A Comparative Study between Response Surface Methodology and Genetic Algorithm in Optimization and Extraction of Leaf Protein Concentrate from *Diplazium esculentum* of Assam

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Abstract: Fern is a seedless vascular plant that reproduces via spores and has various usefulness. This study was carried out to optimize the conditions of leaf protein concentrate extraction using ultrasound from defatted fern type *Diplazium esculentum.* The extraction of defatted fern protein was conducted using ultrasound. Rotatable central composite design (RCCD) of response surface methodology was used for identification of the best condition and extraction yield optimization. An attempt with genetic algorithm optimization was also carried out and revealed that optimized results were of highest desirability as compared to response surface methodology. The final optimum results, by using genetic algorithm was observed to be 21.12 min of sonication time, 56.88 °C temperature, 7.59 pH and 66.2 ml of solvent for an optimum protein yield of 33.79% where desirability value was 1.00. UHPLC analysis of the sample revealed the presence of all the essential amino acids, except tryptophan.

Keywords: Leaf protein concentrate, *Diplazium esculentum*, GA, Optimization, Response surface methodology, Genetic algorithm.

INTRODUCTION

Diplazium esculentum is one of the most commonly consumed edible fern found throughout Asia and Oceania. It is known as *Pucuk paku* in Malaysia, *Paco* in the Philippines, *Dhekia* in Assam, *Dhenkir shaak* in Bengali and *Linguda* in Northern India, referring to the curled fronds [1]. In Thailand it is known as *Phak khut*. It is reported to have mild amounts of fern toxins but no major toxic effects were recorded [2]. This plant has higher amount of bioactive compounds such as antioxidants, vitamins, proteins, carbohydrates and lipids [3]. Studies of plant proteins, as a non-conventional source has been on the increase, due to the new challenges of providing adequate protein for an expanding world population [4]. The world is coming to recognize the grim truth that ultimately the population growth will outstrip food suppliers with apocalyptic results. About 36 million people die every year as a result of hunger on contrary it has been reported, that amino acid compositions of leaf protein concentrate (LPC) is as good or as better than that of many common food stuffs which can solve this major problems in the developing world [5-7]. Proteins are indiscriminating constituent in human diet to sustain human development production of protein is a must. Attempts are being made to develop techniques to evaluate various sources of unconventional protein sources which in turn can diminish protein calorie malnutrition [6]. Proteins play an important role in food processing and food product development, as its functional properties influence consumer acceptability. Both animal and plant proteins are used commercially as functional ingredients. Plant proteins are the most abundant in the world. A number of vegetable proteins have been tried to incorporate in various food products as well as functional and nutritional ingredients [7].

Application of ultrasound in food industry is attracting much attention nowadays [8]. The objective of this study was to optimize the condition of ultrasound assisted extraction technology of proteins from *Diplazium esculentum,* and study its amino acid profile. Response surface methodology is an affective statistical technique for optimizing complex processes. In this study Rotatable central composite design (RCCD) was employed with 5 levels. It is one of the commonly used statistical techniques for designing of experiments for food processes and food formulations.

Response surface methodology (RSM) has been shown to be an effective tool for optimizing processes [4,9-11]. Basically RSM relates product properties by using regression equations that describe interrelations between input parameters and product properties. RSM was used to optimize the conditions for an extruder, producing an amaranth-based snack food [12,13]. RSM was even used to analyze the effect of corn flour, green gram flour, xanthan, guar gum, arabic gum and carboxymethyl cellulose (CMC) on the sensory attributes (expansion ratio) of an extruded

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snack food and found that the responses were affected mostly by changing the levels of corn flour, green gram flour and guar gum and to a lesser extent by changing the levels of xanthan, arabic gum and CMC [10]. RSM was even used to optimize the ingredients and process conditions for preparing *puri*. Some good examples of the appropriate applications of this technique were tried on textured products for the optimization of complex products, properties and many process variables [14-16].

On the other hand, genetic algorithm (GA) is one of the emerging trends in optimization of a complex objective function. This technique is based on genetic simulation of the biological evaluation process followed by crossover and mutation [17]. Natural selection principles and Darwin's species evolution theory are the basis for the development of genetic algorithm. Genetic algorithm is superior over other conventional techniques due to its less susceptibility to be stuck at local minima, requirement of minute knowledge of the process variables and capability of finding optimized conditions under the condition of large space [18]. Existence of living beings based on the competition for appropriate limited natural resources is the main principle of genetic algorithm (GA). Hence, it is one of the sturdier optimization techniques in terms of heftiness and easy customization. Now-a-days it is widely used in the field of food engineering and technology for the evaluation of optimization process [19]. The aim of the present study is to optimize the extraction of leaf protein concentrate from *Diplazium esculentum* by using response surface methodology (RSM) and genetic algorithm (GA).

MATERIALS AND METHODS

Collection and Treatment of Samples

The leaves of *Diplazium esculentum* were collected from Sonitpur district of Assam, India. The leaves were washed with running tap water followed by distilled water and weighed prior to pulping. Leaf protein concentrate was obtained by following the procedure of Fellows [20] with slight modification as illustrated in Figure **1**. The leaf protein concentrate obtained was then hot air oven dried (Advantage Lab, Model: AL01-05-100, Germany) for future chemical and functional property studies.

Protein Extraction

The leaves were ground with distilled water and subjected to pH adjustment with 1N, HCl and 1N, NaOH, which was then subjected to ultrasound in bath sonicator (Bandelin Sonorex, Z659800, Berlin) inbuilt with temperature adjustment. The slurry derived after

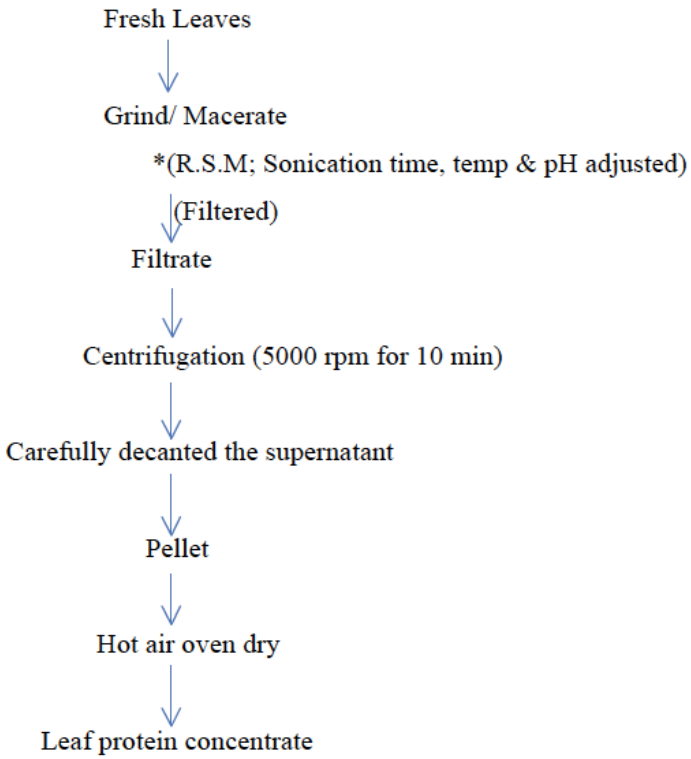

Figure 1: Flow chart of extraction of leaf protein concentrate with slight modification as adopted from Fellows [20].

grinding was then subjected to filtration with muslin cloth. The filtrate obtained was centrifuged at 5000 rpm. The residue obtained was the leaf protein concentrate which was hot air oven dried (Advantage Lab, Model: AL01-05-100, Germany) for 12 h.

Protein Determination

The protein content of the leaf protein concentrate was determined by using Kjeldahl method [21] in Kjeldahl instrument [Pelican Kelplus Kes 12L (Digestion unit) + Kelplus classic DXVA(Distillation unit),India] and multiplying the nitrogen content of the sample with protein conversion factor of 6.25.

Experimental Design

A rotatable central composite experimental design with 4 variables was used to study the response pattern and to determine optimum combination of the variables. The variables were x_1(ultrasonic time), x_2(ultrasonic temperature), x_3(pH) and x_4 (solvent concentration) on the response of protein yield(Y). The process variables and their corresponding levels are shown in Table **1**. The proposed equation for response was as follows:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{j=1}^{4} \beta ii X_i^2 + \sum_{i=1}^{4} \sum_{j=1}^{4} \beta_{ij} X_i X_j$$

Where Y is measured response associated with each factor level combination, β_0 is an intercept and β_1 is regression coefficient computed from the experimental values of Y and X_i is the coded levels of independent variables the terms X_i X_J and X_i^2 represents the interaction and quadratic terms respectively.

Amino Acid Analysis by UHPLC

Samples were hydrolyzed with 4ml of 6N HCl. The solutions were sealed in tubes under Nitrogen and incubated in an oven at 110°C for 24 h. Amino acids were determined by Ultra high-performance liquid chromatography (Thermo Scientific Dionex UHPLC, Model: Ultimate 3000 RSLC [22].

Data Analysis

Data were represented as means of 3 replicated determinations. The responses obtained from each set of experimental design were subjected to multiple non-linear regressions analysis by using design expert version 6.0.11 (Stat-ease, Minneapolis, MN, USA). The fit of polynomial model equation and significances of regression coefficient were evaluated by the coefficient of determination (R^2) p-value and lack of fit respectively. Model verification for checking the predicted response was done by finding percent of variation between the experimental and predicted values.

RESULTS AND DISCUSSIONS

The values of protein yield for different experimental combinations are presented in Table **2**. For determining the effect of independent variables (ultrasonic time, ultrasonic temperature, pH an solvent concentration) on the response (Protein yield) statistical analysis was performed. Multiple regression analysis was done for the evaluation of the developed mathematical model between different factors and response. For estimating the behavior of the response as a function of independent variables, multiple regression coefficients were calculated. For determining the significance terms of the experimental data, ANOVA on each response variable was performed followed by the judgment of experimental data with the calculated F-value [23]. Adequacy of the model was checked and tested by lack of fit test, considering statistical parameters like fitted R^2, predicted R^2, PRESS and adequacy precision. On the basis of non- significant lack of fit ($p>0.05$), higher R^2 value (closer to 1), low PRESS and higher adequacy precision value, the fitted model can be considered as the adequate one for predicting the mentioned response [24, 25].

Table 1: Processes Variables and their Corresponding Levels

Independent variable	Codified	Uncodified	Level				
			-2	-1	0	1	2
Ultrasonic time (min)	x_1	X_1	2.5	15	27.5	40	52.5
Temperature (°C)	x_2	X_2	25	40	55	70	85
pH	x_3	X_3	1	4	7.5	11	14
Solvent concentration (ml)	x_4	X4	15	40	65	90	115

Table 2: RCCD Experimental Design with Process Variables for Sonicated Treatment on Protein Yield

S/No	Time (x_1)	Temp (x_2)	pH (x_3)	Solvent Concentration (x_4)	Protein yield (%)
1	-1	-1	-1	-1	10.61
2	1	-1	-1	-1	10.84
3	-1	1	-1	-1	13.85
4	1	1	-1	-1	10.75
5	-1	-1	1	-1	13.51
6	1	-1	1	-1	20.16
7	-1	1	1	-1	15.13
8	1	1	1	-1	18.44
9	-1	-1	-1	1	8.17
10	1	-1	-1	1	9.11
11	-1	1	-1	1	11.36
12	1	1	-1	1	8.96
13	-1	-1	1	1	11.64
14	1	-1	1	1	18.99
15	-1	1	1	1	15.61
16	1	1	1	1	18.22
17	-2	0	0	0	6.04
18	2	0	0	0	10.29
19	0	-2	0	0	20.75
20	0	2	0	0	23.22
21	0	0	-2	0	9.87
22	0	0	2	0	20.78
23	0	0	0	-2	14.98
24	0	0	0	2	13.32
25	0	0	0	0	31
26	0	0	0	0	35
27	0	0	0	0	34
28	0	0	0	0	32.8
29	0	0	0	0	35.56
30	0	0	0	0	33.24

The values are mean of three replicates.

From Table **3**, it can be observed that statistical parameters like probability (p) values of the model, 4 independent variables, quadratic terms involved in the model were significant, whereas $x_1 * x_2$ and $x_1 * x_3$ were the only significant interaction terms. The lack of fit ($p>0.05$) value of the model was non-significant with p-value of 0.8409, which implies accuracy of the model. The R^2value of the model was 0.9887, whereas the adjusted R^2 and predicted R^2 were 0.9782 and 0.9596, respectively. On the basis of the above results, it can be conferred that the model is adequate and accurate enough for predicting the mentioned response.

The final equation in terms of coded factors was as follows:

Protein Yeild= $33.60+1.00* x_1+0.59* x_2+2.91* x_3-0.61* x_4-6.60* x_1^2-3.14* x_2^2-4.81* x_3^2-5.10* x_4^2-0.92* x_1 * x_2+1.52* x_1 * x_3+0.088*x_1* x4-0.19* x_2 * x_3+0.20* x_2 * x_4+0.35* x_3 * x_4$

Table 3: Analysis of Variance Showing the Linear, Quadratic, Interaction and Lack of Fit of the Response Variables

Source of variation	df	Response variables		
		Protein yield		
		Sequential sum of squares	F-value	p value
Model	14	2346.13	93.9	< 0.0001***
x_1	1	24.18	13.55	0.0022***
x_2	1	8.44	4.73	0.0461*
x_3	1	203.41	113.98	< 0.0001***
x_4	1	8.82	4.94	0.042*
x_1^2	1	1194.75	669.47	< 0.0001***
x_2^2	1	271.28	152.01	< 0.0001***
x_3^2	1	634.56	355.57	< 0.0001***
x_4^2	1	714.44	400.33	< 0.0001***
x_1*x_2	1	13.6	7.62	0.0146*
x_1*x_3	1	36.75	20.59	0.0004***
x_1*x_4	1	0.12	0.07	0.7955
x_2*x_3	1	0.6	0.33	0.5717
x_2*x_4	1	0.64	0.36	0.5594
x_3*x_4	1	2.01	1.13	0.3054
Residual	15	26.77		
Lack of Fit	10	13.28	0.49	0.8409
Pure Error	5	13.49		
Correlation Total	29	2372.9		
R^2	0.9887			
Adjusted R^2	0.9782			
Predicted R^2	0.9596			
Adequate Precision	30.072			
PRESS	95.91			

*Significant at P≤0.05.
**Significant at P≤0.01.
***Significant at P≤0.001.

Whereas the final equation in terms of actual factors was

$$\text{Protein yeild} = -103.68582 + 2.395 X_1 + 1.705 X_2 + 5.708 X_3 + 0.969 X_4 - 0.042 X_1^2 - 0.014 X_2^2 - 0.393 X_3^2 - 0.008 X_4^2 - 0.005 X_1 X_2 + 0.034643 X_1 X_3 + 0.00028 X_1 X_4 - 0.004 X_2 X_3 + 0.001 X_2 X_4 + 0.004 X_3 X_4$$

Effect of Interaction of Various Factors on Protein Yield

Figure **2A** illustrates the effect of time and temperature on protein yield. It can be observed that the protein yield increases with sonication temperature and time. Similar findings were observed on sonication time for the extraction of phenolic compounds from coconut shell [26]. Similar findings for extraction temperature in the extraction of phytic acid from peanut seeds [26,27]. The increase in protein yield with the increase in sonication time might be accredited due to the increase in mass transfer process with the rising trend of sonication time, whereas increase in temperature leads to breakdown of cell wall of the *Diplazium esculentum* plant, which resulted in maximum yield of protein.

The effect of pH, time and temperature on protein yield is presented in Figure **2B** and **D**. In both cases, protein yield increases with the increase of pH,

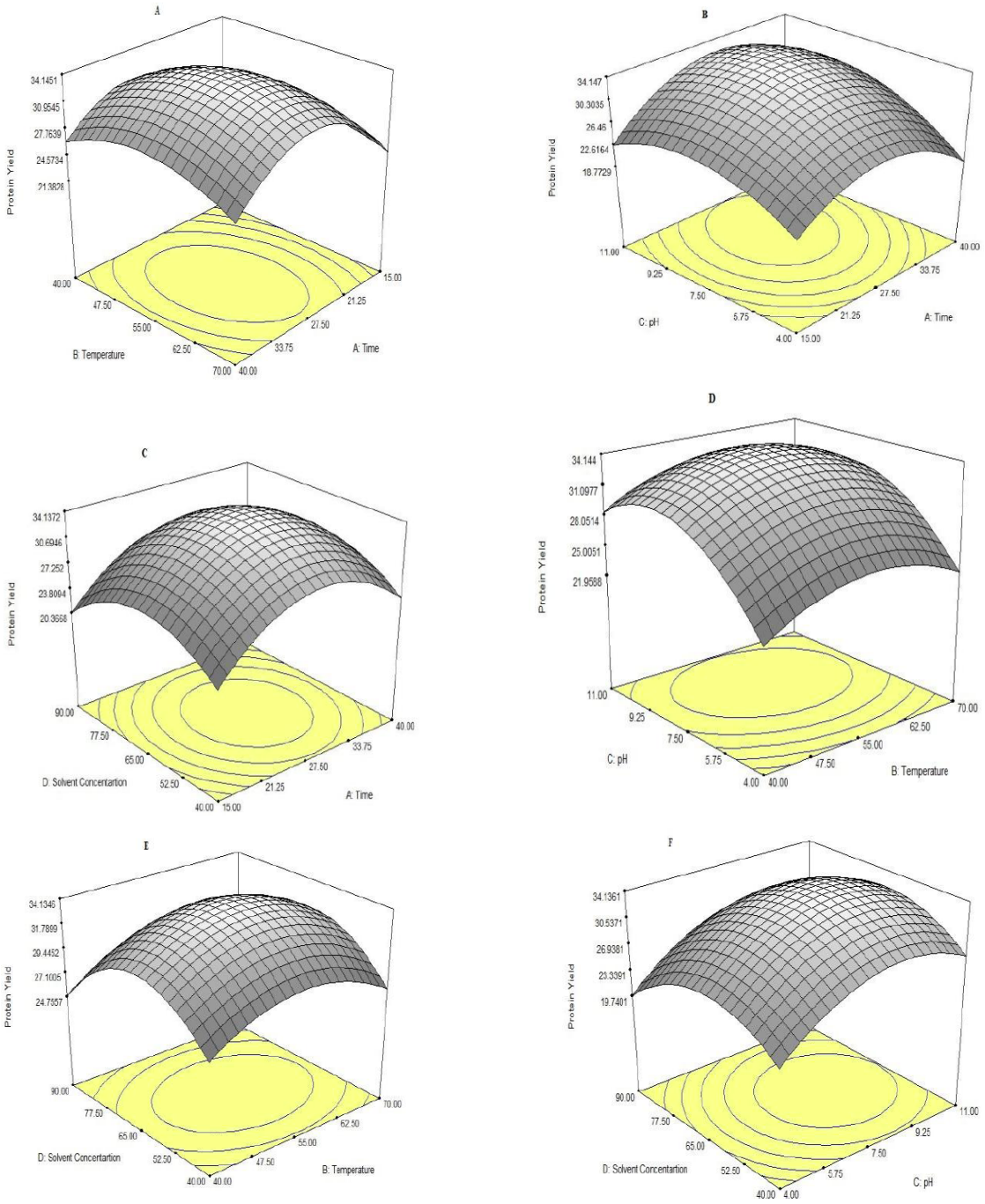

Figure 2: Variation of protein yield against different processing factors.

A) Interaction effect of temperature and time on protein yield **B**) Interaction effect of pH and time on protein yield **C**) Interaction effect of solvent concentration and time on protein yield **D**) Interaction effect of pH and temperature on protein yield **E**) Interaction effect of solvent concentration on protein yield **F**) Interaction effect of solvent concentration and pH on protein yield.

sonication time and temperature respectively. This work can be explained with the works of Irakoze and Sindayigaya [28] where they worked on the optimization of Malted Sorghum. The increase in protein yield with the increasing concentration of pH is due to the fact that protein denaturation decreases with the rise in pH value that resulted in higher amount of protein yield.

Figure **2C** and **E** depict the effect of solvent concentration, time and temperature on protein yield. It can be observed that solvent concentration with the increase of sonication time or temperature increases up to a certain point and then decreases. The increase in protein yield with the increasing concentration of solvent up to a certain point might be due to effective swelling of the leaves, resulting in increased surface

area for solute–solvent contact. But after certain period of time, as the bound water is released from the swelled leaves, the concentration of the solution starts decreasing. As a result of which there is no significant effect of solvent concentration on the protein yield. Similar findings were observed on the effect of solvent concentration for the extraction of rebaudioside A from Stevia leaves [29].

Figure **2F** reveals the effect of pH and solvent concentration on protein yield and it increases with the increase in pH as mentioned earlier, but solvent concentration shows rising effect on the protein yield up to a certain point followed by final decrease in the protein yield upon further increase of its concentration.

Optimization Using Genetic Algorithm and Model Verification

The optimum results can be obtained by using several methods like overlaying of contour plots i.e. graphical method and combining all the responses into one measurement i.e. desirability function [30]. In this study, independent variables were optimized numerically using statistical software Design Expert for RSM (version 6.0.11, Stat-Ease, Inc 2021, East Hanuepin Ave., Suite 480, Minneapolis, MN 55413) and MATLAB-R2012a for genetic algorithm. These two optimization processes were compared on the basis of highest desirability factor. From the numerical analysis of response surface methodology, it was observed that 28.84 min of sonication time, 56 °C temperature, 8.61 pH and 63.86 ml of solvent are the best condition for an optimum protein yield of 34.15%. In this case, highest desirability for the optimization process was 0.952.

The condition and constraints of genetic algorithm is given below:

Maximization of the following Objective Function

$$\text{Protein Yeild} = -103.68582 + 2.395 X_1 + 1.705 X_2 + 5.708 X_3 + 0.969 X_4 - 0.042 X_1^2 - 0.014 X_2^2 - 0.393 X_3^2 - 0.008 X_4^2 - 0.005 X_1 X_2 + 0.034643 X_1 X_3 + 0.00028 X_1 X_4 - 0.004 X_2 X_3 + 0.001 X_2 X_4 + 0.004 X_3 X_4$$

Where, X_1=Time

X_2=Temperature

X_3=pH

X_4=Solvent concentration

Ranges of Process parameters

$15 \leq X_1 \leq 40$

$40 \leq X_2 \leq 70$

$4 \leq X_3 \leq 11$

$40 \leq X_4 \leq 90$

The main parameters in this optimization process were population size, mutation rate, crossover rate and number of generations, respectively (Table **4**). The optimized result obtained by using genetic algorithm and RSM is shown in Table **5**. From the results, it can be observed that the desirability of the optimized result obtained from genetic algorithm is comparatively more than response surface methodology. So, for predicting the optimized process parameters with maximum protein yield, genetic algorithm gave better results. Model verification was done on the basis of per cent of variation between the predicted and actual protein yield. From Table **6** it can be observed that the per cent of variation between the actual and predicted protein

Table 4: Conditions for Genetic Algorithm (G.A)

Sl No	Population size	Mutation rate	Crossover rate	Number of generations
1.	40	1.0	0.8	1000

Table 5: Optimized Result Obtained by Using Genetic Algorithm (GA) and Response Surface Methodology (RSM)

Sl. No	Name of optimization technique	Time (min)	Temperature (°C)	pH	Solvent concentration (ml)	Protein yield (%)	Desirability
1.	GA	21.12	56.88	7.59	66.2	33.79	1.00
2.	RSM	28.84	56.00	8.61	63.86	34.15	0.952

Table 6: Validation of the Optimized Result

	Response obtained from GA	Response obtained from RSM
	Protein yield	Protein yield
Predicted value	33.79	34.15
Actual value	33.89±0.29[a]	33.29±0.18[a]
% Variation	0.29	2.51
Mean difference	0.10	0.86

[a]signifies standard deviation.

yield was less in case of GA as compared to RSM. Comparative results (Tables **5** and **6**), revealed that GA is a superior optimization technique over RSM.

Evaluation of Amino Acid Composition

The amino acid composition in the leaf protein concentrate of *Diplazium esculentum* are presented in Table **7** and Figure **3**. The total amino acid present in *Diplazium esculentum* is 210.81 mg/g which are comparatively high with respect to *Telferia occidentalis* and *Amaranthus hybridu*s [31]. In the present study total amount of essential amino acid is 48.36 mg/g which is also high when compared with *Telferia occidentalis* and *Amaranthus hybridus* [31]. The total amount of sulphur containing amino acid (cysteine and methionine) was found to be 62.56 mg/g which are also high compared to 58 mg/g recommended for infants [32]. Cysteine and methionine residue functions in the catalytic cycle of many enzymes by forming disulphide bonds that contribute in protein structure however the specific function of methionine is not known but a variety of oxidants react with methionine to form methionine sulphoxide which in turn serves as an efficient oxidant scavenger [33]. Amino acids such as glutamine and argenine are also present in high concentration in this particular leaf protein concentrate of *Diplazium esculentum*, which in turn are reported to have health benefitting effects. Glutamine is reported to be present as free amino acid available for circulation and is also present in the intracellular pools. It acts as a precursor for the synthesis of aminoacids, proteins, nucleotides and many biologically important molecules and also in the degradation of skeletal muscles and stimulating glycogen synthesis in the liver [34]. On the other hand arginine improves blood circulation, strengthens immune system, accelerates rate of healing of wounds, improves the burning of excess of fats in the body, reduces cholesterol levels from blood and acts as a biological precursor of nitrous oxide (NO) [35].

Table 7: Amino Acid Compositions in the Leaves of *Diplazium esculentum*

Amino acids	*Diplazium esculentum* (mg/g)
Lysine	2.73±0.12
Threonine	6.08±0.27
Cysteine	41.71±0.18
Valine	2.40±0.31
Methionine	20.85±0.23
Isoleucine	6.18±0.05
Leucine	7.05±0.13
Tyrosine	6.18±0.07
Phenylalanine	8.32±0.03
Histidine	1.8±0.04
Arginine	53.6±0.11
Glycine	1.27±0.02
Alanine	3.90±0.03
Glutamine	46.0±0.22
Asparagine	2.73±0.24

Results are expressed as mean of three replications ± standard deviation.

CONCLUSIONS

In conclusion RSM, GA and UHPLC analysis established *Diplazium esculentum* to be a good candidate of protein source, which is mostly neglected, and ultrasound served the extracting leaf protein concentrate in maximized amount. The optimized conditions for response surface methodology (RSM) were observed to be 28.84 min of sonication time, 56.0 °C temperature, 8.61 pH and 63.86 ml of solvent with a desirability value of 0.952, whereas for genetic algorithm, the optimum results were observed to be 21.12 min of sonication time, 56.88 °C temperature, 7.59 pH and 66.2 ml of solvent with a desirability value of 1.00. In this case the optimum protein yield was 33.79%, whereas for RSM, it was 34.15%, respectively.

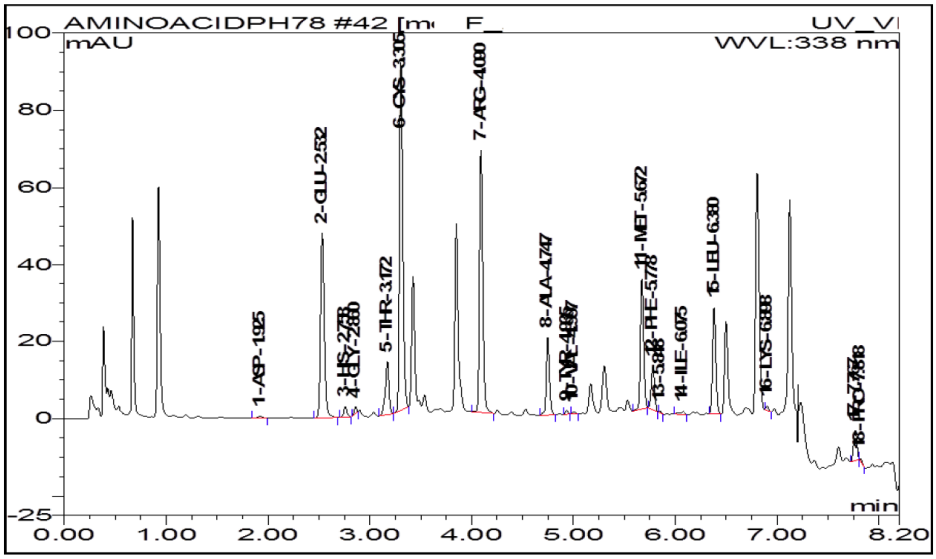

Figure 3: UHPLC chromatogram of LPC from (*Diplazium esculentum*).

Model verification for checking the predicted response was done by finding percent of variation between the actual and predicted values. The percent of variation in case of GA was less than RSM. Hence, it can be concluded that genetic algorithm (GA) is superior over response surface methodology (RSM) in order to find out optimized condition of maximum protein yield. LPC obtained was later used to study the functional properties.

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