OPTIMIZATION OF PROTEIN EXTRACTION FROM FERN FROND USING RESPONSE SURFACE METHODOLOGY (RSM)

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ABSTRACT

Protein extractability from *Nephrolepis biserrata* and *Arthropteris orientalis* was studied under various conditions. The extraction of fern protein was conducted using alkaline, alcohol and saline treatments under various conditions of treatment concentrations and time of agitation. Central composite design of Response Surface Methodology (RSM) was used for identification of the best condition and extraction yield optimization. Results showed that alkaline treatment produced the highest extraction yield with maximum protein recovery of 5.11 mg/mL and 2.03 mg/mL for *Nephrolepis biserrata* and *Arthropteris orientalis* respectively at 0.1 M NaOH concentration and 30 min of agitation time.

Keywords: Fern, *Nephrolepis biserrata*, *Arthropteris orientalis*, Response Surface Methodology, Agitation Time, Solvents Concentration.

INTRODUCTION

Ferns are members of a large and diverse group of plants commonly referred to as the lower plants. It has been estimated that only 5% of all bryophytes have been studied with regard to any phytochemical properties (Asakawa, 2001). There have been a number of protein extraction methods explained and used by different scientists in previous studies (Naushad *et al*., 2010; Hameed *et al*., 2009; Gómez-Brenes *et al*., 1983; Damania *et al*., 1983; Miller *et al*., 1972). The quality of a method however depends on the amount of protein extracted. The protein extraction methods mostly used in the laboratory involves milling samples into powder after being flashed with liquid nitrogen followed by suspending in protein extraction buffer. However, there are various extraction conditions such as pH, solvent types, solvent concentration, extraction time and solvent/flour ratio that may affect the final properties of the extracted protein prior to centrifugation.

Total proteins as well as storage proteins in plants have been studied using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). This study cannot be carried out without the isolation of protein from the plants. Protein isolation is a very important step when incorporating proteins from oil-producing plants into food products. Raw materials with high level of oil need to be defatted prior to protein isolation in order to prevent emulsion formation during protein extraction and produce oil-free protein materials. To obtain samples that are free from fat, raw materials are first defatted with solvents such as hexane (Kumagai *et al*., 2002) and petroleum ether (Sathe *et al*., 2002).

Various extraction conditions such as pH, solvent types (NaOH, NaCl or Ethanol), solvent concentration, extraction time, solvent/flour ratio, may affect the final properties of the extracted protein. Munasinghe and Sakai (2004) demonstrated that NaCl had a significant increase in protein extractability as compared to KCl and LiCl salts. Ragab *et al.* (2004), Lawal (2004), Inyang and Iduh (1996) and Badifu and Akubor (2000) studied the effect of NaCl and pH on the extractability of cowpea proteins, locust bean protein isolate, sesame seed concentrate and African breadfruit kernel flour respectively.

When many factors and interactions affect desired responses in a certain process design, Response Surface Methodology (RSM) becomes an effective tool for optimizing the process. It is a statistical technique used to design experiments that yield the relevant information in the shortest time with the least cost and also obtain rapid and efficient development of new products and processes (Pericin *et al*., 2008). In addition, to analyze the effects of the independent variables, the experimental methodology also generates a

mathematical model that describes the overall process (Batista, 1999).

Response surface experiments aim to recognize the response that can be thought of as a surface over the explanatory variables experimental space, usually uses an experimental design such as Central Composite Rotatable Design (CCRD) to fit an empirical, full second-order polynomial model. The advantages of using RSM have been reported to include reduction in the number of experimental trials needed to evaluate multiple parameters and the ability of the statistical tool to identify interactions. RSM has successfully been applied for the optimization of different extraction conditions (Yi *et al.*, 2011; Tiezheng *et al.*, 2010; Wani *et al.*, 2006).

However, there is not yet any investigation about the optimization of fern protein extraction. No report on the effect of several variables like time, solvent types and solvent concentration on the extractability of fern frond has been documented. The objective of this study was to investigate the effect of various factors or conditions necessary to optimize protein extraction from fern fronds flour for maximum protein content.

MATERIALS AND METHODS

Plant Material

Fern frond, *Nephrolepis biserrata* (Swartz) Schott and *Arthropteris orientalis* (Gmel.) Posth. belonging to the family Davalliaceae were botanically identified in the herbarium of the Biology Deparment of Kwame Nkrumah University of Science and Technology. *Nephrolepis biserrata*, was harvested from a stream along the Mango Road and *Arthropteris orientalis*, harvested from palm trees along Buroburo Road within the Kwame Nkrumah University of Science and Technology, Kumasi-Ghana. After being washed and solar-dried for two weeks, the fern fronds were milled into flour using MPE roller mills (Model: GP-140 Grinder) and passed through 25 μm mesh sieve to obtain fine powder.

Preparation of Defatted Fern Frond Flour

The oil of fern fronds flour was extracted using the cold extraction method by soaking the flour (tied in a cheese cloth) in hexane using a ratio of 1:10 w/v, with respect to flour/solvent for 48 h in an air tight container at room temperature. The oil free fern fronds flour was then air-dried and stored in high density polyethylene bags under refrigeration (4 °C) until when needed.

Experimental Design

The experimental design was conducted using the Design Expert software (version 7.0, Stat Easy Inc., Minneapolis, USA). For alkaline, saline and ethanol treatment, the effect of independent variables i.e., NaOH, NaCl, Ethanol concentration and time, were investigated as shown in Table 1.

Table 1. Constraint for Treatment Conditions

Treatment	Name	Units	Lower limit	Upper limit
Alkaline	NaOH	mol/dm^3	0.01	0.1
	Time	min	30	60
Saline	NaCl	mol/dm^3	0.01	0.1
	Time	min	30	60
Alcohol	Ethanol	%	50	70
	Time	min	10	60

Protein Extraction

The effect of time and NaOH, NaCl and ethanol concentration on the extractability of protein from defatted fern flour were determined by dispersing 0.5 g of sample in 25 mL of the three solvents based on different combinations, as shown in Tables 2 and 3. Samples were agitated at room temperature for the specified extraction periods in an orbital shaker (Gallenkamp orbital shaker, London-UK) at 150 rpm. The solubilized liquor was separated from insoluble material by centrifugation at 2500 rpm for 15 min at room temperature (25 °C). After optimization, the proteins were extracted with the optimum conditions. Concentrated HCl was used to precipitate protein from the supernatant with the optimum treatment and centrifuged at 3000 rpm for 15 min. The precipitate was washed repeatedly with distilled water after which the precipitated proteins were freeze-dried.

Table 2. Actual Level of Independent Variables for NaOH and NaCl Treatments

Runs	Independent variables	
	Solvent concentration (M)	Time (min)
1	0.01	30
2	0.1	30
3	0.01	60
4	0.1	60
5	0.055	45
6	0.055	45
7	0.055	45
8	0.055	45
9	0.01	45
10	0.1	45
11	0.055	30
12	0.055	60
13	0.055	45
14	0.055	45
15	0.05	45
16	0.05	45

Table 3. Actual Level of Independent Variables for Ethanol

Runs	Independent variables	
	Solvent concentration (%)	Time (min)
1	50	10
2	50	60
3	50	35
4	60	35
5	60	35
6	60	35
7	60	35
8	60	10
9	60	60
10	60	35
11	60	35
12	60	60
13	60	35
14	70	10
15	70	60
16	70	35

Protein Determination

Soluble protein in supernatant was estimated by the method of Bradford (1976) using the coomassie protein assay reagent with bovine serum albumin as the standard.

Statistical Analysis

The response data obtained from the standard curve were loaded into the Design Expert (2007) statistical tool and run to generate regression parameters which were studied. The predicted values of protein yields were calculated using regression model and compared with experimental values. The most compatible estimation model among the mean, linear, quadratic and cubic expressions of each response variable were identified based on all the statistical analysis which includes sequential model, sum of squares, lack of fit tests and the model summary statistics. When a model had been selected, an analysis of variance was calculated to assess how well the model represented the data.

RESULTS

The influence of extraction time on protein extractability from defatted fern fronds flour in three different solvents viz: NaOH, Ethanol and NaCl are shown in Tables 4, 5 and 6 respectively. Virtually all of the protein was extracted with increasing concentration of the solvents used indicating that the bulk of fern fronds protein solubilized at a higher concentration.

Table 4. Central Composite Design for NaOH, Variables and Responses

Runs	Independent variables		Dependent variables	
	A: Solvent (M)	B: Time (min)	NB proteins (mg/mL)	AO proteins* (mg/mL)
1	0.01	30	2.023	0.420
2	0.1	30	6.182	2.159
3	0.01	60	3.489	1.307
4	0.1	60	3.068	1.943
5	0.055	45	2.682	1.227
6	0.055	45	3.114	1.886
7	0.055	45	3.261	1.148
8	0.055	45	3.170	1.432
9	0.01	45	1.989	0.761
10	0.1	45	3.841	2.239
11	0.055	30	3.352	1.318
12	0.055	60	3.511	1.398
13	0.055	45	3.284	1.114
14	0.055	45	3.852	1.466
15	0.05	45	3.750	1.295
16	0.05	45	4.966	1.125

* NB = *Nephrolepis biserrata*; AO = *Arthropteris orientalis*

Table 5. Central composite Design for Ethanol, Variables and Responses

Runs	Independent variables		Dependent variables	
	A: Solvent (%)	B: Time (min)	NB proteins (mg/mL)	AO proteins (mg/mL)
1	50	10	1.505	0.201
2	50	60	1.980	0.157
3	50	35	1.749	0.180
4	60	35	2.817	1.778
5	60	35	2.039	2.080
6	60	35	1.786	0.643
7	60	35	1.442	0.792
8	60	10	2.742	1.515
9	60	60	2.335	0.901
10	60	35	2.802	1.126
11	60	35	3.064	1.275
12	60	35	2.561	0.865
13	60	35	2.701	1.136
14	70	10	2.508	3.370
15	70	60	4.749	2.939
16	70	35	5.792	3.232

Table 6. Central Composite Design for NaCl, Variables and Responses

Runs	Independent variables		Dependent variables	
	A: Solvent (M)	B: Time (min)	NB proteins (mg/mL)	AO proteins (mg/mL)
1	0.01	30	1.023	0.625
2	0.1	30	0.830	0.534
3	0.01	60	1.341	0.477
4	0.1	60	0.795	0.500
5	0.055	45	0.420	0.284
6	0.055	45	0.420	0.432
7	0.055	45	0.341	0.330
8	0.055	45	0.705	0.330
9	0.01	45	1.000	0.410
10	0.1	45	0.966	0.261
11	0.055	30	0.545	0.398
12	0.055	60	0.636	0.250
13	0.055	45	0.557	0.489
14	0.055	45	0.739	0.182
15	0.05	45	0.318	0.409
16	0.05	45	0.682	0.364

Alkaline Treatment

Relationship between time and concentration of extracted protein: From Table 4, the extracted protein concentration was 1.99–6.18 mg/mL for *Nephrolepis biserrata* and 0.42–2.24 mg/mL for *Arthropteris orientalis*. The independent variables were analyzed to predict the maximum extracted proteins under the given range (Table 1). The response surface graph for extracted protein from defatted *N. biserrata* as a function of time and NaOH concentration is shown in Figure 1.

Table 7. Optimum Protein Recovery for both Treatments

Runs	Optimum conditions		Maximum protein recovery (mg/mL)	
	Concentration	Time(min)	NB	AO
Alkaline (NaOH)	0.1 M	30	5.11	2.03
Alcohol (Ethanol)	70%	53	5.12	3.02
Saline (NaCl	0.1 M	53	0.83	0.47

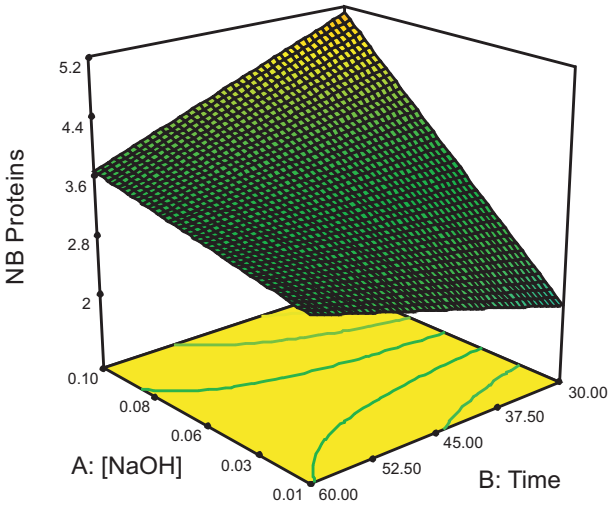

Figure 1. Effect of Time and NaOH Concentration on the Extractability of Proteins from Defatted *Nephrolepis biserrata*

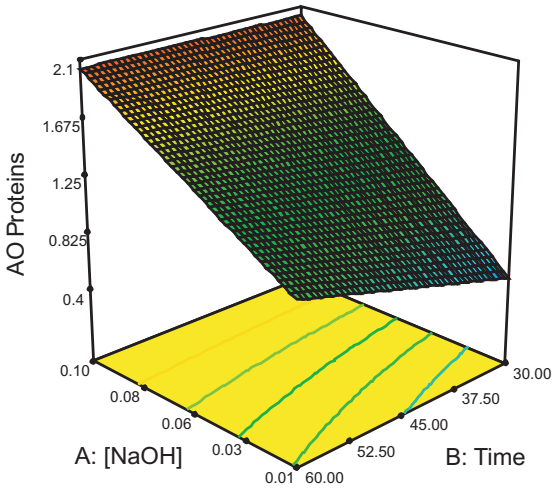

Figure 2. Effect of Time and NaOH Concentration on the Extractability of Proteins from Defatted *Arthropteris orientalis*

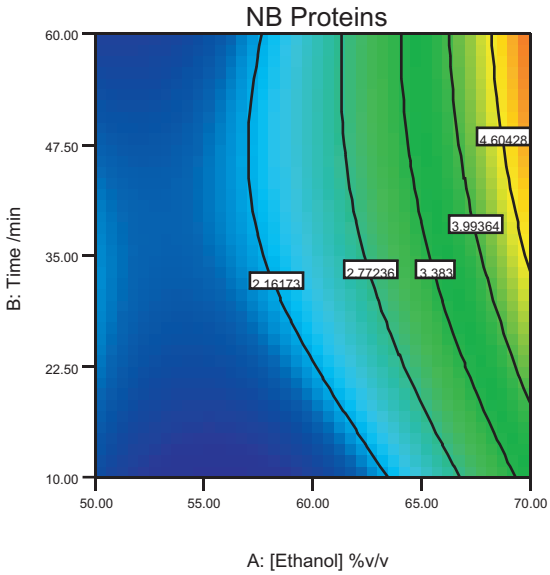

Figure 3. Effect of Time and Percentage Ethanol on Protein Recovery from *Nephrolepis Biserrata*

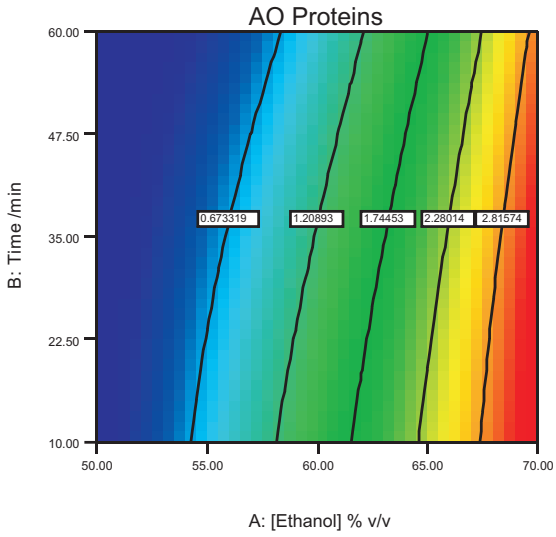

Figure 4. Effect of Time and Percentage Ethanol on Protein Recovery from *Arthropteris Orientalis*

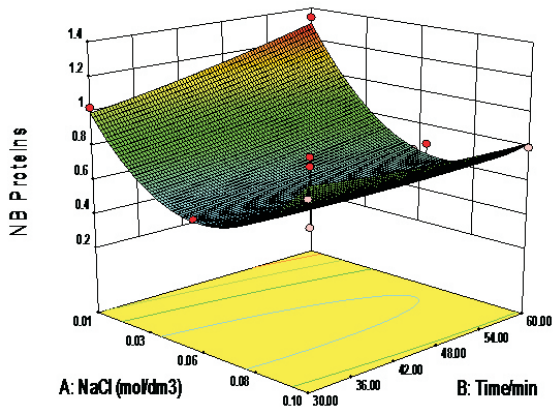

Figure 5. Effect of Time and NaCl Concentration on the Extractability of Proteins from Defatted *Nephrolepis biserrata*

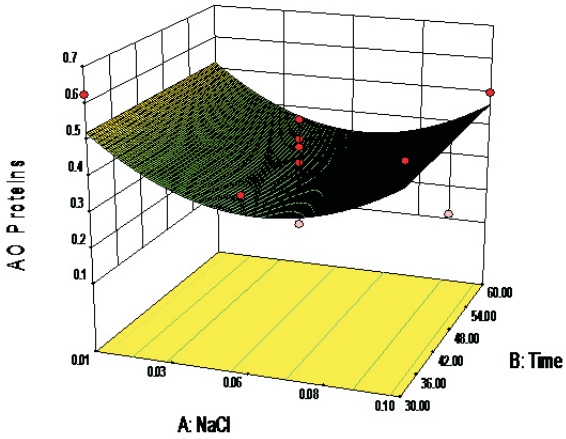

Figure 6. Effect of Time and NaCl Concentration on the Extractability of Proteins from Defatted *Arthropteris orientalis*

Table 8. ANOVA for Response Surface Reduced 2FI Model for NB proteins using NaOH

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Block	0.039551	1	0.039551			
Model	69.54814	5	13.90963	43.3283	<0.0001	significant
A-[NaOH concentration]	1.934573	1	1.934573	6.026168	0.0214	
B-Time	0.103349	1	0.103349	0.32193	0.5755	
C-NaOH	61.09521	1	61.09521	190.3107	< 0.0001	
AB	3.040354	1	3.040354	9.47066	0.0050	
AC	3.374656	1	3.374656	10.51201	0.0034	
Residual	8.025717	25	0.321029			
Lack of Fit	6.121695	13	0.4709	2.96782	0.0343	not significant
Pure Error	1.904022	12	0.158669			
Cor Total	77.61341	31				

Table 9. ANOVA for Response Surface 2FI Model for AO Proteins using NaOH

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob >F	
Block	0.07574	1	0.07574			
Model	10.68107	6	1.780179	48.80653	< 0.0001	significant
A-[NaOH concentration]	1.101928	1	1.101928	30.21118	< 0.0001	
B-Time	0.014732	1	0.014732	0.403897	0.5311	
C-NaOH	7.965945	1	7.965945	218.3995	< 0.0001	
AB	0.122175	1	0.122175	3.349635	0.0797	
AC	1.379175	1	1.379175	37.81236	< 0.0001	
BC	0.097118	1	0.097118	2.662655	0.1158	
Residual	0.875381	24	0.036474			
Lack of Fit	0.401175	12	0.033431	0.845993	0.6116	not significant
Pure Error	0.474206	12	0.039517			
Cor Total	11.6322	31				

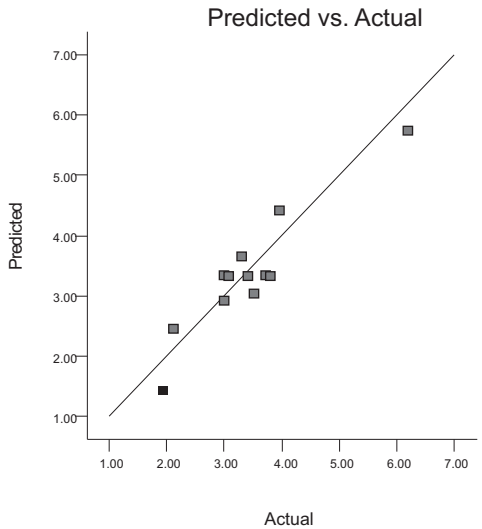

Figure 7. Correlation Between Predicted and Actual Extracted Protein (mg mL^{-1}) from NB

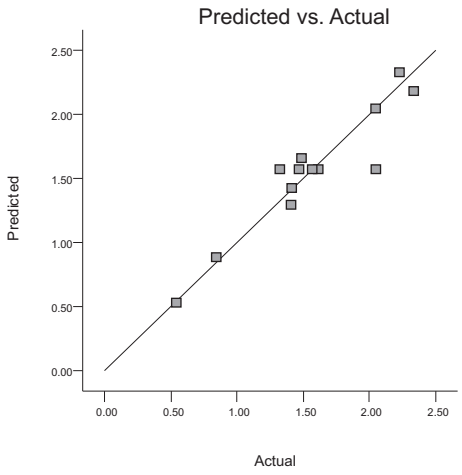

Figure 8. Correlation Between Calculated and Experimentally Extracted Protein from AO

Alcohol Treatment

Relationship between time and concentration of extracted protein: Figure 3 shows values of protein extracted from *Nephrolepis biserrata* by varying time and percentage ethanol while letting the flour/solvent ratio remain constant at 0.5:25 (w/v). Variation in time and percentage ethanol revealed that a maximum amount of extracted protein was obtained when time was more than 35 min and percentage ethanol was 70%. An increase in time of agitation with high concentration of ethanol increased the amount of protein extracted. Maximum protein observed was 5.12 mg/mL and the most favourable condition was 53 min of agitation and 70% concentration of ethanol.

Saline Treatment

Relationship between time and concentration of extracted protein: Results in Table 6 show that the experimental concentration of extracted protein varied from 0.31 to 1.34 mg/mL for *Nephrolepis biserrata* and 0.18 to 0.63 mg/mL for *Arthropteris orientalis*. In general, the best result in terms of protein extraction was obtained using high NaCl concentration without any significant effect on time.

DISCUSSION

Alkaline Treatment

The extracted protein was more pronounced at the high alkaline concentration of 0.1 M (Figure 1) and this was consistent with an observation by Batista (1999) when extracting proteins from hake and monk fish using NaOH. However, increasing the agitation time showed a negative linear effect on extracted protein amount indicating that most of the proteins dissolve in the solution during the first 30 min of agitation, but the protein extraction was low after 45 min. This means that prolonged time of agitation causes protein denaturation which reduces the amount of protein in solution (Eke and Akobundu, 1993).

A similar trend was observed for proteins extracted from *A. orientalis* using NaOH as solvent concentration as compared to *N. biserrata*. Arifin *et al.* (2009) ascertained increased in protein recovery from palm kernel meal as NaOH concentration increase. Eromosele *et al.* (2008) found that extractable African yam bean protein decreased from 17.8 to 14.9% at 0.01 and 0.1 M NaOH respectively. This observation was contrary to that observed in this study. However, there was no effect of time on the amount of protein extracted as shown by the response surface plot from the experimental design in Figure 2. This show that time of agitation is not significant when extracting proteins from *A. orientalis*.

Alcohol Treatment

The optimum conditions for extracted protein recovery from *Arthropteris orientalis* were 70% ethanol and time of 10 min agitation. This yielded 3.32 mg/mL of protein extracted (Figure 4). This revealed that increase in percentage ethanol showed an increasing trend however, increasing time showed a negative linear effect on protein extraction. Hancock *et al.* (1990) observed improvement in protein quality due to increasing ethanol concentration during extraction of over-processed soybean flakes.

Saline Treatment

The relationship between the variables and amount of protein extracted are as shown in Figures 5 and 6 for *N. biserrata* and *A. orientalis* respectively. Variation in time and salt concentration revealed maximum amount of extracted protein of 0.83 mg/mL at time of 53 min of agitation and NaCl concentration of 0.1 M. There was increase in protein extracted as NaCl concentration increased from 0.01 M to 0.1 M. This was significantly different from that observed in Figure 6 for *Arthropteris orientalis* but consistent with study made by Govardhan-Singh *et al.* (2011).

Though the experimental amount of extracted protein from *N. biserrata* ranges from 0.32 mg/mL to 1.34 mg/mL, no significant effect was observed as time of agitation increased from 30 min to 60 min on the extracted protein but as NaCl concentration decreased, the amount of extracted protein also decreased.

When the salt concentration was kept at 0.10 M (Figure 6), maximum amount of extracted protein of 0.47 mg/mL was observed at time of 45 min. The results also revealed that at 0.01 M concentration of NaCl 0.52 mg/mL of protein was extracted and when the salt concentration was

increased from 0.01 M to 0.08 M, the amount of protein extracted decreased to 0.35 mg/mL. But then there was a sharp rise in the amount of protein extracted from 0.35 mg/mL to 0.45 mg/mL peaking at 0.625 mg/mL. This reflection was contrary to the study conducted by Govardhan-Singh *et al.* (2011) who observed a consistent increase in protein extractability (from moringa seed flour) from 37.74 % to 84.72 % at 0.05 M to 0.75 M and a gradual decrease from 84.72 % to 71.21 % at 0.75 M to 2.0 M. This phenomenon could be explained by the fact that at low concentrations of salt, solubility of the protein usually increases slightly (salting in) (Arifin *et al.*, 2009).

At high concentrations of salt, solubility of the proteins dropped sharply as a result of salting out and therefore precipitate. The reason is that at high salt concentrations, the abundance of the salt ions decreased the solvating power of the salt ions thereby decreasing the solubility of the proteins and precipitation resulted (Arifin *et al.*, 2009).

Time of agitation had little or no effect on optimization result. This result agreed with the findings of Thompson (1977) for mung bean proteins, reporting that the time of extraction did not have much influence on nitrogen extractability. Jyothirmayi *et al.* (2006) had also reported that extraction of proteins increased till 35 min after which it remained constant.

Optimum Conditions for the Extraction of Defatted Fern Flour and Model Verification

By considering all the conditions in response to the amount of protein recovered from *N. biserrata* and *A. orientalis* as shown in Table 7, it was evident that NaOH and Ethanol recorded the highest protein recovery for both fern types. *A. orientalis* recorded the lowest amount of protein for all the treatment with as low as 0.47 mg/mL for NaCl.

No protein precipitated for both *N. biserrata* and *A. orientalis* at pH of 1.8 and 13.8 for ethanol extraction but high protein precipitation was observed for NaOH extraction using supernatants of *N. biserrata* and *A. orientalis* at a pH range of 2.3 to 2.5. The absence of protein precipitation upon the addition of concentrated or 0.5 M HCl to the ethanol extract might be due to little or no traces of prolamines in both supernatants of *N. biserrata* and *A. orientalis*. This is because in a less polar solvent such as ethanol, proteins are rarely noticeably soluble (e.g. prolamines) although prolamines are most soluble in alcohols.

By comparing both treatments, it was noticed that the optimization condition of independent variables (0.1 M and 30 min of agitation) for alkaline treatment was most suitable for *N. biserrata* and *A. orientalis* protein extraction. Arifin *et al.* (2009) noticed 0.03 M to be the optimum NaOH concentration when extracting protein from palm kernel meal. Kongo-Dia-Moukala and Zhang (2011) also observed a time of 33 min to be optimum for extracting protein from defatted corn.

Model Fitting

The study utilized RSM to develop a prediction model for optimizing the extraction of protein from defatted fern flour (DFF). The independent and dependent values presented in Table 4 were analyzed to obtain a regression equation that could predict the responses within the given range. The regression equation for protein extraction is as follows:

NB protein extracted (mg/mL) =
$3.43 + 0.93A - 0.25B - 1.14\,AB$ ….. (1)
AO protein extracted (mg/mL) =
$1.44 + 0.64A + 0.13B - 0.28AB$ ….. (2)

Where, A is the coded value of variable NaOH concentration; B is the coded value of variable time.

The plot of experimental values of extracted protein (mg/mL) versus those calculated from Eq. 1 and Eq. 2 indicated a good fit, as presented in Figure 7 and 8. Colour differences in the fit plotted indicated the level of extracted protein which represents red as the highest extracted protein while narrow down to blue colour was the lowest extracted protein. The results of Analysis of Variance (ANOVA) gave a coefficient of determination (R^2) of 0.90 and 0.92; adjusted R^2 of 0.88 and 0.90 for *N. biserrata* and *A. orientalis* respectively. This implies that 90% and 92% of the variations could be explained by the fitted model, indicating the adequacy of the applied model

which is a measure of degree of fit. The Coefficient of Determination observed in this study was similar to that recorded by Badwaik *et al.* (2012) when recovering oil from peanut. Therefore, the developed model could adequately represent the real relationship among the parameters chosen. The F-values for *N. biserrata* and *A. orientalis* for NaOH were 43.33 and 49.44 respectively, meaning the model suggested was significant at 95% probability level as shown in Table 8 and 9.

CONCLUSION

Defatted fern protein was effectively extracted by the alkaline method. The production of protein from defatted fern was optimized using Response Surface Methodology of design-expert software. The extracted protein was significantly influenced by NaOH concentration and time of agitation. The optimized condition was at 0.1 M NaOH concentration and agitation time of 30 min. The protein recovery from *Nephrolepis biserrata* and *Arthropteris orientalis* were 5.11 mg/mL and 2.03 mg/mL respectively. Under optimum conditions, experimental protein value was lower than predicted by second-order model.

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