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Optimization of Production of Conjugated Linoleic Acid from Soybean Oil

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Linoleic acid from soybean oil was used to synthesize conjugated linoleic acid (CLA), and the response surface methodology (RSM) was applied to optimize the process. A temperature of −35 °C and a solvent to oil sample ratio of 8 were suggested for removal of saturated fatty acids by low-temperature crystallization. The ratio of oil sample/urea/methanol suggested was 1:2:5.5 (w/w/v) for removal of oleic acid by urea crystallization. A temperature of 150 °C and a time of 140 min were found to be the optimal conditions in the isomerization for the production of c-9,t-11 and t-10,c-12 CLA isomers.

KEYWORDS: Conjugated linoleic acid (CLA); soybean oil; response surface methodology (RSM); low-temperature crystallization; urea crystallization; isomerization; linoleic acid

INTRODUCTION

Conjugated linoleic acid (CLA) is a mixture of positional and geometrical isomers of linoleic acid (LA) with a conjugated double system. It exhibits many biological effects such as anticarcinogenic activity (*1*−*3*), immune stimulation (*4*), body fat reduction (*5*), cholesterol reduction in blood (*6*), and lowering atherosclerosis (*7*). CLA is widely found in various foods, including dairy products, meat and meat products, certain vegetable products, and some seafoods, its contents ranging from 0.01 to 1.7% (*8*). CLA can be formed through the isomerization of linoleic acid (LA) by bacterium (*9*), oxidation of LA by free radicals (*10*), and heat treatment (*1*). Synthetic methods are commonly used to produce CLA in a large quantity (*11*, *12*). In these methods, LA could be isomerized to form CLA under alkali conditions. Synthetically prepared mixtures contain predominantly the c-9,t-11 CLA (where c = cis and t = trans) and t-10,c-12 CLA isomers in approximately equal amounts (*11*, *13*). It has generally been thought that the c-9,t-11 CLA is the biologically active isomer because of its greater natural abundance in our current food supply and its preferential incorporation into cellular lipid (*2*, *3*).

LA-rich seed oils are good sources for the production of CLA. Safflower, corn and cottonseed oils have been used to isolate LA (*14*). Safflower oil is most commonly used to obtain LA, which is subsequently converted into CLA (*11*, *12*). Safflower oil is relatively expensive and not widely available. Comparatively, soybean oil, the most largely consumed oil in the world, is also a LA-rich oil. Although its LA content is not as high as that of safflower oil, it is cheap and widely available. The use of soybean oil to produce CLA has not yet been studied.

To obtain LA from LA-rich oils, the LA must be released from the triacylglycerols, separated, and then purified. Low-temperature crystallization (*14*), urea precipitation (*11*, *12*), and column chromatography (*15*) were used alone or in combination to achieve this purpose. To our knowledge, to date, the reported studies of the isolation of LA and synthesis of CLA have been based on one-factor-at-a-time experiments. The results of one-factor-at-a-time experiments often ignore interactions between factors that are present simultaneously (*16*). Response surface methodology (RSM) is a set of mathematical and statistical methods for modeling phenomena and finding combinations of experimental factors that will lead to optimum responses (*17*). The present work aims at producing CLA from soybean oil using RSM to optimize the process.

MATERIALS AND METHODS

Materials. Soybean oil was obtained from a local oil company (Uni-President Food Co.). Chemicals, solvents, and CLA standards were purchased from Sigma Chemical Co. (St. Louis, MO). The chemicals and solvents were of the purest reagent grades.

Saponification of Soybean Oil. Soybean oil (500 g) was refluxed for 1 h with a solution of potassium hydroxide (115 g) in ethanol (400 mL) and water (125 mL). After partial cooling and addition of crushed ice (0.5 L) followed by sulfuric acid (4 M, 600 mL), the mixture was transferred to a separatory funnel (*18*). After separation, the aqueous lower layer was extracted with ether (200 mL). The ether extract was combined with the major organic layer and washed with water (2 × 100 mL). The solvent was subsequently removed on a rotary evaporator, and then the extract was placed in a vacuum oven and maintained at 50 °C for 5 h to remove the residual solvent (**Figure 1**).

Experimental Designs and Statistical Analyses. A central composite rotatable design (CCRD) coupled with response surface meth-

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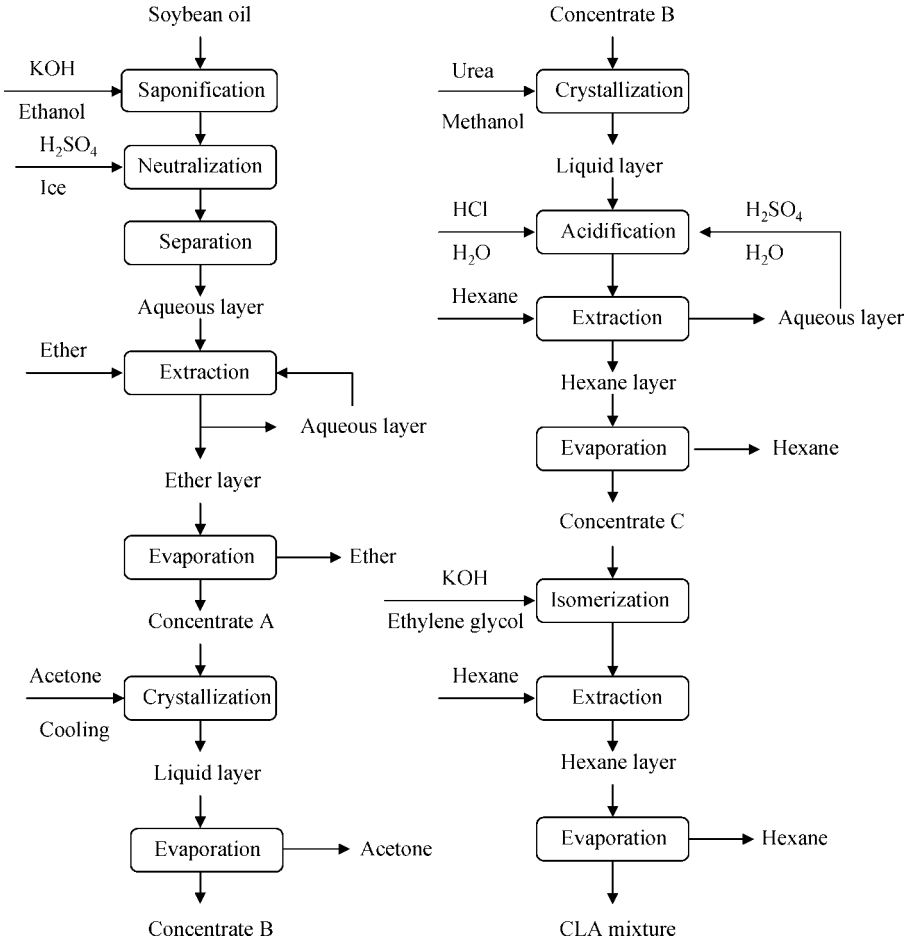

Figure 1. Flowchart for the production of conjugated linoleic acid from soybean oil.

Table 1. Coded and Decoded Levels of the Parameters Used in the Experimental Design for Low-Temperature Crystallization, Urea Crystallization, and Isomerization of Linoleic Acid

	low-temp crystallization		urea crystallization		isomerization of linoleic acid	
coded level[a]	temp (°C)	acetone (mL)	urea (g)	methanol (mL)	temp (°C)	time (min)
(–1)	–50	120	10	55	135	120
(+1)	–20	200	30	105	165	180
(–√2)	–56	103.4	5.9	44.7	129	108
(+√2)	–14	216.6	44.1	115.4	171	192
0	–35	160	20	80	150	150

[a] A two-factor and second-order central composite rotatable design was used. The design consists of three portions of data points in the coded matrix: (1) cube points corresponding to the levels of (–1) and (+1), (2) star points corresponding to (-√2) and (+√2), and (3) center points corresponding to (0) and (0) for each factor.

odology (RSM) (*19*) was employed to study the effects of low-temperature crystallization of saturated fatty acids, urea crystallization of oleic acid, and alkali isomerization of LA to CLA. The statistical analyses were conducted using Statistica for Windows (StatSoft, Tulsa, OK).

Low-Temperature Crystallization of Saturated Fatty Acids. A portion of saponified and solvent-removed oil (20 g) was mixed with acetone and stored in a refrigerator overnight. The temperature and acetone volume used followed the experimental designs in **Table 1**. The solution was filtered through a Büchner funnel kept at each corresponding storage temperature. The fatty acids were recovered from the filtrate by evaporating the solvent on a rotary evaporator, and then the fatty acids were placed in a vacuum oven and maintained at 60 °C for 5 h to remove the residual solvent (**Figure 1**).

Urea Crystallization of Oleic Acid. LA extracts (5 g) were crystallized in urea dissolved in warmed methanol. The temperature and methanol volume used followed the experimental designs in **Table 1**. The mixture was heated to effect complete solution, and then it was allowed to cool slowly to room temperature. The well-defined needles of urea complexes were put in a refrigerator overnight at 5 °C. Vacuum filtration was used to recover purified LA contained in the mother liquor. The mother liquor was transferred to a separatory funnel and acidified to pH <2 with HCl (6 N, 200 mL) and deionized water (dH_2O) (200 mL). LA was extracted with hexane (100 mL). The aqueous phase was reacidified with sulfuric acid (6 N, 50 mL) and dH_2O (30 mL), followed by extraction with hexane (100 mL). Hexane fractions were combined. The hexane phase was washed with 30% (v/v) methanol/dH_2O (3 × 50 mL) and then with dH_2O (3 × 50 mL). The washed hexane phase was dried over anhydrous sodium sulfate, and the hexane was removed using a rotary evaporator (**Figure 1**) (*12*).

Alkali Isomerization of LA to CLA. Ethylene glycol (100 g) was added to a flat-bottom flask (500 mL) equipped with a branched-hollow stopper. Nitrogen was bubbled through the ethylene glycol. The flask was placed in an oil bath and heated to the designated temperature. The flask was removed from the oil bath, and 26 g of KOH was added with magnet stirring; then the flask was reheated to the previous temperature. As soon as the KOH was dissolved and evenly mixed, the flask was removed from the oil bath and 50 g of LA-containing sample was added. The flask was replaced in the oil bath and reheated to the designated temperature and time as shown in **Table 1**. The flask was removed from the oil bath and cooled to room temperature with cold tap water. Methanol (100 mL) was added. The solution was transferred to a separatory funnel and acidified (pH <2) with 125 mL of 6 N HCl. After dilution with 100 mL of dH_2O, the CLA was extracted with 100 mL of hexane. The hexane extract was washed with 30% methanol in dH_2O (3 × 100 mL) and then with dH_2O (3 × 100 mL) (*20*). Anhydrous sodium sulfate was added to remove water. The

Table 2. Effects of Temperature and Acetone Volume on the Removal of Palmitic Acid and Loss of Linoleic Acid by Low-Temperature Crystallization

	palmitic acid		linoleic acid	
factor[a]	effect[b]	*p* value	effect[b]	*p* value
temp (L)	0.0841**	0.0002	4.09**	0.0002
temp (Q)	0.0201	0.1278	−2.93**	0.0025
acetone (L)	0.0136	0.2261	1.06	0.0883
acetone (Q)	−0.0085	0.4795	0.39	0.5250
temp (L) × acetone (L)	0.027	0.8539	−1.16	0.1696

[a] L, linear term; Q, quadratic term. [b] **, significant effects at $p < 0.01$.

hexane was removed under vacuum rotary evaporation (**Figure 1**). The CLA was stored under refrigeration at −20 °C until use.

Fatty Acid Analysis. The fatty acids were methylated in 4% HCl−methanol at 60 °C for 20 min (*20*), and their methyl esters were analyzed by gas chromatography (GC). A Hitachi G-3000 gas chromatograph equipped with a flame ionization detector (Tokyo, Japan) and an SP-2560 (Supelco, Bellefonte, PA) fused-silica capillary column (100 m × 0.25 mm i.d.) was used. The carrier gas was hydrogen, and the column flow was 1 mL/min. The injector and detector temperatures were 250 and 300 °C, respectively. The oven temperature was programmed from 180 to 220 °C at a rate of 1 °C/min (*21*). The computer software (SISC Chromatography Data System, Davis, CA) was used for the integration and calculation of the peak areas in the gas chromatograms. The fatty acids were identified and quantified by comparing their retention times and peak areas with those of the corresponding standards.

RESULTS AND DISCUSSION

Low-Temperature Crystallization of Saturated Fatty Acids. Low-temperature solvent fractionation has been used to separate higher melting triacylglycerols (TAG) from complex mixtures of TAG because TAG molecules can form more stable crystals within shorter periods (*22*). Acetone and hexane are frequently used solvents for solvent fractionation of fats or oils, of which acetone is most extensively used because different numbers of saturated acyl groups and double bonds of TAG exhibit different solubilities in this solvent (*23*). In this study, acetone was used as a solvent to remove saturated fatty acids according to the low-temperature crystallization method.

The fatty acid composition of soybean oil used in this work is the following: C16:0 (11.88 ± 0.27%), C18:0 (4.61 ± 0.32%), C18:1 (22.89 ± 0.21%), C18:2 (53.81 ± 0.25%), and C18:3 (6.81 ± 0.01%). Palmitic acid (PA, C16:0) and stearic acid (C18:0) are the main saturated fatty acids in soybean oil. After low-temperature crystallization, the stearic acid could be removed completely at the temperature below −14 °C on the basis of GC analyses. The contents of PA and LA in the mother liquor were used as indicators of the effectiveness of saturated fatty acid removal and the loss of LA in this process. **Table 2** shows the statistical analyses of ANOVA on the effects of temperature and acetone volume on the removal of saturated fatty acids and the loss of LA. For the effect on the removal of saturated fatty acids, the linear term of temperature [temperature (L)] had more of an effect than the quadratic term [temperature (Q)], and it was significant at $p < 0.01$. The positive effect meant when the temperature was increased, more PA would be retained in the mother liquor. The effect of acetone volume at either linear term [acetone (L)] or quadratic term [acetone (Q)] was not significant ($p > 0.05$). The high *p* value (0.853) of the product of [temperature (L) × acetone (L)] indicated that no interaction effect was significantly present between these two parameters at $p < 0.05$.

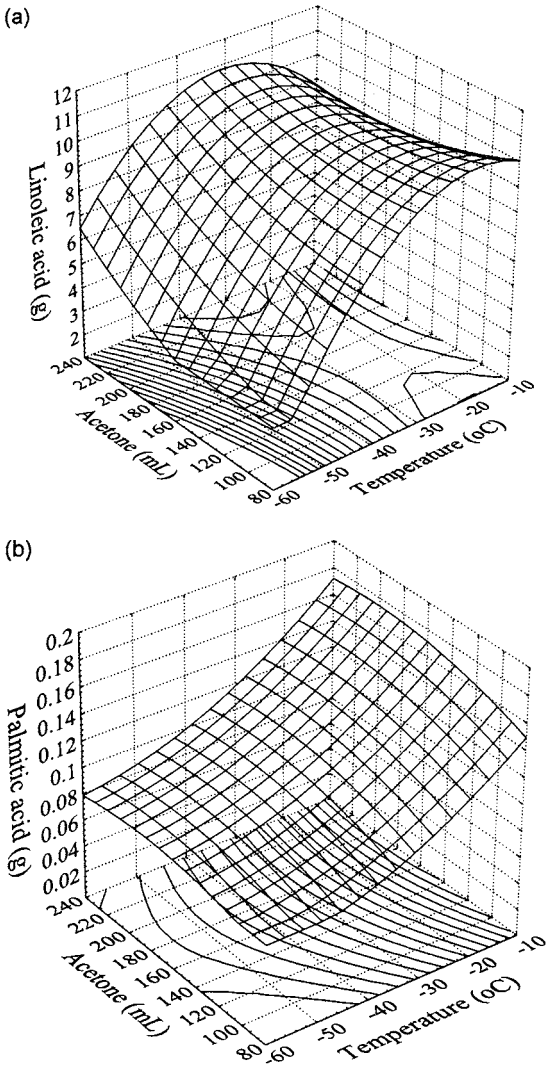

Figure 2. Response surfaces and contour plots of the contents of linoleic acid (**a**) and palmitic acid (**b**) in the mother liquor with different treatments of temperature and acetone volume.

On the other hand, for the effect on the loss of LA, both temperature (L) and temperature (Q) had significant effects, and the former had more influence on the loss of LA. Therefore, the net positive effect indicated that more LA would stay in the mother liquor as the temperature increased. The effect of acetone (L) or acetone (Q) was not significant at $p < 0.05$, but acetone (L) was significant at $p < 0.1$. However, the *p* value of acetone (Q) was 0.52. This revealed that acetone (L) had a greater effect than acetone (Q). In contrast to acetone (L) on the PA removal ($p = 0.22$), the effect of acetone (L) on the loss of LA ($p = 0.08$) was significant at $p < 0.1$. This result showed that acetone volume had more of an effect on the loss of LA than on the removal of PA at $p < 0.1$. No interaction effect of temperature and acetone volume [(temperature (L) × acetone (L)) on the loss of LA was observed because of the *p* value (0.16) >0.05.

From **Figure 2a**, the trend was observed that the higher temperature and the more solvent were used, the more LA would be retained in the mother liquor. On the contrary, the higher temperature and the more solvent were used, the less PA could be removed from the mother liquor (**Figure 2b**). Therefore, a compromise must be reached when suitable conditions are determined. In the present study, a temperature of −35 °C and a solvent to oil sample ratio of 8 were suggested. Under these

Table 3. Effects of Urea Amount and Methanol Volume on the Removal of Oleic Acid and Loss of Linoleic Acid by Urea Crystallization

	oleic acid		linoleic acid	
factor[a]	effect[b]	*p* value	effect[b]	*p* value
urea (L)	–0.6970**	0.0005	–1.9172**	0.0000
urea (Q)	0.3006**	0.0041	0.5578**	0.0007
methanol (L)	0.2223	0.0770	0.6610**	0.0031
methanol (Q)	0.0041	0.9734	–0.1879	0.2785
urea (L) × methanol (L)	–0.4032*	0.0339	–0.1795	0.3972

[a] L, linear term; Q, quadratic term. [b] *, significant effects at $p < 0.05$; **, significant effects at $p < 0.01$.

conditions, 98% of PA could be removed, and the content of LA in the mother liquor was 70%. The residual PA could be completely removed at the next urea crystallization stage.

Urea Crystallization of Oleic Acid. Urea can form crystalline inclusion compounds with fatty acids. The formation of inclusion compounds with fatty acids depends on their degree of unsaturation; the more unsaturated, the less will be the likelihood of their inclusion into the urea crystals (*23*). In this work, urea was used to separate oleic acid (OA) from LA. The OA and LA contents in the mother liquor were used to evaluate the effectiveness of OA removal and the loss of LA, respectively. For the effect on the removal of OA, **Table 3** shows that the linear term of urea amount [urea (L)] and the quadratic term [urea (Q)] had significant effects ($p < 0.01$). The net negative effect of urea amount meant when the urea amount increased, more OA would be removed from the mother liquor. In contrast, the linear term of methanol volume [methanol (L)] or quadratic term [methanol (Q)] did not show a significant effect ($p > 0.05$). The product of [urea (L) × methanol (L)] was significant at $p < 0.05$, which meant there was an interaction effect between these two variables. The results indicated urea amount was the primary determining factor for the removal of OA.

For the effect on the loss of LA, urea (L), methanol (L), and urea (Q) had significant effects ($p < 0.05$) on the loss of LA. Of them, urea (L) had the most effect followed by methanol (L) and urea (Q). The net negative effect of urea amount meant when the amount of urea was increased, more LA would be lost from the mother liquor, whereas the positive effect of methanol (L) indicated that the greater methanol volume was used, the more LA would be retained in the mother liquor. By summing these effects, it was showed that urea amount had more of an effect than methanol volume on the loss of LA. No interaction effect of urea amount and methanol volume [urea (L) × methanol (L)] on the loss of LA was found as indicated by the *p* value (0.39) >0.05.

The ratio of methanol volume to urea amount used in the urea crystallization of LA was a fixed value in most studies (*12*, *18*, *24*). To our knowledge, the changing effects of methanol volume to urea amount have not been reported. In this study, methanol volume showed a significant effect on the loss of LA. In addition, urea amount and methanol volume also exhibited an interaction effect on the removal of OA. From **Figure 3b**, the more urea used, the more OA could be removed, but simultaneously the more LA would be included in the urea complex and lost from the mother liquor as shown in **Figure 3a**. By overlapping the two contour plots in **Figure 3** and considering the yield and cost, the ratio of oil sample (grams) to urea (grams) to methanol (milliliters) suggested was 1:2:5.5. Under these conditions, the yield of LA-containing oil was 54%

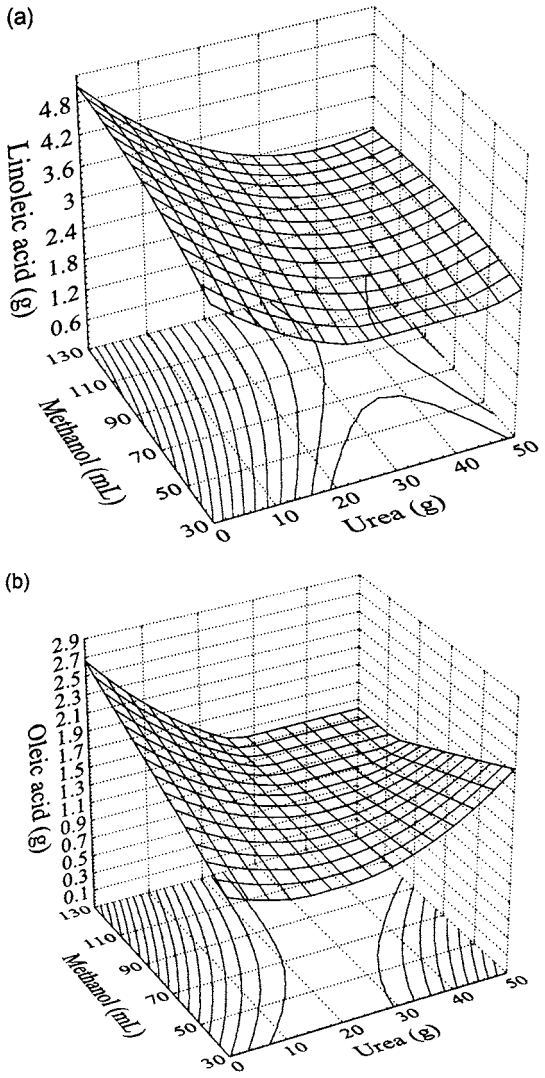

Figure 3. Response surfaces and contour plots of the contents of linoleic acid (**a**) and oleic acid (**b**) in the mother liquor with different treatments of urea amounts and methanol volume.

and LA purity was 82.2% by urea crystallization once. More urea crystallizations might remove more OA and increase LA purity, but the yield would be greatly reduced.

Alkali Isomerization of Linoleic Acid to CLA. Temperature and time were the two main parameters used in many studies for chemical synthesis of CLA (*11*, *12*, *20*). In the present study, both variables were also used to evaluate their effects on the alkali isomerization of LA to CLA. c-9,t-11 CLA and t-10,c-12 CLA were two major isomers by chemical synthesis reported in the literature. Therefore, we observed the changes of these two isomers to determine the most suitable conditions to synthesize the CLA in this work. The contents of c-9,t-11 CLA and t-10,c-12 CLA mixture together and c-10,c-12 CLA alone in the isomerized oil samples were used as indicators for completion of the conversion of LA to CLA under different treatments of temperature and time. **Table 4** shows that the linear term of temperature [temperature (L)] did not show a significant effect on the formation of c-9,t-11 CLA and t-10,c-12 CLA, but the quadratic term [temperature (Q)] had a significant effect ($p < 0.01$). The negative effect meant when the temperature decreased, greater amounts of both isomers would be produced with the prerequisite that the temperature is sufficient to convert LA to CLA. In contrast, the linear term of time [time (L)] or the quadratic term [time (Q)] did not show

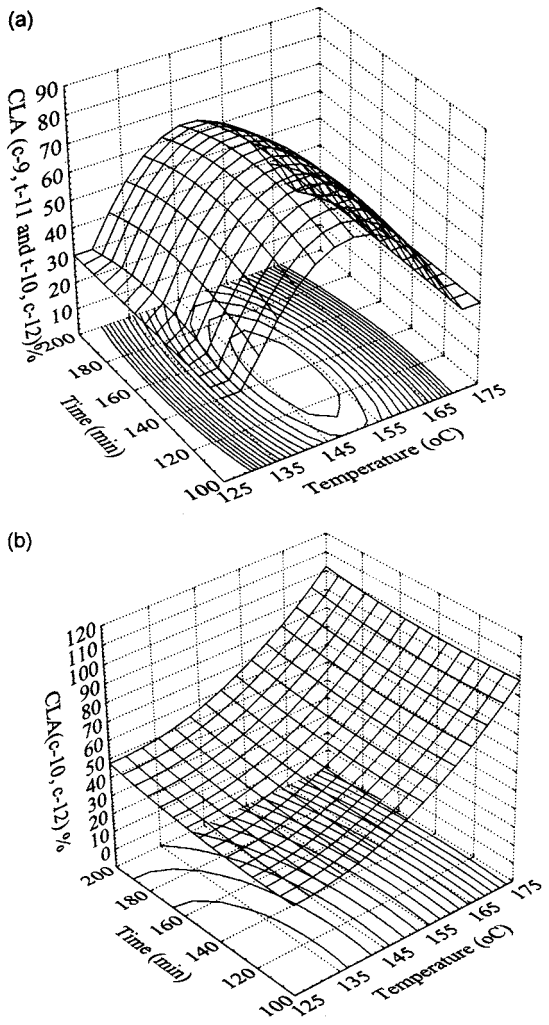

Figure 4. Response surfaces and contour plots of the contents of c-9,t-11 and t-10,c-12 CLA mixture (**a**) and c-10,c-12 CLA (**b**) in the oil samples after isomerization with different treatments of temperature and time.

Table 4. Effects of Temperature and Time on the Isomerization of Linoleic Acid to Conjugated Linoleic Acid (CLA)

	CLA (c-9,t-11 and t-10,c-12)		CLA (c-10,c-12)	
factor[a]	effect[b]	*p* value	effect[b]	*p* value
temp (L)	−8.3556	0.42	51.5803**	0.0005
temp (Q)	−62.0038**	0.0079	15.6711	0.0795
time (L)	−4.8480	0.6355	6.1404	0.2889
time (Q)	−7.4374	0.5873	3.2885	0.6493
temp (L) × time (L)	−5.2200	0.7151	−1.4100	0.8517

[a] L, linear term; Q, quadratic term. [b] **, significant effects at $p < 0.01$.

a significant effect ($p > 0.05$). The product of [temperature (L) × time (L)] was not significant at $p < 0.05$, which meant there was no interaction effect between these two variables. The results indicated that temperature, as shown by the relatively large negative value of temperature (Q) in **Table 4**, was the primary determining factor for the formation of these two isomers. From **Figure 4a**, the surface and contour plots show that the stationary point can be obtained. By statistical analysis, the critical values of temperature and time calculated were 150 °C and 140 min, respectively, and the predicted response (the total content of both isomers) was 65.5%. After regression analysis, the prediction model could be established by the function of $z = -3204.7279 + 41.9273x - 0.1378x^2 + 2.0287y - 0.0041y^2 - 0.0058xy$ (where z = response, x = temperature, and y = time). R^2 was 0.87. The "lack of fit" test was not significant at $p < 0.05$, which means the model is fit.

For the effect on the formation of c-10,c-12 CLA, temperature (L) had a significant effect ($p < 0.01$), but temperature (Q) did not show a significant effect ($p > 0.05$). The positive effect meant that when the temperature was increased, more c-10,c-12 CLA would be produced in the prerequisite as stated above. In contrast, neither time (L) nor time (Q) showed a significant effect ($p > 0.05$). The product of [temperature (L) × time (L)] was not significant at $p < 0.05$, which meant there was no interaction effect between these two variables. The results revealed that temperature was the primary determining factor for the formation of c-10,c-12 CLA, as shown by the relatively large positive value of temperature (L) in **Table 4**. The ratio of c-9,t-11 CLA to t-10,c-12 CLA by chemical synthesis reported in many studies was ~1 (*11*, *13*). However, the compositions of the CLA isomers would change at different temperature and time treatments (*11*). In this study, the ratio of these two isomers was similar to that of the results reported for temperatures of <160 °C. When the temperature and time were increased, both isomers decreased and the c-10,c-12 CLA increased as shown in **Figure 4b**. It has been reported that as the temperature was set at 180 °C and the time was increased, both methyl esters of c-9,t-11 CLA and t-10,c-12 CLA decreased and the *trans*-form CLA such as t-10,t-12 CLA increased. The length of reaction was reduced using a higher temperature, but the di-trans isomers increased and other isomers appeared (*11*). The highest temperature used in this work was relatively lower compared to the reported study (*11*); therefore, a cis−cis isomer such as c-10,c-12 CLA was the predominant isomer in our conditions, although other cis−trans or trans−trans isomers might also be present in lesser amounts. Longer time and higher temperature not only affected the composition of CLA isomers, but they also affected oil color during isomerization. We found that the oil color became dark as longer time and higher temperature were employed. Using the optimal conditions stated above in the isomerization, a CLA mixture could be obtained with a yield of 90.5 ± 2% based on the initial weight of LA-containing oil (urea treated) or 18.3 ± 1.4% based on the initial weight of soybean oil used.

In conclusion, optimal critical values could not be obtained from the contour plots in the low-temperature or urea crystallization as done in the isomerization due to some unknown or unexpected operating factors or reasons that have to be compromised such as yield, purity, cost, and color. The response surface methodology still provided a valuable means to help us understand the relative or interaction effects of the possibly influential parameters on the synthesis of CLA. In addition, RSM also helped us to achieve relatively optimal conditions in each complex process within our study range. Most importantly, the study provided useful information in using soybean oil to synthesize CLA.

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