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Optimization of extraction conditions for carotenoids from black gram husk using response surface methodology

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Abstract
Black gram (*Vigna mungo*) husk, a by-product of pulse milling industry, contains bioactive compounds such as carotenoids, phenolic compounds etc. In the present study, extraction of carotenoids from black gram husk was optimized using a three process variable, three level Box–Behnken design of RSM with solvent to solid ratio (10-30 mL/g), extraction temperature (20 to 50 °C) and extraction time (2 to 8 h). The total carotenoids content of the husk extracts ranged between 2939.58 – 3941.68 µg/100g. The model showed a satisfactory coefficient of R^2 (0.991). Solvent to solid ratio, extraction temperature and the interaction of extraction temperature and time significantly ($p \leq 0.05$) affected the total carotenoids content. The optimum extraction conditions obtained was - 27.28 mL/g solvent to solid ratio, 30 °C extraction temperature and 6 h time. The predicted total carotenoids content was 3964 µg/100g and the result was validated. This study showed valorisation potential of black gram husk for extraction of carotenoids under optimized conditions using RSM.

Keywords: Black gram, husk, by-product, carotenoids, RSM

1. Introduction
Black gram (*Vigna mungo*) is one of the important pulse crops in India which is used for preparation of variety of food products such as cooked dhal, idli, hopper, papad and waries etc. after dehulling. During milling of black gram into dhal about 25% is left as a by-product comprising of husk (almost 9%) and *chuni* (16%) and is presently used as cattle feed. By-products from different food processing industries which were traditionally treated as environmental pollutants are being recognized as good sources for obtaining valuable components. The husk of black gram has been reported to contain the highest concentration of bioactive compounds such as carotenoids, polyphenols and dietary fibers among different milled fractions of black gram (Girish *et al*., 2012) [6] which indicates its potential application as nutraceuticals and as functional food ingredients in various processed foods.
Carotenoids are a class of natural red, yellow, and orange tetraterpenoid pigments which are universally synthesized by all terrestrial and aquatic photoautotrophs, including plants, microalgae, and macroalgae. These are water-insoluble, moderately soluble in organic solvents and completely fat-soluble. Colored fruits and vegetables are the major dietary source of carotenoids in the human diet. For centuries these have been widely used for colorant purposes in many types of foods, drinks and beverages, confectionery, food supplements and even drugs (Dewick, 2009) [4]. In recent years there is increasing focus on their other bioactivities also such as an antioxidants, antitumor agents, cardiovascular disease preventers, and immune system modulators etc. In the fruits, carotenoids are generally present in higher amounts, in the surface of tissues (i.e. external pericarp and peel) and seeds, which are normally rejected by consumers and food and biotechnological industries (Ayala-Zavala *et al*., 2011) [2]. Girish *et al*. (2012) [6] has also reported higher concentration of carotenoids in the husk of black gram, generally left as a by-product, than in the cotyledon part. Thus, recovery of carotenoids from food industrial wastes seems to be of the utmost importance. It is widely accepted that for extraction of bioactive compound, specific extraction techniques should be used in accordance with the source material. Therefore, for the extraction of carotenooids-liposoluble compounds, from wastes and by-products, the most commonly used techniques is the extraction with organic solvents, singly or even in association with other procedures for maximum extraction efficiency.

Wang and Liu (2009) [9] investigated different solvents/compositions for maximum extraction of carotenoids from rapeseed and reported the solvent (Petroleum ether: acetone, 1:1) as the most efficient one.

The black gram husk has been investigated for antioxidant activity (Girish *et al*., 2012) [6], as peroxidase enzyme source (Ajila & Prasada Rao, 2009) [1] and as source of dietary fiber (Kamani & Meera, 2021) [7] etc. The literature survey also indicated that carotenoids content of black gram husk has been assessed by many researchers. However, limited studies have been carried out to optimize the extraction conditions for the carotenoids from black gram husk. Hence, the present study was undertaken to optimize the extraction conditions for carotenoids from black gram husk using response surface methodology.

2. Materials and Methods

2.1 Raw material and chemicals

Black gram (cv. Mash 14) was procured from Directorate of Seed Research, PAU, Ludhiana, and dehulled using the standard procedure to obtain the milling fractions *viz*. dal (gotta + split), husk and *chuni*. The husk fraction was taken and finely ground (60 mesh size) for further experimental use. The chemicals and reagents used for the analyses were of Analytical grade.

2.2 Experimental design

The optimum carotenoids extraction from black gram husk was investigated by using a three process variables, three level Box–Behnken design of response surface methodology (RSM) and independent variables namely, solvent to solid ratio (X_1, 10-30 mL/g), extraction temperature (X_2, 20 to 50 °C) and extraction time (X_3, 2 to 8 h). The husk sample was extracted with the solvent (petroleum ether: acetone, 1:1) in a flask, protected from light, with constant agitation according to the extraction duration and at the specified temperatures. The response variable recorded was total carotenoids (Y, mg/100 g). A second order polynomial model was used for predicting the response. The best possible combination of these variables was obtained through the response surface methodology (RSM) and desirability function analysis.

2.3 Determination of total carotenoids

The total carotenoids extracted at different solid to solvent ratio, extraction temperatures and extraction time were determined according to the method of Dauqan (2011) [3] by using Ultraviolet-Visible (UV-vis) spectrophotometer. The total concentration of carotenoids was calculated by using the following equation, described by Rodriguez-Amaya and Kimura (2004) [8], (Eq. 1):

$$\text{Total carotenoid content } (\mu g/100g) = \frac{A\,(\text{total}) \times \text{vol.(mL)} \times 10^6}{A \times 10^3 \times \text{Sample wt.(g)}} \times 1000 \quad (1)$$

Where A is the absorbance value of extract at 450 nm; and A is the extinction coefficient of carotenoids (=2592).

2.4 Statistical analysis

The software 'Design Expert' (Version 8.0.5, Stat-Ease, Minneapolis, MN) was used for experimental design, data analysis and quadratic model building. Five replicates at the center of the design were used to allow the estimation of a pure error sum of squares. One-way ANOVA analysis was employed to test the significance of difference between variables. The experimental data were fitted to a second-order polynomial model to express the response variables as a function of independent variables and obtain the regression coefficients. The generalized second-order polynomial model (Eq. 2) which was employed in the RSM analysis, is given as hereunder:

$$Y = \beta_0 \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i<j=1}^{3} \beta_{ij} X_i X_j, \quad (2)$$

where, Y is the response variable; β_0, β_i, β_{ii} and β_{ij} are regression coefficients for the intercept, linear, quadratic, and linear-by-linear interaction terms, respectively and X_i, X_j are denotes the coded values of independent variables. With the help of 'Design Expert' software, the response surface and contour plots were generated while keeping a variable constant in the second-order polynomial model.

2.5 Validation of model

The predictive equations of RSM, generated by the Design Expert software, was used for obtaining the optimum extraction conditions for carotenoids. The optimum conditions were employed for extraction of carotenoids and carotenoids content was determined. The predicted and experimental values were compared in order to validate the model.

3. Results and Discussion

3.1 Fitting the response surface models and response surface analysis

Table 1 presents the independent variables and the coded and actual values of their lower, middle, and upper design points, selected for the optimization purpose using RSM.

Table 1: Coded and actual values of the independent variables used for optimization

Independent variable	Unit	Symbol	Level		
			Lower (-1)	Middle (0)	Higher (+1)
Solvent to solid ratio	mL/g	X_1	10	20	30
Temperature	°C	X_2	20	35	50
Time	h	X_3	2	5	8

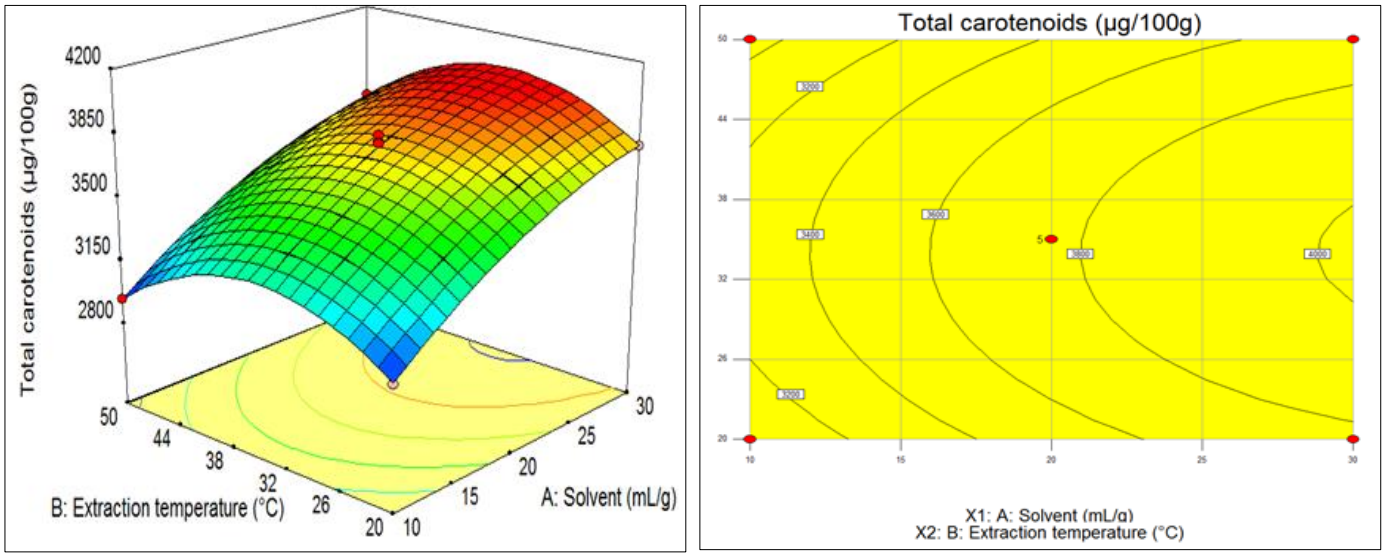
The solvent (petroleum ether: acetone, 1:1) was used in the present study as it was established as the most efficient one for maximum extraction of carotenoids by Wang and Liu (2009) [9] from rapeseed. The Table 2 shows the design and the analytical values of extract yield and carotenoids content as obtained under different sets of experimental conditions.

Table 2: Three factor, three level Box-Behnken experimental design and total carotenoids content (µg /100 g) of black gram husk

Run	Factor 1	Factor 2	Factor 3	Response
	Solvent/solid ratio	Temperature	Time	Total carotenoids (µg/100g)
1	20	35	5	3780.41
2	20	35	5	3679.67
3	20	50	8	3148.15
4	20	20	8	3533.67
5	20	50	2	3440.53
6	20	35	5	3749.40
7	30	50	5	3676.72
8	20	35	5	3787.67
9	10	35	8	3197.79
10	30	35	2	3856.34
11	10	20	5	3009.08
12	10	50	5	2939.58
13	20	35	5	3826.70
14	30	35	8	3941.68
15	10	35	2	3151.75
16	30	20	5	3748.35
17	20	20	2	3248.07

The non-significant lack of fit for carotenoids content (p = 0.8175 > 0.05) indicated that the regression equations were well fitted with the experimental results. It indicated that the quadratic regression models were appropriate for predicting the effect of these parameters on the responses. The relationships between independent and response variables are presented in terms of three-dimensional representation of the response surfaces as generated by the models.

The results of the response surfaces for carotenoids content were in the range of 2939.58 – 3941.68 µg/100g. The solvent to solid ratio, extraction temperature and the interaction of extraction temperature and time significantly affected the total carotenoids content (Y, µg/100g) of the extracts. The model showed a satisfactory coefficient of R^2 (0.991). The response surface and contour plots (Fig. 1) were generated as a function of solvent to solid ratio (10-30 mL/g) and extraction temperature (20-50 °C) while keeping the extraction time constant at 5 h.



a) b)

Fig 1: (a) Response surface and (b) Contour plot for interaction effect of solvent to solid ratio and extraction temperature on total carotenoids content

The regression equation for the carotenoids content was obtained after removing the non-significant factors and is as given below (Eq. 3).

$$Y = +3.76+0.37X_1-0.042X_2 +0.016X_3 -0.0005X_1X_2+0.009X_1X_3-0.14X_2X_3-0.11X_1^2-0.31X_2^2-0.11X_3^2 \quad (3)$$

Where, Y represents the response variable (total carotenoids), and X_1, X_2 and X_3 are significant process variables. The nature of extraction solvent is an influential parameter for extraction of any compound from a matrix. The nature of carotenoids vary from non-polar (e.g. β-carotene) to polar (e.g. lutein). For extraction of carotenoids, the polarity of a solvent is of utmost importance because it affects the ability of the solvent to dissolve the particular carotenoids (Warkoyo & Saati, 2011) [10]. The increase in carotenoids content with increasing solvent to solid ratio may be attributed to the mass transfer principles which state that the concentration gradient is a driving force for mass transfer and thereby allows a higher extraction of solids by solvent (Tan *et al*., 2011) [11]. Similar effect of solvent concentration has also been reported by Dianursanti (2020) [5].

3.2 Optimization of experimental conditions and verification of model: Table 3 presents the optimum extraction conditions for carotenoids from black gram husk which were determined from the numerical optimization of RSM with respect to the desired response goal. From the model it was obtained that under the extraction conditions of 27.28 mL/g solvent to solid ratio, 30°C extraction temperature and time of 6 h, the predicted total carotenoids content was 3964 µg/100g with desirability of 1.0. The verification of predicted values, via experiments under numerically optimized conditions, indicated that experimental values were reasonably close to the predicted values confirming that the predicted model was valid and adequate.

Table 3: Numerical optimization for the and comparison of predicted and observed experimental values for the response variables

Variable	Goal	Experimental limit		Optimum value	Desirability
		Min	Max		
Independent variables					
Solvent to solid ratio (mL/g)	in range	10	30	27.28	1.0
Temperature (ºC)	in range	20	50	30.31	
Time (h)	in range	2	8	5.91	
Response variables				Predicted value	Observed value*
Total carotenoids content (µg/100g)	Maximize	2939.58	3941.68	3964.0	3982.6 ± 10.03

* Mean ± Standard deviation (n=3)

4. Conclusions

The present study concluded that the black gram husk can be a potential source of carotenoids by using the extraction conditions as optimized using Response surface methodology. The three factor, three level Box Behnken Design provided the optimized extraction conditions as solvent to solid ratio of 27.28 mL/g, 30 °C extraction temperature and 6 h extraction time with desirability of 1.0. The solvent to solid ratio, extraction temperature and the interaction of extraction temperature and time were found to be the significant factors for the total carotenoids content of the extracts. Under the optimized conditions the software predicted total carotenoids content of 3964.0 µg/100g for which validation experiment was also carried out. The results indicated that the experimental value was reasonably close to predicted values and hence confirmed the validation and adequacy of the model.

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