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Response surface-optimized synthesis of *cis*-9,*trans*-11-octadecadienoic acid through dehydration of castor oil

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Abstract

Cis-9,*trans*-11-octadecadienoic acid (*9c,11t*-18:2), is the most abundant and biologically active isomer of conjugated linoleic acid that is of interest due to its anticarcinogenic and anticholesterolemic properties. To further study the metabolic pathways and physiological effects of *9c,11t*-18:2, it is desirable to obtain substantial amounts of this compound in a relatively pure form, ideally by simple methods from readily accessible materials. The optimization of reaction conditions (including reaction temperature, time and amount of catalyst) for *9c,11t*-18:2 production by dehydration of castor oil as starting material was investigated using response surface methodology (RSM). This optimization process was done by application of KOH as an inexpensive dehydrating reagent. It was found that the temperature of dehydration and amount of catalyst were the most effective factors in CLA isomer composition and yield. When the reaction temperature was increased, the yield of *9c,11t*-18:2 decreased whereas with increasing in catalyst amount, it was increased. Time had a moderate impact on CLA production and with increasing in time, CLA yield was affected slightly. The temperature, 50°C; catalyst amount, 1.8 g and reaction time, 5.25 h were found to be the optimum points to achieve the maximum yield of *9c,11t*-18:2 (53.93%).

Introduction

Conjugated linoleic acid (CLA) is a collective term which refers to a mixture of positional and geometric isomers of linoleic acid with conjugated double bonds (Wang *et al*., 2007). In the recent years, there have been considerable interests in conjugated linoleic acids, owing to their unique biological properties. It is believed that CLA isomers have revealed a wide range of biological activities including reduction in body fat, enhanced bone mineralization, antioxidative, anticarcinogenic, antiatherosclerotic, antiadipogenic, antidiabetogenic, and immune modulating effects (Kim *et al*., 2003; Norhayati *et al.*, 2011). CLA is a unique fatty acid because it is present in food from animal sources such as dairy foods and meats (Lie *et al*., 1997).

Commercial CLA is produced by alkaline isomerization of linoleic acid-rich oils and tends to contain an equimolar mixture of the *9c,11t*- and *10t,12c*-isomers (Pariza *et al*., 2001; Rainer and Heiss, 2004). Other potential ways to produce conjugated fatty acids include the isomerization of linoleic acid using bacteria, such as *Lactobacillus plantarum* and dehydration of ricinoleic acid. Production of conjugated fatty acids converted from linolenic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and experimental evaluation of their physiological activities *in vitro* and *in vivo* have also been reported. The methods may contribute to the preparation of a conjugated fatty acid fraction with maximal physiological activity (Kishino *et al.*, 2002; Kishino *et al.*, 2003).

The fatty acid, *9c,11t*-18:2 sometimes called CLA, is the most abundant positional and geometric isomers that exist in nature. The *cis*-9,*trans*-11-18:2 isomer is believed to be the principal isomer responsible for the anticarcinogenic effects of CLA mixtures. In order to study the biological properties of *9c,11t*-18:2 isomer in detail, it is desirable to obtain substantial amounts of this compound in a relatively pure form, ideally by simple methods and readily accessible substrates (Berdeaux *et al*., 1997).

Castor oil is an economical source of ricinoleic acid so that 88% of its total fatty acids is ricinoleic acid. Even though castor oil is inedible, it has long been an article of commerce mainly due to the versatility of the oil (Ando *et al.*, 2004; Nagao and Yanagita, 2005; Ogunniyi, 2006). This oil can be dehydrated to give semi-drying or drying oil which is used extensively in paints and varnishes. During the dehydration process, the hydroxyl group of ricinoleic acid (C18:1; 12-hydroxy-9-*cis*-octadecenoic acid) is replaced by a double bond and different products are

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formed, i.e., the nonconjugated and the conjugated linoleic acids (Ramamurthi *et al*., 1998).

Production of CLA via dehydration of ricinoleic acid is an efficient reaction, but there are many factors affecting the process and usually an expensive dehydration reagent named 1,8-diazabicyclo-[5.4.0]-undec-5-ene (DBU) is used as catalyst. This fact necessitates more study on development of potential ways to obtain substantial amounts of the relatively pure isomer to be commercially exploited for industrial applications and incorporation into food formulations. Then the objective of this study was to optimize the reaction parameters including time, temperature, and amount of catalyst by response surface methodology (RSM) to obtain maximum yield of CLA isomer (*9c,11t*-18:2) produced using KOH instead of DBU as a cheaper catalyst.

Materials and Methods

Chemical reagents and samples

Castor oil was purchased from local market (Isfahan, Iran). All solvents/ chemicals used were of analytical grade and obtained from Merck (Darmstadt, Germany). Pure fatty acids as standard including palmitic, stearic, oleic, linoleic and linolenic acids were purchased from the Sigma Chemical Co. (St Louis, MO).

Determination of fatty acids profile in castor oil

The methyl esters of castor oil were prepared based on the method described by Goli *et al.* (2009). One hundred microliter of sodium methoxide (0.5M) was added to 50 µl sample in 1ml n-hexane. The mixture was shaken vigorously for 15 min in room temperature and allowed to stand and separate. Hexane phase was removed and 1µL of this phase was injected to GC.

The gas chromatographic analysis of fatty acid methyl esters (FAMEs) was performed on an Agilent 6890N gas chromatograph equipped with a flame ionization detector. The column used was a HP-5 (30 m, 0.32 mm i.d., 0.25 µm film thickness) type. The temperature program consisted of 180ºC for 1min and then increasing the temperature to 210ºC at a rate of 1.3ºC/min and holding for 5min, then increasing to 250ºC at a rate of 5ºC/min and holding for 10 min. Temperatures of injector and detector were 230 and 250ºC respectively. Ultra high purity helium was used as the carrier gas.

Transesterification of castor oil

Transesterification of castor oil was carried according to Berdeaux *et al.* (1997). The oil (50 g) was dissolved in dichloromethane (50 mL), and then methanol (100 mL) containing fresh sodium (0.5 g). The solution was refluxed for 10min, then poured into water (250 mL) that contained 12N hydrochloric acid (8 mL). The aqueous layer was extracted with hexane (3 × 200 mL), and the hexane layer was washed with water (100 mL) that contained potassium bicarbonate (2%). The sample was then dried over anhydrous sodium sulfate.

Isolation of methyl ricinoleate by countercurrent distribution (CCD)

Isolation of methyl ricinoleate was performed according to Berdeaux *et al*. (1997). Hexane and 90% aqueous methanol were shaken together to obtain equilibrated layers. The upper layer (3 × 300 mL) and lower layer (10 × 150 mL) were used in three separatory funnels. Fifty grams of prepared FAMEs was shaken with 300 mL hexane and 150 mL 90% aqueous methanol in the first separating funnel. The layers were separated, and the methanol phase was removed. The hexane phase was then washed with fresh 90% aqueous methanol. The methanol (mobile) phases were sequentially passed through two more separating funnels, each containing 300 mL hexane (stationary phase) and finally these methanol phases were combined together. Methanol phases were concentrated under reduced pressure on a rotary evaporator (50°C). A turbid oil was formed which extracted with hexane (3 × 250 mL), water (425 mL) and saturated brine (175 mL). The combined organic layers were dried and concentrated under vacuum with a rotary evaporator (40°C).

Preparation of methyl 12-mesyloxy-octadec- 9-enoate (MMOE)

Preparation of MMOE was performed by mixing ricinoleic acid methyl ester (30 g), methanesulfonyl chloride (24 ml), triethylamine (30 ml) and dichloromethane (300 ml), and stirring for 45 min on an ice water bath. The reaction mixture was diluted with 750 ml of dichloromethane and successively washed with 150 ml of HCl solution (2N) and twice with 300 ml of distilled water. The mixture was then dried over anhydrous sodium sulfate, filtered, and the solvents were removed in a rotary evaporator. MMOE production was confirmed by FTIR (JASCO FT/IR- 680 plus).

Production of CLA via dehydration of MMOE catalyzed by KOH

KOH-dependent dehydration of MMOE was accomplished in absolute ethanol. In brief, desirable amount of KOH (according to RSM runs) was dissolved in 20 ml of absolute ethanol in a screw-cap flask (100 ml) followed by adding 0.5 g MMOE.

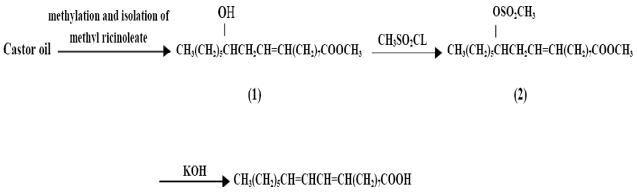

Figure 1. Production of conjugated linoleic acids. (1) Castor oil was converted to fatty acid methyl esters followed by isolation of ricinoleic acid methyl ester (2) methyl ricinoleate was then used for preparation of methyl 12-mesyloxy-octadec-9-enoate (MMOE) (3); conversion of MMOE to conjugated linoleic acids by potassium hydroxide (KOH).

A gentle stream of nitrogen gas was blown inside the flask for few minutes to remove residual air and after closing the cap, it was heated with constant stirring for a specific time (according to RSM runs) on oil bath. The flask was then removed and cooled to room temperature. In the next step 10 ml of distilled water was added to the flask and the solution was then transferred to a 250 ml separation funnel. The mixture was acidified with 10 ml of HCl solution (6N) and extracted three times with 40 ml of hexane. The combined hexane extract was then washed with 15 ml of NaCl solution (0.9%) and dried over anhydrous sodium sulfate. hexane was removed in a rotary evaporator and the CLA isomers in reaction medium were identified by GC. The reaction scheme adopted for the synthesis of *9c,11t*-18:2 is shown in Figure 1.

Detection of CLA isomers in reaction medium by GC

The methyl esters of CLA were prepared based on the method described by Goli *et al.* (2008). For detection of CLA isomers, 7 ml BF_3 was added to 300 mg of oil and after mixing it was left in room temperature for 30 min with occasionally shaking. Then 15 ml saturated brine was added and shaken vigorously. After adding 6 ml n-hexane, this phase was removed and 1 µL was injected to GC. The gas chromatographic analysis of FAME was performed on an Agilent 6890N gas chromatograph at the same condition as described before.

Experimental design for RSM

Before RSM was applied, approximate conditions for *9c,11t*-18:2 isomer synthesis, namely, reaction temperature, reaction time and amount of catalyst in gram, were determined by varying one independent variable at a time while keeping the others constant. An appropriate range for each independent variable was determined for RSM. A three-level, three-variable D-optimal design was adopted to optimize

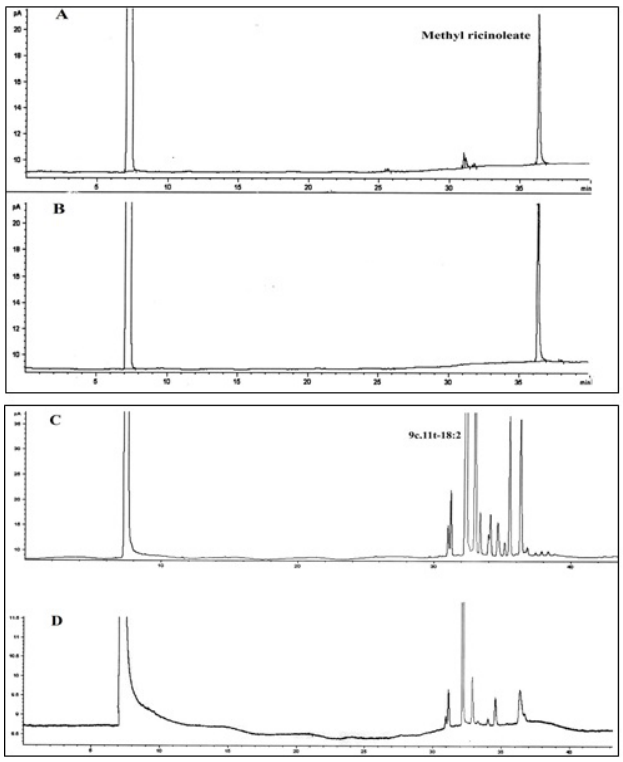

Figure 2. GC chromatogram of fatty acids profile in castor oil (A) before and (B) after purification of ricinoleic acid. GC chromatogram of CLA oil (C) before and (D) after urea crystallization.

the reaction conditions for maximum production of *9c,11t*-18:2 from chemical reaction. The experimental plan was designed and the results obtained were analyzed using Design Expert version 7.1.6 (Stat-Ease Inc., Minneapolis, MN) software to build and evaluate models. Reaction temperature (X_1: 50, 80, 110◦C), time of reaction (X_2: 4, 8, 12 h) and amount of catalyst (X_3: 1, 1.4, 1.8 g) were used as the variables to maximize the response (*9c,11t*-18:2(%)). The experimental design included 18 experiments of three variables each at three levels (−1, 0, +1).

Crystallization with urea for increasing the purity of 9c,11t-*18:2 isomer*

Urea adduct formation was done by dissolving FAME (20 g) in a hot solution of urea in methanol (20 g urea/100 mL methanol). After cooling under nitrogen with occasional swirling, the flask was left overnight at 4°C. The urea adducts and nonadduct fractions were separated by filtration through a Buchner funnel. FAME from each fraction was dissolved in hexane for GC analysis.

Results and Discussion

Fatty acids profile of castor oil and purification of methyl ricinoleate

GC analysis of the transesterified castor oil

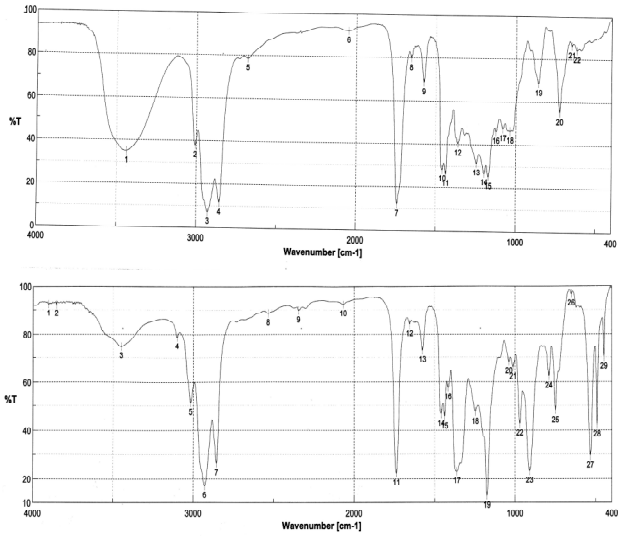

Figure 3. FTIR spectra of the transesterified castor oil before (A) and after preparation of MMOE (B).

revealed a mixture of 87.57% methyl ricinoleate, 1.32% 16:0, 0.51% 18:0, 5.16% 18:1, 4.29% 18:2, and 1.12% 18:3 fatty acids. Pure methyl ricinoleate could be isolated on a silica chromatographic column by a desirable developing solvent, or by preparative HPLC (Gunstone and Said, 1971), but these methods are only suitable on the milligram to gram scale. Recently, Berdeaux *et al.* (1997) and Tassignon *et al.* (1994) have used an efficient CCD method for purify methyl ricinoleate from castor oil esters quickly on a large scale (100 g). In this study, purification step yielded 43.7 g methyl ricinoleate from 50 g castor oil with a purity of about 99% (Figure 2).

Confirmation of MMOE production by FTIR

The FTIR spectra afford information on the functional groups of the sample. Figure 3 shows the infrared spectrum of the transesterified castor oil before and after preparation of MMOE. It is believed that when methyl ricinoleate is transformed to the mesylate, the hydroxyl group was modified to become a better leaving group (Figure 1) that can undergo competitive elimination or substitution, depending on the experimental conditions (Berdeaux *et al*., 1997).

The band of hydroxyl functional group near 3449 cm^{-1} was observed in the infrared spectrum of transesterified castor oil. As the conversion of ricinoleic acid to MMOE advances, the concentration of hydroxyl group in the sample decreases because the hydroxyl group converts to OS_2OCH_3 and so that the related band in spectrum would become small.

Selection of factor levels and experimental design

According to the preliminary experiments, as the amount of catalyst increased, the *9c,11t*-18:2 content also increased (unpublished data). However, it was observed that using higher amount of KOH may lead to a product with a dark color. Therefore, for KOH content, the selected points were 1.0, 1.4, and 1.8 g of KOH. *9c,11t*-18:2 content increased with increasing temperature up to 110°C and above this temperature, additional isomers were produced. Then the lower, middle, and upper points for the reaction temperature were chosen as 50, 80, and 110°C. As reported by Gunstone and Said (1971), the heating time of 12 h with a polycyclic base DBU or 1.5-diazabicyclo [4.3.0]- undec-non-5-ene (DBN) made 100% elimination of ricinoleic acid and production of mainly the *9c,11t*-octadecadienoate isomer, though a reaction time of 4 h may be sufficient. So the reaction times of 4, 8, and 12 h were chosen as the lower, middle, and upper points, respectively.

Table 1. Results of analysis of variance, regression coefficients and P-values for *9c,11t*-18:2

Source	Sum of squares	Degree of freedom	Mean square	F- value	P- value
Model	172.00	3	57.33	89.53	< 0.0001
Residual error	8.97	14	0.64		
Lack- of- fit	2.30	10	0.23	0.14	0.9947
Pure error	6.66	4	1.67		
Total	180.96	17			

Variables	Regression coefficient	P- value
Intercept	49.20	<0.0001
X_1	-1.98	<0.0001
X_2	0.13	0.5443
X_3	2.84	<0.0001

Model fitting

The actual set of experiments performed (experimental runs 1–18) and the yield of *9c,11t*-18:2 was obtained. For creating response surfaces, the data obtained based on the above design was fitted to a first-order polynomial equation of the form. The coefficients of independent variables determined for the linear polynomial model for the *9c,11t*-18:2 yield is given in Eq. 1:

$$Y = 49.20 - 1.98X_1 + 0.13X_2 + 2.84X_3 \quad (1)$$

The models were found to agree with the data at the probability level of 95%. The accuracy of the

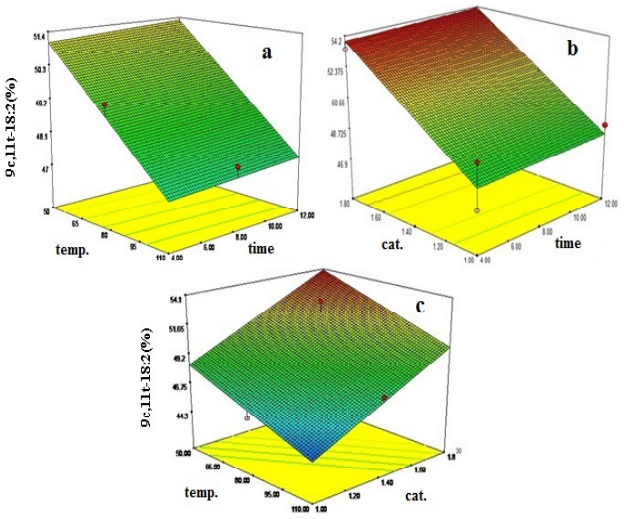

Figure 4. Response surface plot for the effect of temperature and time at center level of catalyst (a), effect of catalyst and time at center level of temperature (b) and effect of catalyst and temperature at center level of time (c) on the *9c,11t*-18:2%.

model was evaluated by coefficient of determination (R^2 and adjusted R^2 values). Values of R^2 and adjusted R^2 were 0.9505 and 0.9398 respectively. Analysis of variance (Tables 1) also showed that the regression model for *9c,11t*-18:2 production was statistically good with a significance level of $p < 0.0001$ and the models had no significant ($p > 0.05$) lack of fit. Thus, well-fitting models for *9c,11t*-18:2 production were successfully established.

Parameters effect on 9c,11t-*18:2 production*

As shown in Table 1, it can be found that the variable with the most significant effect on the *9c,11t*-18:2 yield was the linear term of catalyst ($p < 0.001$), followed by the linear terms of temperature ($p < 0.001$) and time.

Temperature

The temperature of the dehydration reaction was an important factor in the CLA isomer composition and yield of conversion. The results showed the negative linear effect of temperature on *9c,11t*-18:2 isomer content as by increasing of the temperature from 50ºC to 110ºC the content (%) of *9c,11t*-18:2 was decreased about 5% (Figure 4) and we also observed some extra peaks at higher temperature. The major possible reason for this change is that by increasing in temperature, production of other CLA isomers especially total *t*, *t*-18:2 isomers were increased and therefore *9c,11t*-18:2 isomer which was the desirable isomer decreased. In fact, because trans double bonds in CLAs are thermodynamically more stable than cis double bonds, the level of *t,t*-CLAs is higher with increasing reaction temperature and longer reaction times.

Catalyst

Amount of catalyst played an important role in dehydration of MMOE and strongly affects the *9c,11t*-18:2 yield. As shown in Figure 4, KOH content had positive linear effect, so increasing of KOH content from 1 to 1.8 g enhanced the *9c,11t*-18:2 content by about 6%. Then higher amount of catalyst is desirable but it should be mentioned that using high level of catalyst can lead to obtain a dark colored product that is not desirable. Some researchers showed that dehydration of castor oil by other catalysts (such as sodium bisulfate, sodium bisulfite) at higher temperature in comparison with this study also led to CLA production but in limited amounts. On the other hand, with phosphoric acid (0.1% w/w) at 280°C for 5 h, satisfactory proportions of CLA were obtained (54% of total FA) with a majority of 9-*cis*,11-*trans* isomer (61% of total CLA), although *9t, 11t* isomer was predominant by using sulfuric acid as catalyst (Villeneuve *et al*., 2005)

Time

With p-values of more than 0.05 (Table 1), time had an insignificant effect on *9c,11t*-18:2. Figure 4 shows that reaction time had a positive linear effect on CLA content and slight increase in CLA content observed with increase in time from 4 to 12 h (about 0.5%). Gunstone *et al.* (1971) showed that heating for 12 h with DBU or DBN gave 100% elimination and mainly the *9c,11t*-18:2 isomer, though a reaction time of 4 h may be sufficient. Berdeaux *et al.* (1997) reported that after 4 h at 110°C with DBU in toluene, a mixture containing mainly *9c,11t*-18:2, accompanied by *9c,11c, 9t,11t-, 9c,12t* and *9c,12c*-18:2 isomers was obtained. They showed that increasing the reaction time above 4 h did not modify the ratio between the different isomers, and only a slight increase in the amount of *9t,11t*-18:2 was noticeable which is in agreement with the data of this work.

Process optimization of CLA production

The reaction conditions would be considered optimum if the *9c,11t*-18:2 yields reached maximum value. The response surface plots in any two of three independent variables can be observed in Figure 4. Figure 4a denotes the surface plot of the CLA yield as a function of time and temperature at catalyst amount of 1.4 g. At high temperature, the yield of CLA is small while increasing in time changed the CLA% insignificantly. However, based on the response surface plot which was constructed for center level of temperature (Figure 4b) and center level of time

(Figure 4c) it is clear that the amount of catalyst and temperature had positive and significant effects on CLA formation.

The uncoded coordinate of stationary point for the CLA yield was 50ºC, 11.44 h and 1.8 g of KOH. At the optimum point, the maximum predicted value of the CLA yield was 54.13%. On the other hand at the same condition with 5.25 h reaction time, the predicted CAL yield was 53.93% that is a little different from the reaction time of 11.44 h. It was mentioned before that time has a moderate impact on CLA production and with increasing in time, CLA yield was affected slightly, so in order to operate conveniently and saving time, this parameter was changed slightly however the CLA yield was not affected so much. The adequacy of the model predicted was examined by performing independent experiments at the optimal conditions. Verification results revealed that the predicted values from the model were reasonably close to observed values (Observed value, 54.39% and residual ricinoleic acid, 9.2%).

Urea crystallization

One step urea crystallization was employed to enrich *9c,11t*-18:2 from its geometric and positional isomers. When a ratio of urea/FAME equal to 1:1 (w/w) was used, some isomers other than *9c,11t*-18:2 formed crystalline adducts, while *9c,11t*-18:2 primarily remained in the mother liquor. Thus, urea complexation with 20 g of FAME and 20 g of urea gave 12 g of a nonadduct fraction and 8 g of an adduct fraction. As shown in Figure 1 (C and D), GC analysis of the nonadduct fraction showed an increase in the amount of *9c,11t*-18:2 to 76.59% after just one step urea crystallization (compared to 54.39% in the origin CLA oil). Villeneuve *et al.* (2005) evaluated urea fractionation on FFA of dehydrated/ isomerized castor bean oil and their results showed an increase about 5.6% in CLA content with a majority of *9c,11t*-18:2 (60.8%) in mother liquor. Berdeaux *et al.* (1997) showed that two urea crystallizations produced a product containing 83% methyl *cis*-9,*trans*-11-octadecadienoate.

Conclusion

A simple method was developed to prepare *9c,11t*-18:2 from methyl ricinoleate that can be applied on a commercial scale. Dehydration of MMOE is the most attractive alternative to produce CLA. The starting material, castor oil, is relatively inexpensive. In this study KOH was used as an efficient dehydrating reagent and results showed that this method has the potential of being scaled up to produce an inexpensive and efficient CLA. The resulting oil contained more than 87% CLA and the conversion efficiency for ricinoleic acid to *9c,11t*-18:2 by KOH was ca. 62%. The oil contained CLA with *9c,11t*-18:2 isomer being predominant (more than 54% of CLA fraction) in contrast to alkali isomerization of linoleic acid that produces only about 45% of this CLA isomer. Therefore in producing *9c,11t*-18:2 as the most active isomer, dehydration of MMOE catalyzed by KOH would be a preferred method to produce CLA. Optimal reaction conditions were: 1.8 g of KOH as catalyst, 50°C of reaction temperature, 5.25 h of reaction time and just two reaction parameters including temperature and amount of catalyst has significantly effects on CLA%. Finally, urea fractionation can be also applied efficiently on resulting oil to remove some other CLA isomers produced in reaction medium and obtain FFA products containing high level of *9c,11t*-18:2 isomer.

Acknowledgments

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