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Optimization of Thermal Degradation of Chlorophyll in Amaranth Leaves Puree Using Response Surface Methodology (RSM)

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Abstract : Amaranth (*Amaranthus tricolor*) is highly nutritious leafy vegetables containing higher amount of vitamins and minerals. The color of green leafy vegetables is due to the presence of chlorophyll molecule which gets affected during heat treatment. In the present wok, kinetics of thermal degradation of chlorophyll a (Cha) and chlorophyll b (Chb) in Amaranth leaf puree were studied at pH values of 5.6, 6.6 and 7.6 and temperatures of 70 to 90^{o}C for time 0 to 60 min. Nonlinear regression equation based on response surface method (RSM) has been developed for determining the concentration of chlorophyll a and chlorophyll b and comparison has been made with the experimental data for different parameters that describes the thermal preservation studies on Amaranth leaf puree considering the influence of temperature, pH and time. The adequacy of the proposed equation has been performed with normal probability plot of the residuals for Cha and Chb and coefficient of regression (R^2).
Keywords : Amaranth, Chlorophyll, Nonlinear Regression Equation, Response Surface Method, Thermal Degradation.

Introduction

Amaranth comes under Amaranthus plant family having multi-purpose use of crop which can supply grains and leafy vegetables of high nutritional quality as a food and animal feed and also cultivated as an ornamental plant[1]. It is popularly grown leafy vegetable in tropical regions of the world like India, Africa, Bangladesh, Sri lanka and the Caribbean[2]. Amaranth leaves having rich source of protein, vitamins, minerals[3,4] and is used as herbal remedies effective in stopping diarrhea and hemorrhagic problems. Color of green vegetables is due to the presence of chlorophyll which is of many types, though the formation of chlorophyll a and chlorophyll b are typically found in higher plants in an approximate ratio of 3:1[5]. Color of chlorophyll a appears blue-green while for chlorophyll b having yellow-green[6]. Thermal stabilities and structure of chlorophyll a and b were found to be different and chlorophyll a was reported to be thermally less stable than chlorophyll b[7-9].

Since color is a major, sensory characteristic in determining the product acceptability, it is of critical importance to the food industry to prevent or minimize pigments degradation during processing[10]. Thermally processed green vegetables exhibit inferior color as compared to fresh ones. Several researchers[7,11] have reported the color changes (from bright green to olive-brown) during processing and attributed it mostly to conversion of chlorophylls to pheophytins[8]. Various methods for preservation of green color of vegetables have been proposed. Investigations have been carried out to maintain pH at high levels during heat treatment and storage. This procedure was beneficial after processing but eventually during storage the conversion of

chlorophyll to pheophytin occurs, causing drastic color changes[9]. The use of alkaline additives to retard the formation of pheophytins from chlorophylls by replacement of hydrogen ions has been studied for a number of years. Numerous patents[11] have been obtained for the addition of alkaline compounds to conventionally heat-processed green vegetables. The metal chlorophyll derivatives form bonds, which are more resistant to acid and heat than naturally occurring magnesium chlorophyll complexes[12].

Color degradation of green leafy vegetables can be predicted from kinetic models; usually first order and a variety of different models have been developed[13-15] while for the appearance of browning can be evaluated by first and zero order kinetic models[16,17]. Several researchers have studied the rate of conversion of chlorophyll extent[18,19] and others were investigated the stability of chlorophyll at various pH levels using high temperature short time process (HTST)[20-22]. In continuation of this work nonlinear equation has been developed on the frame work of response surface method (RSM) for concentration of Cha and Chb.

RSM is the combinations of three different parameters such as design of experiment (DoE), regression modelling techniques and optimizing process[23]. Experimental designs in combination with RSM help to visualize the relationship between responses (output) and factor levels for locating highest response values[24]. Even having complex interactions, it is helpful in evaluating the relative significance of several affecting factors[25]. RSM is an approach applied in the field of production and optimization of various products equivalent to chemical and enzymes industries[26]. This technique requires minimum time of experimentation and was found more effective than conventional methods.

The objective of the study was to evaluate the kinetics of thermal degradation at various temperatures (70 to 90°C) & pH (5.6 to 7.6) of Amaranth leave puree. An attempt has been made to develop a nonlinear regression equation based on RSM for concentration of Cha and Chb and a comparision is made between the results obtained using modeled equations and experimental. The adequacy of nonlinear equations has been checked by normal probability plot and coefficient of regression.

Experimental

All chemicals were of AR grade purchased from Fisher Scientific Ltd, India. Fresh Amaranth leaves were obtained from a local market in Raipur. The leaves were de-stemmed, washed, drained and blanched in hot water at 85 ± 5ºC for 3.5 min. Sample were taken out and cooled immediately in chilled water. Chilled leaves after removing excess amount of water were comminuted in wet grinder afterwards the puree was sieved (14 mesh aperture size) and was stored in deep freezer (-7^{0}C) until further use. The pH of the puree (5.6, 6.6 and 7.6) was measured using pH meter. Adjustment of pH was done using buffered solution by combining 0.1 M citric with 0.2 M disodium hydrogen phosphate (Mcllvaine's buffer) to the desired pH ±0.05[22].

Thermal treatment

Kinetics of thermal degradation of amaranth leaves was studied using mild thermal treatment under pasteurization condition of 70, 80 and 90ºC using water bath for a residence time of 0 to 60 min for different pH ranging from (5.6-7.6). The puree sample of approximately 1.5-2 gm were sealed in vials and immersed in a water bath for times (0, 10, 20, 30, 40 and 60 min). Time taken by the sample to reach the desired temperature (come up time) was considered when the exact temperature of the water bath was reached. Come-up time was determined by inserting thermocouple in the vials. The samples were transferred in a container containing chilled water just immediately after the thermal treatment to stop the degradation rate.

Chlorophyll estimation

2±0.5 gm of puree was taken for chlorophyll extraction with 80% acetone (V/V) using centrifuge (Remi Lab Equipments, India). 30 ml supernatant extract was collected in 50 ml volumetric flask. 10 ml by volume of this sample was taken in three different flasks of 25 ml. In the first flask, volume was made up to 25 mL by 80 % acetone. In the second flask, 1 mL of 0.5 M oxalic acid in acetone was added and mixed. It was then allowed to stand for 90 min. The flasks volume was then made up to 25 mL with 80 % acetone. In the third flask, 0.32 mL of 12 N HCL was added and the flask was allowed to stand for 30 min, following which 0.23 mL of ethanolamine was added and volume was made up with 80% acetone. Sample was filtered through Whatman filter paper no. 1(125mm). Percent absorbance of clear pigment solution was measured at both 664 and 646.5 nm using spectrophotometer (Systronic, India Ltd., India). Concentrations of chlorophyll *a*, chlorophyll *b* and

total chlorophyll were calculated by the method of Jones et al.[12] All experiments were performed in triplicate and an average value has been used in order to monitor the degradation of chlorophyll with time.

Response Surface Method

RSM is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing process[25]. For the practical application of response surface method (RSM), it is necessary to develop an approximating model for the true response surface. A second order nonlinear response surface model (for three input variables), obtained based on regression analysis is given by

$$y_i = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1^{\,2} + \beta_5 x_2^{\,2} + \beta_6 x_3^{\,2} + \beta_7 x_1 x_2 + \beta_8 x_2 x_3 + \beta_9 x_3 x_1 + \varepsilon \quad (1)$$

Here, y_i is the observed value of concentration of chlorophyll a and b, the term nonlinear is used because equation (1) represents nonlinear relationship between dependent and independent variables and it gives better approximation with the experimental results.

$\beta_0, \beta_1, \beta_2 \ldots\ldots \beta_n$ are regression coefficients and x_1, x_2, x_3 are coded variables usually defined to be dimensionless with mean zero and standard deviation. The natural variables (time, pH and temperature) are converted into coded variables using the following relationship:

$$x_{i1} = \frac{\xi_{i1} + [\max(\xi_{i1}) + \min(\xi_{i1})]/2}{[\max(\xi_{i1}) - \min(\xi_{i1})]/2} \quad (2)$$

where ξ_{i1} represents natural variables with maximum and minimum values. The method of least squares is used to estimate the regression coefficients in a multilinear regression model. Myers and Montgomery[25], presented the multilinear regression equation in the form of matrix:

$$y = X\beta + \varepsilon \quad (3)$$ And

the least squares estimators b are given by,

$$b = (X'X)^{-1} X'y \quad (4)$$

Where X is the coded matrix corresponding to the variable parameters (time, pH and temperature), X' is the transpose matrix of X and y is the experimental observed value of Cha and Chb.

Residual analysis

Analysis of residuals is conducted to examine if the regression equations provide an adequate approximation to the true system and verify that none of the least square regression assumptions is violated. The residuals is defined by

$$e_i = y_i - \hat{y}_i \quad (5)$$

i = 1,2,3…..n where, y_i is the concentration of chlorophyll a and b obtained using experimental procedure (observed value) and $\hat{y}_i$ is the predicted value from regression model. The residuals play an important role in judging model adequacy. If the residual plot falls along a straight line, then the normality assumption is satisfied. Further, computed values of the coefficient of regression (R^2) and the adjusted coefficient of regression (R^2_{adj}) also give description of adequacy of fitted model. Regression coefficients are calculated by the equations given below:

$$R^2 = 1 - \frac{\sum_{i=1}^{n} e_i^2}{\sum_{i=1}^{n} y_i^2 - \frac{(\sum_{i=1}^{n} y_i)^2}{n}} \quad (6)$$

$$R_{adj}^2 = 1 - \frac{n-1}{n-p}(1-R^2) \quad (7)$$

where n is the total number of sample points and p is the number of model parameters. The value of R^2 varies from 0 to 1 and value close to 1 indicates good agreement with the fitted model explained by the regression equations.

Results and discussion

Chlorophyll Estimation

The degradation of chlorophyll is higher at lower pH values and higher temperatures. It was found that in the temperature range of 70 to 90 °C there has been uniform decrease in *k* values of both Cha and Chb (Table 1). Though it is recommended to apply strong thermal treatment to achieve commercial sterility, it is noticed that this leads to higher color degradation. However, it can be lowered by altering the pH of the puree (at higher pH values lower thermal degradation was found).

Table 1. Experimental rate constant value (*k*) for degradation of Cha and Chb of Amaranth leave puree at different temperature and pH

Temperature (ºC)	pH	*k* (Cha) (min^{-1})	*k* (Chb) (min^{-1})
70	5.6	0.012	0.010
80		0.016	0.014
90		0.027	0.024
70	6.6	0.008	0.007
80		0.014	0.012
90		0.016	0.014
70	7.6	0.007	0.006
80		0.011	0.009
90		0.014	0.011

Arrhenius equation[27] describes temperature dependence by relating the reaction rate constant k to the reciprocal of absolute temperature as

$$k = k_o \exp\left(\frac{-E_a}{RT}\right) \quad (9)$$

E_a is activation energy (kJ/mol), R is the universal gas constant (8.314 J/mol.K), k is reaction rate constant at T (absolute temperature) and k_o is frequency factor.

The logarithms of rate constants (*k*) were plotted against 1/T (where T is temperature in K) (Fig 1 and 2). Kinetic parameters (k,E_a) are useful to predict the quantity change that occur during thermal processing. Lower the value of k better is the stability of color. Higher is the E_a value, greater is the heat sensitivity of visual color degradation during thermal processing. It has been found that the activation energies of both Cha and Chb are comparable. Activation energies for Cha are 41.84, 36.07, and 31.77 kJ/mol at pH of 5.6, 6.6 and 7.6 respectively; while for Chb were 40.83, 31.85 and 27.30 kJ/mol at pH of 5.6, 6.6 and 7.6, respectively.

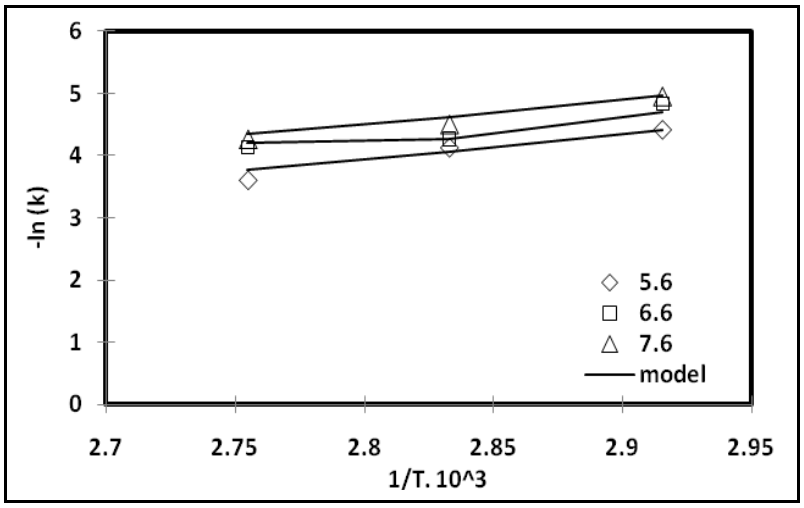

Figure 1. Arrhenius plot of Cha degradation of Amaranth leaves puree under different pH conditions

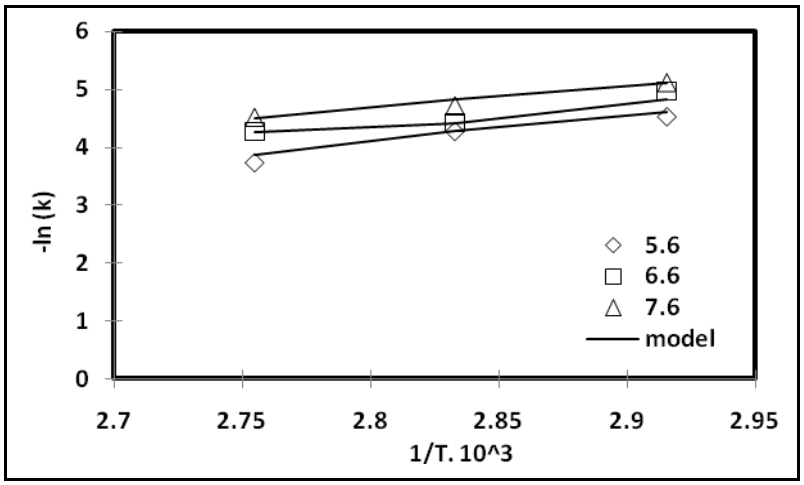

Figure 2. Arrhenius plot of Chb degradation of Amaranth leaves puree under different pH conditions

Lower E_a value at higher pH signifies that the puree is more heat stable at higher pH. The values are in agreement by the work by Koca et al.[22], studied the effect of pH (5.5-7.5) and temperature (70-100 ^oC) on chlorophyll degradation and color loss in blanched green peas. Activation energies in this case were found to be for –a value ranged from 8.13 to 12.0 kcal/mol, and for -a/b values ranged from 8.77 to 12.0 kcal/mol with varying pH values at 70, 80, 90 and 100°C. Esther et al.[28] used curry leaf paste for the study of thermal degradation kinetics of color at temperature ranges from 50 to 90 ºC up to 60 min. Thermal degradation of color obeys first order reaction kinetics and corresponding activation energies were found to be 18.44±3.2 and 17.203±5.9 kJ/mol. Lixia et al.[29] studied the kinetics of color degradation of chestnut kernel during thermal treatment at 50 to 70 ºC for 0 to 160 min at 3 ºC of storage for five months using computer vision system (CVS). First order reaction model showed a good fit for L^* and b^* values and R^2 ranges from 0.923 to 0.977. The activation energies during thermal treatment of chestnut were found to be 287.16 and 347.48 kJ/mol while during storage were found 89.18 and 78.47 kJ/mol respectively. Color degradation (L*, a*& b* value) of African cowpea leaves during thermal treatment (55-80 ºC per 90 min) were investigated[30]. Thermal degradation of the green color in the leaves is of first order reaction kinetics followed by Arrhenius equation having activation energy of 88.78±3.21 kJ/mol.

Regression equations

Above study suggests that degradation of chlorophyll due to thermal effects is the function of time, pH and temperature. The variation of Cha and Chb as a function of different parameters such as time, pH and temperature is presented in equation (11) and (12). The values of Cha and Chb calculated from the above equations and presented in Table 2 and 3.

Table 2. Typical predicted values of chlorophyll concentration from regression model, values obtained through experiments and residuals concentration values for Cha

time (min)	pH	temperature (°C)	Cha (y_i) (observed value)	Cha ($\hat{y}_i$) (predicted value)	Residuals (e_i) $y_i - \hat{y}_i$
0	5.6	70	2.81	2.85	-0.04
10	5.6	70	2.59	2.55	0.04
20	5.6	70	2.32	2.26	0.06
30	5.6	70	1.97	1.99	-0.02
40	5.6	70	1.61	1.74	-0.13
60	5.6	70	1.28	1.30	-0.02
0	5.6	80	2.32	2.31	0.01
10	5.6	80	2.06	2.01	0.05
20	5.6	80	1.79	1.72	0.07
30	5.6	80	1.48	1.45	0.03
40	5.6	80	1.14	1.20	-0.06
60	5.6	80	0.85	0.75	0.10
0	5.6	90	1.92	1.87	0.05
10	5.6	90	1.59	1.56	0.03
20	5.6	90	1.26	1.27	-0.01
30	5.6	90	0.95	1.00	-0.05
40	5.6	90	0.63	0.75	-0.12
60	5.6	90	0.34	0.30	0.04
0	6.6	70	3.18	3.18	0.00
10	6.6	70	2.93	2.90	0.03
20	6.6	70	2.75	2.64	0.11
30	6.6	70	2.48	2.39	0.09
40	6.6	70	2.17	2.16	0.01
60	6.6	70	1.88	1.76	0.12
0	6.6	80	2.65	2.69	-0.04
10	6.6	80	2.31	2.41	-0.10
20	6.6	80	2.06	2.14	-0.08
30	6.6	80	1.87	1.89	-0.02
40	6.6	80	1.46	1.66	-0.20
60	6.6	80	1.11	1.26	-0.15
0	6.6	90	2.27	2.29	-0.02
10	6.6	90	1.98	2.00	-0.02
20	6.6	90	1.71	1.74	-0.03
30	6.6	90	1.56	1.49	0.07
40	6.6	90	1.32	1.26	0.06
60	6.6	90	1.03	0.85	0.18
0	7.6	70	3.61	3.65	-0.04
10	7.6	70	3.37	3.39	-0.02
20	7.6	70	3.13	3.15	-0.02
30	7.6	70	2.89	2.93	-0.04
40	7.6	70	2.68	2.72	-0.04
60	7.6	70	2.31	2.36	-0.05
0	7.6	80	3.11	3.20	-0.09
10	7.6	80	3.01	2.94	0.07
20	7.6	80	2.83	2.70	0.13
30	7.6	80	2.61	2.47	0.14
40	7.6	80	2.39	2.27	0.12
60	7.6	80	1.95	1.90	0.05

0	7.6	90	2.88	2.85	0.03
10	7.6	90	2.62	2.58	0.04
20	7.6	90	2.34	2.34	0.00
30	7.6	90	2.05	2.11	-0.06
40	7.6	90	1.85	1.90	-0.05
60	7.6	90	1.4	1.53	-0.13

Table 3. Typical predicted values of chlorophyll concentration from regression model, values obtained through experiments and residuals concentration values for Chb

time (min)	**pH**	**temperature (°C)**	**Chb (y_i) (observed value)**	**Chb ($\hat{y}_i$) (predicted value)**	**Residuals (e_i) $y_i - \hat{y}_i$**
0	5.6	70	1.18	1.19	-0.01
10	5.6	70	1.12	1.07	0.05
20	5.6	70	0.98	0.97	0.01
30	5.6	70	0.85	0.88	-0.03
40	5.6	70	0.75	0.79	-0.04
60	5.6	70	0.63	0.65	-0.02
0	5.6	80	1.08	1.01	0.07
10	5.6	80	0.96	0.89	0.07
20	5.6	80	0.84	0.77	0.07
30	5.6	80	0.68	0.67	0.01
40	5.6	80	0.59	0.57	0.02
60	5.6	80	0.47	0.41	0.06
0	5.6	90	0.84	0.87	-0.03
10	5.6	90	0.72	0.74	-0.02
20	5.6	90	0.62	0.62	0.00
30	5.6	90	0.41	0.50	-0.09
40	5.6	90	0.31	0.40	-0.09
60	5.6	90	0.18	0.21	-0.03
0	6.6	70	1.39	1.46	-0.07
10	6.6	70	1.25	1.33	-0.08
20	6.6	70	1.19	1.22	-0.03
30	6.6	70	1.11	1.11	0.00
40	6.6	70	0.98	1.01	-0.03
60	6.6	70	0.88	0.84	0.04
0	6.6	80	1.19	1.23	-0.04
10	6.6	80	1.06	1.10	-0.04
20	6.6	80	0.96	0.97	-0.01
30	6.6	80	0.88	0.85	0.03
40	6.6	80	0.68	0.75	-0.07
60	6.6	80	0.55	0.55	0.00
0	6.6	90	1.04	1.05	-0.01
10	6.6	90	0.93	0.90	0.03
20	6.6	90	0.81	0.76	0.05
30	6.6	90	0.71	0.64	0.07
40	6.6	90	0.6	0.52	0.08
60	6.6	90	0.39	0.31	0.08
0	7.6	70	2.10	2.09	0.01
10	7.6	70	1.98	1.95	0.03
20	7.6	70	1.88	1.82	0.06
30	7.6	70	1.74	1.70	0.04

40	7.6	70	1.64	1.59	0.05
60	7.6	70	1.38	1.39	-0.01
0	7.6	80	1.81	1.81	0.00
10	7.6	80	1.68	1.66	0.02
20	7.6	80	1.52	1.53	-0.01
30	7.6	80	1.34	1.39	-0.05
40	7.6	80	1.19	1.27	-0.08
60	7.6	80	1.00	1.05	-0.05
0	7.6	90	1.56	1.57	-0.01
10	7.6	90	1.43	1.42	0.01
20	7.6	90	1.27	1.27	0.00
30	7.6	90	1.11	1.13	-0.02
40	7.6	90	0.98	0.99	-0.01
60	7.6	90	0.75	0.76	-0.01

The predicted values from regression equations and experimental results are compared and predicted values are in good agreement with experimental values. The response surface equation for the concentration of Cha and Chb obtained from nonlinear regression are as follows:

$$y_{Cha} = 1.8941 - 0.7158x_1 + 0.5106x_2 - 0.4524x_3 + 0.0802{x_1}^2 + 0.0694{x_2}^2 + 0.0461{x_3}^2 + 0.0654x_1x_2 + 0.0433x_2x_3 - 0.0064x_3x_1 \quad (11)$$

$$y_{Chb} = 0.8534 - 0.3396x_1 + 0.3615x_2 - 0.2359x_3 + 0.0396{x_1}^2 + 0.1774{x_2}^2 + 0.0204{x_3}^2 - 0.0389x_1x_2 - 0.0497x_2x_3 - 0.0299x_3x_1 \quad (12)$$

With reference to the above equations (11) and (12) the predicted values for concentration of Cha and Chb are used to calculate rate constant (*k*) (Table 4). In Fig.1 and Fig.2 the line represents the model plots for Cha and Chb at different pH and temperature.

Table 4. Model rate constant value (k) for degradation of Cha and Chb of Amaranth leave puree at different temperature and pH

Temperature (°C)	pH	*k* (Cha) (min^{-1})	*k* (Chb) (min^{-1})
70	5.6	0.012	0.010
80		0.017	0.014
90		0.023	0.021
70	6.6	0.009	0.008
80		0.014	0.012
90		0.015	0.014
70	7.6	0.007	0.006
80		0.010	0.008
90		0.013	0.011

The slope of Arrenhius plot is multiplied with gas constant (*R*) to get activation energy of Cha and Chb. The activation energy for Cha were observed 38.35, 32.07, and 29.07 kJ/mol for different pH of 5.6, 6.6 and 7.6 and for Chb were 33.68, 31.34 and 26.61 kJ/mol respectively. The predicted activation energy of Cha and Chb are compared with the experimental values. The adequacy of proposed nonlinear response surface equations is evaluated from residual analysis and coefficients of regression (R^2) are discussed in subsequent section.

Analysis of residuals and regression

In order to examine the adequacy of proposed response surface equations, normal probability versus residuals plot is obtained for Cha and Chb. The residuals defined in equation (5) play an important role in judging model adequacy. A check of the normality assumption made by constructing a normal probability plot of the residuals and if the residuals plot fall along a straight line, signifies satisfactory assumption[25]. Concentration of chlorophyll a and b obtained from response surface equations and compared with experimental results and results are found in good agreement. Tables 2 and 3 represent typical results of the concentration of chlorophyll a and b of experimental and predicted values and residuals for Cha and Chb respectively. It is observed that the experimental values corresponding to Cha varies (max. & min) from 3.61 to 0.34 µmol/lt and 2.098 to 0.18 µmol/lt for Chb whereas the predicted values for Cha varies from 3.65 to 0.29 µmol/lt and 2.086 to 0.21 µmol/lt for Chb respectively. The values from predictions using regression equation for Cha and Chb are quite close and comparable. In addition, a check of the normality assumptions is made by the construction of normal probability plot of the residuals and typical results for Cha and Chb are presented in Figures 3 and 4. It is observed that the residual plot falls along a straight line indicating satisfactory assumptions of normality. In addition, computed values of coefficients of regression (R^2) and adjusted R^2 also gives description of adequacy of fitted model. For a good model value of R^2 and adjusted R^2 should be close to 1. The calculated values of Cha and Chb are in the range of 0.988 indicating adequacy of the regression equations.

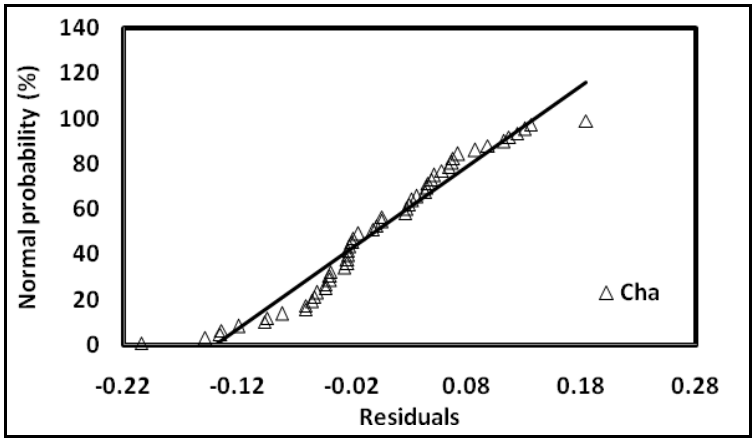

Figure 3. Normal probability plot of the residuals for Cha

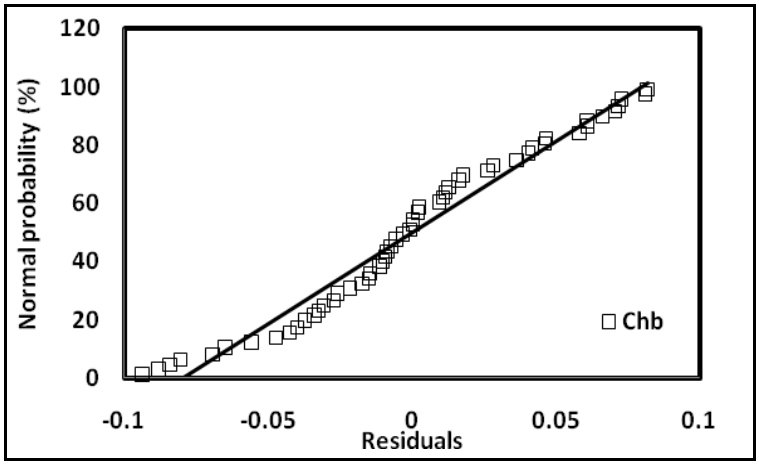

Figure 4. Normal probability plot of the residuals for Chb

Conclusion

In this paper an approach to use nonlinear regression equation based on response surface method was suggested. Considering three different modes of parameters (time, pH and temperature) in the evaluation of

concentration of Cha and Chb, predicted value was compared with the experimental values. Thermal degradation kinetic was studied at different temperatures and pH values. First order degradation was found and lower degradation was found at higher pH (7.6) and lower temperature (70°C). Degradation of chlorophyll a was found higher than chlorophyll b. Activation energy was computed by Arrhenius equation and was found to be higher at lower pH for both chlorophyll molecules. The concentrations, reaction rate constant (*k)* and activation energy (E_a) of Cha and Chb using proposed model (nonlinear regression equation) was found very close to the experimental values. The adequacy of modeled equation is checked by normal probability plot and was found satisfactory assumptions as the residual plot falls along a straight line for both Cha and Chb. Coefficients of regression (R^2) and adjusted R^2 also gives description of adequacy of fitted model and was found 0.988 which is close to 1.

Nomenclature:

Cha = Chlorophyll a (µmol/l)
Chb = Chlorophyll b (µmol/l)
E_a = Activation Energy (kJ/mol)
k = Rate constant (min^{-1})
t = time (min)
T = temperature (K)
R^2 = Regression coefficient
M = Molarity
N = Normality

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