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Full Length Research Paper

Process optimization for the enrichment of α-linolenic acid from silkworm pupal oil using response surface methodology

Jun Wang[1], Fu-An Wu[1,2]*, Yao Liang[1] and Meng Wang[1]

[1]School of Biotechnology and Environmental Engineering, Jiangsu University of Science and Technology, Zhenjiang 212018, P.R. China.
[2]Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, P.R. China.

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α-Linolenic acid (ALA) was widely used in food and pharmaceutical industry, as an important medical material. In the present study, the mixed fatty acids (MFAs) extracted from desilked silkworm pupal oil were used raw materials for enriching ALA, using urea clathration method; their preparation process was optimized by response surface methodology (RSM). The research results indicated that the enrichment of ALA produced, by using urea clathration method from desilked silkworm pupal oil was effectively enhanced by RSM. Three parameters of the process for enriching ALA were optimized as follows, using RSM: the ratio of saturated urea/EtOH solution to MFAs was 2:1 (v/v); clathration temperature, 4°C and clathration time, 2 h. The adequately high R^2 value, 0.9674 (adjusted R^2 = 0.9086) indicated the statistical significance of the model. The purity of ALA after optimization was 31.87%, while predicted value, 35.60%. In conclusion, enrichment conditions optimization to enhance purity of ALA from the MFAs of desilked silkworm pupal oil can be easily and effectively done by RSM. The developed enrichment process of ALA indicated desilked silkworm pupal oil as a potential raw material for producing ALA.

Key words: α-Linolenic acid (ALA), desilked silkworm pupal oil, urea clathration method, enrichment process optimization, response surface methodology (RSM).

INTRODUCTION

In 2006, the production of dry mulberry cocoon in China was reported as 740,390 tons. Hence, 444,234 tons of dry desilked silkworm pupae are available in China per year (Wei et al., 2009), and China has become the largest producing country in the world. However, most of the desilked silkworm pupae are used only as fertilizer and a small proportion is used as constituents of chicken and fish feed, or even regarded as industrial waste (Wei et al., 2009). Therefore, lots of desilked silkworm pupae have not been fully utilized. Indeed, disposal of desilked silkworm pupae is a serious problem because the putrilages of the waste are toxic due to their corruption; thus, governments must enforce the legislation of controlling this waste disposal to avoid its harmful effects to the environment. Recently, the chemical compositions of desilked silkworm pupae have attracted considerable attentions in the world, and desilked silkworm pupae are considered to be a good source of a large number of bioactive substances. The by-products of silk-reeling industry, desilked silkworm pupae, are known for their nutritional value due to the have high protein and fat in them. The neutral lipid of desilked silkworm pupae was considered to be a good source of α-linolenic acid (ALA). This is based on numerous literature reports on fatty acid composition of neutral lipids, predo- minantly of silkworm pupae, *Bombyx mori* L. (Nakasone and Ito, 1967; Zhou and Han, 2006), although there were variations in the level of ALA (traces to 40% of total fatty acids) reported so far in the *B. mori* L. silkworm pupae (Shanker et al., 2006). In view of the pharmaceutical application of ALA, the preparation of ALA from desilked silkworm pupae is thus of essential importance.

*Corresponding author. E-mail: fuword@163.com. Tel: 86-511-8561-6571.

ALA is a member of polyunsaturated fatty acids PUFAs) widely distributed in plant (Ihara-Watanabe et al., 2000) and animal (Salazar-Vega et al., 2009) materials, which is very important for human nutrition and disease prevention (Christensen et al., 2005). The importance of PUFAs in human nutrition and disease prevention was scientifically recognized three decades ago. Both omega-3 (ω3) and omega-6 (ω6) PUFA are precursors of hormone-like compounds known as eicosanoids, which are involved in many important biological processes in the human body (Shahidi and Wanasundara, 1998). Nowadays, ALA is used to prevent a variety of diseases such as cardiovascular (Roupas et al., 2006), hypertension, inflammatory and autoimmune disorders (Ferrucci et al., 2006), depression and certain disrupted neurological functions (Christensen et al., 2005). This is because it can produce eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the body by a series of chain elongation and desaturation (Trattner et al., 2008). Therefore, ALA is a PUFA with prominent bioactivity and is used widely in the medicine, food and cosmetic industries.

Extraction technology of crude PUFAs from bio-material have developed very rapidly (Robles et al., 1998), such as sterilization (Hou et al., 2008), centrifugation, hydrodistillation, low pressure solvent extraction, soxhlet solvent extraction, ultrasound solvent extraction (Pereira et al., 2008), extraction by imidazolium-based ionic liquids containing silver tetrafluoroborate (Li et al., 2009), extraction by novel -complexing sorbent (Li et al., 2009) and supercritical carbon dioxide extraction processes (Rutkowska and Stolyhwo, 2009; Wei et al., 2009). However, a critical challenge for preparation of ALA is the enrichment step, which is the key step in the process. The methods generally used to obtain PUFAs-rich fractions from natural oils are based on differences in the polarity and/or spatial configuration of the fatty acids (FAs) present in the extract. These differences, mostly associated with the number of double bonds in the carbon chain, can enable the separation of PUFAs with respect to their degree of unsaturation. The common methods of producing PUFAs concentrates include winterization, fractional distillation, urea inclusion, high-performance liquid chro- matography and argentated silica gel chromatography (Sajilata et al., 2008). Usually, urea clathration method was found to be rapid, easy and reliable, so the enrichment of ALA by urea clathration method from desilked silkworm pupae oil is a good way to provide natural medical material. Therefore, enrichment of ALA from desilked silkworm pupae oil is the basic research because of its high additional value application in food and pharmaceutical industries. At the same time, processing and stabilizing desilked silkworm pupae could represent two advantages: a solution of the environmental problem derived from a great desilked silkworm pupae disposal and the obtaining of stabilized ALA as natural medical material.

The limitations of classical method of process parameters optimization can be overcome by the application of statistical based approach. RSM, an extensively used statistical technique for media optimization, is a collection of statistical techniques which uses design of experiments for building models (Shah et al., 2004), evaluating the effect of factors and searching for the optimum conditions (Wang et al., 2009). To the best of our knowledge, up till now, there is no information in the literature on enrichment processing study of ALA from desilked silkworm pupae oil used by RSM. Therefore, in the present work, we have used a central composite rotatable design (CCRD) of response surface methodology for process conditions optimization to enrich the purity of ALA from desilked silkworm pupae oil.

MATERIALS AND METHODS

Plant materials

Desilked silkworm pupae were collected in May 2009, at Jiangsu Province in China and authenticated as the desilked silkworm (*B. mori* L.) pupae by Prof. Fu-An Wu (Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, P. R. China). Sample was dried at 60℃, powered by an herb disintegrator (Qinzhou Sanyang Package Equipment Co., Ltd) and then sieved (60mesh).

Chemicals and reagents

Standard of ALA was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All reagents used were of analytical grade, except methanol and acetonitrile, which were of HPLC grade purchased from TEDIA Co. (Fairfield, OH, USA). All water solutions were prepared with Ultrapur water (Premier MFG Systems, Phoenix, AZ, USA) filtered through a 0.45 μm membrane filter.

The standard of ALA was accurately weighed, and the stock solution of 0.90 mg ml^{-1} concentration was prepared by dissolving it in methanol. It was then diluted to appropriate concentration ranges for the establishment of calibration curves. All solutions were stored at 4℃.

Analytical methods

HPLC-UV was performed using HITACHI Pump L-7100 with a UV-VIS Detector L-7420 (Techcomp Ltd., Shanghai, China) and N-2000 wo station (Hangzhou Mingtong S&T Ltd., Hangzhou, China).

Separation in HPLC-UV was on HC-C_8 column (250 × 4.6 mm i.d.; 5 μm; Angilent, Waldbronn, Germany) kept at 30℃, with a mobile phase consisting of acetonitrile: water (90:10, v/v), at a flow rate of 1.0 ml·min^{-1}, detected at 242 nm.

The derivatization reagents of MFAs were bromoacetophenone and triethylamine (Mehta et al., 1998). Sample of ALA was accurately weighed and dissolved in the 10 ml tube; then, 50 μl of acetone solution of 20 mg ml^{-1} bromoacetophenone and 25 mg ml^{-1} triethylamine were added, respectively. The tube was closed, and then shaken in a water bath shaker at 100℃ for 15 min. After the solution was cooled to room temperature, 70 μl of acetic acid was added to the tube. The tube was closed again, and then shaken in a water bath shaker at 100℃ for 5 min. Then, the acetone in the tube was removed and 500 μl methanol was added and dissolved. All solutions were filtered through a 0.45 μm filter before injection. All samples were determined in triplicate.

Preparation of crude MFAs from desilked silkworm pupae oil

Crude desilked silkworm pupae oil (100 g) was saponified by refluxing for 1 h at 65℃. This was done under the protection of nitrogen, using a mixture of NaOH (1.6 g) and 95% (v/v) aqueous ethanol (400 ml) until the whole mixture turned into a clear homogeneous solution. The rest ethanol was recovered under reduced pressure. To dissolve the saponified mixture, distilled water (100 ml) was added and the aqueous layer containing saponified matter was acidified (pH = 3 - 4) with 10% HCl. The mixture solution was shaken and held on for 15 min; then water layer was emitted. Petroleum ether (60 - 90℃) 100 ml was added to dissolve oil layer. Distilled water (100 ml × 3) was added to wash the oil layer until it turned neutral. The petroleum ester layer, containing free fatty acids, was then dried over anhydrous magnesium sulphate and the solvent was removed at 50℃ to recover free fatty acids (65 g).

Enrichment process of ALA from crude MFAs by urea clathration method

Some urea and alcohol were mixed in the three-mouth 500 ml flask in certain primary freezing temperature scope. Crude MFAs were dissolved by alcohol (10 ml) and then added to additional funnel to slowly flow into the mixture in 10 - 15 min. The rest oil was washed by alcohol (95%, 10 ml). Meanwhile, the mixture was stirred to keep the certain temperature for some time, until the solution was clear under the slow flow rate of the nitrogen. Then the mixture was transferred into conical flasks when the reaction finished.

Experimental design and optimization by response surface methodology

The initial assays were based on a 3^3 factorial design, with three different ratios of saturated urea/EtOH solution to MFAs, as well as three different clathration reaction temperatures and times. The composition of the model was established from these preliminary assays (Wang et al., 2009). The model composition corresponded to an orthogonal 3^3 design, following the methodology of Box-Behnken response surface design. For statistical calculation, the variables were coded according to Canettieri et al. (2007) and Shah et al. (2004). The statistical model was based on the RSM whose equation was determined by analysis of linear multiple regression, using the software STATISITICA 6.0 (Statsoft, USA). The purity of ALA, produced by urea clathration method from crude MFAs of desilked silkworm pupae oil was taken as the dependent variable or response of the design experiments. The statistical significance of the regression coefficients was determined by T test. The variables were correlated by empirical models.

RESULTS AND DISCUSSION

Crude MFAs in desilked silkworm pupae determined by HPLC-UV

On the basis of the UV/VIS, spectra of the derivates of MFAs from desilked silkworm pupae oil reacted with bromoacetophenone and triethylamine by the UV detector in the range of 190 to 800 nm. Experimental results indicate that the PUFAs have the same UV absorption peaks about 242 nm; so 242 nm was selected for monitoring. Figure 1 shows the HPLC chromatographic profiles of the derivates of ALA and crude MFAs sample from desilked silkworm pupae oil was detected at 242 nm.

The chromatograms of the derivates of ALA and crude MFAs sample from desilked silkworm pupae oil is shown in Figure 1; peak shape of the derivates of MFAs in the sample from desilked silkworm pupae oil was symmetrical. Under the optimized isocratic elution conditions, the derivates of crude MFAs from desilked silkworm pupae oil reacted with bromoacetophenone. Triethylamine was separated with resolution greater than gradient elusion model when acetonitrile: water (90:10, v/v) was used as mobile phase at a flow rate of 1.0 ml·min^{-1}. Each component in the samples analyzed was identified by comparing its retention time with that of respective standard and those data reported in literature (Mehta et al., 1998). Retention time for the derivates of ALA (α-C18:3): C18:2, C16:0, C18:1 and C18:0 in crude MFAs from desilked silkworm pupae oil were 8.9, 11.1, 13.7, 14.5 and 19.6 min, respectively. Quantification was carried out by integration of the peaks, using external standards. These results show that MFAs from desilked silkworm pupae oil are mainly composed of five components: α-C18:3, C18:2, C16:0, C18:1 and C18:0.

Calibration data for ALA

Peak of the derivate of ALA can be well separated from other derivates of crude MFAs components from desilked silkworm pupae oil. ALA in the samples analyzed was identified by comparing its retention time with its standard. Quantification was carried out by integration of the peaks using external standards (Figure 2).

The results of calibration data for ALA are shown in Figure 2. Response (peak area, Y) was then plotted against concentration (X, mg) and the five-point calibration curves were found to be linear, as least squares regression gave a correlation coefficient. The equation of ALA was $Y = 2.73*10^9 X - 6.55*10^5$ ($r^2 = 0.9936$), which indicated that the developed analytical method is precise and sensitive for determining the content of ALA in test samples.

Process optimization for the enrichment of ALA from crude MFAs in desilked silkworm pupae oil by urea clathration method and validation of statistical model

Preliminary studies were performed in order to determine the required parameters ranges for the enrichment process of ALA from crude MFAs in desilked silkworm pupae oil. The results show that the ratio of saturated urea /EtOH solution to MFAs, clathration temperature and clathration temperature were three key process parameters for enrichment of ALA from crude MFA in desilked silkworm pupae oil. In the comprehensive consideration of the enrichment process parameters of ALA, 1.0:1 - 2.0:1, 0 - 8℃ and 1 - 3 h were selected the most suitable process parameters range for the enrich- ment of ALA from crude MFA in desilked silkworm pupae oil. The coded values of independent variables are given in Table 1.

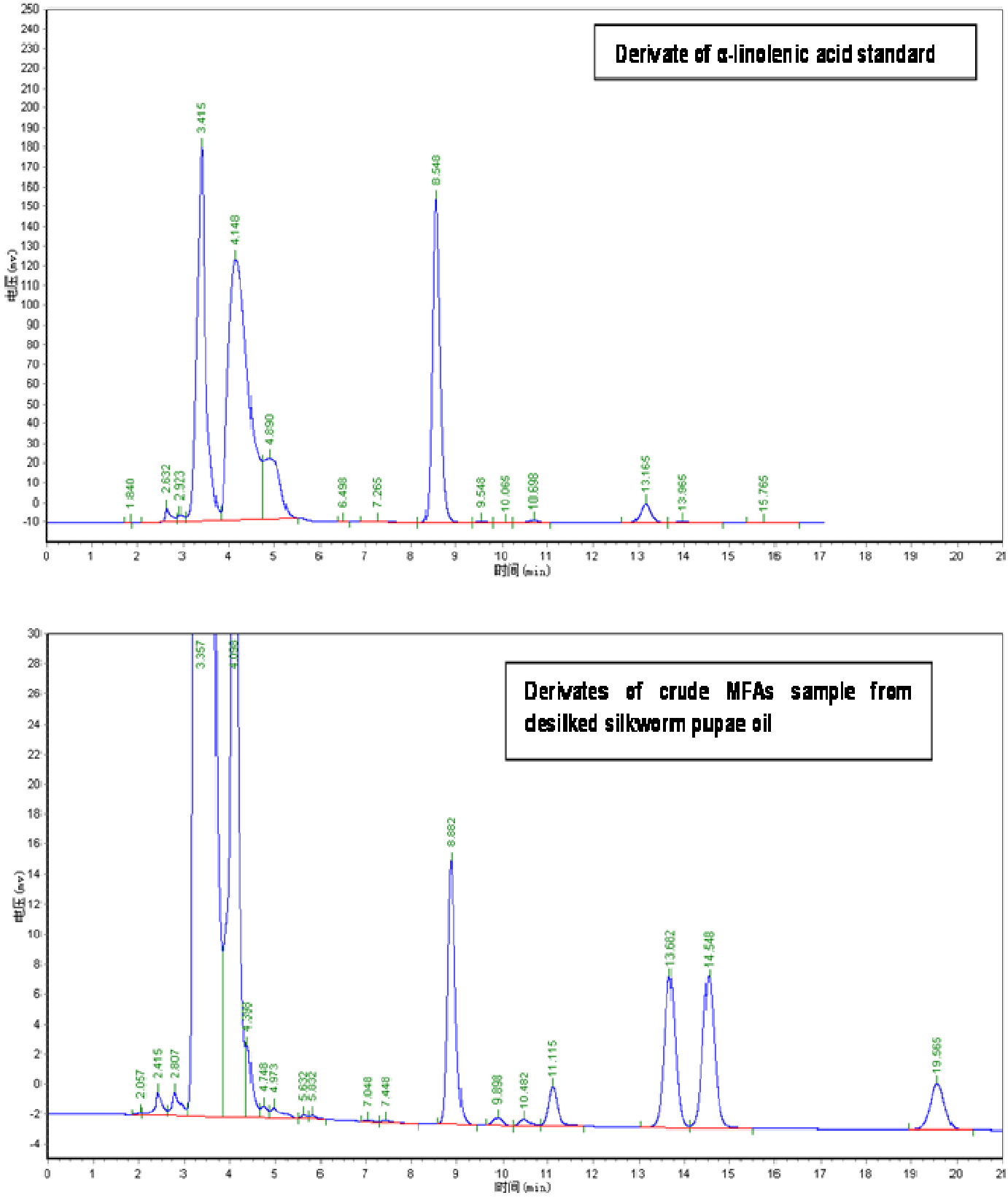


Figure 1. HPLC-UV profiles of the derivates of ALA standard and crude MFAs sample from desilked silkworm pupae oil. Retention time for the derivates of ALA (α-C18:3), C18:2, C16:0, C18:1 and C18:0 in crude MFAs from desilked silkworm pupae oil were 8.9, 11.1, 13.7, 14.5 and 19.6 min, respectively.

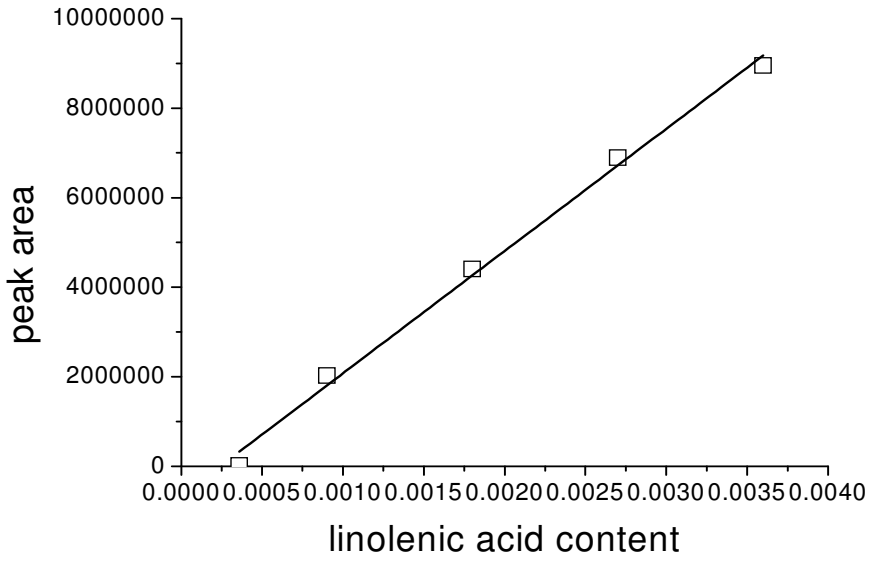


Figure 2. Calibration data for ALA (n = 5).

Table 1. Coded values of independent variables.

No.	Variables	Coded values		
		- 1	0	1
1	Clathration temperature (°C)	0	4	8
2	Clathration time (h)	1	2	3
3	Ratio of saturated urea /EtOH solution to MFAs	1:1	1.5:1	2:1

The design of experiments through urea clathration method and the respective experimental and predicted purity of ALA from crude MFAs in desilked silkworm pupae oil are given in Table 2. The results on estimated effects, standard errors (SE), T test and significance level for the model representing purity of ALA from crude MFA in desilked silkworm pupae oil are presented in Table 2.

Data were analyzed by non-linear multiple regression, using STATISTICA software (Statsoft, v. 6.0). After regression analysis, the second-order response model of purity of ALA was obtained which is given Equation (1).

$$Y\ (\%) = -31.865 + 4.9625X_1 + 5.225X_1^2 + 0.0325X_2 + 7.765X_2^2 + 1.0325X_3 + 0.49X_3^2 - 3.075X_1X_2 + 4.05X_1X_3 - 1.415X_3X_2 \quad (1)$$

The simple model expressed by Equation (2) was generated as:

$$Y\ (\%) = -31.865 + 4.9625X_1 + 5.225X_1^2 + 7.765X_2^2 - 3.075X_1X_2 + 4.05X_1X_3 \quad (2)$$

A low value of the coefficient of variation indicates the very high degree of precision and a good reliability of the experimental values (Del Castillo et al., 1996). The fit of the model was also expressed by the coefficient of determination R^2, which was found to be 0.9674, indicating that 96.74% of the variability in the response could be explained by the model. The solution was obtained by submitting the levels of the factors into the regression equation.

The ANOVA for response surface quadratic model is summarized in Table 3. The model F-value of 16.47 and the low P-value of 0.00068 implied the model was extremely significant.

To determine the most adequate operating conditions and analyze the enrichment process of ALA, the response surfaces were plotted using Equation (3) for three possible combinations, the response surface and contour diagrams of purity of ALA as a function of : (a) X_1 and X_2, (b)X_1 and X_3, (c) X_2 and X_3 are presented in Figure 3.

The simultaneous analysis of so many plots is a complex task, if practical short cuts, taking advantage of prior knowledge of the process, are not adopted (Myers et al., 2004). In Figure 3, the main factors affecting purity of ALA from MFAs in desilked silkworm pupae oil by urea clathration method were the ratio of saturated urea/EtOH solution to MFAs, clathration reaction temperature and

Table 2. Response surface methodology (RSM) design of independent variables and their corresponding experimental and predicted purity of ALA from crude MFAs in desilked silkworm pupae oil.

No.	X_1	X_2	X_3	Purity of ALA (%) experimental*	Purity of ALA (%) predicted	Yield of ALA (%) experimental*
1	-1	- 1	0	27.30	26.95	40.90
2	1	-1	0	12.60	10.87	30.60
3	- 1	1	0	19.00	20.73	46.00
4	1	1	0	16.60	16.96	41.40
5	- 1	0	- 1	28.00	28.10	38.90
6	1	0	- 1	24.80	26.27	40.20
7	- 1	0	1	35.60	34.13	45.60
8	1	0	1	16.20	16.11	49.80
9	0	- 1	- 1	23.00	23.26	41.00
10	0	1	- 1	27.85	26.03	41.30
11	0	- 1	1	22.20	24.03	44.20
12	0	1	1	21.39	21.13	44.60
13	0	0	0	31.63	31.87	49.80
14	0	0	0	33.30	31.87	44.17
15	0	0	0	30.67	31.87	40.40

* Values indicate mean of dupilicate observations.

Table 3. Variance analysis of regression equation.

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*P*-value
Model	619.95	9	68.88	16.47	0.00068
Residual	20.92	5	4.18		
Lack of fit	17.37	3	5.79	3.27	0.024
Pure error	3.54	2	1.77		
Total	640.87	14			
R-Squared = 0.9674, Adjusted R-Squared = 0.9086					

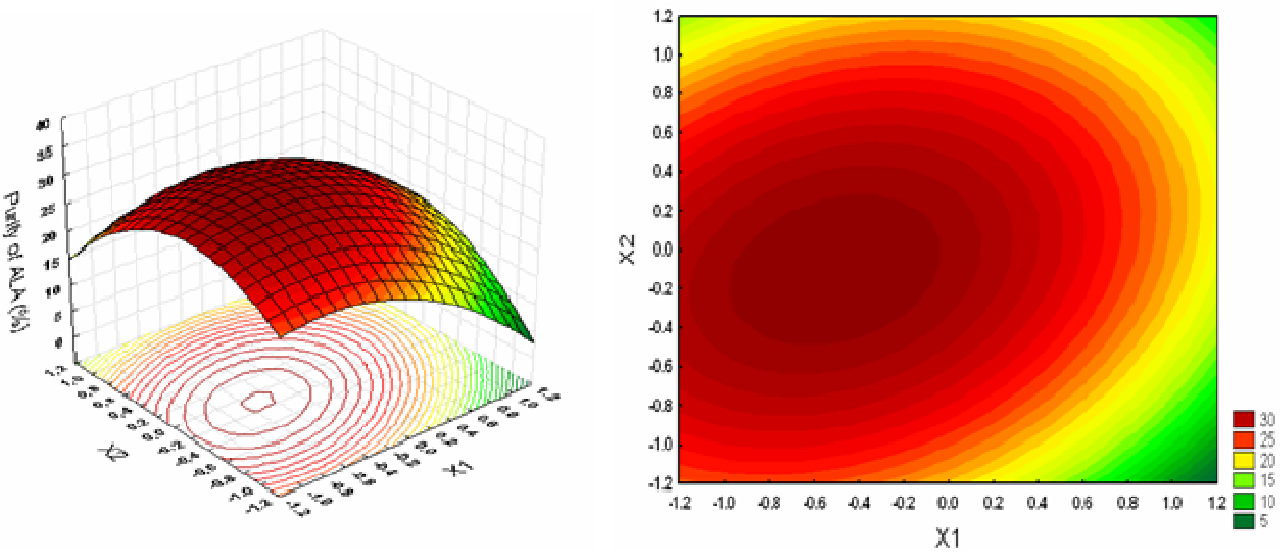
time, respectively. For example, from Figure 3 it can be seen that low temperature and time to MFAs in the process of urea clathration reaction lead to a low purity of ALA. Therefore, clathration reaction temperature and time can be selected at an appropriate value range.

The optimum values were found by solving the regression equation analytically (Shah et al., 2004; Wang et al., 2009). The solution was obtained by submitting the levels of the factors into the regression equation (Equation (1)). In view of the yield of ALA (Table 2), the optimal clathration reaction conditions for the enrichment of ALA from desilked silkworm pupae oil were calculated as follows: clathration temperature, 4℃; clathration time, 2 h and the ratio of saturated urea/EtOH solution to MFAs, 2:1. The predicted response (35.60%) was experimentally verified (31.87%, n = 3). The agreement between predicted value and experimental value of purity of ALA confirms the significance of the model. This indicated that, in addition to establishing optimal conditions for operation, the present methodology also makes it possible to predict purity of ALA when the system is disturbed in some way. This is useful not only for the additional knowledge supplied about the process, but also for the potentials for process control. Therefore, under optimal conditions of urea clathration reaction it is inevitable to have a large increase in purity of ALA.

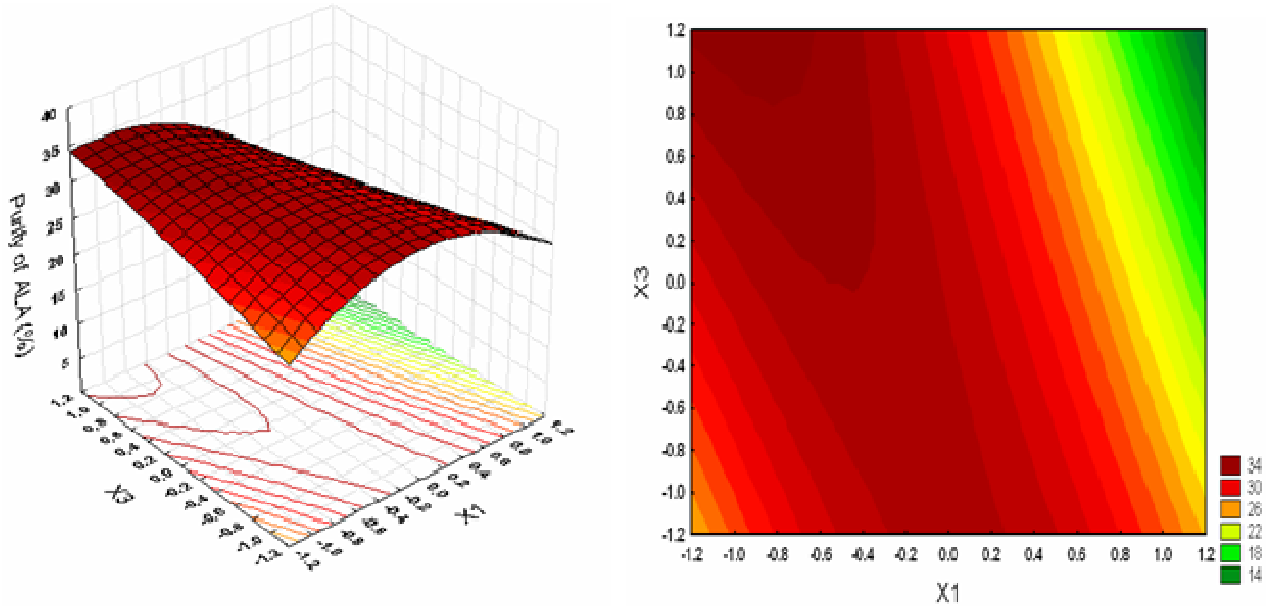
Model adequacy checking

In order to check approximation between the fitted model and actual enrichment process system, the residuals from the least squares fit were investigated to judge model adequacy. The plots of residuals versus the predicted values were depicted in Figure 4 and the correlation analysis of the observed values and predicted values calculated by developed RSM model is shown in Figure 5.

The residuals were distributed randomly on the display, indicating that the variance of the original experimental results was constant for all values of responses, which was preferably based on the judge of adequacy (Canettieri et al., 2007). As a result (Figure 4), it can be concluded that



(a) X_1: clathration reaction temperature (˚C) , X_2: clathration reaction time (h).



(b) X_1: clathration reaction temperature (˚C), X_3: the ratio of saturated urea /EtOH solution to MFAs.

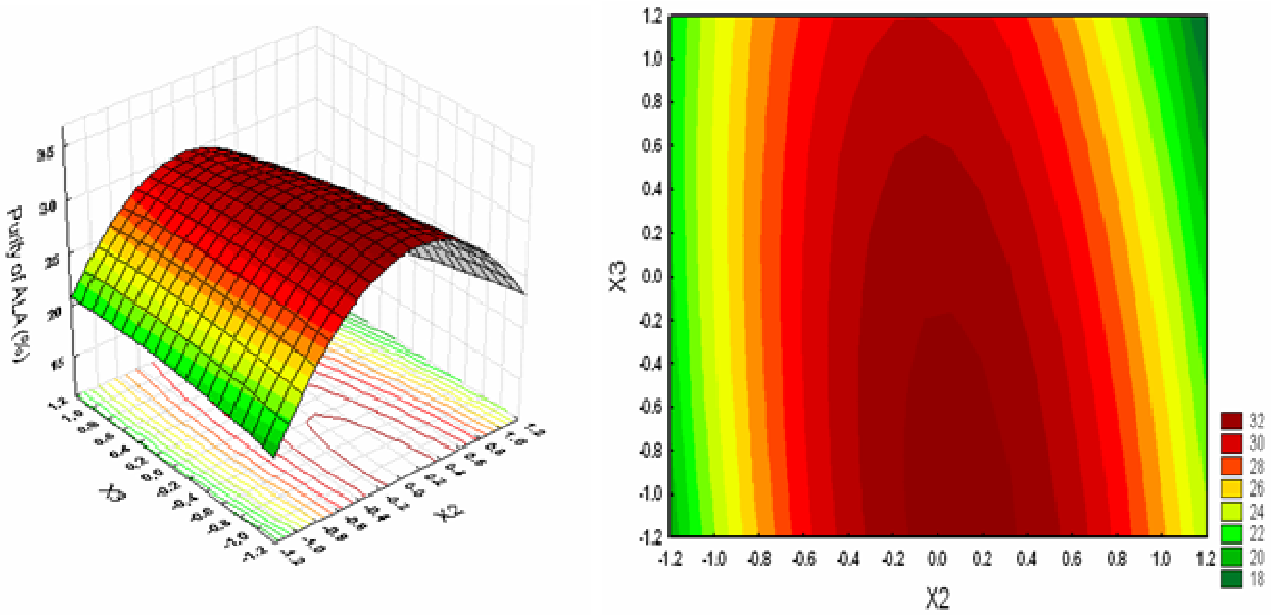
this fitted model is adequate to reveal the enrichment process characteristics of ALA from desilked silkworm pupae oil by RSM.

Figure 5 represented the comparative parity plot between the observed response and the predicted response for RSM prediction. The distribution of scattered points reflected how accurately the predicted response explained the observed response.

Figure 5 showed that the correlation coefficient of scattered points in RSM prediction approached is very high. As a result, the RSM model fitted the experimental data with an excellent accuracy.

Conclusion

The optimal enrichment process of ALA from desilked silkworm pupae oil with urea clathration method was successfully achieved by RSM in this study. Three process parameters for enrichment of CA were optimized as follows by using CCRD of RSM: the ratio of saturated urea /EtOH solution to MFAs = 2:1 (V/V); clathration temperature,



(c) X_2: clathration reaction time (h), X_3: the ratio of saturated urea /EtOH solution to MFAs

Figure 3. Response surface and contour diagrams of purity of ALA as a function of: (a) X_1 and X_2, (b) X1 and X_3, (c) X_2 and X_3.

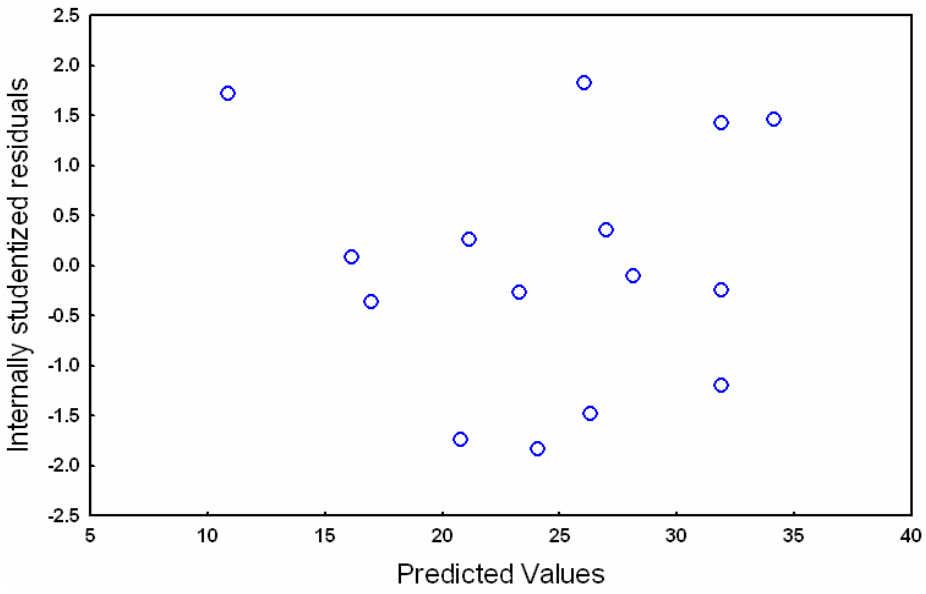


Figure 4. Plot of Internally studentized residuals versus predicted values.

4℃ and clathration time, 2 h; the adequately high R^2 value, 0.9674 (Adjusted R^2 = 0.9086) which indicated the statistical significance of the model. The purity of ALA after optimization was 31.87% while predicted value, 35.60%. RSM as an effective method proved to be quite adequate for the design and optimization of the enrichment process of ALA from desilked silkworm pupae oil and the developed RSM model was constant for all values of responses.

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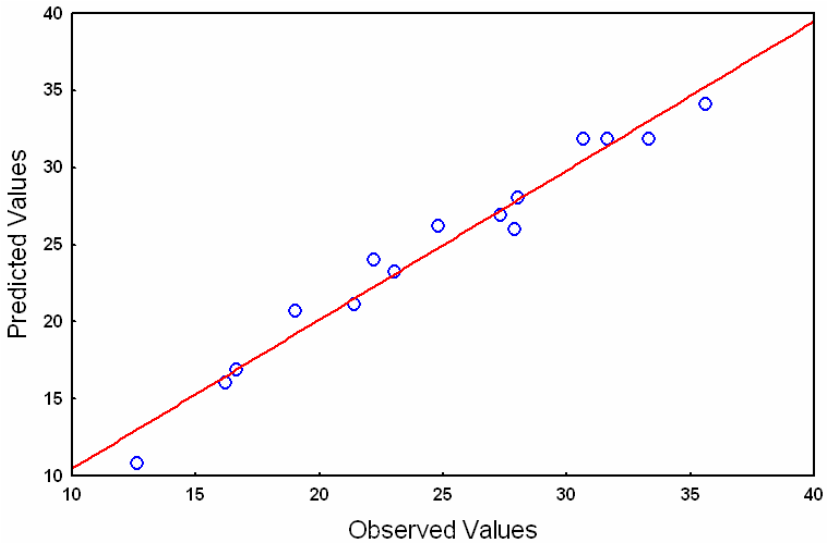

Figure 5. Correlation of testing RSM model.

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