PAPER • OPEN ACCESS

Research on some factors affecting extraction of chlorophyll from mulberry leaves (Morus alba)

To cite this article: Q H Tran *et al* 2019 *IOP Conf. Ser.: Mater. Sci. Eng.* **479** 012004

View the article online for updates and enhancements.

Research on some factors affecting extraction of chlorophyll from mulberry leaves (Morus alba)

Q H Tran[1], T Q Pham[1], H T Vu[2], D X Le[3], O T Tran[4], A Q Ngo[1], T D Nguyen[5], B T Hoang[3] and S T Do[1, 6]

[1] Institute of Chemistry, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Street, Cau Giay District, Hanoi, Vietnam;
[2] Faculty of Hydro-Meteorology, HCMC University of Natural Resources and Environment, Le Van Sy Street, Tan Binh District, Ho Chi Minh City, Vietnam;
[3] Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Street, Cau Giay District, Hanoi, Vietnam;
[4] Vietnam National University of Agriculture, Gia Lam District, Hanoi, Vietnam;
[5] NTT Institute of High Technology, Nguyen Tat Thanh University, 300A Nguyen Tat Thanh, District 4, Ho Chi Minh City, Vietnam;

[6] E-mail: dosyvnn@gmail.com

Abstract. The purpose of this report is to investigate some factors affecting extraction of chlorophyll from mulberry leaves (Morus alba). In the three (3) types of solvents used (acetone, ethanol, and methanol), 90% acetone was the appropriate solvent to extract chlorophyll from mulberry leaves giving a total chlorophyll concentration of 0.341%, in which chlorophyll achieved a concentration of 63%. Some factors such as temperature, material/solvent ratio, extraction time were also investigated. This survey was studied for optimum use a Box-Wilson central composite design consisting of 03 independent variables (temperature, materials/solvent ratio and extraction time) and two dependent variables (total chlorophyll and chlorophyll a ratio). Second-order polynomial equations were obtained for the responses, which fitted well with the experimental data. Select the optimal conditions are T=26.75 ºC, material/solvent ratio 12.67 %, τ = 24.3 h. Optimal results are found out conditions to obtain the highest chlorophyll total ($Y_{1(opt)}$ = 0.354%) while chlorophyll a ratio at the desired concentration $Y_2 >$ 60%.

1. Introduction

Chlorophyll present in the chloroplasts of plants and accountable for the color of leaves. It plays the most important role in the photosynthesis which takes place in the leaves of plants and algae. The photosynthetic process uses the light energy to convert CO_2 from the atmosphere to carbohydrates and O_2 [1,2]. The evolution process takes place in the two following steps: (i) the chlorophyll molecules absorb energy from light; (ii) this energy is transfer to the chloroplasts and is used to convert CO_2 into carbohydrate. The overall reaction for photosynthesis is:

$$6CO_2 + 6H_2O + h\nu \rightarrow C_6H_{12}O_6 + 6O_2$$
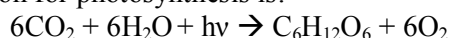
This overall chemical equation for photosynthesis is deceptively simple. However, a series of complex reactions must occur in a coordinated manner for the synthesis of carbohydrates. To produce

a sucrose molecule, $C_6H_{12}O_6$, plants require about 30 distinct proteins that work within a complicated membrane structure.

Chlorophyll, known as an antioxidant compound, has been found in plants including their green leaves, stems, flowers and roots [3]. In medicine, chlorophyll is used as nutritional approaches in blood sugar reduction, detoxification, digestion, excretion and lowering of allergens. It is also used as a green pigment because its nontoxicity for humans and superiority in hygiene, which compared to artificial synthetic pigment. Therefore, worldwide research focus has shifted to the investigation of extraction of pigment groups from cheap natural materials and the use of by-products to collect pigment groups.

White Mulberry (*Morus alba* L., *Moraceae*) is derived from China and widely cultivated in subtropical and temperate regions of the world (including Vietnam). Their leaves are used as food for silkworm and cattle, and their fruits are used as supplemental food for human and poultry. In addition, mulberry was known as a famous medicinal plant in traditional medicine because of their antibacterial, anti-inflammatory, antidiarrheal, anti-inflammatory, and anti-oxidant properties [4,5]. The studies by Flaczyk and his workers show that mulberry leaves contain not only valuable phenolic, flavonoids compounds but also high levels of chlorophyll [6]. Vietnam has a long-established tradition of growing mulberry in relatively large quantity [7]. Therefore, using mulberry leaves to extract chlorophyll will bring the best quality and efficiency in comparison with the use of other types of leaf.

In this paper, we investigate some factors affecting the extraction of chlorophyll from mulberry leaves (Morus alba) in order to develop a highly efficient chlorophyll extraction process. Moreover, input factors affecting the efficient chlorophyll extraction process conducted using response surface methodology (RSM) were also investigated.

2. Materials and methods

2.1. Materials
Mulberry leaves were purchased at Viet Hung sericulture research station, Vietnam Sericulture Research Center, Vu Thu district, Thai Binh province.

2.2. Methods
Method of preliminary processing of raw material samples: material leaves, after rinsing gently under tap water, were soaked with distilled water to remove dirt and other water-insoluble impurities. After that, the materials were retrieved and drained completely. Chlorophyll extraction was performed with a solvent to prevent aging, causing chlorophyll loss in the material.

2.2.1. Sample preservation method. Raw materials were wrapped in silver foil, put in a PE plastic bag, which was then vacuumed and stored in the dark at -18 ºC to prevent chlorophyll from being destroyed. Frozen samples were kept for 7 days prior to the analysis.

2.2.2. Sample processing. During the sample processing, silver foil was used to wrap the extraction container to minimize the loss of chlorophyll. The equipment and tools were cleaned with acetone by acetone-distilled water to ensure stable chlorophyll concentrations between the experiments.

Estimation of chlorophyll content: The chlorophyll content in leaves was estimated in accordance with US standards SERAS-SOP. 2030, Lichtenthaler, H.K [8–10], chlorophyll is extracted in 80% acetone and the absorption at 663 nm and 645 nm are measured by a spectrophotometer. The amount of chlorophyll is calculated using the absorption coefficients.

The sample of the leaf was finely cut and mixed; 1g of tissue was weighed and ground to a fine pulp with the addition of 20 ml of 80% acetone. This solution was centrifuged at 5000 rpm for 5 minutes and the supernatant was transferred to a 100-ml volumetric flask. The procedure was repeated until the residue becomes colorless. The mortar and pestle were washed thoroughly with 80% acetone and clear washings were collected in the volumetric flask. This was made to 100 ml with 80% acetone.

This absorbance was recorded at 645, 663 and 652 nm against the solvent (80% acetone) blank. The amount of chlorophyll present in the extract mg chlorophyll per g tissue was calculated using the following equations:

$$\% \text{ chlorophyll a} = \left[12.7 \text{x}(A_{663}) - 2.69 \text{x}(A_{645})\right] \text{x} \frac{V}{10^6 \text{ xW}} \text{x } 100\% \qquad (1)$$

$$\% \text{ chlorophyll b} = \left[22.9 \text{x}(A_{645}) - 4.68 \text{x}(A_{663})\right] \text{x} \frac{V}{10^6 \text{xW}} \text{x} 100\% \qquad (2)$$

$$\% \text{ chlorophyll total} = \left[20.2 \text{x}(A_{645}) + 8.02 \text{x}(A_{663})\right] \text{x} \frac{V}{10^6 \text{xW}} \text{x} 100\% \qquad (3)$$

$$\text{Chla ratio (\%)} = (\% \text{ chla/ total chl}) * 100\% \qquad (4)$$

where, A is absorbance at specific wavelength, V is final volume of chlorophyll extract 80% acetone, and W is fresh weight of tissue extracted.

2.2.3. Method of chlorophyll extraction in laboratory. 100 g of raw materials put into the blender (manufactured by Philips) and milled within 1–2 minutes until the ingredients are finely ground. After that, the milling solution is poured into a dark-colored glass bottle of 1000 ml, followed by addition of solvent (acetone, methanol, ethanol) to reach a volume of 1000 ml. The concentration of solvents for each experiment ranged from 70 to 100%. Glass bottles were enclosed by silver foil and kept under dark conditions at -4 ℃ for appropriate duration (16–24 h) for each experiment. Subsequently, the sample was filtered and centrifuged at 6000 rpm for 10 minutes. Supernatant were recovered for use in the following experimental steps.

2.2.4. Statistical Procedures. Response surface methodology, in particular, the Box-Wilson central composite design [8] was employed to estimate the effect of 3 reaction parameters (temperature, materials/solvent ratio, extraction time) on chlorophyll total output and Chla ratio. Procedures for the construction of the 15 orthogonal design matrix, for the mathematical-statistical treatments, and for the determination of optimal conditions followed instructions described by Pham Hong Hai [11]. Regression validation was performed using the Student t-test and the Fisher *F-test.*

3. Results and discussion

3.1. Effects of type and percentage of solvent on chlorophyll content
Table 1 and Figure 1 showed the extracted chlorophyll content (%) from mulberry leaves (*Morus alba*) by different solvents and at different concentrations. The ratio of chlorophyll a to total chlorophyll also evaluated. The results of Table 1 and Figure 1 showed that methanol, acetone and ethanol are suitable solvents for the extraction of chlorophyll. The chlorophyll content of mulberry leaves ranges from 0.241 to 0.341% with different solvents (methanol, acetone and ethanol).

Based on the results obtained, it can be said that chlorophyll a (Chla), chlorophyll b (Chlb), total chlorophyll concentrations and Chla ratio vary with the solvent used and corresponding concentrations. Acetone 90% is the solvent that induced the highest total chlorophyll, achieving 0.341% compared to that of Chla of 63.3%. This result is in line with works of other authors such as Karsten *et al*. [12], who successfully used 90% acetone to extract chlorophyll in algae, and Ronen et al., [13], who also used 90% acetone to extract chlorophyll under conditions of cold and dim light.

Among the three solvents, acetone and methanol gave balanced results of Chla ratio, ranging between 56 and 62%, while ethanol achieved the highest Chla ratio (64–65%). According to some previous studies [14,15], the chlorophyllase enzyme still retains its active fraction at different concentrations of solvents that cause chlorophyll to be converted to other isomers. On the other hand, the activity of chlorophyll b reductase also converts chlorophyll b into chlorophyll. It is possible that the activity of these two enzymes during extraction (immersion, sample milling, extraction by solvents at different concentrations) has led the difference in the amount and percentage of obtained chlorophyll.

Table 1. Effects of different types of solvent on chlorophyll a, b and total chlorophyll.

| Solvent	Symbol	Chla (%)	Chlb (%)	Chlorophyll total (%)	Chla ratio (%)
Acetone	Ac70	0.151	0.107	0.258	58.471
	Ac80	0.157	0.109	0.266	58.959
	Ac90	0.216	0.125	0.341	63.373
	Ac100	0.154	0.114	0.268	57.395
Ethanol	Et70	0.154	0.117	0.271	56.825
	Et80	0.177	0.093	0.270	65.591
	Et90	0.194	0.096	0.290	66.874
	Et100	0.166	0.122	0.288	57.647
Methanol	Me70	0.145	0.149	0.294	49.366
	Me80	0.138	0.132	0.270	51.058
	Me90	0.151	0.095	0.246	61.556
	Me100	0.143	0.098	0.241	59.301
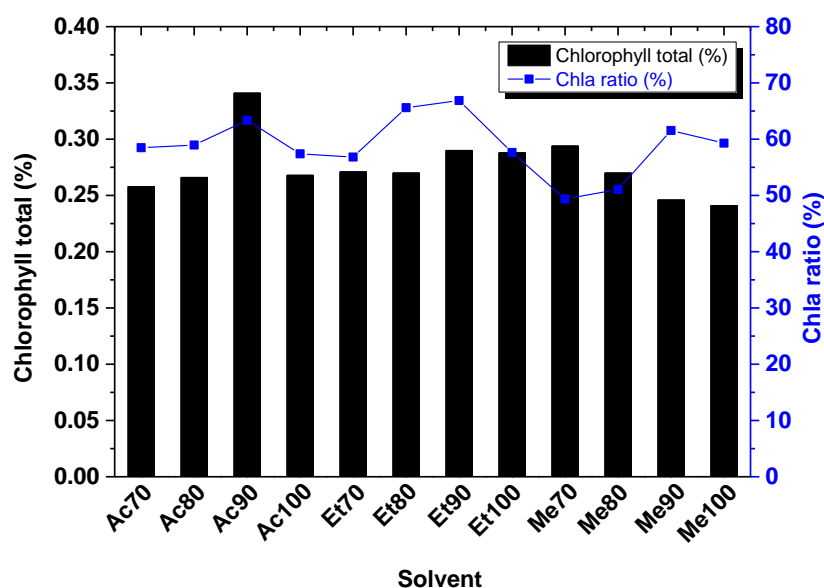
Figure 1. Effects of type and percentage of solvent on the extraction of chlorophyll from mulberry leaves.

3.2. Effects of temperature on chlorophyll extraction process

The following experiments were performed with the selected solvent of 90% acetone. Temperature also affects the chlorophyll extraction process. Leaching process was carried out at different conditions of temperature ranging from 20 to 40 ºC. The results are shown in Table 2 and Figure 2. Increasing temperature promotes chlorophyll extraction process from the material. However, in this case, due to the extreme sensitivity to heat of chlorophyll, chlorophyll is converted into a new compound-pheophytin (dark olive), reducing the amount of chlorophyll in the material. Therefore, when the temperature exceeds 35 ºC, the chlorophyll content and the chla ratio decrease and achieves 48.87% at 40 ºC.

**Table 2**. Effects of different conditions of temperature on chlorophyll a, b and total chlorophyll.

Temp	Chla (%)	Chlb (%)	Chlorophyll total (%)	Chla ratio (%)
10	0.120	0.104	0.224	53.439
20	0.137	0.103	0.240	56.893
25	0.216	0.125	0.341	63.373
30	0.207	0.128	0.335	61.782
35	0.179	0.126	0.305	58.749
40	0.119	0.125	0.244	48.870
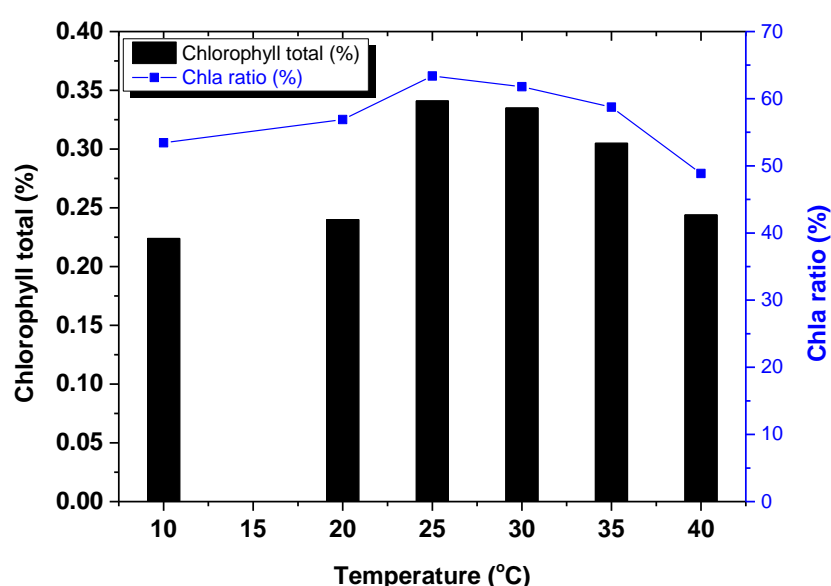
Figure 2. Effects of different conditions of temperature on the extraction of chlorophyll from mulberry leaves.

3.3. Effects of solvent/materials ratio on chlorophyll contents

The appropriate solvent/material ratio for the chlorophyll extraction process also determined. We carried out 5 extraction trials using 90% acetone with the ratio of 5%, 10%, 15%, 20% and 25% (v/w). The results are shown in Table 3 and Figure 3. Based on the results of statistical analysis (Table 3 and Figure 3), it is revealed that the ratio of solvent/material has a significant effect on the chlorophyll extraction efficiency. The ratio of 5% and 10% gave the highest extraction efficiency of, 0.341 and 0.340%, respectively, in which chla ratio was 63.3% and 60.7%. When increasing the material to 15, 20, 25%, the extraction efficiency decreases gradually. The cause of this change is due to the insufficiency of solvent for the full dissolution and extraction of chlorophyll from the cell. Therefore, when increasing the amount of acetone, the chlorophyll content increases considerably which also, in turn, improve its antioxidant activity.

However, given that the chlorophyll content in the mulberry leaves is constant, the excessive solvent could cause a balance between phases, resulting in the impairment of chlorophyll extraction efficiency. Therefore, we selected the appropriate material/solvent ratio of 10% in subsequent analyses.
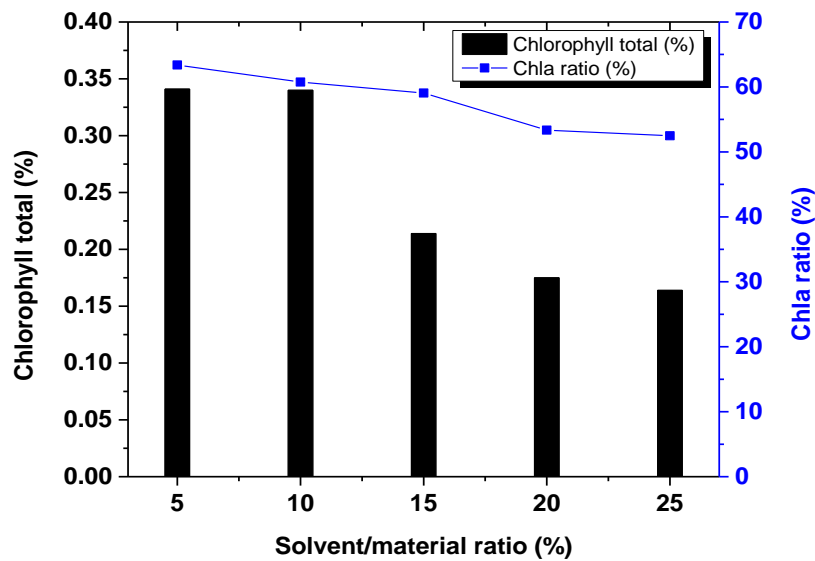
Figure 3. Effects of solvent/material ratio on the extraction of chlorophyll from mulberry leaves.

Table 3. Effects of different solvent/materials ratio on chlorophyll a, b and total chlorophyll.

Ratio	Chla (%)	Chlb (%)	Chlorophyll total (%)	Chla ratio (%)
5%	0.216	0.125	0.341	63.373
10%	0.207	0.133	0.340	60.752
15%	0.126	0.087	0.214	59.066
20%	0.093	0.081	0.175	53.356
25%	0.086	0.078	0.164	52.488

3.4. Effects of the extraction time on chlorophyll contents

The immersion duration of the material in the solvent also investigated. Visually, the effect of the extraction time on chlorophyll content fluctuated. The results are shown in Table 4 and Figure 4. Based on the results in Table 4 and Figure 4, the amount of chlorophyll obtained ranged from 0.214% to 0.341%. The chlorophyll content and chla ratio attained the maximum level of 34.1% and 63.3%, respectively, at 24 h; At 0 h, the chlorophyll content was lowest (21.4%). For chla ratio, the lowest point (52.5%) was reached at 36 h.

Table 4. Effects of the extraction time on chlorophyll a, b and total chlorophyll.

Time	Chla (%)	Chlb (%)	Chlorophyll total (%)	Chla ratio (%)
0h	0.120	0.093	0.214	56.256
6h	0.135	0.089	0.224	60.357
12h	0.152	0.099	0.251	60.632
18h	0.186	0.118	0.304	61.268
24h	0.216	0.125	0.341	63.373
30h	0.168	0.111	0.278	60.167
36h	0.143	0.129	0.273	52.579
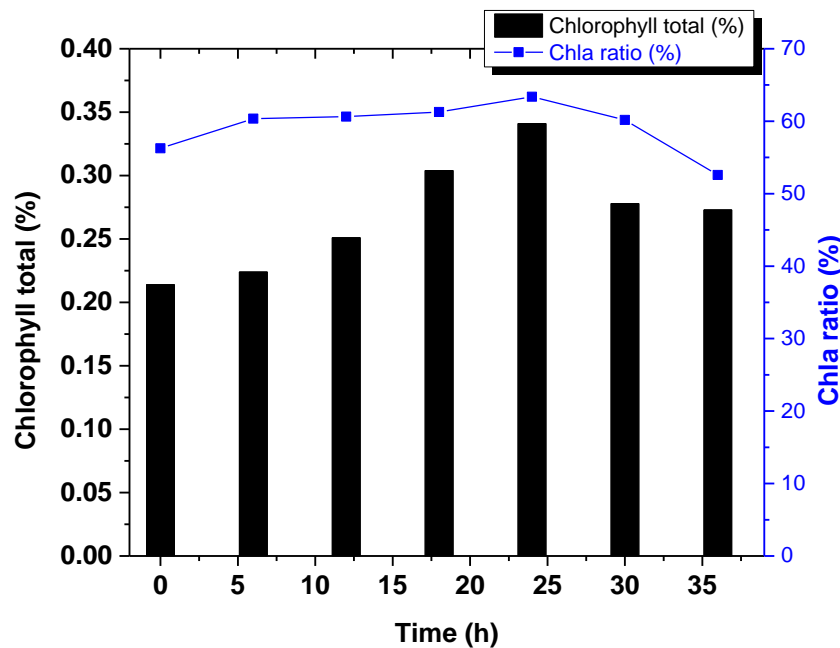
Figure 4. Effects of the extraction time on the extraction of chlorophyll from mulberry leaves.

3.5. Optimization and experimental planning

Response Surface Methodology was chosen to optimize the chlorophyll extraction process from the mulberry leaves. Based on our prior experiments, 03 reaction parameters, including temperature (X_1), materials/solvent ratio (X_2), reaction time (X_3), were chosen as independent variables for the design of experiments (Table 5) because of their observed effects on the outcome of the process. The outcome (concentration) of total chlorophyll (Y_1, %) and Chla ratio (Y_2, **%**) were chosen as dependent variables. For statistical calculations, the variables X_i were coded as x_i according to Equation (5):

$$x_i = (X - \bar{x}_i)/(\Delta x_j) \quad (i = 1, 2, 3\ldots, k) \tag{5}$$

where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, $\bar{x}_i$ is the real value of the independent variable at the center point and Δx_j is a step change.

Table 5. Real values of the independent variables at their corresponding levels in the design.

Variables		Variable range (Δ)	Coded levels				
			-α	-1	0	+1	+α
T, ^{0}C	(X_1)	5	18.9	20	25	30	31.1
Material/ solvent ratio, (%)	(X_2)	5	3.9	5	10	15	16.1
t, h	(X_3)	6	16.7	18	24	30	31.3

Table 6. Design matrix and experimental results.

No.	Real variable			Code variable			Objective function	
	X_1	X_2	X_3	X_1'	X_2'	X_3'	Y_1(%)	Y_2 (%)
1	20	5	18	-1	-1	-1	0.182	52.458
2	30	5	18	+1	-1	-1	0.202	54.398
3	20	15	18	-1	+1	-1	0.215	55.768
4	30	15	18	+1	+1	-1	0.268	58.837
5	20	5	30	-1	-1	+1	0.199	53.285
6	30	5	30	+1	-1	+1	0.264	57.981
7	20	15	30	-1	+1	+1	0.270	59.643
8	30	15	30	+1	+1	+1	0.338	62.813
9	18.9	10	24	-1.215	0	0	0.241	56.893
10	31.1	10	24	+1.215	0	0	0.326	60.664
11	25	3.9	24	0	-1.215	0	0.257	57.312
12	25	16.1	24	0	+1.215	0	0.341	63.343
13	25	10	16.7	0	0	-1.215	0.279	60.632
14	25	10	31.3	0	0	+1.215	0.331	62.116
15	25	10	24	0	0	0	0.327	62.561

The experimental matrix consisted of 15 experiments with a coefficient of α = 1.215; this model was built on the Design expert 7.0 software as described by Pham Hong Hai [11]. We use Design Expert 7.0 software to process the data in Table 6 and get results below:

$$Y_1 = 0.34 + 0.028\,X_1 + 0.032\,X_2 + 0.024\,X_3 - 0.04\,X_1^2 - 0.03\,X_2^2 - 0.025\,X_3^2 \qquad (6)$$

where, R-Squared = 0.9852 and Adj-R-Squared = 0.9585

$$Y_2 = 62.99 + 1.59\,X_1 + 2.40\,X_2 + 1.28\,X_3 - 2.95\,X_1^2 - 1.90\,X_2^2 - 1.19\,X_3^2 \qquad (7)$$

where, R-Squared = 0.9824 and Adj-R-Squared = 0.9506.

Using algorithms FLEXIPLEX of the nonlinear plan in the original values of the initial value of variable, we select the optimal conditions are $x_1^{opt} = 0.35$, $x_2^{opt} = 0,533$ and $x_3^{opt} = 0.048$ corresponding to the values of T=26,75°C, materials/solvent ratio of 12.67 %, τ = 24.3 h. Optimal conditions are found out to obtain the highest chlorophyll total ($Y_{1(opt)}$ = 0.354%) while chlorophyll a ratio at the desired concentration $Y_2 > 60\%$.

4. Conclusions

The results of the research on some factors affecting the extraction of chlorophyll from mulberry leaves (Morus alba) indicated that 90% acetone is a suitable solvent for the chlorophyll extraction process. We selected the chlorophyll extraction temperature of 25 °C, the appropriate material / solvent ratio of 5–10% and the extraction time of 24 h. Applied Design Expert methods to find a mathematical model of the process consists of 2 objective functions. Select the optimal conditions are T = 26.75 °C, materials/solvent ratio 12.67 %, τ = 24.3 h. Optimal results are found out conditions to obtain the highest chlorophyll total ($Y_{1(opt)}$ = 0.354%) while chlorophyll a ration at the desired concentration $Y_2 > 60\%$.

References

[1]   A Khalyfa, S Kermasha, I Alli 1992 Extraction, purification, and characterization of chlorophylls from spinach leaves *J. Agric. Food Chem.* **40** 215–220

[2]   M Meroni, M Rossini, L Guanter, L Alonso, U Rascher, R Colombo, J. Moreno 2009 Remote sensing of solar-induced chlorophyll fluorescence: Review of methods and applications *Remote Sens. Environ* **113** 2037–2051

[3]   B Srichaikul, R Bunsang, S Samappito, L Butkhup, G 2011 Bakker, Comparative Study of Chlorophyll Content in Leaves of Thai Morus alba Linn *Species, Plant Sci. Res.* **3** 17–20

[4]   Shahid-ul-Islam, L J Rather, F Mohammad 2016 Phytochemistry, biological activities and potential of annatto in natural colorant production for industrial applications – A review *J. Adv. Res.* **7** 499–514

[5]   J Liu, H Qiu, Z Zhu, T Zou 2015 Antibacterial, anti-inflammatory, and antioxidant effects of Yinzhihuang injection *Biomed. Mater. Eng.* **26** S2123–S2132

[6]   E Flaczyk, J Kobus-Cisowska, M Przeor, J Korczak, M Remiszewski, E Korbas, M Buchowski 2013 Chemical characterization and antioxidative properties of Polish variety of Morus alba L. leaf aqueous extracts from the laboratory and pilot-scale processes *Agric. Sci.* **04** 141–147

[7]   TXB Phung, TH Hoang, TD Nguyen 2014 Isolation, determination and activity of mulberrofuran k and steppogenin from mulberry leaves *J. Med. Mater.* **14** 207–210

[8]   P Horcajada, et al. 2010 Porous metal-organic-framework nanoscale carriers as a potential platform for drug deliveryand imaging *Nat. Mater.* **9** 172–178

[9]   A Gitelson, M N Merzlyak 1994 Quantitative estimation of chlorophyll-a using reflectance spectra: Experiments with autumn chestnut and maple leaves *J. Photochem. Photobiol. B Biol.* **22** 247–252

[10]   A D Richardson, SP Duigan, GP Berlyn 2002 An evaluation of noninvasive methods to estimate foliar chlorophyll content *New Phytol.* **153** 185–194

[11]   HH Pham, KC Ngo 2007 The data analysis and planning of experiments in chemical research *Nat. Sci. Technol. Publ. House.* 89–104

[12]   R Schumann, N Häubner, S Klausch, U Karsten 2005 Chlorophyll extraction methods for the quantification of green microalgae colonizing building facades *Int. Biodeterior. Biodegradation.* **55** 213–222

[13]   R Ronen, M Galun 1984 Pigment extraction from lichens with dimethyl sulfoxide (DMSO) and estimation of chlorophyll degradation *Environ. Exp. Bot.* **24** 239–245

[14]   J Barrett, S W Jeffrey 1964 Chlorophyllase and Formation of an Atypical Chlorophyllide in Marine Algae. *Plant Physiol.* **39** 44–7

[15]   J Barrett, SW Jeffrey 1971 A note on the occurrence of chlorophyllase in marine algae *J. Exp. Mar. Bio. Ecol.* **7** 255–262