Research Article

Optimization of germination buckwheat for alkaloid extracted using response surface methodology

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ABSTRACT

This study applied response surface methodology and synthetic evaluation method to optimize the alkaloid content of buckwheat sprouts. Solid-liquid (1:10-1:30), ethanol concentration (60%-80%), extraction temperature (20℃-60℃) and extraction time (20-40 min) were considered as treatment factors in the optimization. All treatment variables shown significant effects on the alkaloid content of buckwheat sprouts (P<0.05). A second order polynomial model produced a satisfactory fitting of the experimental data considering the alkaloid content (R^2_{adj}=0.8803, S=0.094). Considering the feasibility, the best conditions for the alkaloids content were identified as solid-liquid ratio 1:15, ethanol concentration70%, extraction temperature 45℃ and extraction time 20 min, under these conditions the alkaloids content was 1.35 mg/g. This process control study provided baseline data for developing industry of buckwheat sprouts.

Key words: Buckwheat, Germination, Alkaloid, Response surface methodology, Ultrasonic extraction

INTRODUCTION

Buckwheat has two main species including common buckwheat (*Fagopyrum esculentum Moench*) and tartary buckwheat (*Fagopyrum tataricum Gaertn*), which is a nutritious and pharmaceutically important plant. They are rich in functional ingredients, such as, flavonoid compounds (mainly rutin and quercetin), phenolic acids, amino acids, minerals, vitamins and crude fibers. Buckwheat and its products have high antioxidant activity and reducing capillary vessel brittleness, improving microcirculation, so buckwheat attracted wide attention in recently years [1, 2].

During germination (Fig.1), many significant changes were observed in the biochemical and physical aspects that resulted in the reduction of undesirable substances and the improvement of nutritional values [3]. Due to the buckwheat germination can enhance the digestibility of buckwheat protein as well as improve the quality of protein and the nutritional value of fatty acid composition, the germinated buckwheat has been considered as a new vegetable. Michał Sˊwieca had studied on the impact of elicitation and the phenylpropanoid pathway feeding on the nutritional quality, the potentially bioaccessible phenolics and the antioxidant capacity of young buckwheat sprouts [4]. Jianxiong Hao had studied the slightly acidic electrolyzed water(SAEW) treatment could promote the rutin accumulation of germinated buckwheat by influencing phenylpropanoid secondary metabolic pathway instead of the inhibition of rutin degrading enzyme (RDE) activity [5]. All of the above have proved that buckwheat sprouts were further studied by more and more researchers.

Alkaloids is a nitrogen-containing compound in plants, Eva Tavcˇar Benkovic researched that isolation, analysis and structures of phototoxic fagopyrins from buckwheat, he founded two new Alkaloids (fagopyrin A and fagopyrin E) [6]. Alkaloids of buckwheat sprouts will gradually payed attention by scientists. In this paper, the best extraction process of buckwheat alkaloid was researched.

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Fig. 1. Germination buckwheat seeds

EXPERIMENTAL SECTION

Materials
Buckwheat seeds were collected from Shanxi Academy of Agricultural Sciences during its availability of the season. Chemicals including bromocresol green, potassium hydrogen phthalate, hydrochloric acid, sodium hydroxide, sodium carbonate, ethanol, chloroform. atropine sulfate were obtained from Sigma Aldrich (Gillingham, Dorset, UK). Other chemicals were of analytically pure**.**

Germination seeds
Approximately 50.0 g of buckwheat were sanitized by soaking for 3 min in a sodium hypochlorite solution 0.1 mol/L. Seeds were rinsed three times with deionized water. The resulting seeds were spread uniformly into the dish for germination. The germination process was carried out in a incubator with the temperatures 25℃ and the relative humidity about 80% to 95%[7]. Germination was conducted in dark conditions. Sprouts were harvested in the proper germination times, then immediately washed three times with deionized water. Samples were immediately placed into plastic bags and stored at -18℃.

Alkaloids Extraction
The extraction of Alkaloids was carried out according to Hadi and Bremner [8] and extraction of Alkaloids was done by the ultrasonic assisted ethanol extraction method. Centrifuge at 4000 rpm, 15 min. The filtrate was rotary evaporated to a syrup state. The crude alkaloid mixture was then separated from neutral, acidic materials, and water soluble ingredients by hydrochloric acid solution (2%), then basification of the aqueous solution using sodium carbonate (5%, w/v) and re-extracted with Chloroform. Dilute with Chloroform to volume 25 mL and absorbance was measured at 415.5 nm. The results were indicated as mg atropine sulfate equivalent.

Determination of alkaloids content
Weighed accurately 1.0 g sample, ultrasonic extracted in the appropriate parameter, then centrifugated at 4000 rpm, 15 min, under the room temperature. The final extract was filtered and the filtrate was concentrated under vacuum. The crude alkaloid mixture was then separated from neutral, acidic materials, and water soluble ingredients by extraction with aqueous hydrochloric acid (5%) followed by chloroform extraction, then basification of the aqueous solution using sodium carbonate (5%, w/v) and re-extracted with chloroform, and evaporated in volume to 5 mL. Pipetted 1 mL concentrate to 10 mL test tube, and added chloroform to 6 mL, then putted 4 mL Bromocresol Green in the mixture. The mixed solution was transferred to a separatory funnel, then added 15 mL chloroform, shocked 3 min,   pipette chloroform layer to the 25 mL volumetric flask, diluted with ethanol to volume 25 mL and absorbance was measured at 415.5 nm. The results were expressed as mg atropine sulfate equivalent.

Effect of different experiment factors and levels on the alkaloid content
In this experiment, the factors of solid-liquid ratio, ethanol concentration, extraction temperature and extraction time were considered, and each factor have five levels, they were shown in table 1.

Table 1 The experimental factors and levels

Levels	Solid-liquid ratio	Ethanol concentration (%)	Extraction temperature (℃)	Extraction time (min)
1	1:10	60	20	20
2	1:15	65	30	25
3	1:20	70	40	30
4	1:25	75	50	35
5	1:30	80	60	40

Experiment designs and statistical analysis
Response surface methodology (RSM) was used to investigate the effects of process variables (Solid-liquid ratio, ethanol concentration, extraction temperature and extraction time) on the alkaloids content [9].

A Box-Behnken Design including 29 experimental runs formed by four variables and three levels was used. The experimental data were fitted to a second order polynomial model.

The data were statistically analyzed by means of a stepwise multiple regression using Design Expert 8.0.6 to yield equations for optimizing the process variables. Significant effects of independent variables on each response was determined by ANOVA.

RESULTS AND DISCUSSION

Effect of different solid-liquid ratio on the alkaloid content
As the Fig. 2 shown, alkaloids content was increased according to the increase of solid-liquid ratio, in the rang from 1:10 to 1:15, and then with the increase of the solid-liquid ratio, alkaloid content in reduced. In this study, alkaloid content reached to the maximum 0.95 mg/g, when its solid-liquid ratio was 1:15.
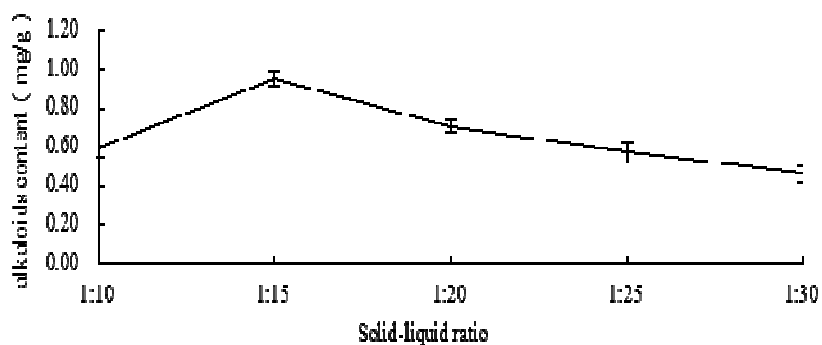

Fig. 2. Effect of different Solid-liquid ratio content

Effect of different ethanol concentration on the alkaloid content
Zhao[10] hold that fats and sugars in the sample will be extracted when given a higher concentration ethanol, the total alkaloid content will be affected. Alkaloids dissolution rate decreased, when the ethanol concentration is lower. According to the Fig. 3, ethanol concentration increased at first and then decreased. When ethanol concentration was 70%, alkaloid content reaches the maximum, which was 1.06 mg/g.
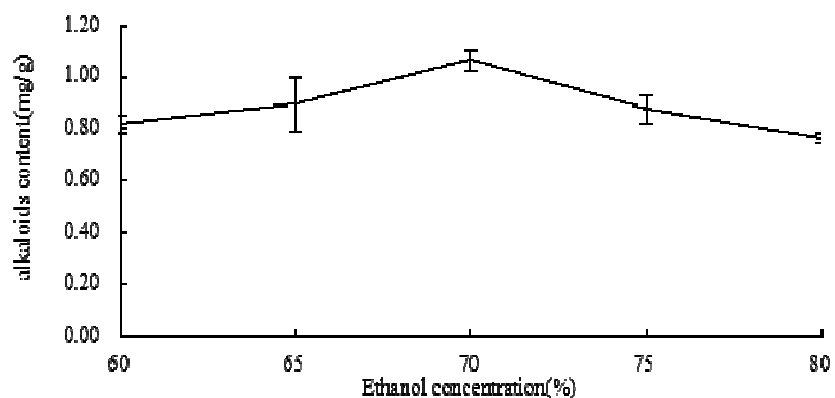
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Fig. 3.   Effect of different Ethanol concentration content

Effect of different extraction temperature on the alkaloid content

As HANG[11] reported that thermally unstable alkaloids will be broken. The Fig. 4 shown, in the range of temperature of 20 ℃ to 50 ℃ the higher temperature, the less alkaloids content. But, the alkaloids content suddenly decreased, when the temperature higher than 50 ℃. The result is that alkaloid content reached to the maximum 1.20 mg/g, when temperature is 50 ℃.
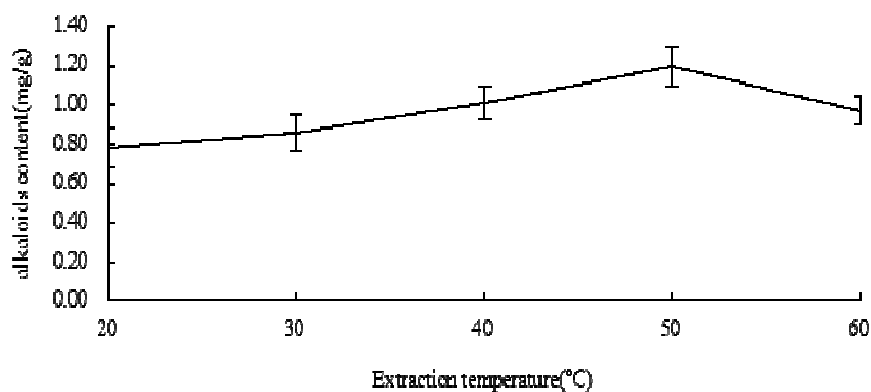

Fig. 4. Effect of different Extraction temperature content

Effect of different extraction time on the alkaloid content

YU Miao[12] researched the internal structure of alkaloids was damaged, when extraction time was too long. As the Fig.5 shown that with the increase of extraction time, the alkaloid content increased first and then decreased. The reason is that longer extraction time may damage the internal structure of alkaloids, and then reduced the alkaloid content of buckwheat sprouts.In this study, when extraction time was 20 min, alkaloid content reaches the maximum, which was 1.10 mg/g.
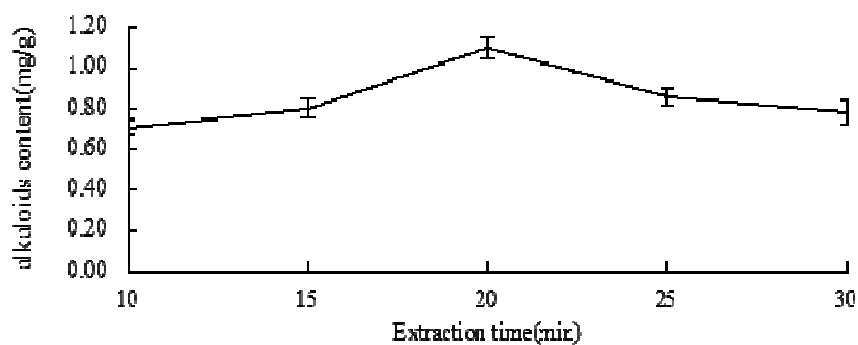

Fig. 5. Effect of different Extraction time content

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Fitting the Response surface models
In this study, the values obtained experimentally for response variable was compared against the reduced equations and replicates values, indicating a satisfactory model. Experimental values of response variables for response surface design was shown in table 2, and ANOVA evaluation of prediction models was shown in table 3, which were obtained using F tests to verify the significance of each effect or interaction. All the factors were presented significant ($p<0.05$) quadratic effect on alkaloid content of buckwheat sprouts. Removed the insignificant, the equation could be expressed as:

Alkaloids content $= 1.34 + 0.06A + 0.092B + 0.067C - 0.084D + 0.19AD - 0.12BD - 0.32A^2 - 0.26B^2 - 0.31C^2 - 0.27D^2$

where A, B, C and D were solid-liquid, ethanol concentration, extraction temperature and extraction time, respectively.

Total desirability value was 61.4% in operational conditions (solid-liquid ratio 1:15, ethanol concentration 71.18%, extraction temperature 48.85℃, extraction time 19.11 min) . Considering the feasibility, the best conditions for the alkaloids content were identified as solid-liquid ratio 1:15, ethanol concentration70%, extraction temperature 45℃, extraction time 20 min, under these conditions the alkaloids content was 1.35 mg/g.

As shown in Fig.6-11, at the given solid-liquid ratio, ethanol concentration and extraction temperature, alkaloids content increased and then decreased . The same conclusion came to the solid-liquid ratio, ethanol concentration and extraction temperature. The reason for these appearance is that the higher temperature and ethanol concentration may destroy the structure of alkaloids, the longer time of extraction and higher solid-liquid ratio will result in a waste of resources. Therefore , optimal combination factors should be identified to achieve minimal impact on the alkaloids content .

Table 2 Experimental values of response for response surface design

Experimental Runs	variables				Alkaloids content （mg/g）
	Solid-liquid ratio	Ethanol concentration (%)	Extraction temperature (℃)	Extraction time （min）	
1	1:10	70	50	15	0.85
2	1:15	70	40	25	0.99
3	1:15	70	50	20	0.86
4	1:20	70	40	20	0.75
5	1:15	70	40	15	0.66
6	1:10	70	40	20	1.39
7	1:10	70	50	25	0.89
8	1:15	75	50	25	0.63
9	1:15	70	60	25	0.68
10	1:10	65	50	20	0.72
11	1:15	70	60	15	0.62
12	1:15	65	50	25	0.75
13	1:15	75	60	20	0.81
14	1:15	70	50	20	0.9
15	1:20	75	50	20	1.33
16	1:20	65	50	20	0.61
17	1:15	70	50	20	1.42
18	1:15	65	60	20	0.75
19	1:20	70	50	15	0.81
20	1:10	75	50	20	0.94
21	1:10	70	60	20	0.49
22	1:15	75	50	15	0.34
23	1:20	70	50	25	1.33
24	1:15	65	50	15	0.98
25	1:15	75	40	20	0.72
26	1:15	65	40	20	1.21
27	1:20	70	60	20	0.59
28	1:15	70	50	20	0.69
29	1:15	70	50	20	1.08

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Table 3 ANVOA evaluation for response variable coefficient of models

Source	Sum of squares	df	Mean Square	F-Value	P-value	
Model	1.96	14	0.14	15.71	< 0.0001	significant
A	0.043	1	0.043	4.85	0.0448	
B	0.1	1	0.1	11.54	4.3×10^{-3}	
C	0.053	1	0.053	5.99	0.0282	
D	0.085	1	0.085	9.55	8×10^{-3}	
AB	2.25×10^{-3}	1	2.25×10^{-4}	0.025	0.8759	
AC	3.60×10^{-3}	1	3.60×10^{-3}	0.4	0.5351	
AD	0.14	1	0.14	15.8	1.4×10^{-3}	
BC	0.027	1	2.70×10^{-2}	3.06	0.1022	
BD	0.06	1	6.00×10^{-2}	6.74	0.0211	
CD	0.024	1	0.024	2.70	0.1227	
A^2	0.66	1	0.66	74.66	< 0.0001	
B^2	0.44	1	0.44	49.77	< 0.0001	
C^2	0.61	1	0.61	68.95	< 0.0001	
D^2	0.49	1	0.49	54.65	< 0.0001	
Residual	0.12	14	8.90×10^{-3}			
Lack of Fit	0.099	10	9.87×10^{-3}	1.52	0.3643	not significant
Pure Error	0.026	4	6.48×10^{-3}			

Independent variables: A-solid-liquid ratio, B-ethanol concentration,
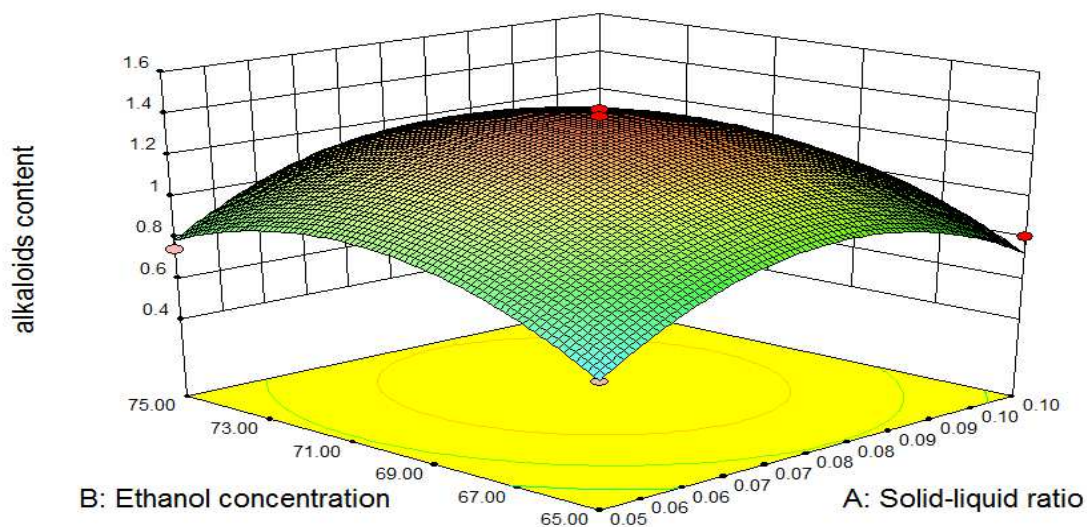*C-extraction temperature, D-extraction time*

Fig. 6.   Response surface and contour plots for alkaloids content at constant extraction temperature (50℃), extraction time (20 min), respectively
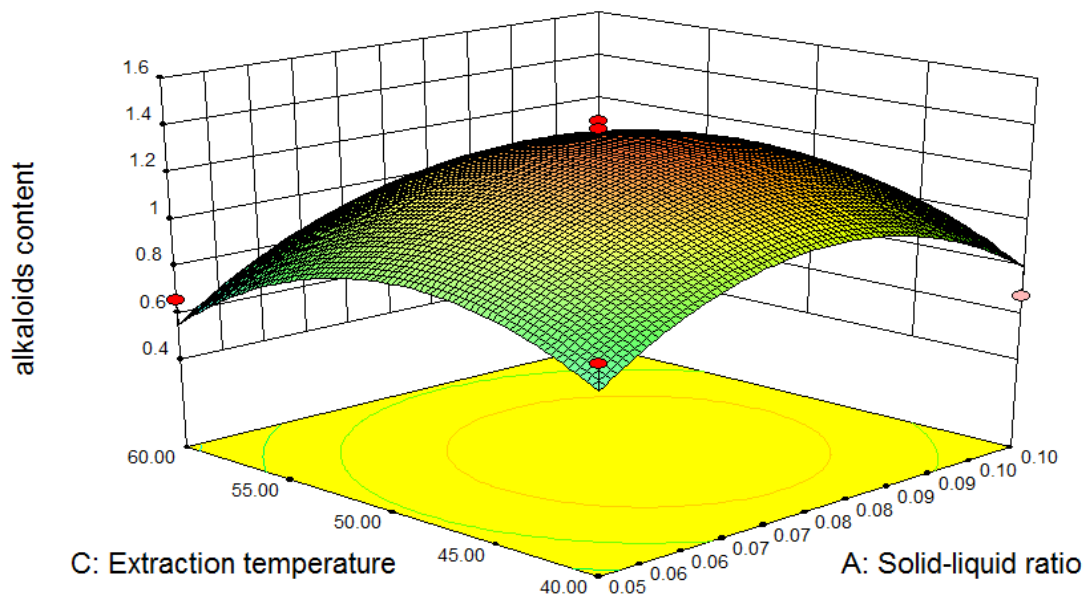
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Fig. 7. Response surface and contour plots for alkaloids content at constant ethanol concentration (70%), extraction time (20 min), respectively
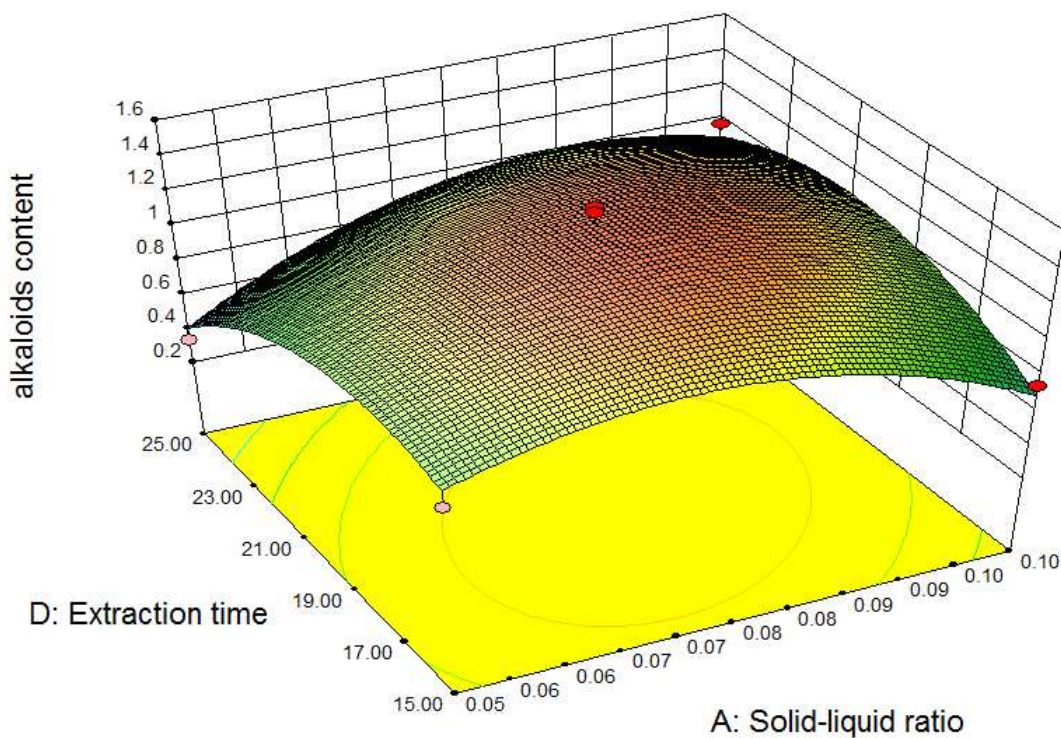

Fig. 8. Response surface and contour plots for alkaloids content at constant ethanol concentration (70%), extraction temperature (50℃), respectively
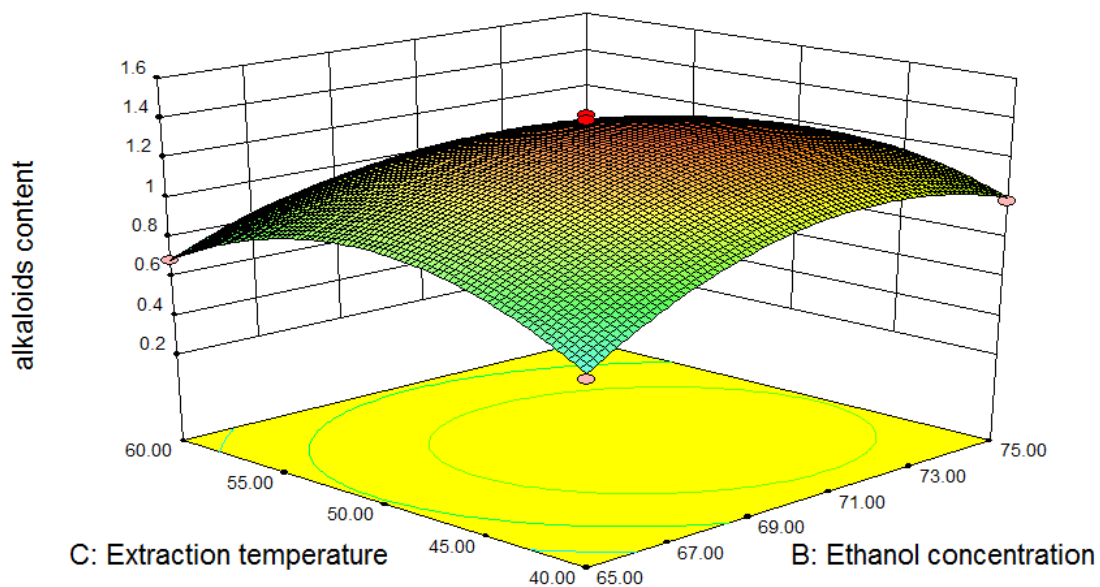
Fig. 9. Response surface and contour plots for alkaloids content at constant solid-liquid ratio (1:15), extraction time (20 min), respectively
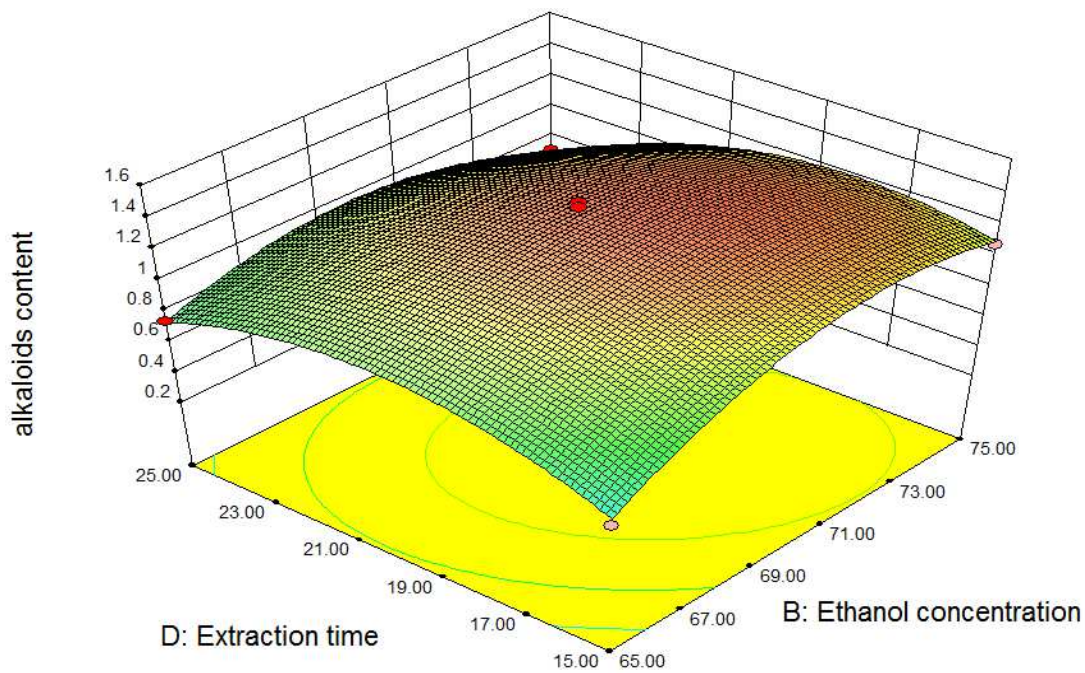

Fig. 10.   Response surface and contour plots for alkaloids content at constant solid-liquid ratio (1:15), extraction temperature (50℃), respectively
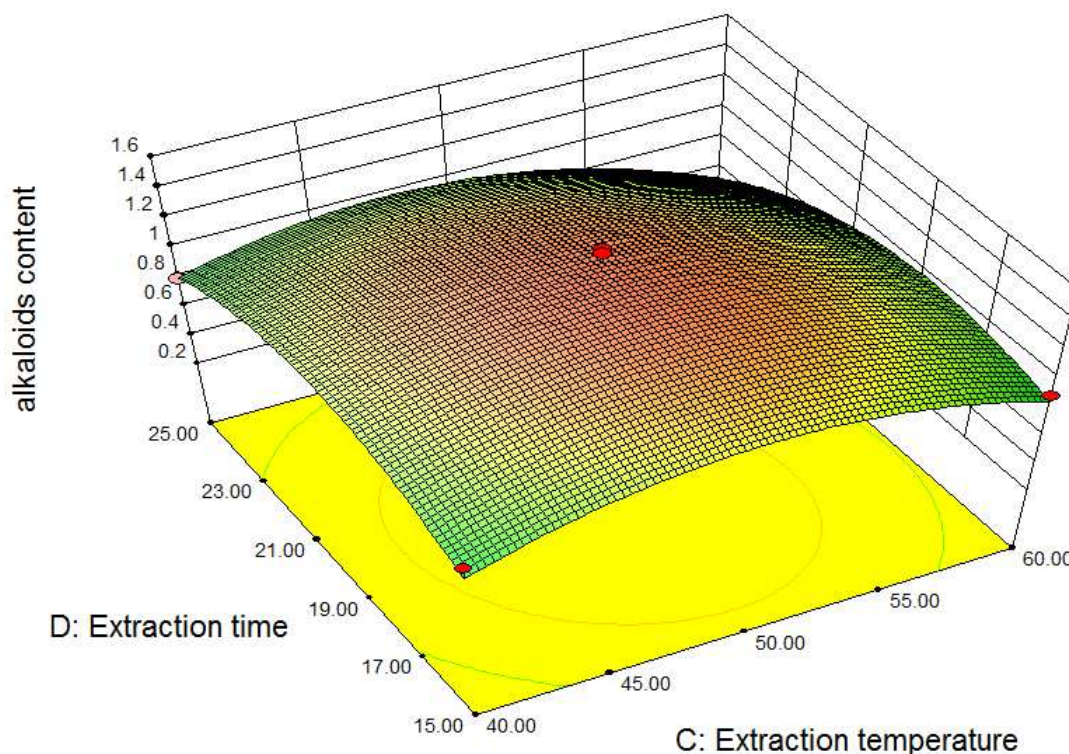
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Fig. 11.   Response surface and contour plots for alkaloids content at constant solid-liquid ratio (1:15), ethanol concentration (70%), respectively

CONCLUSION

In this study, Alkaloids of buckwheat sprouts was extracted using ultrasonic extraction, and response surface methodology design was employed to optimize the variables, including olid-liquid ratio, ethanol concentration, extraction temperature and extraction time. All these factors exhibited significantly quadratic effect on alkaloids contents. The optimum condition was identified as solid-liquid ratio 1:15, ethanol concentration 70%, extraction temperature 45℃, extraction time 20 min, under these conditions the alkaloids content was 1.35 mg/g. This quality and process control study provided the baseline data made a great potential for industrial usage.

REFERENCES

[1] DG Lee; IS Jang; KE Yang. *J. Acta Pharm*, **2016**, 66(2), 297-302.
[2] LS Lee; EJ Choi; CH Kim. *J. Journal of Cereal Science*, **2016**, 68, 181-186.
[3] G Zhang; Z Xu; Y Gao. *J. Journal of Food Science,* **2015**, 80(5), 1111-1119.
[4] Świeca; Michał. *J. Food Chemistry,* **2016**, 192, 625-632.
[5] H Jianxiong; W Tongjiao; Li Huiying. *J. Food Chemistry,* **2016**, 201, 87-93.
[6] T Benković; Ž Dušan; F Miha. *J. Food Chemistry,* **2014**, 143, 432-439.
[7] Z Yiming; W Hong; C Linlin. *J. Food Chemistry*, **2015**, 186, 244-248.
[8] S Hadi; B John; Bremner. *Molecules J. Molecules,* **2001**, 6(2),117-129.
[9] W Gang; D Yun; X Xin. *J. Food Control*, **2016**, 59,743-749.
[10]Z Qiang;Y Si-jiu;W Ting-pu. *J. Acta Prataculturae Sinica,***2012**, 04,206-214.
[11] Z Jian-guang; X Yan; L Yuan.   *J. Food Science*, **2013**, 10,11-15.
[12] Y Miao; B Yun-jiao; D Qichang. *J. Chinese Traditional and Herbal Drugs*, **2013**, 10, 1286-1289.