = -36.53639 - 0.14944X_1 + 6.33336X_2 + 278.16237X_3 + 0.00027X_1^2 - 0.07523X_2^2 + 112.19199X_3^2 - 17.00592X_2X_3 + 0.00207X_1X_2 + 0.11013X_1X_3$	85%[b]
0.5 M NaCl	$Y = 38.64595 + 0.11873X_1 + 1.44375X_2 + 2.48074X_3 - 0.00045X_1^2 - 0.03365X_2^2 - 63.56903X_3^2 - 0.08762X_1X_3 + 0.00033X_1X_2$	31.5% (N.S.)[c]
0.01 M NaOH	$Y = 48.96948 + 0.39551X_1 - 0.02174X_2 + 51.34482X_3 - 0.00076X_1^2 + 0.00222X_2^2 - 593.71779X_3^2 - 0.75376X_1X_3 - 0.00378X_1X_2 + 3.07833X_2X_3$	52%[b]
buffer, pH 7.0	$Y = 147.05243 - 0.17413X_1 - 3.96342X_2 - 528.62530X_3 + 0.00063X_1^2 + 0.06150X_2^2 + 1306.9031X_3^2 + 6.93078X_2X_3 + 0.00068X_1X_2 - 0.03684X_1X_3$	55%[b]
70% ethanol	$Y = 9.50503 - 0.10078X_2 - 48.09529X_3 - 0.01334X_1 + 0.00001X_1^2 + 0.00079X_2^2 + 65.53496X_3^2 + 0.59697X_1X_3 + 0.40756X_2X_3 + 0.00014X_1X_2$	77%[b]

[a] X_1 = time of extraction (min), X_2 = temperature of extraction (°C), X_3 = meal-to-solvent ratio. [b] Significant at $P \leqslant 0.01$. [c] Not significant.

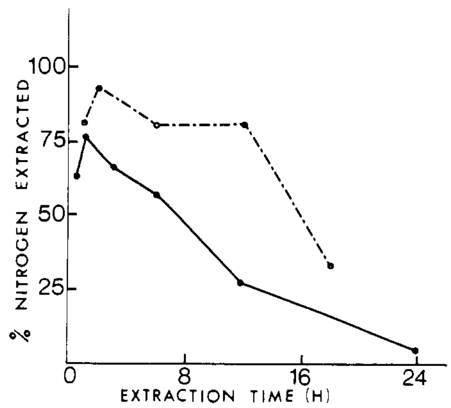

Figure 1. Effect of extraction time on the extractability of proteins of cowpea (*Vigna unguiculata*) in 0.01 M sodium hydroxide: (- -) multiple extractions and (–) single extractions.

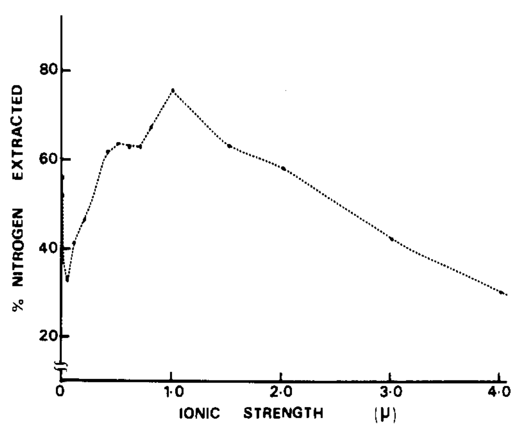

Figure 2. Effect of ionic strength (pH 7.0) on extractability of cowpea proteins.

at 20000g for 20 min at 4 °C and nitrogen determined in the supernatant.

(iii) Calculations. Stepwise multiple regression analysis was done to establish equations for predicting nitrogen extractability for each of the solvents.

To request three-dimensional surfaces on a two-dimensional plane, a digital plotting system was used. This program was used to evaluate the regression equations.

(*d*) *Nonprotein Nitrogen Determination.* This was determined by precipitating the proteins at 25 °C with 0.8 M trichloroacetic acid (Cl_3CCOOH). The Cl_3CCOOH was added until no further precipitation occurred, after which the mixture was centrifuged at 20000g for 15 min. The nitrogen remaining in the supernatant was determined.

RESULTS AND DISCUSSION

Effect of Extracting Conditions on Nitrogen Extractability. Preliminary experiments using 0.01 M sodium hydroxide revealed that extraction time had a marked effect on nitrogen extractability (Figure 1). The highest yield for single extractions (76%) was obtained after 1 h, beyond which there was a reduction in the nitrogen extracted. This decrease may be due to denaturation and coagulation of the proteins arising from the formation of foam during the prolonged extraction. There is thus no need to extend extraction time since most of the extractable proteins are removed at shorter extraction times. Multiple extractions yielded more nitrogen; probably as a result of increased meal-to-solvent ratio during multiple exposures.

The data shown in Figure 1 indicate that 93.4% of the total nitrogen in cowpea flour is extractable with 0.01 M sodium hydroxide. This value compares well with those reported by Smith et al. (1959) and Powrie (1961) for kidney beans and navy beans, respectively.

Cowpea proteins showed a characteristic solubility curve with changing ionic strength at the same pH (Figure 2).

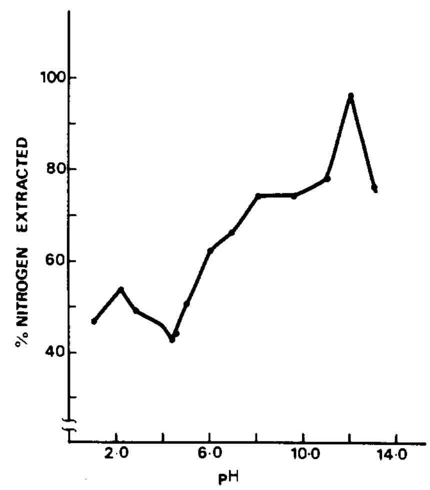

Figure 3. Extractability of cowpea proteins as a function of pH (constant ionic strength, 1.0).

Table III. Effect of Variables Used in Regression Models for Cowpea Nitrogen Extraction in Water

variable	F
time	3.81
temperature	42.84[a]
MSR	6.05[b]
time^2	2.38
temperature^2	19.95[a]
MSR^2	0.14
temperature × MSR	33.30[a]
time × temperature	0.97
time × MSR	0.15

[a] Significant at $P \leqslant 0.01$. [b] Significant at $P \leqslant 0.05$.

Table IV. Predicted Maximum Nitrogen (%) Extractable at Different Meal/Solvent Ratios

solvent	meal/solvent ratio 0.05	0.08	0.125	0.17	0.20
water					
max. N extractable (%)	75.47	66.67	56.71	51.09	49.77
temp. of extraction (°C)	35.00	33.40	28.20	23.40	19.80
time of extraction (min)	30.00	30.00	30.00	30.00	30.00
0.5 M NaCl					
max. N extractable (%)	62.30	61.77	60.79	59.56	58.60
temp. of extraction (°C)	22.20	22.20	22.20	22.20	22.20
time of extraction (min)	135.00	130.10	126.00	122.00	122.00
0.01 M NaOH					
max. N extractable (%)	82.96	79.21	72.20	67.08	63.38
temp. of extraction (°C)	15.00	15.00	15.00	35.00	35.00
time of extraction (min)	198.00	185.00	164.40	88.80	76.20
buffer, pH 7.0					
max. N extractable (%)	79.98	72.06	67.43	71.51	77.17
temp. of extraction (°C	15.00	15.00	35.00	35.00	35.00
time of extraction (min)	240.00	240.00	240.00	240.00	240.00
70% ethanol					
max. N extractable (%)	5.99	5.04	4.00	3.96	4.00
temp. of extraction (°C)	15.00	15.00	35.00	35.00	35.00
time of extraction (min)	30.00	30.00	240.00	240.00	240.00

A sharp decrease in nitrogen extractability at low ionic strength was observed, the lowest extraction occurring at an ionic strength of 0.05. There is, however, an increase in solubility as the ionic strength is increased from 0.05 to 1.0 after which there is a gradual decrease. Hermansson (1972, 1973) reported a similar curve for a commercial soybean protein isolate. She offered a physicochemical explanation for these changes, which could be applied to cowpea proteins. Thus, the sharp decrease in solubility (Figure 2) at low ionic strengths may be due to the formation of ionic bonds (a) within the protein molecule and (b) between adjacent protein molecules leading to the formation of aggregates. The observed increase in solubility from ionic strength 0.05 to 1.0 and the decrease from 1.0 to 4.0 are a result of "salting-in" and "salting-out" effects.

Extractability of cowpea proteins, like other plant proteins, was affected by the pH of the extracting solvent (Figure 3). An isoelectric pH of 4.4 is shown. Although cowpea proteins exhibit a characteristic pH–solubility curve, it is clear (Figure 3) that even at the isoelectric point about 40% of the protein was still extractable. This does not agree with the data previously reported for other legume proteins (Grant et al., 1976; Quinn and Beuchat, 1975; Flink and Christiansen, 1973; Pant and Tulsiani, 1969; Smith et al., 1959). Unlike cowpeas, most of these legumes showed a protein extractability of about 10% or less at their isoelectric point. This higher extraction at the isoelectric pH (Figure 3) may be due to (a) the presence of neutral salts in the extracting buffers or (b) the composition of the proteins since some proteins albumins and some globulins may not precipitate at the isoelectric pH.

Establishing Optimum Conditions for Extracting Cowpea Proteins in Different Solvents. For each of the five solvents multiple regression equations (Table II) were established to predict the amount of nitrogen extractable under varying meal–solvent ratios (MSR), temperatures, and times of extraction. Variables affecting cowpea nitrogen extractability was evaluated from the data.

Eighty-five percent of the variation observed in extracting cowpea nitrogen with water could be explained by the regression equations. The regression model (Table II) showed a strong dependence of nitrogen extractability on the temperature of extraction and MSR (Table III). Other variables showing statistical significance ($P \leq 0.01$) were temperature squared and the interaction between temperature and MSR. Thus using water as the extracting solvent, a change in the MSR can affect the influence of temperature on the extractability of cowpea nitrogen.

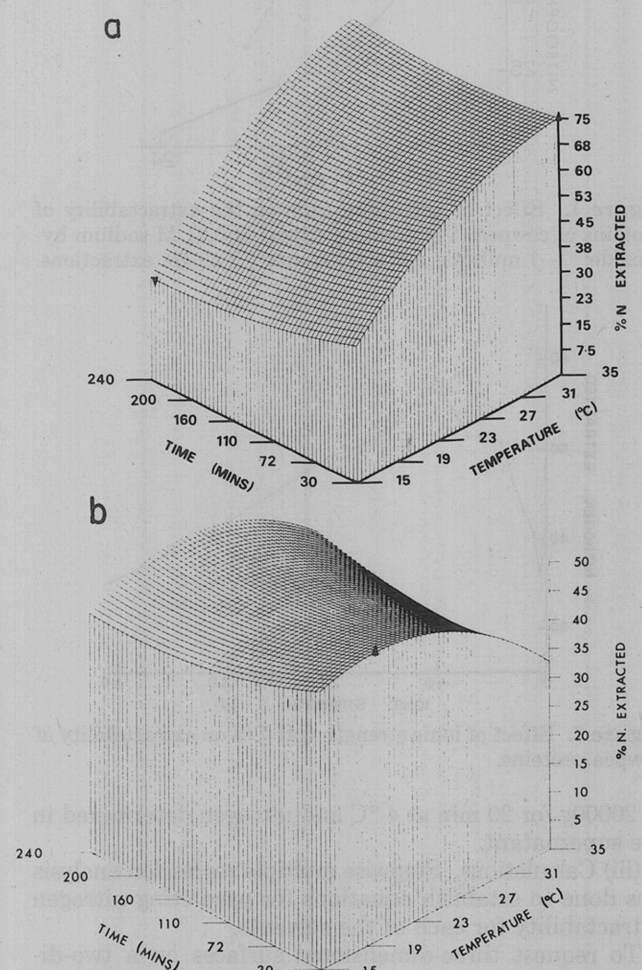

Figure 4. Effect of temperature and time of extraction on nitrogen extractability of cowpea flour in water at meal/solvent ratio of (a) 0.05, (b) 0.2. Arrows indicate maximum (↑) and minimum (↓) points.

To illustrate the regression equation graphically, MSR was held constant and the effect of time and temperature on nitrogen extractability plotted (Figure 4). These two

Table V. Regression Equations for Predicting Nitrogen Extractability from Defatted Cowpea Flour

solvent	equation[a]	R^2
0.5 M NaCl	$Y = 58.55698 - 0.03227X_1 - 0.20339X_2 + 281.64753X_3 - 0.00012X_1^2 + 0.02956X_2^2 - 331.19795X_3^2 - 15.92666X_2X_3 + 0.38221X_1X_2 + 0.00094X_1X_3$	60%[b]
0.01 M NaOH	$Y = 35.27753 - 0.61862X_1 + 4.91028X_2 + 64.69620X_3 + 0.00113X_1^2 - 0.09237X_2^2 + 0.01194X_2^2 - 12.81420X_2X_3 + 0.59378X_1X_3$	67%[b]
buffer, pH 7.0	$Y = 104.62350 - 0.44125X_1 - 2.30515X_2^2 + 257.84956X_3 + 0.00039X_1^2 + 0.04619X_2^2 - 17.26775X_2X_3 + 0.01192X_1X_2 + 0.55485X_1X_3$	69%[b]
70% ethanol	$Y = 9.77096 + 0.00513X_1 - 0.30638X_2 - 19.09388X_3 + 0.00570X_2^2 + 3.0404X_3^2 - 0.0001X_1X_2 + 0.00849X_1X_3 - 0.07058X_2X_3$	87%[b]
water	$Y = 113.19204 + 0.04615X_1 - 3.98951X_2 - 465.00904 - 0.00034X_1^2 + 0.09350X_2^2 + 1894.54028X_3^2 - 7.17898X_2X_3 + 0.43829X_1X_3 - 0.00175X_1X_2$	61%[b]

[a] X_1 = time of extraction (min), X_2 = temperature of extraction (°C), X_3 = meal-to-solvent ratio. [b] Significant at $P \leqslant 0.01$.

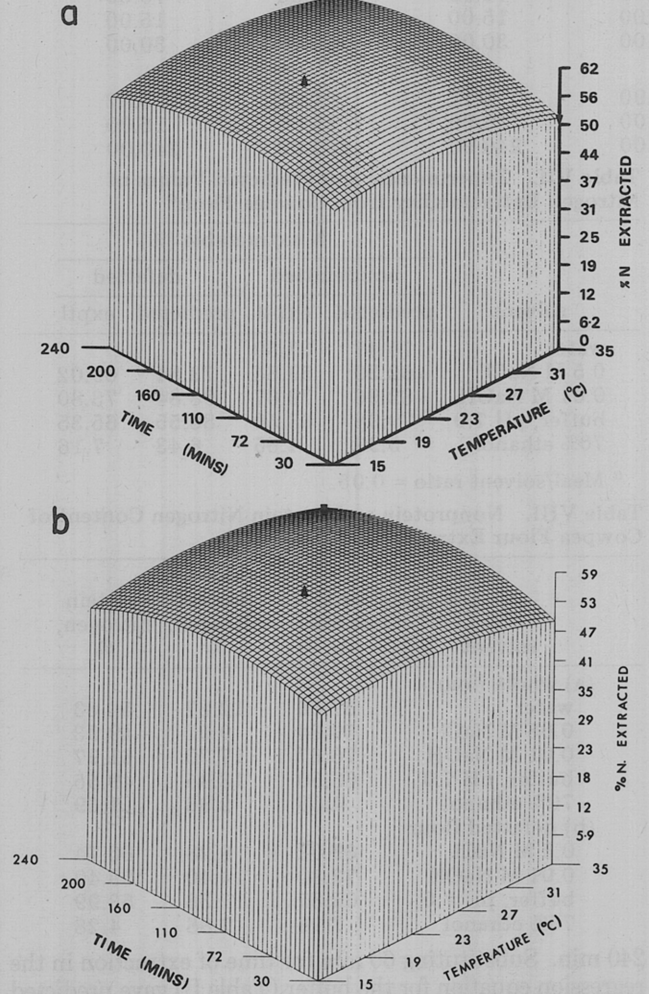

Figure 5. Effect of temperature and time of extraction on nitrogen extractability of cowpea flour in 0.5 M NaCl at meal/solvent ratio of (a) 0.05, (b) 0.2.

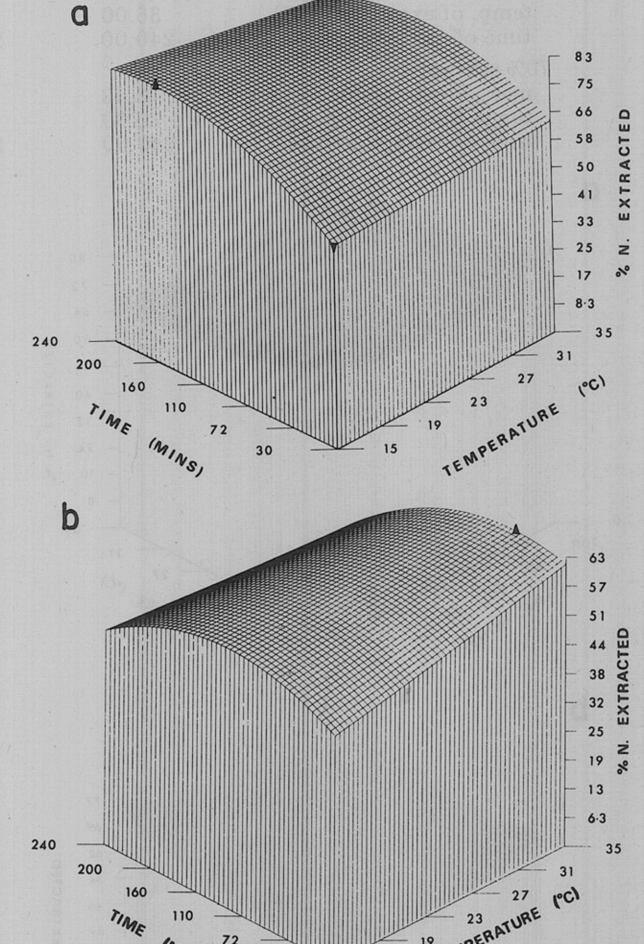

Figure 6. Effect of temperature and time of extraction on nitrogen extractability of cowpea flour in 0.01 M NaOH at a meal/solvent ratio of (a) 0.05, (b) 0.20.

graphs show clearly the influence of temperature and MSR on the extractability of nitrogen. For a MSR of 0.05 (Figure 4a), the effect of temperature is almost linear. The same effect was found at MSR of 0.08. With an increase in MSR to 0.125, the temperature showed a curvilinear effect while at the highest MSR (0.2), the effect was distinctly curved (Figure 4b).

The maximum extractable nitrogen at each MSR was obtained from the regression equation (Table IV). The highest extractable nitrogen occurred at the lowest MSR (0.05). The conditions for optimal extraction and the amount of nitrogen extracted, however, changed with MSR. Using water as a solvent, there was a decrease in nitrogen extractability with increasing MSR. Similar analyses were performed on the data for the other solvents. Variables showing significant effect on nitrogen extractability depended on the solvent.

For extraction with 0.5 M NaCl, the square of time and temperature of extraction showed significant effects ($P \leq 0.05$) on nitrogen extractability but the effect of MSR was not significant. This may be seen in Figure 5 where a change in MSR from 0.05 to 0.2 did not affect the shape of the response surface curve. The predicted maximum nitrogen extractable at different MSR is shown in Table IV. Unlike the water model, changing MSR from 0.05 to 0.2 did not change appreciably the maximum extractable nitrogen.

Time of extraction was the most significant variable ($P \leq 0.05$) when 0.01 M NaOH was used for cowpea nitrogen extraction. The response surface plots (Figure 6) indicate that while temperature of extraction showed a linear response on extractability of nitrogen, time of extraction showed a curved response. The conditions for optimal extraction of nitrogen varied with MSR (Table IV). Like

Table VI. Predicted Maximum Nitrogen (%) Extractable from Defatted Cowpea Flour at Different Meal/Solvent Ratios

solvent	meal/solvent ratio 0.05	0.08	0.125	0.17	0.20
0.5 M NaCl					
max. N extractable (%)	73.84	65.50	64.15	64.41	64.53
temp. of extraction (°C)	35.00	35.00	15.00	15.00	15.00
time of extraction (min)	80.40	130.00	122.00	193.80	240.00
0.01 M NaOH					
max. N extractable (%)	98.84	91.59	80.96	71.92	66.89
temp. of extraction (°C)	35.00	35.00	33.40	30.20	28.20
time of extraction (min)	240.00	240.00	240.00	240.00	240.00
buffer, pH 7.0					
max. N extractable (%)	86.55	80.15	74.85	75.50	76.00
temp. of extraction (°C)	35.00	35.00	15.00	15.00	15.00
time of extraction (min)	240.00	240.00	30.00	30.00	30.00
70% ethanol					
max. N extractable (%)	6.43	5.90	5.11	4.33	3.82
temp. of extraction (°C)	15.00	15.00	15.00	15.00	15.00
time of extraction (min)	240.00	240.00	240.00	240.00	240.00

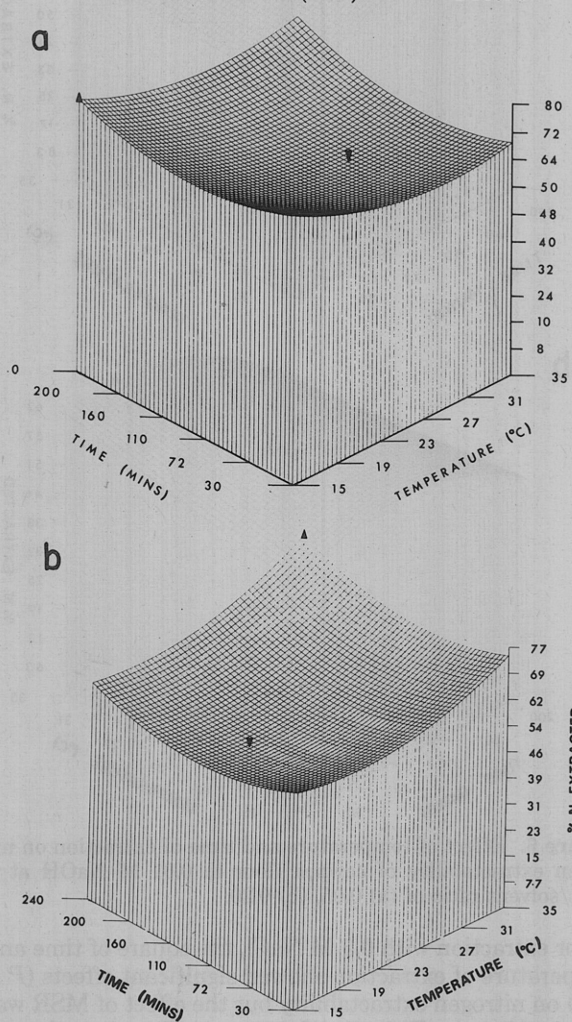

Figure 7. Effect of temperature and time of extraction on nitrogen extractability of cowpea flour in phosphate buffer at a meal/solvent ratio of (a) 0.05, (b) 0.20.

the water model, nitrogen extractability in 0.01 M NaOH decreased with increasing MSR. The maximum nitrogen extracted, 82.96%, was obtained at MSR of 0.05.

Temperature of extraction, MSR, and their square terms were the most significant variables when phosphate buffer was used for extraction. Graphically this was shown as a highly curved response due to temperature (Figure 7). Time of extraction had little effect. The predicted maximal nitrogen extractable, 79.98%, was shown to occur at a MSR of 0.05, a temperature of 15 °C and a time of

Table VII. Experimental and Predicted Values of Nitrogen Extractability from Cowpea Flour[a]

solvent	N extractable (%) nondefatted predicted	nondefatted exptl	defatted predicted	defatted exptl
water	75.47	67.50		
0.5 M NaCl	62.30	59.84	73.84	69.02
0.01 M NaOH	82.96	79.67	98.84	73.80
buffer, pH 7.0	79.98	63.50	86.55	65.35
70% ethanol	5.99	4.55	6.43	7.16

[a] Meal/solvent ratio = 0.05.

Table VIII. Nonprotein and Protein Nitrogen Content of Cowpea Flour Extracts

solvent	total N extracted, %	nonprotein nitrogen, %	protein nitrogen, %
(a) whole flour			
water	67.50	12.87	54.63
0.05 M NaCl	59.84	9.91	49.93
0.01 M NaOH	76.67	17.70	61.97
buffer, pH 7.0	63.50	13.95	49.55
70% ethanol	4.55	2.86	1.69
(b) defatted flour			
0.5 M NaCl	69.02	8.28	60.74
0.01 M NaOH	73.80	14.40	59.40
buffer, pH 7.0	65.35	11.36	53.99
70% ethanol	7.16	2.88	4.28

240 min. Substituting 60 min for time of extraction in the regression equation for the buffer (Table II) gave predicted nitrogen extractable as 75.79% at a MSR of 0.05 and temperature of 15 °C. This shows that a change in time of extraction from 240 min to 60 min would cause little reduction in the predicted nitrogen extractable.

Nitrogen extractions in 70% ethanol showed highly significant ($P \leq 0.01$) first order terms which is reflected in the response surface plots (Figure 8). At both higher and lower meal–solvent ratios a linear response due to time and temperature of extraction was observed which gave a table-top appearance to the response surface plots (Figure 8). Extractions with 70% ethanol gave the lowest yield of nitrogen at any of the five MSR's used.

Nitrogen extractability using defatted cowpea flour showed improved regression equations (Table V) in the form of increased R^2 for most of the solvents. The regression equations were represented graphically as done with the nondefatted flour and the maximum extractable nitrogen predicted (Table VI). A relatively higher nitrogen extractability was predicted from the equations. Djang et al. (1953), working with mung beans (*Phaseolus aureus*),

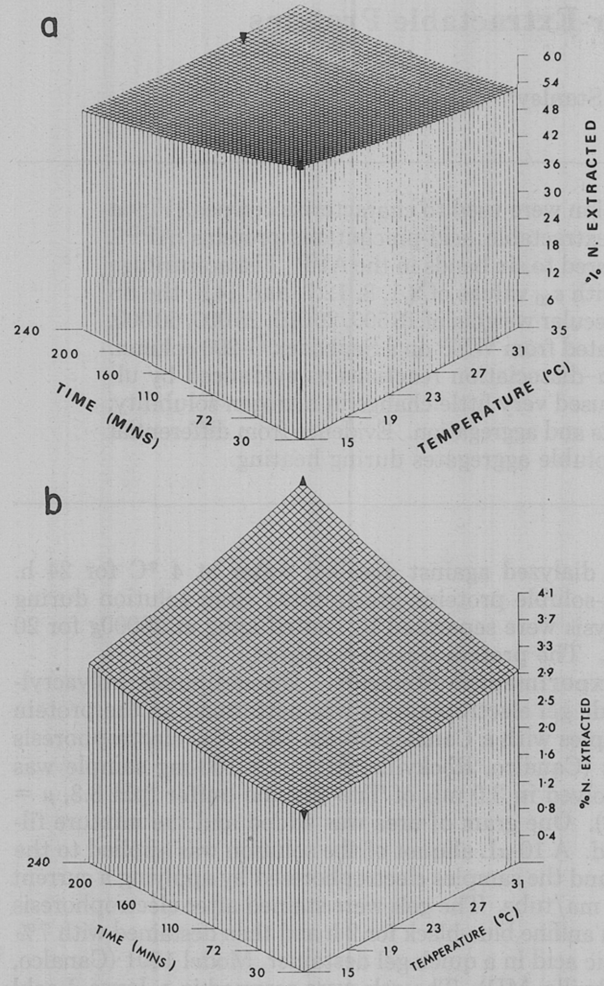

Figure 8. Effect of temperature and time of extraction on nitrogen extractability of cowpea flour in 70% ethanol at a *meal-to-solvent ratio of* (a) 0.05, (b) 0.20.

reported a similar effect.

To check the predictability of the regression equations, the extractions were repeated using the optimal conditions given by the regression analysis. The experimental values (Table VII) showed good agreement with the predicted values for most of the solvents.

One advantage in having these regression equations is that the time and temperature of extraction and the meal–solvent ratio can be changed at will and the expected yield of nitrogen estimated to suit any extraction conditions. For example, the regression analysis showed that using water, the maximum extractable nitrogen (75.47%) occurred at 35 °C, after extracting for 30 min at MSR of 0.05. If an extraction temperature of 25 °C was preferred, the predicted maximum nitrogen to be extracted will be 65.16%. The experimental value obtained after extracting at these conditions was 63.50%, showing reasonable agreement with the predicted value. Thus, the regression equations are useful tools in predicting the extractability of cowpea protein nitrogen within the limits considered in the experimental design.

All the solvents investigated extracted some amount of nonprotein nitrogen (NPN) as shown in Table VIII. Because of the heterogeneity of the group of substances classified as NPN, it is not surprising that a wide variation was detected in their extractability in different solvents (Table VIII). The relatively low NPN extracted from the defatted cowpea flour by all the solvents may be due to the loss of some of the NPN during the defatting process. The values obtained compare well with those reported in the literature for other legumes (Smith et al., 1959; Wood and Cole, 1973).

After removing the contribution made by NPN, the protein nitrogen extracted was found to vary, 0.01 M sodium hydroxide extracting the highest amount. One interesting observation was the relatively high amount of protein nitrogen extracted by water as compared to that by 0.5 M sodium chloride (Table VIII). Legume proteins are considered to be mainly globulins, i.e., soluble in salt solutions. However, Smith et al. (1959) have shown that a number of seeds, especially in the legume family, contain nearly as much water-soluble as salt-soluble protein, or even more. Soybean, for example, was shown to have 84.1% of the total nitrogen extractable with water as compared to 72.3% extractable by 0.5 M sodium chloride. From the data, it can be concluded that cowpea proteins, like soybean, showed a higher solubility in water than in 0.5 M sodium chloride solution. The high extractability in water may be due to the presence of salts in the seed.

CONCLUSIONS

Response surface methodology technique proved to be a useful tool in establishing optimum conditions for extracting cowpea protein nitrogen. Conditions for extracting the proteins in different solvents differ and for this reason *a universal condition of extraction cannot be established.* In general, a decrease in meal–solvent ratio increased protein extraction in all solvents. This however results in a dilute solution. The conditions established from the analyses can be used as guidelines in establishing conditions on cowpea extraction in the solvents used.

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