FORMATION AND CONFIGURATION TRANSFORMATION OF CONJUGATED LINOLEIC ACID BY RHODIUM HETEROGENEOUS CATALYST AND EXTRUSION

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Technology

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การเกิดและการเปลี่ยนโครงสร้างของ CONJUGATED LINOLEIC ACID

โดยตัวเร่งปฏิกิริยาวิวิธพันธุ์โรเดียมและกระบวนการเอกซ์ทรูชัน

นางพัชรินทร์ ภักดีฉนวน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

สาขาวิชาเทคโนโลยีอาหาร

มหาวิทยาลัยเทคโนโลยีสุรนารี

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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Conjugated linoleic acid (CLA) มีคุณสมบัติเป็นสารป้องกันการเกิดมะเร็ง ป้องกันการเกิดการอุดตันของหลอดเลือด และลดปริมาณไขมันสะสม ตัวเร่งปฏิกิริยาวิวิธพันธุ์โรเดียมสามารถกระตุ้นการเกิดปฏิกิริยา isomerization เปลี่ยนกรดไขมันชนิดไลโนเลอิกในน้ำมันถั่วเหลืองเป็น CLA ในรูปไตรกลีเซอร์ไรด์ เพื่อศึกษาและทำนายสภาวะที่เหมาะสมในการเกิด CLA ได้ใช้การวางแผนการทดลองแบบ central composite rotatable design ด้วย 3 ตัวแปร 5 ระดับ ประกอบด้วย อุณหภูมิ อัตราเร็วการกวนผสม และเวลาในการทำปฏิกิริยา พบว่าอุณหภูมิและเวลาในการทำปฏิกิริยามีผลโดยตรงต่อการเพิ่มของ CLA โดยปฏิกิริยาที่อุณหภูมิ 200 องศาเซลเซียส อัตราเร็วของการกวนผสม 200 รอบต่อนาที เป็นเวลา 49 นาที มีผลให้ปริมาณ CLA เพิ่มขึ้นจาก 0.63 มิลลิกรัมต่อกรัมของน้ำมันถั่วเหลืองเริ่มต้นเป็น 202.42 มิลลิกรัมต่อกรัมน้ำมัน โดยพบไอโซเมอร์ *cis9,trans11* และ *trans10,cis12* ซึ่งมีรายงานว่าเป็นไอโซเมอร์ที่มีคุณสมบัติข้างต้นในปริมาณสูง การศึกษาความเป็นไปได้ของการเกิด isomerization บนตำแหน่งของไตรกลีเซอร์ไรด์พบว่า กรดไขมันชนิดไลโนเลอิกสามารถเปลี่ยนเป็น CLA ได้ทุกตำแหน่ง

การใช้กระบวนการเอกซ์ทรูชันเป็นตัวแทนของการแปรรูปอาหารโดยใช้ความร้อน พบว่าการเพิ่มขึ้นและการเปลี่ยนรูปร่างของ CLA เป็นผลจากอุณหภูมิและค่าทอร์ค การเอกซ์ทรูชันที่อุณหภูมิผลิตภัณฑ์ 150 องศาเซลเซียส และค่าทอร์ค ร้อยละ 70 ทำให้ปริมาณ CLA เพิ่มจาก 1.17 เป็น 7.75 มิลลิกรัมต่อกรัมน้ำมัน การเอกซ์ทรูชันที่สภาวะนี้ยังทำให้เกิด CLA ชนิด *trans/trans* ต่ำที่สุดและผลิตภัณฑ์มีการขยายตัวสูงที่สุด

สาขาวิชา เทคโนโลยีอาหาร
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PATCHARIN PAKDEECHANUAN : FORMATION AND CONFIGURATION TRANSFORMATION OF CONJUGATED LINOLEIC ACID BY RHODIUM HETEROGENEOUS CATALYST AND EXTRUSION. THESIS ADVISOR : ASSOC. PROF. KANOK-ORN INTARAPICHET, Ph.D. 107 PP. ISBN 974-533-363-8

CONJUGATED LINOLEIC ACID/RHODIUM HETEROGENEOUS CATALYST/ ISOMERIZATION/EXTRUSION

Conjugated linoleic acid (CLA) has been reported in animals to have anticarcinogenic, antiatherogenic and body fat reduction activities. Rhodium heterogeneous catalyst was able to catalyze isomerization of linoleic acid in soybean oil to triacylglycerol-CLA. A central composite rotatable design with 5 levels of 3 variables, namely reaction temperature, stirring speed and reaction times, was used to determine maximum CLA yield. Formation of CLA during isomerization was greatly dependent on reaction temperature and time rather than stirring speed. CLA content of soybean oil increased from 0.63 to 202.42 mg/g oil when isomerization was done at 200^{o}C, for 49 min with a stirring speed of 200 rpm. This isomerization condition also provided high proportion of the beneficial isomers *cis9,trans11* and *trans10,cis12*. Investigation of acyl selection during isomerization indicated that linoleic acid at any position in triacylglyceride could possibly be isomerized to CLA.

Extrusion process was used as a case study for thermal processing. Extrusion temperatures and torques influenced formation and configuration of CLA. Extrusion at a product temperature of 150^{O}C and 70% torque affected an increase in CLA contents,

from 1.17 mg/g of oil in feeds to 7.75 mg/g of oil in corn extrudates with a minimum formation of *trans/trans* CLA and highest expansion of the extrudates.

School of Food Technology

Academic Year 2004

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Advisor's Signature _____

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Patcharin Pakdeechanuan

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LIST OF ABBREVIATIONS

Ag^{+}-HPLC	=	silver ion high performance liquid chromatography
BF_3	=	Borontrifluoride
BMI	=	Body mass index
c	=	*Cis*
^{o}C	=	Degree Celsius
$CaCl_2$	=	Calcium chloride
CLA	=	Conjugated linoleic acid
CLAME	=	Conjugated linoleic acid methyl ester
Cytb5	=	Cytochrome b-5
DAG	=	Diacylglycerol
DMBA	=	7,12-dimethybenz(a)anthracene
DMOX	=	Dimethyloxazoline
FAME	=	Fatty acid methyl ester
FAS	=	Fatty acid synthase
Fig.	=	Figure
g	=	Gram
GC	=	Gas chromatography
GC-DD-FTIR	=	Gas chromatography direct deposit fourier transform Infrared
GC-FTIR	=	Gas chromatography-fourier transform infrared
GC-MS	=	Gas chromatography-mass spectrometry

LIST OF ABBREVIATIONS (Continued)

GI tract	=	Gastro-intestinol tract
GLM	=	General linear model procedure
h	=	Hour
HCl	=	Hydrochloric acid
HDL-cholesterol	=	High density lipoprotein cholesterol
Ir	=	Iridium
Kg	=	Kilogram
kJ	=	Kilojoule
M	=	Molar
mg	=	Milligram
MAG	=	Monoacylglycerol
min	=	minute
mL	=	Milliliter
mm	=	millimeter
µm	=	Micrometer
µmol	=	Micromole
mu	=	Mass unit
m/z	=	Mass to charge
N	=	Normal
NaCl	=	Sodium chloride
Ni	=	Nickel
nm	=	nanometer

LIST OF ABBREVIATIONS (Continued)

NMR	=	Nuclear magnetic resonance
Os	=	Osmium
Pd	=	Palladium
Pt	=	Platinum
PPAR	=	Peroxisome proliferators-activated receptor
PUFAs	=	Polyunsaturated fatty acids
Rh	=	Rhodium
$RhCl(PPh_3)_3$	=	tris-triphenylphosphine chlororhodium
rpm		round per minute
Ru	=	Ruthenium
SME	=	specific mechanical energy
t	=	*Trans*
TAG	=	triacylglycerol
TBARS	=	Thiobarbituric acids
TLC	=	Thin layer chromatography
UV	=	Ultraviolet

CHAPTER I

INTRODUCTION

Conjugated linoleic acid (CLA; CH_3 $(CH_2)_5CH=CHCH=CH(CH_2)_7COOH$)) is a polyunsaturated fatty acid found naturally in ruminant food products. It is a mixture of positional and geometrical conjugated dienoic isomers of linoleic acid formed as intermediates during the reticulolumen isomerization of linoleic acid and through the endogeneous desaturation of *trans11* octadecenoic acid. CLA has gained considerable attention over the last decade due to the possible beneficial effects of dietary CLA, as well as the formation of CLA by biological, chemical and thermal reactions. In animal models, CLA has been shown to exhibit anti-carcinogenic, anti-atherogenic, immune modulator and body fat reducing activities. The *cis9*,*trans11* isomer is the main CLA occurring naturally in food. This isomer, together with *trans10,cis12*, is considered to be biologically active, while was recently extended to include the minor isomers *trans*9,*trans11* and *cis9*,*cis11*.

Although, recommendations to achieve health benefits for a human with a 70 kg body weight specify a CLA intake of at least 3 g/day, whereas the CLA content in food is not higher than 12 mg/g of fat. Today, CLA is commercially available as a supplement in gel capsules. Alkaline isomerization of linoleic acid is a general method that has been used for commercial CLA production. This method provides predominantly *cis9*,*trans11*-18:2 and *trans10*,*cis12*-18:2 isomers. However, a disadvantage of alkaline isomerization is the use of excess strong basic potassium

hydroxide or sodium methoxide in the reaction. Besides, CLA in the free fatty acid form in this method is easily oxidized in air.

Therefore, CLA formation by using other methods have been proposed. This dissertation reported the possibility of increasing CLA formation by using heterogeneous rhodium as a catalyst in the transformation reaction and the extrusion process as a case study for thermal processing to increase CLA content. Chromatographic methods including TLC, GC and HPLC were used to investigate the content and configuration of total CLA and individual isomers during the reactions.

1.1 Research objectives

1. To study the formation and configurative transformation of CLA by an isomerization process using a heterogeneous catalysts,
2. To study the effects of extrusion parameters, temperatures and torques on the content and configuration transformation of CLA in the extrudates.

1.2 Research hypothesis

1. Heterogeneous catalyst under optimum isomerization condition is able to provide high activity and selectivity of the isomerization of fatty acids in soybean oil to CLA.
2. Thermal and mechanical energies of extrusion can affect the formation and content of CLA in corn extrudates.
3. Extrusion parameters can alter the configuration of CLA isomers.

1.3 Scope and limitation of the study

1. Formation of CLA by heterogeneous catalyst

   Rhodium, platinum, and palladium heterogeneous catalysts were used for preliminary studies, and the catalyst that provided the highest activity was chosen. A central composite rotatable design with 5 levels of 3 variables namely, reaction temperature, reaction time and stirring speed was used to investigate the maximum yield of CLA formation. CLA was analyzed by HPLC and GC and individual CLA isomer were identified by GC-MS techniques. *Sn*-positions of triacylglycerol in isomerized oil were investigated to monitor possibility of the conversion of fatty acids in soybean oil to CLAs.

2. Formation of CLA by extrusion

   Corn meal was extruded at different temperatures and torques. Corn extrudates were analyzed for CLA content and configuration by GC and HPLC techniques.

3. Alteration of CLA positional configuration by extrusion

   Corn meal mixed with alkaline isomerized oil was extruded at different temperatures and torques. Corn extrudates were analyzed for configuration transformation and positions on the acylglycerol of CLA by HPLC techniques.

1.4 Expected results

1. The optimum isomerization condition for the maximum CLA formation using a heterogeneous catalytic reaction can be predicted.

2. The possibility of conversion of linoleic acid to CLA in the various triacyl *sn*-glycerol positions is revealed.
3. The effect of extrusion parameters on the formation and configuration alteration of CLA is understood and applicable.

CHAPTER II

LITERATURE REVIEW

Conjugated linoleic acid (CLA) is a collective term describing the positional and geometrical conjugated dienoic isomers of linoleic acid. It was found in food products in 1935 and reported to be an intermediate in biohydrogenation of C18-unsaturated fatty acids by rumen anaerobic gram positive bacteria, *Butyrivibrio fibrisolvens*, *Ruminococcus albus* and *Eubacterium sp.* in 1967 (Feller et al. 1967). Subsequently, Pariza and Hargraves (1985) found an anticarcinogenic property, which was the first time that chemoprotective property of CLA was mentioned. It was identified from grilled ground beef and showed mutagenesis modulator activity which inhibited initiation of mouse epidermal tumors by 7, 12- dimethylbenz(a)anthracene (DMBA). From then on, CLA has gained considerable attention and further research showed highly effective anticarcinogenic properties of CLA against cancer induced by known carcinogens in different animal's organs. For example, CLA was able to inhibit benzo(a)pyrene-induced mouse forestomach tumors (Ha, Storkson, and Pariza, 1990), was affective against DMBA-induced mammary tumorigenesis in rats (Ip, Chin, Scimeca, and Pariza, 1991), inhibited 2-amino-3-methyl imidazo [4,5-f] quinoline-induced lung and large intestine carcinogenesis and finally was effective against 2-amino-3-methyl imidazo[4,5-f] quinoline-induced colon carcinogenesis (Liew, Schut, Chin, Pariza, and Dashwood, 1995). CLA also has been shown to have other chemoprotective properties in animal models, such as inhibition of cholesterol induced

atherosclerosis (Kim and Liu, 1999; Yamasaki, Kishihara, Ikeda, Sugano, and Yamada, 1999; V.C. Gävino, G. Gävino, Lablanc, and Tuchweber, 2000) fat deposition reduction (Brodie, Manning, Ferguson, Jewell, and Hu, 1999; Yamasaki et al., 1999; Park, Storkson, Albright, Liu, and Pariza, 1999) improvement of type II diabetes mellitus and immunomodulating capability (Gnädig, Rickert, Sébédio, and Steinhart, 2001).

The functional properties of CLA are due to chemical and physiological effects, which are different from those of all-*cis*, nonconjugated polyunsaturated fatty acids. The double bonds in CLA exhibited ^{13}C NMR signals and were identified for 20 different CLA isomers (Bernas et al., 2003). These are *cis,cis*; *trans,trans*; *cis,trans* and *trans,cis* isomers of the 7,9; 8,10; 8,11; 10,12 and 11,13-C18:2 diene acids. However, only 2 isomers, *cis9,trans11* and *trans10,cis12*-CLA (Fig. 2.1) have been shown to have biological properties by numerous researchers.

(a) (b)

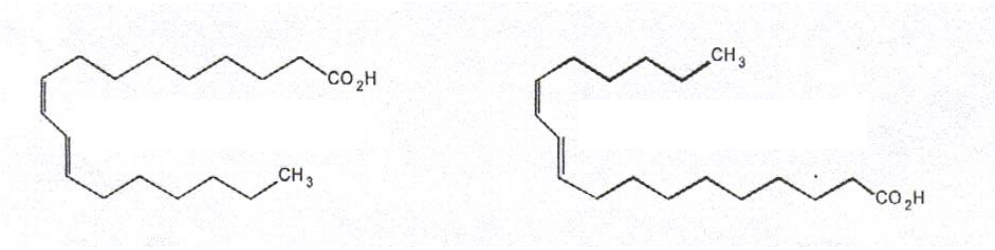


Fig. 2.1 Configuration of (a) *cis*9,*trans*11 and (b) *trans*10,*cis*12-CLA

CLA content in animal products is usually higher than in plant materials because of bioisomerization by ruminal bacteria as mentioned above. CLA concentrations range from 2.9 – 11.3 mg/g of fat in dairy products and 3.1 – 8.5 mg/g

of fat in beef, whereas the CLA content in fat from nonruminants and vegetable oils is only 0.6 – 0.9 mg/g of fat (Table 2.1).

Table 2.1 CLA content of ruminant animal products and plant materials

Types	Content (mg/g fat)	Source*
Vegetable oils	0.60-0.90	A
Dairy products	2.90–11.30	A
Cheese	3.59-7.96	B
Fermented dairy product	3.82-4.66	B
Nonfat frozen dairy dessert and non fat yogurt	0.60-1.70	B
Beef fat	3.10–8.50	A

*Source A; Decker (1995)

B; Lin, Boylston, Chang, Luedecke, and Shultz (1995)

Based on research using animal models, human with a 70 kg body weight has to take in at least 3 g CLA per day to achieve the beneficial properties (Pariza and Hargraves, 1985) whereas the CLA in natural food is not higher than 12 mg/g of fat. Therefore, synthetically prepared CLA is now available in commerce.

2.1 Biological properties of CLA

Biological beneficial properties of CLA have mainly been demonstrated in animal models. CLA appears to act differently compared to its parent linoleic acid, but

the mechanisms of the actions still remain unclear. Some biological properties are discussed as followed:

2.1.1 Anticarcinogenesis

The anticarcinogenic property of CLA was first identified in the mouse skin multistage carcinogenesis model. Ha et al. (1987) isolated CLA isomers from grilled ground beef and applied them to the dorsal area of the mouse skin prior to initiating cancer with 7,12-dimethybenz(a)anthracene (DMBA), a known carcinogen. The result showed that CLA treatment inhibited tumor incidence by approximately 20% more than linoleate at 16 weeks postpromotion.

Additional evidence of carcinogenesis inhibition by CLA has been found in benzo(a)pyrene-induced mouse forestomach neoplasia according to Ha, Storkson, and Pariza (1990). Presumed tumors, 1 mm or larger, were counted using a dissecting microscope, followed by histological examination for the confirmation of neoplasia. Histological examination of the benzo(a)pyrene-induced forestomach tumors revealed that there were papillomas with or without focal areas of epidermal hyperplasia as shown in Table 2.2. The CLA treatment significantly reduced the number of tumors, whereas linoleic acid had no such effect ($P<0.025$). They also revealed that the body weight and food intake were not affected by CLA. Therefore, caloric restriction known to reduce tumor risk was not a factor in the reduction of forestomach neoplasia by CLA.

Ip, Singh, Thompson, and Scimeca (1994) reported CLA as a chemoprotection of mammary carcinogenesis. In their experiment, rats were fed with a diet containing the feeding regimen 0.05, 0.10, 0.25 or 0.5 % of CLA 2 weeks before introducing

DMBA and continuing for 9 months. As shown in Fig. 2.2, CLA added to the diet significantly reduced mammary tumor yield in rats given a low dose of DMBA.

Table 2.2 Comparison of olive oil, CLA, and linoleic acid on inhibition of benzo(a)pyrene-induced forestomach neoplasia in female mice

Treatment	Tumor incidence (%)	Tumors /mouse	Body weight (g/mouse)	Food Intake (kcal/wk/mouse)
Olive oil	90.9[a]	3.6 ± 0.5[a]	31.5 ± 0.7	87.1 ± 3.00
CLA	70.9 [b]	1.4 ± 0.5 [b]	33.2 ± 0.9	90.7 ± 3.25
Linoleic acid	78.9 [a]	3.5 ± 1.3 [a]	32.7 ± 0.8	95.7 ± 3.39

[a-b] Means in the columns followed by different letters are significantly different ($p<0.025$)

Source: Ha et al. (1990)

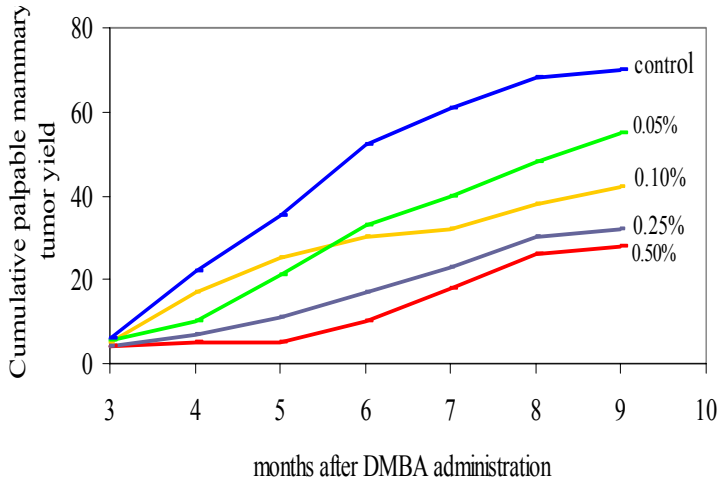


Fig. 2.2 Cumulative appearance of palpable mammary tumors as a function of time after DMBA administration in rats fed

Source: Ip et al. (1994)

In addition, Ip et al. (1994) also suggested that the *cis*9,*trans*11-CLA may be the most important isomer for anticarcinogenesis, became only the *cis*9,*trans*11-CLA isomer was preferentially incorporated into the forestomach membrane phospholipids when compared with other isomers.

There were several reports that proposed the cellular mechanisms for anticarcinogenesis by CLA. The first proposal was revealed by Zu and Schut in 1992. They suggested that CLA was involved in the reduction of DNA adducts. CLA inhibited 2-Amino-3-methylimidazo[4,5-f] quinoline (IQ)-DNA adduct formation by more than 57% in mice in many organ as shown in Fig. 2.3.

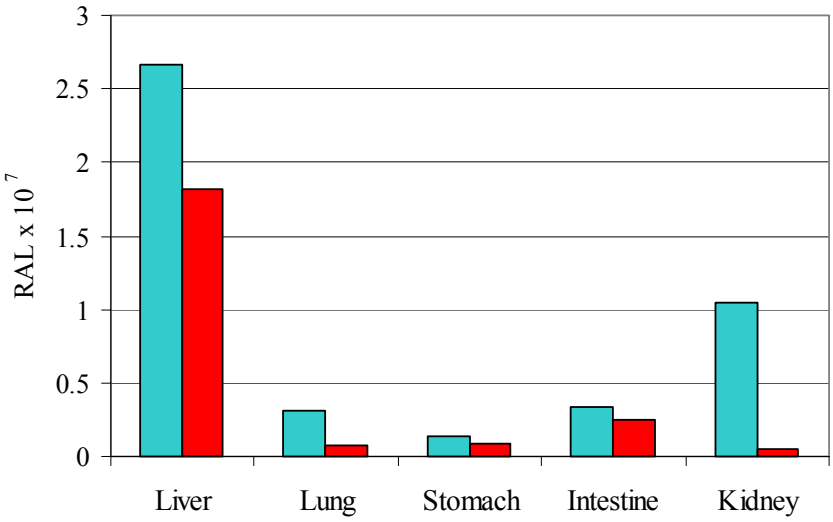


Fig. 2.3 Individual IQ-DNA adduct formation with ( ) or without ( ) CLA pretreatment in mice

Source: Zu and Schut (1992)

In addition, CLA was found to compete with other polyunsaturated fatty acids (PUFAs) for incorporation into membrane phospholipids. Cook, Miller, Park, and

Pariza, (1993) showed that dietary CLA (0.5% w/w) partially displaced arachidonate in rat fat pads tissue. They proposed that CLA-induced reduction of phospholipid arachidonate was likely to inhibit eicosanoid synthesis and tumorigenesis. However, the effects of dietary CLA on eicosanoid synthesis are not yet known.

Recently, CLA was reported to induce apoptosis in animal breast-cancer cells as evidenced by a reduction in expression of the anti-apoptotic (Ip et al., 1999). Furthermore, CLA was found to inhibit oestrogen-receptor positive MCF-7 mammary cancer (Durgam and Fernandes, 1997) whilst linoleic acid had the opposite effect.

Nevertheless, the mechanism of anticarcinogenesis of CLA are still not clear and further cellular studies are needed to be carried.

2.1.2 Anti-atherosclerosis

CLA has been shown to be highly effective in lowering cholesterol and preventing atherosclerosis in animals. Gävino et al. (2000) reported the effect of an atherogenic diet supplemented with CLA and linoleic acid on plasma lipids, weight gain and food intake of male hamsters. The result showed that CLA lowered plasma triglycerides and total cholesterol after 2 and 6 weeks of feeding as compared to linoleic acid but did not affect HDL–cholesterol level (Table 2.3).

Table 2.3. Effect of dietary CLA and linoleic acid on plasma total triglycerides, total cholesterol, HDL-cholesterol and non-HDL-cholesterol of hamsters

Plasma lipids	Weeks	CLA (mmol/L)	Linoleic acid (mmol/L)
Triglyceride	2	4.6 ± 1.3^{a}	8.1 ± 2.4^{b}
	6	4.3 ± 2.2^{a}	9.9 ± 2.4^{b}
Total cholesterol	2	4.9 ± 0.6^{a}	6.6 ± 0.5^{b}
	6	4.5 ± 0.5^{a}	6.5 ± 1.0^{b}
HDL-cholesterol	2	1.4 ± 0.2^{ns}	1.8 ± 0.4^{ns}
	6	1.6 ± 0.5^{ns}	1.7 ± 0.5^{ns}
Non-HDL-cholesterol	2	3.5 ± 0.5^{a}	4.8 ± 0.6^{b}
	6	2.9 ± 0.7^{a}	4.9 ± 1.3^{b}

[a-b] Mean in the rows followed by different letters are significantly different ($p<0.05$)

[ns] Not significantly different ($p>0.05$)

Source: Gävino et al. (2000)

2.1.3 Body fat reduction

CLA is claimed to be a body fat reducer because it possibly reduces fat cell size in animal models. Azain, Hausman, Sisk, Flatt, and Jewell (2000) investigated the reduction in fat pad size in rats fed CLA. Growing female rats were fed diets containing 0, 0.25 and 0.5 g/100 g diet for 5 weeks to determine the effects on growth performance and fat mass. The result showed that CLA reduced retroperitoneal pad weight from 0.68 to 0.59 and 0.51 g in rats fed 0.25 and 0.5 % of CLA, respectively ($P<0.05$) (Table 2.4). It was noted that final body weight and carcass weight were not

significantly affected. The reduction in adipose tissue mass in response to dietary CLA was accounted for by a decrease in cell size (cell diameter) rather than a change in cell number (cells/pad), which is consistent with metabolic changes, ie. decreased lipid deposition and increased lipolysis. The main effect of dietary CLA in the retroperitoneal pad accounted for a 26 % reduction in pad weight in rats fed for 7 days ($P<0.05$).

Clinical studies of body fat reduction by CLA were done by Choi et al., (2000). The effect of the *cis*9,*trans*11 and *trans*10,*cis*12 isomers on lipid composition and gene expression during the differentiation of mouse 3T3-L1 preadipocytes were determined. The result demonstrated that treatment of 3T3-L1 cells with the *trans*10,*cis*12 isomer of CLA reduced the expression of the SCD1 in a dose-dependent (10-100 μmol/L) manner. However, the expression of other adipocyte genes, such as adipose P2 (aP2), SCD2, fatty acid synthase (FAS), CCAAT enhancer binding protein (C/EBP) and peroxisome proliferators-activated receptor (PPAR2), were not significantly affected. The downregulation of the SCD1 mRNA expression corresponded to a decrease in SCD protein and enzyme activity and caused smaller lipid droplet. In conclusion the study showed that the mechanism of *trans*10,*cis*12 action on SCD gene expression could involve decreased SCD mRNA stability and/or gene transcription whereas *cis*9,*trans*11 isomer did not alter adipocyte gene expression.

Table 2.4 Effect of dietary conjugated linoleic acid on animal growth

	Diet		
	Control	CLA 0.25 %	CLA 0.50 %
Body weight, g			
Initial	158[ns]	158[ns]	158[ns]
Final	206[ns]	209[ns]	202[ns]
Carcass weight, g	133.9[ns]	138.6[ns]	132.1[ns]
Retroperitoneal pad			
Weight, g	0.68[a]	0.59[b]	0.51[c]
Cells/pad, n x10^6	1.09[ns]	1.22[ns]	1.31[ns]
Cell diameter, μm	68.0[ab]	64.3[a]	61.2[b]
40-69 μm, %	67.5[a]	74.9[ab]	80.9[b]
70-240 μm, %	23.4[a]	16.4[b]	11.5[b]

[a-b] Mean in the rows followed by different letters are significantly different ($p<0.05$)

[ns] Not significantly different ($p>0.05$)

Source: Azain et al. (2000)

By contrast, studies on CLA supplementation in human showed no significant reduction of body fat. A human double-blind, placebo-controlled study was carried out on overweight or obese volunteers (BMI between 27.5 and 39.9 kg/m^2) to test the influence of CLA on body composition (Berven et al., 2000). The volunteers received either 3.4 g of CLA/day or 4.5 g of olive oil/day during twelve weeks. No differences between the CLA-supplemented and the control group were found in blood parameters (blood lipids, hematology, liver enzymes, blood electrolytes, creatinine and lactate

dehydrogenase) or clinical vital signs (blood pressure and heart rate). In the CLA-group, mean body weight was reduced by 1.1 kg and body fat mass decreased by 0.9 kg, but the data were not significantly different (P>0.05) from the control. Supporting data from at least three other reports also showed no significant reduction of body weight or body fat by CLA diets (Zambell et al., 2000; Medina et al., 2000). Gnädig et at. (2001) suggested that the contradictory findings could be due to the differences in dose of CLA used in the experiments. High doses of CLA was used in the animal model, and the study was carried out on growing animals but in human studies only dietary amounts of CLA were used in adults.

2.2 CLA synthesis

2.2.1 CLA synthesis in ruminant

Isomerization and desaturation were found to be involved with the CLA synthesis in ruminants. The isomerization of C18-unsaturated fatty acid was first reported as a source of CLA by Feller et al. (1967). Linoleate isomerase (EC 5.2.1.5) in ruminal bacteria is responsible for forming *cis*9,*trans*11-CLA from the *cis*9,*cis*12 double bonds of linoleic acid. This enzyme is bound to the bacterial cell membrane of the principal rumen bacteria, *Butyrivibrio fibrisolvens, Ruminococcus albus* and *Eubacterium sp.* (Feller et al., 1967). In 1967, Kepler and Tove studied the purification and properties of a linoleate isomerase from *Butyrivibrio fibrisolvens.* Their result showed that the optimum pH for the linoleate isomerase was 7.0-7.2 in a phosphate buffer system. The enzyme did not require the addition of nucleotide cofactors (CoA, ATP, MG^{++}, ADP, AMP or NAD^{+}) or the presence of a hydrogen atmosphere.

At present, there are several articles showing that isomerization is not the main mechanism of CLA synthesis in ruminants. Ntambi (1999) reported that 90% of CLA was synthesized by desaturation of *trans*11-C18:1 to *cis*9,*trans*11-CLA by $^{9}\Delta$-desaturase. The enzyme is located in the endoplasmic reticulum membrane of the mammary gland and adipose tissue. It catalyses the Δ^{9} desaturation of a spectrum of fatty acyl-CoA substrates by a set of microsomal electron transport proteins composed sequentially of NADH cytochrome b5 reductase, cytochrome b5 and the terminal desaturase (Fig. 2.4). Desaturase is the rate-limiting component in this reaction. Its activity is regulated by different factors such as diet, hormones, temperature, metal, proximal proliferators, vitamin A, and developing processes (Ntambi, 1999).

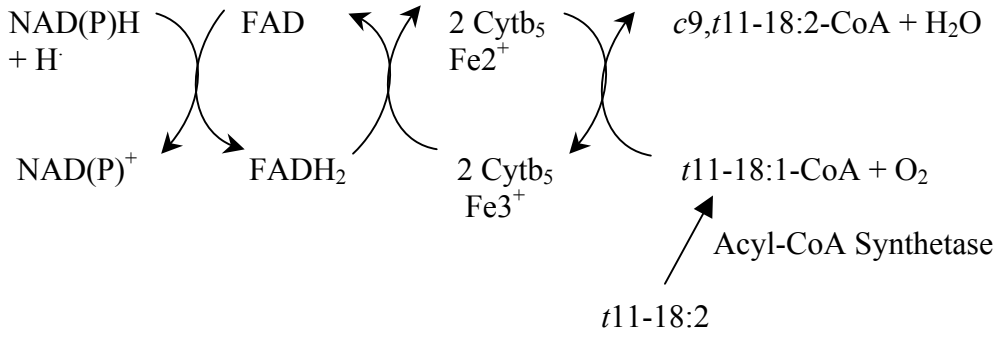


Fig. 2.4 Biochemical pathway of desaturation of *trans*11-18:1 to *cis*9,*trans*11-18:2
Source: Ntambi (1999)

In conclusion, polyunsaturated fatty acids (PUFAs) in form of glycolipids or triacylglycerols in the feed are hydrolyzed to fatty acids by microbial lipases. From then on, they are isomerized and hydrogenated to *cis*9,*trans*11-CLA, *trans*11-C18:1, C18:0 and other octadecamonoenoic acids by ruminal bacteria. After that, fatty acids

will flow through the GI tract and be absorbed into the blood (Fig. 2.5). In the mammary gland, the desaturase enzyme will desaturate *trans*11-C18:1 to *cis*9,*trans*11-CLA which accounts for 90% of the *cis*9,*trans*11-CLA synthesis here (Ntambi, 1999).

Therefore, formulating diets high in PUFAs from either plant oil or animal fat will affect the CLA content in ruminant-derived food products. However, Stanton et al. (1997) revealed that feeding of various sources of PUFAs including CLA-supplements possibly increased CLA content approximately by only up to 8 mg/g of fat in dairy products (Table 2.5).

Table 2.5 Enhancement of CLA content in milk by manipulating diets

Feed (% of dry matter)	Total CLA contents in milk (mg/g oil)	Sources
1.5% of rapeseed	5.23	A
3% of rapeseed	7.89	A
12% of extruded soybean oil	6	B
3% of canola oil	5	C
3% of canola oil + 0.4% of CLA	6	C
3% of soybean oil + 0.4% of CLA	6	C

*Sources A; Stanton et al. (1997)

B; Chouinard, Corneau, Barbano, Metzger, and Bauman (1999)

C; Loo and Herbein (2003)

An increase in the CLA content in meat products was also found by manipulating diets. Ivan et al. (2001) fed cows with sunflower seed oil (6% of dry

matter) for 49 days and studied changes of CLA in muscle and subcutaneous fat. The result showed increasing CLA contents in meat from 3.67 mg/g of fat in the non-sunflower seed oil diet group to 5.2 mg/g of fat in the sunflower seed oil diet group. Similarly results showed that CLA in subcutaneous fat increased from 5.4 mg/g of fat in the non-sunflower seed oil diet group to 7.2 mg/g of fat in the sunflower seed oil diet group.

There are some animal feeds that can possibly be used to enhance CLA content. A study of in milk fat by Bauman et al. (1999) revealed that only plant oil, fish oil, pasture, forage and processed oil seed (ground, roasted, micronized, flake and extruded) can be used, as shown in Table 2.6. These sources have available linoleic acid for ruminal bacteria to give great response and a dose dependency in increasing the content of CLA.

Table 2.6 Dietary factors that affect CLA contents in milk fat

Dietary factors	Content of CLA in milk fat
Unsaturated and saturated fat	Increased by addition of unsaturated fat
Type of plant oil	Increased with oils high in PUFAs
Processed seeds	Increased
High–oil corn grain and silage	Minimal effect
Marine algae	Increased
Pasture	Higher than on conserved forages
Growth stage of forage	Increased with less mature forage
CLA supplement	Dose-dependent increase

Source; Bauman et al. (1999)

2.2.2 Enhancing of CLA by processing

Studying the increase of CLA in processed food has gained considerable attention after finding physiological benefits of CLA. Initial reports showed an increase of CLA in processed cheeses (Ha, Grimm, and Pariza, 1989). From then on, an enhancement of CLA formation at elevated temperature in several dairy products was reported (Table 2.7).

It is assumed that CLA formation in processed food involves the oxidation of linoleic acid. Ha et al. (1989) explained that heat treatment activated oxidation and the radicals from the oxidation reacted with proton from hydrogen donors, such as proteins, and then rearranged to form conjugated diene structures. Therefore, the main factors affecting enhancement of CLA in processed foods are temperature, lipid content and protein content. Moreover, air and starter cultures might affect the CLA formation, which was observed in cheddar cheese processing (Lin, Boylston, Luedecke, and Shultz, 1999)

Table 2.7 CLA concentrations in processed dairy products

Products	Fat content	CLA concentration (mg/g fat)	
	(%)	Unprocessed raw material	Finished product
Butter (salted)	80	6.11[a]	8.11[b]
Butter (unsalted)	80	6.11[a]	7.82[b]
Yogurt	0.05	4.40[a]	5.25[b]
Cheese (cheddar)	32	4.84[a]	5.02[a]

[a-b] Mean in the rows followed by different letters are significantly different ($p<0.05$)

Source: modified from Shantha, Ram, O'Leary, Hicks, and Decker (1995)

2.2.3 Chemical reaction

2.2.3.1 Alkaline isomerization

A prototropic shift mechanism was proposed by Ingold, Shoppee, and Thorpe (1926) and applied to a heated alkaline process for CLA formation by Moore (1939). At present, alkaline isomerization is mainly used for commercial production of CLA. In general, safflower and sunflower oils are widely used for CLA production because of high linoleic acids content. These synthetic mixtures were reported to contain two major isomers; *cis*9,*trans*11-18:2 and *trans*10,*cis*12-18:2 with are estimated amount of conjugated acids of about 75% by using the original method of Nichols et al. (1951). After modification a yield of more than 90% was reported (Zu and Schut, 1992).

Fig. 2.6 shows the isomerization of linoleic acid, $CH_3(CH2)_4CH=CH\text{-}CH_2\text{-}CH=CH(CH_2)_7COOH$, to conjugated isomers by the heated alkaline method. When a proton is removed from the center methylene of linoleic acid, there remains a negative ion, which can be represented by a resonance hybrid of the structure.

$$CH_3(CH2)_4CH=CH\text{-}\overline{C}H_2\text{-}CH=CH(CH_2)_7COOH$$

The resonance would ionize and promote the prototropic shift. When the bond involved in the shift is *cis*, the new bond formed is assumed predominantly to be *trans* configuration. When the bond involved in the shift is *trans*, the new bond formed can be assumed to be either in the *cis* or *trans* configuration. As indicated, it is predicted that the predominant isomers formed during the alkaline isomerization of *cis*-*cis*-linoleic acid will be *cis*9,*trans*11 and *trans*10,*cis*12-18:2 (Nichols *et al*., 1951).

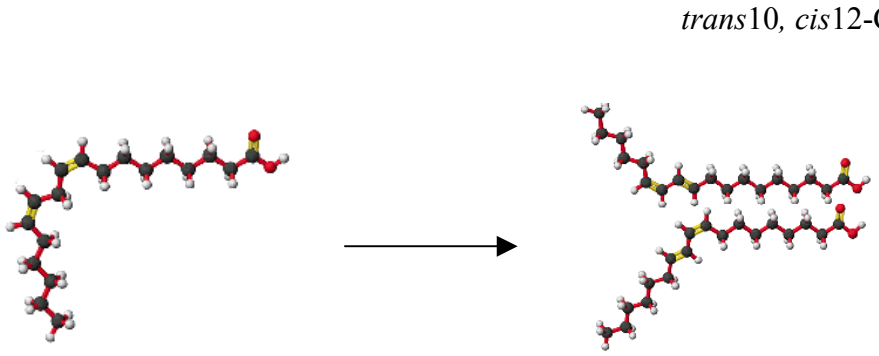


Fig.2.5 Isomerization of linoleic acid to *cis*9, *trans*11 and *trans*10, *cis*12-CLA

2.2.3.2 Homo- and heterogeneous catalysts

Other methods for conjugating of diene stuctures by chemical reactions have been attempted by using homogeneous and heterogeneous catalysts. Homogeneous transition metal catalysts such as Wilkinson's catalyst $RhCL(PPh_3)_3$ (tris-triphenylphosphine chlororhodium) have been widely used to study the isomerization of double bonds in organic compounds including linoleic acid (Adlof, 1999). Beside Wilkinson's catalyst, $[RhCl(C_8H_{14})_2]_2$ and $RhCl.2H_2O$ have been used (Larock, Dong, Chung, Reddy, and Ehlers, 2001) to provide high yields of isomerization of linoleic acid to CLA. However, it is difficult to separate soluble homogeneous catalysts from the product.

Heterogeneous catalysts possibly can isomerize diene double bonds as well. Nickel, palladium, platinum and rhodium are very active catalysts for hydrogenation of triglycerides of unsaturated acids and also have the tendency to catalyze isomerizations

and double bond migrations (Ertl, 1997). In addition, they are easy to use and separate from the isomerized oil product.

Mossoba, McDonald, Armstrong, and Page (1991) found the *cis,trans* and *trans,cis* isomers of linoleic acid to be present in hydrogenated soybean oil and margarine from a hydrogenation process using a Nickel catalyst. Based on Mossoba et al. (1991) work, M.O. Jung, Yoon, and M.Y. Jung, (2001) used 0.1% of a commercial selective nickel catalysts for studying the effects of temperature (170$\pm$2, 190$\pm$2, and 210$\pm$2^{o}C) and agitation rate (300, 500 and 700 rpm) on the formation of total conjugated linoleic acids during oil hydrogenation. The data showed that by increasing the hydrogenation temperature from 170 to 210^{o}C, the quantity of CLA obtained was about 2.6 times higher. As the agitation rate decreased, the CLA formation increased but the time to reach the maximum CLA content also increased. The maximum CLA contents in soybean oil obtained during hydrogenation at 210^{o}C with agitation rates of 300, 500 and 700 rpm were 162.82, 108.62, and 66.15 mg total CLA/g oil, respectively. However, toxicity of the Nickel catalyst, the high temperature process and a fire-hazardous filtration stage were the main disadvantages. In addition, under these severe conditions secondary products may be formed by thermal decomposition and these could be harmful (Savchenko and Makaryan, 1999).

Bernas et al. (2003) applied heterogeneous supported metal catalysts (Rutinium (Ru), Nickel (Ni), Palladium (Pd), Platinum (Pt), Rhodium (Rh), Iridium (Ir) and Osmium, (Os)) for isomerization of linoleic acid at 8-120^{o}C in a solvent system. The reaction that took place is shown in Fig. 2.7. Linoleic acid is isomerized to CLA and also hydrogenated to monounsaturated octadecenoic acids (oleic acid, elaidic acid, *cis*-vaccenic acid, and *trans*-vaccenic acid). CLA is possibly hydrogenated to

monounsaturated octadecenoic aicd as well as further hydrogenated to stearic acid (n-octadecanoic acid). Isomerization and hydrogenation are two competing parallel reactions. The presence of chemisorbed hydrogen increased the conversion but reduced the isomerization activity. In the experiment, Ru and Ni showed the best conjugation properties (53 and 37%) whereas Pd favored the double bond hydrogenation reaction.

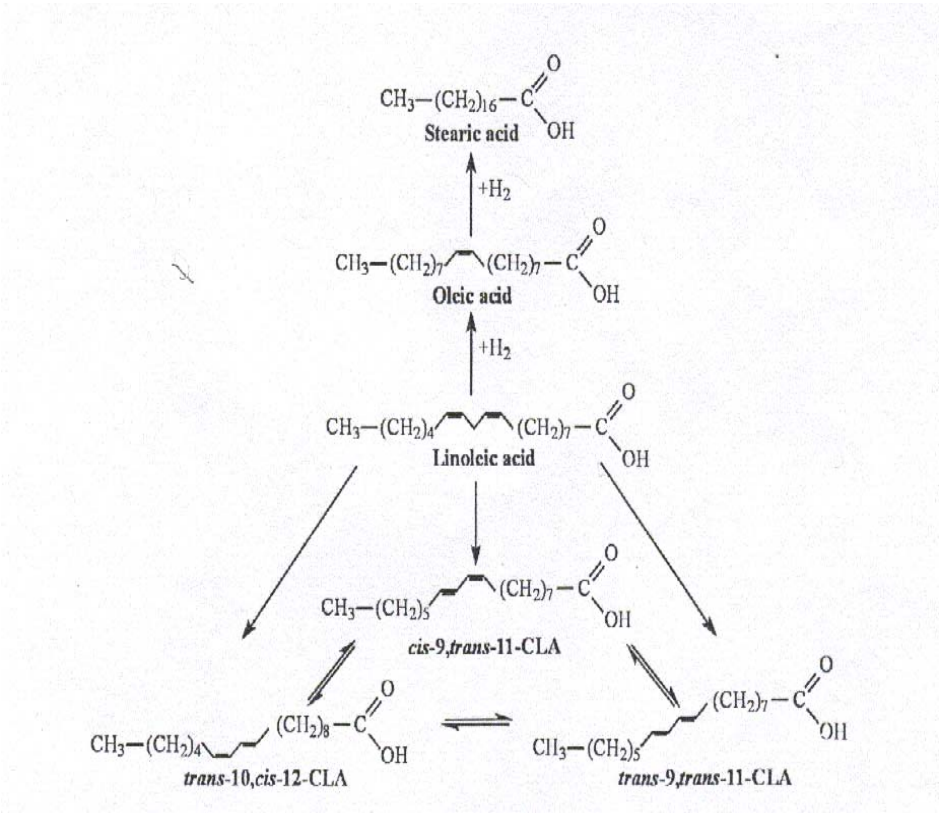


Fig. 2.6 Reaction scheme for isomerization and hydrogenation of linoleic acid

Source: Bernas et al. (2003)

2.3 CLA analysis

There are 14 possible positional isomers counting from carbon 2,4 to carbons 15,17. Each positional isomer has four geometric isomers (*cis,cis*; *cis,trans*; *trans,cis*

and *trans,trans*) for a total of 56 possible isomers. The double bond positions of CLA isomers actually identified in rumen fat range from 6,8-18:2 to12,14-18:2 in most of the possible geometric configurations for a total of about 20 isomers (Sehat et al., 1998).

The combination of gas chromatography (GC) and silver ion high performance liquid chromatography (Ag^+-HPLC) offers the best separation of these isomers with complementary identification by GC-mass spectrometry (GC-MS) and GC-fourier transform infrared (GC-FTIR) analyses. These four analyses are discussed bellow.

2.3.1 Gas chromatography

GC is often used in the analysis of CLA in the form of fatty acid methyl esters. A 100 m cyanopropylsilicone capillary GC column provides good separation for the analysis of the closely related geometrical and positional isomers of CLA. A shorter (50 or 60 m) column is more prone to show interference by methoxylation artifacts. Roach, Mossoba, Yurawecz, and Kramer (2002) showed that methoxylation artifacts eluted after the CLA region on a 100 m CP-Sil 88 or SP 2560 column but they may elute with the CLA in a 50 m column. Thus, 100 m cyanopropylsilicone capillary GC columns (CP-Sil 88, Varian, Palo Alto, CA; SP 2560, Supelco Inc., Bellefonte, PA; BPX70, SGE, Melborne, Australia) are widely used and recommended (Roach et al., 2002).

The elution order on this polar column, CP-Sil 88 or SP 2560, is *cis*,*trans*/*trans*,*cis* followed by all *cis* and finally, all *trans* CLA (Fig. 2.8) (Roach et al., 2002). Many of the CLA isomers overlapped with in each geometric group and also between the *cis,trans* and *cis,cis* group. Mossoba et al. (1999) showed that elution

time of *cis,trans* CLA isomers increased as the Δ value of the *cis* double bond increased in the molecule. For a pair of *cis,trans* isomers, in which the *cis* double bond has the same Δ value, the isomer with the lower Δ *trans* value eluted first. This means that for the same positional isomer, the *cis,trans* will elute before the *trans,cis* geometric isomer. The observed elution time of *cis,cis* isomers increased with increasing Δ values, while that of *trans,trans* CLA isomers increased with decreasing Δ values, and *t*10,*t*12- to *t*7,*t*9-C18:2 do not separate.

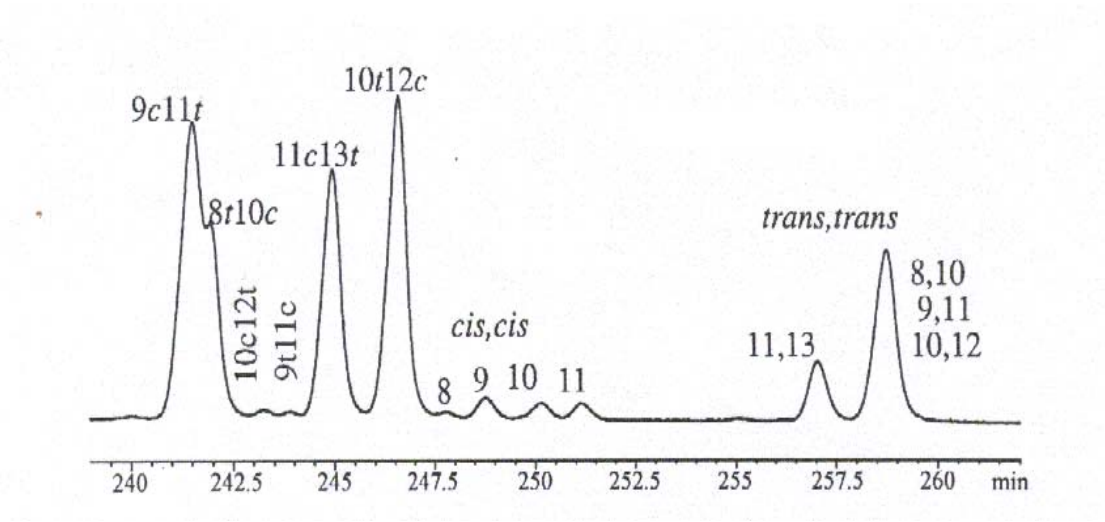


Fig. 2.7 Partial GC chromatogram of the CLA isomers using 100 m CP-Sil 88 capillary column

Source: Roach et al. (2002)

The elution order of each CLA isomer on a 100 m CP-Sil 88 column is shown in table 2.8. These separations are evident only when the relative concentration of the different isomers is similar. Whenever the relative concentration is uneven a number of

CLA isomers will be masked by the predominant isomers. Identification of CLA peaks can be confirmed by GC-MS and GC-FTIR.

Table 2.8 Elution order of positional and geometrical CLA isomers on a 100 m CP-Sil 88 capillary gas chromatographic column

Cis,trans-18:2	*Cis,cis*-18:2	*Trans,trans*-18:2
*c*7,*t*9	*c*7,*c*9*	*t*12,*t*14
*c*8,*t*10	*c*8,*c*10	*t*11,*t*13
*t*7,*c*9	*c*9,*c*11	*t*10,*t*12
*c*9,*t*11	*c*10,*c*12	*t*9,*t*11
*t*8,*c*10	*c*11,*c*13	*t*8,*t*10
*c*10,*t*12	*c*12,*c*14	*t*7,*t*9
*t*9,*c*11		
*c*11,*t*13		
*t*10,*c*12		
*c*12,*t*14		

* CLA isomers shown in parentheses are predicted.

Source: Roach et al. (2002)

2.3.2 High performance liquid chromatography

A silver ion HPLC procedure was developed and applied to the separation of CLA isomers. The conjugated diene structure of CLA has a distinctive UV spectrum and exhibits a characteristic absorbance at 234 nm whereas non-conjugated FAME respond only poorly at this UV wavelength. When combined with silver ion-HPLC

column using 0.1% acetronitrile in hexane as the mobile phase, it is possible to separate and quantify the groups of CLA isomers in form of methyl ester. CLA isomers were resolved on the basis of double bond configuration and position of the conjugated diene functional group in the fatty acid chain. To date, the chromatographic resolution of complex mixture of CLA isomers obtained by Ag^{+}-HPLC is substantially better than those reported by GC on polar phases, or by HPLC on C_{18} or silica columns.

In the Ag^{+}-HPLC method, the CLA mixture is separated into three groups of *trans,trans*; *cis,trans/trans,cis* and *cis,cis*-18:2 isomers. The observed elution time revealed that retention within each group of geometric CLA increased as the Δ values decreased (Mossoba et al., 1999) (Table 2.9). Sehat et al. (1999) reported that each of the three groups of geometric isomers was shown to contain at least four major positional CLA isomers, 8,10; 9,11; 10,12 and 11,13-18:2 (Fig. 2.9). In addition, increasing the number of Ag^{+}-HPLC columns in series from 1 to 6 improved the resolution of methyl esters of CLA and was able to separate *trans*12,*trans*14; *trans*7,*trans*9; *cis*12,*trans*14 and *trans*7,*cis*9 (Sehat et al., 1999). Furthermore, this system was able to separate *trans*11,*cis*13 from *cis*11,*trans*13 as well. However, they suggested that three Ag^{+}-HPLC columns in series appeared to be the best compromise to obtain satisfactory resolution of most CLA isomers.

An analysis of underivatized CLA was proposed by Cross, Ostrowska, Muralitharan, and Dunshea (2000). They did not methylate CLA to methyl ester but directly analyzed it in the free fatty acid form. Silver ion was used and small concentrations of acetic acid and acetronitrile in hexane were used as the mobile phase and varied to optimize retention times. In the experiment, two mechanisms were

involved, acetronitrile dissolved the CLAs off the Ag^+ adsorption sites whereas acetic acid removed the acids from the hydrogen bonding sites. A mobile phase of 2.5% acetic acid and 0.025% acetronitrile in hexane was found to be the optimum mobile phase for CLA isomers separation.

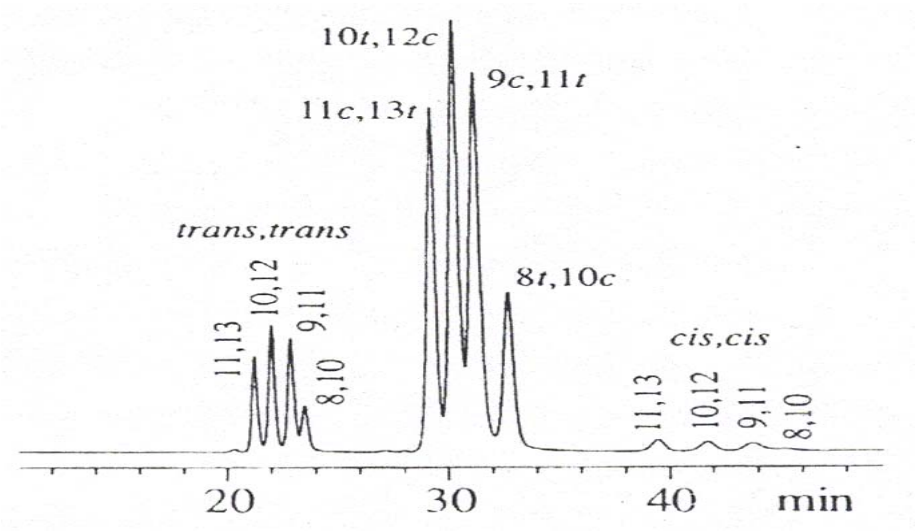


Fig. 2.8 Ag^+-HPLC chromatogram using two columns in series

Source: Sehat et al. (1999)

Table 2.9 Elution order of positional and geometrical CLA methyl ester by Ag^+-HPLC

Trans,trans-18:2	*Cis,trans*-18:2	*Cis,cis*-18:2
*t*12,*t*14	*c*12,*t*14	*c*12,*c*14
*t*11,13t	*c*11,*t*13	*c*11,*c*13
*t*10,*t*12	*c*10,*t*12	*c*10,*c*12
*t*9,*t*11	*c*9,*t*11	*c*9,*c*11
*t*8,*t*10	*c*8,*t*10	*c*8,*c*10
*t*7,*t*9	*c*7,*t*9	*c*7,*c*9

Source; Mossoba et al. (1999); Sehat et al., (1999)

2.3.3 Gas chromatography-infrared spectroscopy

GC-FTIR can provide specific information about the nature of functional groups and confirm the configuration of double bonds (*cis,trans*, *cis*,*cis* or *tran,trans*) for complex mixtures of CLA geometric isomers (Mossoba, 2001). Conjugated fatty acids give rise to weak but unique bands that make their geometric isomers easily distinguishable due to the number, position and configuration of double bonds. The corresponding GC direct deposit FTIR (GC-DD-FTIR) data for CLA DMOX derivatives were: *cis,trans*: 3020 and 3002 cm^{-1}; *cis,cis*: 3037 and 3005 cm^{-1}; and *trans,trans*: 3017 cm^{-1} (Fig. 2.10). By contrast, the band position found for non-conjugated FAME were 3035 and 3005 cm^{-1} (one or two trans double bonds), 3010 cm^{-1} (*cis* double bond), 3018 cm^{-1} (two or three *cis* double bonds), 3035, 3010, and 3005 cm^{-1} (*cis* and *trans* double bonds separated by more than one methylene group), 3035, 3018, and 3005 cm^{-1} (*cis* and *trans* double bonds separated by a single methylene group) (Mossoba, 2001).

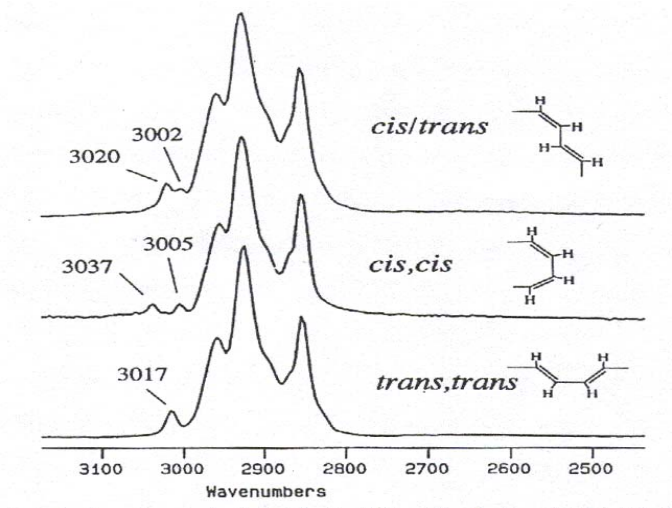


Fig. 2.9 Expanded GC-DD-FTIR hydrocarbon stretch spectral region for CLA DMOX geometric isomers

Source: Mossoba (2001)

2.3.4 Gas chromatography-mass spectroscopy (GC-MS)

Double bond positions of CLA can be recognized by GC-Mass spectrometry with fatty acids in the form of dimethyloxazoline (DMOX). The reagent used for DMOX derivatives react with free carboxylic acids or methyl ester functional groups providing highly diagnostic mass spectra. The DMOX CLA spectra are characterized by a loss of 15 from the molecular ion (m/z 333) and successive losses of 14 except for the conjugated diene system, which may be located in the chain by its characteristic loss sequence of 12, 14 and 12. Carbons in the chain allyl to the conjugated diene system are favored radical sites and produce more abundant fragmentation than other positions in the chain. These abundant fragment ions flanking a loss sequence of 12, 14 and 12 facilitate assignment of the positions of the double bonds in the carbon chain (Fig. 2.11).

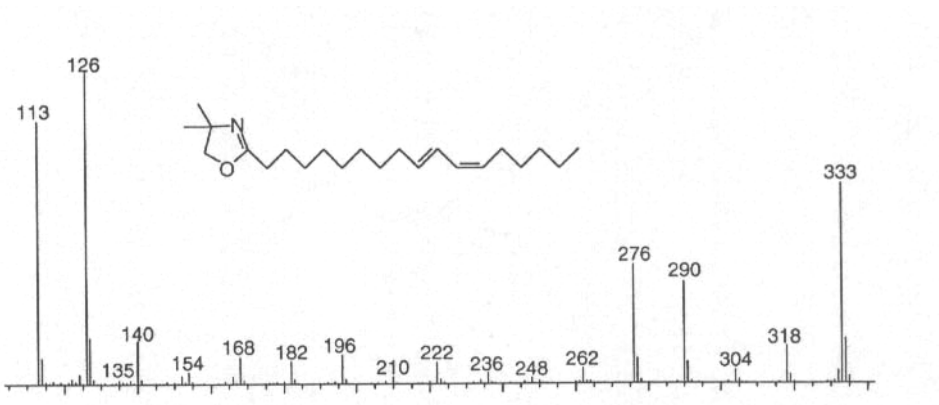


Fig. 2.10 EI mass spectrum of trans11, cis13-CLA DMOX derivative

Source: Roach (2002)

If the letters “n” and “m” are used to indicate the locations of the conjugated double bonds in the carbon chain of the CLA molecule, then n-1, n-2, m+1, m+2, and m+3 identify nearby carbons in the chain relative to the conjugated double bonds. The distinctive conjugated diene loss sequence of 12, 14, and 12 occurs between carbons n-1 and n, n and m-1, and m-1 and m (Roach, 2002). The fragment ions for CLA isomers with double bond positions from C-6 to C-15 are shown in Table 2.10.

Table 2.10 Diagnostic DMOX CLA fragment ions for CLA isomers

Isomer	n-2	n-1	n	m-1	m	m+2	m+3
6,8	140	154	166	180	192	220	234
7,9	154	168	180	194	206	234	248
8,10	168	182	194	208	220	248	262
9,11	182	196	208	222	234	262	276
10,12	196	210	222	236	248	276	290
11,13	210	224	236	250	262	290	304
12,14	224	238	250	264	276	304	318
13,15	238	252	264	278	290	318	332

Source: Roach (2002)

The predictable pattern of these fragments makes it possible to search GC-MS data records of DMOX derivatized extracts for individual CLA isomers of interest.

References

Adlof, R.O. (1999). The lindlar-catalyzed reduction of methyl santalbate: A facile preparation of methyl 9-cis,11-trans-octadecadienoate-9,10-d_2. **J. Am. Oil Chem. Soc.** 76: 301-304.

Azain, M.J., Hausman, D.B., Sisk, M.B., Flatt, W.P., and Jewell, D.E. (2000). Dietary conjugated linoleic acid reduces rat adipose tissue cell size rather than cell number. **J. Nutr.** 130: 1548-1554.

Bauman, D.E., Baumgard, L.H., Corl, B.A., and Griinari, J.M. (1999). Biosynthesis of conjugated linoleic acid in ruminants. **Proceeding of the American Society of Animal Science** 1-15.

Bernas, A., Kumar, N., MäkArevela, P., Kul'kova, N.V., Holmbom, B., Salmi, T., and Murzin, D.Y. (2003). Isomerization of linoleic acid over supported metal catalysts. **Appl. Catal.** 6483: 1-19.

Berven, G.A., Bye, A., Hais, O, Blankson, H., Fagerton, H., Thom, E., Wadstein, J., and Gudmunsen, O. (2000). Safety of conjugated linoleic acid (CLA) n overweight or obese hyman volunteers. **Eur. J. Lipid Sci. Technol.** 102: 455-462.

Brodie, A.E., Manning, V.A., Ferguson, K.R., Jewell, D.E., and Hu, C.Y. (1999). Conjugated linoleic acid inhibits differentiation of pre- and postconfluent 3T3-L1 preadipocytes but inhibits cellproliferation only in preconfluent cells. **J. Nutr.** 129: 602-606.

Choi, Y., Kim, Y., Han, Y., Park, Y., Pariza, M.W., and Ntambi, J.M. (2000). **Nutrient-Gene Expression** 1920-1924.

Cook, M.E., Miller, C.C., Park, Y., and Pariza, M. (1993). Immune modulation by altered nutrient metabolism: nutritional control of immune-induced growth depression. **Poultry Sci.** 72: 1301-1305.

Chouinard, P.V., Corneau, L., Barbano, D.M., Metzger, L.E., and Bauman, D.E. (1999). Conjugated linoleic acids alter milk fatty acid composition and inhibit milk fat secretion in dairy cows**. J. Nutr.** 129: 1579-1584.

Cross, R.F., Ostrowska, E., Muralitharan, M., and Dunshea, F.R. (2000). Mixed mode retention and the use of competing acid for the Ag^{+}-HPLC analysis of underivatized conjugated linoleic acids. **J. High Resol. Chromatogr.** 23 (4): 317-323.

Decker, E.A. (1995). The role of phenolics, conjugated linoleic acid, carnosine, and pyrroloquinoline quinone as nonessential dietary antioxidants. **Nutr. Res.** 53 (3): 49-58.

Durgam, V.R., and Fernandes, G. (1997). The growth inhibitory effect of conjugated linoleic acid on MCF-7 cells is related to estrogen response system. **Cancer Lett.** 116: 121-130.

Ertl, G. (1997). **Handbook of Heterogeneous Catalysts.** Weinheim: VCH Verlagsgesellschaft mbH.

Gävino, V.C., Gävino, G., Lablanc, M.J., and Tuchweber, B. (2000). An isomeric mixture of conjugated linoleic acids but not pure cis-9,trans-11-octadecadienoic acid affects body weight gain and plasma lipids in hamsters. **J. Nutr.** 130: 27-29.

Gnädig, S., Rickert, R., Sébédio, J.L., and Steinhart, H. (2001). Conjugated linoleic acid (CLA): physiological effects and production. **Eur. J. Lipid Sci. Technol.** 103: 56-61.

Ha, Y.L., Grimm, N.K., and Pariza, M.W. (1987). Anticarcinogens from fried ground beef: heat altered derivatives of linoleic acid. **Carcinogenesis** 8: 1881-1887.

Ha, Y.L., Grimm, N.K., and Pariza, M.W. (1989). Newly recognized anticarcinogenic fatty acids: Identification and quantification in natural and processed cheese**. J. Agric. Food Chem.** 37(1): 75-81.

Ha, Y.L., Storkson, J., and Pariza, M.W. (1990). Inhibition of benzo(a)pyrene-induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. **Cancer Res.** 50: 1097-1101.

Ingold, Shoppee and Thorpe. (1926). **J. Chem. Soc.** 1477 referenced by Nichols, P.L., Herb, Jr.S.F., and Riemenschneider, R.W. (1951). Isomers of conjugated fatty acids. I. Alkaline-isomerized linoleic acid. **J. Chem. Soc.** 73: 247-252.

Ip, C., Chin, S.F., Scimeca, A., and Pariza, M.W. (1991). Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. **Cancer Res.** 51: 6118-6124.

Ip, C., Singh, M., Thompson, J., and Scimeca, A. (1994). Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. **Cancer Res.** 54: 1212-1215.

Ip, C., Banni, S., Angioni, E., Carta, G., McGinley, J., Thompson, H., Barbano, D., and Bauman, D. (1999). Conjugated linoleic acid-enriched butter fat alters mammary gland morphogenesis and reduces cancer risk in rats. **J. Nutr.** 129: 2135-2142.

Ivan, M., Mir, P.S., Koenig, K.M., Rode, L.M., Neill, L., Entz, T., and Mir, Z. (2001). Effects of dietary sunflower seed oil on rumen protozoa population and tissue concentration of conjugated linoleic acid in sheep**. Small Ruminant Res.** 41: 215-227.

Jung, M.O., Yoon, S.H., and Jung, M.Y. (2001). Effect of temperature and agitation rate on the formation of conjugated linoleic acids in soybean oil during hydrogenation process. **J. Agri. Food Chem.** 49 (6): 3010-3016.

Kepler, C.R., and Tove, S.B. (1967). Biohydrogenation of unsaturated fatty acids. **J. Biol. Chem.** 242 (24): 5686-5691.

Kim, Y.J., and Liu, R.H. (1999). Selective increase in conjugated linoleic acid in milk fat by crystallization. **J. Food Sci.** 64 (5): 792-795.

Larock, R.C., Dong, X., Chung, S., Reddy, C.K., and Ehlers, E. (2001). Preparation of conjugated soybean oil and other natural oils and fatty acids by homogeneous transition metal catalysis. **J. Am. Oil Chem. Soc.** 78 (5): 447-453.

Liew, C., Schut, H.A.J., Chin, S.F., Pariza, M.W., and Dashwood, R.H. (1995). Protection of conjugated linoleic acids against 2-amino-3-methylimidazo[4,5-f]quinoline-induced colon carcinogenesis in the F344 rat: a study of inhibitory mechanisms. **Carcinogenesis** 16: 3037-3043.

Lin, H., Boylston, T.D., Chang, M.J., Luedecke, L.O., and Shultz, T.D. (1995). Survey of the conjugated linoleic acid contents of dairy products. **J. Dairy Sci.** 78: 2358-2365.

Lin, H., Boylston, T.D., Luedecke, L.O., and Shultz, T.D. (1999). Conjugated linoleic acid content of cheddar-type cheeses as affected by processing. **J. Food Sci.** 64 (5): 874-878.

Loo, J.J., and Herbein, J.H. (2003). Dietary canola or soybean oil with two levels of conjugated linoleic acids (CLA) alter profiles of 18:1 and 18:2 isomers in blood plasma and milk fat from dairy cows. **Ani. Feed Sci. and Tech.** 103: 63-83.

Medina, E.A., Horn W.F., Kiem, N.L., Havel, P.J., Banito, P., Kelly, D.S., Nelson, G.J., and Erickson, K.L. (2000). Conjugated linoleic acid supplementation in humans: effects on circulating leptin concentration and appetite. **Lipids** 35: 783-788.

Moore. (1939). **Biochem. J.** 33: 1635. referenced by Nichols, P.L., Herb, Jr.S.F., and Riemenschneider, R.W. (1951). Isomers of conjugated fatty acids. I. Alkaline-isomerized linoleic acid. **J. Chem. Soc.** 73: 247-252.

Mossoba, M.M., McDonald, R.E., Armstrong, D.J., and Page, S.W. (1991). Identification of minor C18 triene and conjugated diene isomers in hydrogenated soybean oil and margarine by GC-MI-IR spectroscopy. **J. Chromatogr. Sci.** 29: 324-330.

Mossaba, M.M., Kramer, J.K.G., Yurawecz, M.P., Sehat, N., Roach, J.A.G., Eulitz, K., Fretsche, J., Durgan, M.E.R., and Ku, Y. (1999). Impact of novel methodologies on the analysis of conjugated linoleic acid (CLA). Implication of CLA feeding studies. **Fett/Lipid** 101:235-243.

Mossoba, M.M. (2001). Application of gas chromatography-infrared spectroscopy to the confirmation of the double bond configuration of conjugated linoleic acid isomers. **Eur. J. Lipid Sci. Technol.** 103: 594-932.

Nichols, P.L., Herb, Jr.S.F., and Riemenschneider, R.W. (1951). Isomers of conjugated fatty acids. I. Alkaline-isomerized linoleic acid. **J. Chem. Soc.** 73: 247-252.

Ntambi, J.M. (1999). Regulation of stearoyl-Co-A desaturase by polyunsaturated fatty acids and cholesterol. **J. Lipid Res.** 40: 1549-1558.

Pariza, M.W., and Hargraves, W.A. (1985). A beef-derived mutagenesis modulator inhibits initation of mouse epidermal tumors by 7, 12-dimethylbenz[a]anthracene. **Carcinogenesis** 8: 591-593.

Park, Y., Storkson, J.M., Albright, K.J., Liu, W., and Pariza, M.W. (1999). Evidence that the trans-10, cis-12 isomer of conjugated linoleic acid induces body composition changes in mice**. Lipids** 34: 235-241.

Roach, J.A.G., Mossoba, M.M., Yurawecz, M.P., and Kramer, J.K.G. (2002). Chromatographic separation and identification of conjugated linoleic acid isomers. **Analytica Chimica Acta.** 465: 207-226.

Savchenko, V.I., and Makaryan, I.A. (1999). Palladium catalyst for the production of pure margarine. **Palatinum Metals Rev.** 43 (2): 74-82.

Sehat, N., Kramer, J.K.G., Mossoba, M.M., Yurawecz, M.P., Roach, J.A.G., Eulitz, K., Morehouse, K.M., and Ku, Y. (1998). Identification of conjugated linoleic acid isomers in cheese by gas chromatography, Silver ion high performance liquid chromatography and Mass spectral reconstructed ion profiles. Comparison of chromatographic elution sequences. **Lipids** 33 (10): 963-970.

Sehat, N., Rickert, R., Mossoba, M.M, Kramer, J.K.G., Yurawecz, M.P., Roach, Adlof, R.O., Morehouse, K.M., Fritsche, J., Eulitz, H.D., Steinhart, H., and Ku, Y. (1999). Improved separation of conjugated fatty acid methyl esters by silver ion high-performance liquid chromatography. **Lipids** 34: 407-413.

Shantha, N.C., Ram, L.N., O'Leary, J., Hicks, C.L., and Decker, E.A. (1995). Conjugated linoleic acid concentrations indairy products as affected by processing and storage. **J. Food Sci.** 60 (4): 695-720.

Stanton, C., Lawless, F., Kjellmer, G., Harrington, D., Devery, R., Connolly, J.F., and Murphy, J. (1997). Dietary influence on bovine milk *cis*-9, *trans*-11-conjugated linoleic acid content. **J. Food Sci.** 62(5): 1083-1086.

Yamasaki, M., Kishihara, K., Ikeda, I., Sugano, M., and Yamada, K. (1999). A reccommended esterification method for gas chromatographic measurement of conjugated linoleic acid. **J. Am. Oil Chem. Soc.** 76 (8): 933-938.

Zambell, K.L., Keim, N.L., Van Loan, M.D., Gale, B., Benito, P., Kelly, D.S., and Nelson, G.J. (2000). Conjugated linoleic acid supplementation in humans: effects on body composition and energy expenditure. **Lipids** 35: 777-782.

Zu, H.X., and Schut, H.A.J. (1992). Inhibition of 2-amino-3-methylimidazo[4,5-f]quinoline-DNA adduct formation in CDF mice by heat-altered derivatives of linoleic acid. **Food Chem. Tox.** 30 (1): 9-16.

CHAPTER III

EFFECTS OF RHODIUM HETEROGENEOUS CATALYST AND ISOMERIZATION CONDITIONS ON LINOLEIC ACID CONJUGATION OF SOYBEAN OIL

Abstract

Rhodium heterogeneous catalyst was used to catalyze isomerization of linoleic acid in soybean oil to conjugated linoleic acid (CLA). A central composite rotatable design with 5 levels of 3 variables, namely reaction temperature, stirring speed and reaction time, was used to determine maximum CLA yield. Formation of CLA during isomerization was greatly dependent on reaction temperature and time. CLA content of soybean oil increased from 0.63 to 202.42 mg/g oil when isomerization was done at 200^{o}C, for 49 min with a stirring speed of 200 rpm. Analysis of triacylglycerol positions showed that linoleic acid at any position in a triacylglyceride could possibly be isomerized to CLA.

3.1 Introduction

Conjugated linoleic acid (CLA; CH_3 $(CH_2)_5CH=CHCH=CH(CH_2)_7COOH$) refers to a mixture of positional and geometrical isomers of linoleic acid with conjugated double bonds. It is found predominantly in meat and dairy products due to isomerization of linoleic acid to CLA in ruminant animal by gram-positive bacteria, such as *Butyrivibrio fibrisolvens, Ruminococcus albus* and *Eubacterium sp.* (Kepler, and Tove, 1967). It has been reported that health benefits of CLA may include anticarcinogenesis (Ha, Storkson, and Pariza, 1990; Ip, Chin, Scimeca, and Pariza, 1991; Schultz, Chew, and Seaman, 1992), antiatherosclerosis (Gavino, Gavino, Lablanc, and Tuchweber, 2000), enhancing immune function (Cook, Miller, Park, and Pariza, 1993) and body fat reduction (Brodie, Manning, Ferguson, Jewell, and Hu, 1999; Park, Storkson, Albright, Liu, and Pariza, 1999). Commercially, CLA is mainly produced by alkaline isomerization of linoleic acid. This isomerization method has been known as diene conjugation since 1951 (Nichols, Herb, and Riemenschneider, 1951) in which *cis*9,*trans*11-18:2 and *trans*10,*cis*12-18:2 fatty acids are predominant isomers from the synthesis. However, using an excess of strong basic potassium hydroxide or sodium methoxide is disadvantageous (Bernas et al., 2003). Furthermore, CLA in its chemical form of a free fatty acid is easily oxidized in air (Yang, Leung, Huang, and Chen, 2000). Other methods for conjugating diene stuctures include using homogeneous and heterogeneous catalysts. Homogeneous transition metal catalysts such as $RhCL(PPh_3)_3$, $[RhCl(C_8H_{14})_2]_2$ and $RhCl{\cdot}2H_2O$, have been used to study the isomerization of double bonds in organic compounds including linoleic acid (Adlof, 1999; Larock, Dong, Chung, and Reddy, 2001). However, it is difficult to separate soluble homogeneous catalysts from the final product.

Heterogeneous catalysts used for double bond hydrogenation not only facilitate hydrogenation but also have the tendency to catalyze isomerizations and double bond migrations (Ertl, 1997). Mossoba, McDonald, Armstrong, and Page (1993) and Jung, M.O. Yoon, and Jung, M.Y. (2001) showed the potential of conjugation of unsaturated fatty acid in edible oil using a heterogeneous nickel catalyst. Palladium, platinum and rhodium are also active catalysts for hydrogenation and possible isomerization. In addition, they are easy to use and separate from the isomerized oil product. From the preliminary studies on the potential use of palladium, platinum and rhodium catalysts for the conjugation of fatty acids, rhodium showed to be the most effective catalyst for conjugation of dienes at high temperature, and ethylene glycol was a good medium in the reaction.

This paper presents a study of the effect of isomerization conditions using a heterogeneous catalyst (Rh on carbon) on the formation of CLA in soybean oil. Silver impregnated high performance liquid chromatography (Ag^+HPLC), gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) were used to determine and identify the CLA isomers. Triacyl-*sn*-glycerols in isomerized soybean oil were partially hydrolyzed with pancreatic lipase and analyzed by TLC and Ag^+HPLC for determining CLA position in the triacyl-*sn*-glycerols.

3.2 Materials and methods

3.2.1 Materials

Soybean oil was purchased from Wal*Mart Store Inc., Bentonville, AR, USA, Lot No. 030203CAA. Three standard CLA methyl ester isomers *cis*9,*cis*11; *cis*9,*trans*11 and *trans*9,*trans*11 (98% purity), were purchased from Matreya, Inc.

(Pleasant Gap, PA, USA) and kept at –20^{o}C. Rhodium, 5% on carbon, was supplied by Strem Chemicals, Inc. (Newburyport, MA, USA), and C_{17} methyl ester, 2-amino-2-methyl-1-propanol (95%) and pancreatic lipase (triacylglycerol lipase; EC 3.1.1.3) were purchased from Sigma-Aldrich Chemicals, Inc. (St. Louis, MO, USA). Silica TLC plates F254, 25x25 cm were purchased from Merck KGaA (Darmstadt, Germany). Sodium methoxide, ethylene glycol, acetonitrile and hexanes were supplied by Fisher Co. (Fair Lawn, NJ, USA). All chemicals and solvents were reagent grade.

3.2.2 Experimental Design

A central composite rotatable design (Cochran, and Cox, 1992; see Chapter 6) with 3 variables at 5 levels, namely reaction temperature (146, 160, 180, 200 and 214^{o}C), stirring speed using a magnetic bar controlled by hot plate heater (100, 200, 300, 400 and 500 rpm) and reaction time (0, 49, 120, 191 and 240 min) was used to determine maximum yield of CLA. Model of the analysis for 3x-variables is shown in Table 3.1, the matrix of the actual test runs are presented in Table 3.2.

Table 3.1 Model and variable ranges of central composite rotatable design

Variables	Levels				
	- 1.682	-1	0	1	1.682
Temperature (^{O}C)	146	160	180	200	214
Speed (level)	1	2	3	4	5
Time (min)	0	49	120	191	240

Source: Cochran, and Cox (1992)

Table 3.2 Model and actual test runs of the experiment

Test run	Model			Actual		
	Temperature (^{o}C)	Speed (level)	Time (min)	Temperature (^{o}C)	Speed (level)	Time (min)
1	-1	-1	-1	160	2	49
2	+1	-1	-1	200	2	49
3	-1	+1	-1	160	4	49
4	+1	+1	-1	200	4	49
5	-1	-1	+1	160	2	191
6	+1	-1	+1	200	2	191
7	-1	+1	+1	160	4	191
8	+1	+1	+1	200	4	191
9	-1.682	0	0	146	3	120
10	+1.682	0	0	214	3	120
11	0	-1.682	0	180	1	120
12	0	+1.682	0	180	5	120
13	0	0	-1.682	180	3	0
14	0	0	+1.682	180	3	240
15	0	0	0	180	3	120

3.2.3 Isomerization conditions

The isomerization was performed by mixing 5 g of soybean oil with 10 g of ethylene glycol in a reaction flask. After the mixtures were heated to the set point temperature, 0.015% of rhodium catalyst was added. After reacting the mixture for the selected reaction time, the isomerized oil was separated from ethylene glycol and catalyst by centrifuging at 2000 × g for 15 min and filtering through a 0.2 μm filter. The isomerized oil was purged with nitrogen and kept at –20^{o}C. The isomerized soybean oil was analyzed for iodine value by iodometric titration according to AOCS

official method Ca 2a-47 (American Oil Chemists Society, 1999). Fatty acid profiles and CLA were analyzed by GC and HPLC, respectively. CLA isomers were identified by spiking of 3 standard CLAs in Ag^+ impregnated HPLC analyses. In addition, molecular ion and characteristic fragmentation pattern of 4,4-dimethyloxazoline (DMOX) CLA derivatives were analyzed by GC/MS and compared with reference literatures (Sehat et al., 1998; Fritsche et al., 2001). The positions of the CLA in triacylglycerols (TAG) were investigated by partially hydrolyzing the CLA-TAG with pancreatic lipase. The triacylglycerol position was analyzed and confirmed by using TLC and HPLC techniques.

3.2.4 Preparation of conjugated linoleic acid methyl ester (CLAME)

Approximately 30 mg of isomerized soybean oil was placed into a 15-mL reaction tube fitted with a Teflon-lined screw cap. Two mL of 0.5 M sodium methoxide and 1 mL of internal standard (2.02 mg/mL of C_{17} methyl ester in hexane) were added. The tube was flushed with nitrogen then heated at 50^{o}C for 20 min with occasional shaking. After methylation was completed, 10 ml of HPLC grade water was added. The solution was transferred to a 40-mL centrifuge tube and 6 mL of hexane was added for CLAME extraction. The solution was centrifuged at 2000 × g at 10^{o}C for 20 min. The hexane layer was dried over sodium sulfate and analyzed by GC and HPLC.

3.2.5 CLAME analysis by HPLC

A Perkin Elmer HPLC equipped with a 20-μL Rheodyne injection loop, an UV detector set at 233 nm and two ChromSpher 5 lipids analytical (4.6 mm i.d. x 250 mm stainless steel, 5 μm particle size, Chrompack) silver-impregnated columns with guard

column were used in series. HPLC separation was performed isocratically with the mobile phase of 0.1% acetonitrile in hexane freshly prepared and at the flow rate of 1.0 mL/min.

3.2.6 CLAME and FAME analysis by GC

CLA and fatty acid methyl esters were analyzed by GC (8500 GC, Perkin-Elmer, Norwalk, CT, USA) equipped with a 100 m x 0.25 mm fused silica capillary column (SP2560, Supelco Inc, Bellefonte, PA, USA). Injector and detector temperatures were 240^{o}C. The column temperature was kept at 75^{o}C for 1 min, then increased at 20^{o}C /min to 185^{o}C and held at 185^{o}C for 15 min, then increased at 4^{o}C /min to 220^{o}C and held at 220^{o}C for 45 min.

3.2.7 4,4-dimethyloxazoline (DMOX) derivatives

CLAME samples were collected from HPLC fractions and hydrolyzed to free fatty acids by using 10 mL of 0.5N KOH in methanol at 80^{o}C for 40 min. After hydrolysis, 10 mL of water was added. The mixture was adjusted to pH 3 with 1N HCl and salted out with NaCl. The mixture was transferred to a 40-mL centrifuge tube and then 10 mL of petroleum ether was added and centrifuged at 2000 × g at 10^{o}C for 20 min. The petroleum ether layer containing free fatty acids was dried over sodium sulfate and concentrated under nitrogen gas to 1 mL. The sample was then placed into a screw cap reaction tube and a three-fold amount of 2-amino-2-methyl-1-propanol (Sehat et al., 1998) was added. The tube was purged with nitrogen, then heated at 170^{o}C for 5 h. At the completion of the reaction, 10 mL of HPLC water was added. The mixture was transferred to a 40-mL centrifuge tube and then 10 ml of petroleum ether

was added. Two mL of saturated NaCl was added to break the emulsion. The mixture was centrifuged at 2000 × g at 10^{o}C for 20 min. The petroleum ether layer was dried over sodium sulfate and concentrated under nitrogen gas. DMOX derivatives were analyzed by GC-MS.

3.2.8 DMOX derivatives analysis by GC-MS

DMOX derivatives were analyzed by GC-MS (GC: Varian, Star 3400DX; MS: Varian, Saturn 2000, Palo Alto, CA, USA) equipped with a 100 m x 0.25 mm fused silica capillary column (SP2560, Supelco Inc, Bellefonte, PA, USA). Injector and transfer line temperatures were held at 220ºC. The column temperature was kept at 75ºC for 1 min, then increased at 20ºC /min to 185ºC and held at 185ºC for 15 min, then increased at 4ºC/min to 220ºC and held at 220ºC for 45 min.

3.2.9 Statistical analysis

Results obtained from the central composite rotatable design were analyzed by using general linear model procedure (GLM) for regression analysis (SAS Institute, 1989). An independent sample t-test was conducted to identify differences among means. A $p<0.05$ was considered statistically significant.

3.2.10 Partial hydrolysis of TAG for CLA positional analysis

Twenty five milligrams of isomerized soybean oil were hydrolyzed with 6 mg of pancreatic lipase in 2.5 mL of 10 M Tris-HCl pH 8, 0.25 mL of 2.2% w/v $CaCl_2$ and 0.625 mL of 0.05% w/v sodium-taurocholate at 40ºC for 12 min (Valle, Enrique, and Rafael, 2000) . The reaction was stopped by adding 5 mL of ethanol and 5 mL of 6.0 N

HCl. The mixture was transferred into a 40-mL centrifuge tube, 10 mL of diethyl ether was added and centrifuged at 2000 × g at 10°C for 20 min for extraction. The diethyl ether layer was dried over sodium sulfate and concentrated under nitrogen gas. CLA positions in triacyl-*sn*-glycerol were analyzed by TLC and HPLC.

3.2.11 Triacyl-sn-glycerol positional analysis by TLC and HPLC

The hydrolysate and standards (tripalmitolein, 1-monolinolein and linoleic acid) were spotted on a silica gel TLC plate. The developing solvent was hexane/diethyl ether/acetic acid (70:30:1 v/v). To detect the positions of the standards and hydrolysates, the TLC plate was partially covered with a glass plate and exposed to iodine vapors. Unexposed TAG fractions were scrapped off the plates and extracted from silica with diethyl ether. Triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG) and free fatty acids (FFAs) fractions were methylated by BF_3/Methanol (Cross, Ostrowska, Muralitharan, and Dunshea, 2000) and analyzed for CLA by HPLC.

3.3 Results and discussions

3.3.1 CLA profiles of Rh catalyst isomerized oil

Ag^+HPLC chromatographic separation of CLAME showed 15 peaks in 3 groups of CLA isomers; *trans*,*trans*; *cis*,*trans*/*trans*,*cis* and *cis*,*cis*-18:2. Peak identifications for 3 CLAs were confirmed to be *trans*9,*trans*11-18:2, *cis*9,*trans*11-18:2 and *cis*9,*cis*11-18:2 by spiking with standard CLAs of 98% purity (see Fig. 6.1, chapter 6). Further identification of other CLA peaks was done by analyzing DMOX derivatives from Ag^+HPLC fractions by GC-MS and using reference literatures (Sehat

et al., 1998; Fritsche et al, 2001). The DMOX derivative spectra showed molecular ions of m/z 333 which is a molecular ion of the DMOX derivative of all CLA isomers (Spitzer, Marx, and Pfeilsticker, 1994). The mass spectra consisted of a series of even-mass ions separated by 14 mass units due to losses of methylene units as shown in Fig. 3.1. The even-mass homologous series m/z 126+14 mu is interrupted in the region of the double bonds. The mass spectrum A shows a mass difference of 12 mu between m/z 196/208 and m/z 222/234, whereas the spectrum B shows 12 mu gaps between m/z 210/222 and m/z 236/248, according to conjugated double bonds in position 9,11 and 10,12 respectively (Sehat et al., 1998; Spitzer et al., 1994; Roach, Mossoba, Yurawecz, and Kramer, 2002).

(A)

(B)

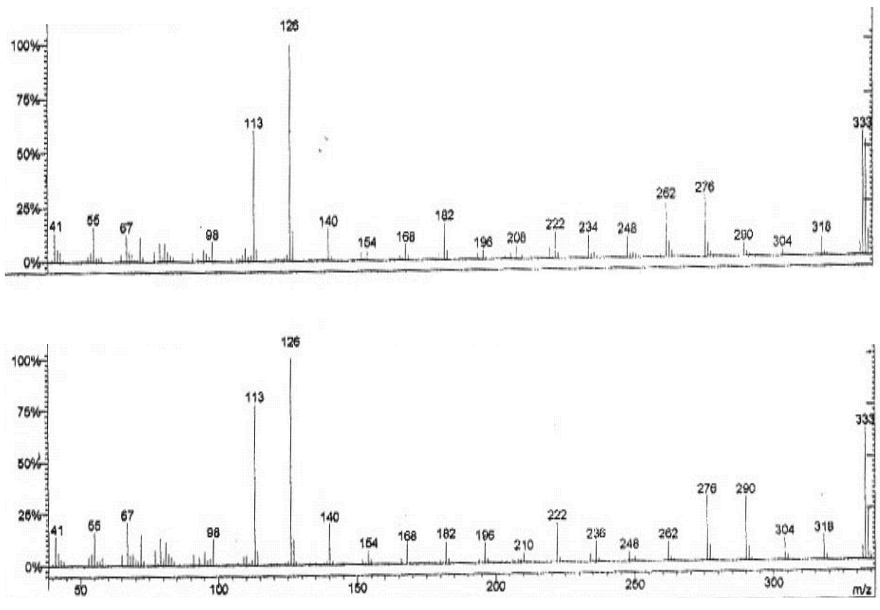


Fig. 3.1 Mass spectra of the 4,4-dimethyloxazoline (DMOX) derivatives of (A) Δ9,11-18:2 and (B) Δ10,12-18:2.

From the results of CLA standard spiking, MS of DMOX derivatives and information from references, it can be concluded that the *trans*,*trans*-18:2 was the first eluted group followed by *cis*,*trans*/*trans*,*cis*-18:2 and then *cis*,*cis*-18:2. The *trans*,*trans* isomers were separated into 6 peaks identified as (1) *t*12,*t*14; (2) *t*11,*t*13; (3) *t*10,*t*12; (4) *t*9,*t*11; (5) *t*8,*t*10 and (6) *t*7,*t*9. The *cis*,*trans*/*trans*,*cis* isomer group contained 5 isomers and was the major group of CLA in the isomerized oil; (7) *c*12, *t*14/*t*12,*c*14; (8) *c*11,*t*13/*t*11,*c*13; (9) *c*10,*t*12/*t*10,*c*12; (10) *c*9,*t*11/*t*9,*c*11and (11) *c*8,*t*10/*t*8,*c*10 as shown in Fig. 3.2. The last CLA group on Ag^{+}HPLC chromatogram included the *cis*,*cis* isomers. They separated into 4 peaks as (12) *c*11,*c*13; (13) *c*10,*c*12; (14) *c*9,*c*11; and (15) *c*8,*c*10.

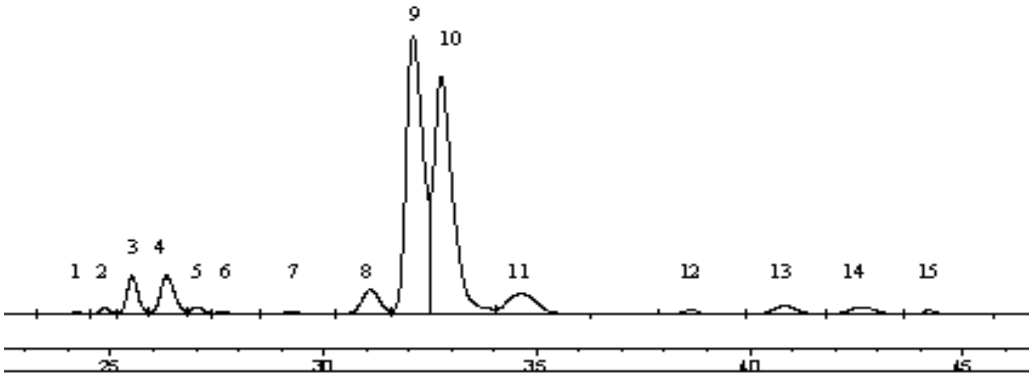


Fig. 3.2. Ag^{+}HPLC chromatogram of isomerized soybean oil. The individual isomers are (1) *t*12,*t*14; (2) *t*11,*t*13; (3) *t*10,*t*12; (4) *t*9,*t*11; (5) *t*8,*t*10; (6) *t*7,*t*9; (7) *c*12, *t*14/*t*12,*c*14; (8) *c*11,*t*13/*t*11,*c*13; (9) *c*10,*t*12/*t*10,*c*12; (10) *c*9,*t*11/*t*9,*c*11; (11) *c*8,*t*10/*t*8,*c*10; (12) *c*11,*c*13; (13) *c*10,*c*12; (14) *c*9,*c*11; and (15) *c*8,*c*10.

3.3.2 Effects of isomerization conditions on the CLA contents

Three types of heterogeneous catalyst, palladium, platinum and rhodium on carbon, were used for preliminary test for the isomerization of soybean oil. Variation of catalyst concentrations (0.004-0.015%), agitation rates (300-600 rpm), type of hydrogen sources (hydrogen gas, hydrogen atmosphere and ethylene glycol), reaction temperatures (40-220^{o}C) and reaction time (15 min - 24 h) were investigated. It was shown that the rhodium catalyst on carbon was more effective for isomerization than palladium and platinum. Using hydrogen gas as the hydrogen source favored hydrogenation rather than isomerization, whereas using ethylene glycol as the hydrogen source was optimal for the conversion of linoleic acid to CLA, which might involve the concentration of chemisorbed hydrogen (Schultz, Chew, and Seaman, 1992). Furthermore, the isomerization did not occur at low temperature, and long reaction times induced hydrogenation rather than conjugation. Thus, the study design included high reaction temperatures for less than 4 h. CLA concentrations were determined by integration of GC peak areas using C_{17} fatty acid as an internal standard. The original soybean oil contained palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid contents of 122.80, 42.60, 223.80, 550.30 and 67.70 mg/g oil, respectively, and a total CLA concentrations of 0.63 mg/g oil, which increased to 2.33 mg/g oil (treatment 13) by simply being heated from room temperature to 180ºC (Table 3.3).

Table 3.3. Effects of Isomerization conditions on iodine values and CLA contents of isomerized soybean oil

Treatment	Temperature (^{o}C)	Speed (rpm)	Time (min)	Total CLA (mg/g of oil)	Iodine Value
1	160	200	49	112.67±7.45	120.19±0.80
2	200	200	49	202.42±22.07	115.53±0.64
3	160	400	49	129.82±13.36	118.85±1.04
4	200	400	49	181.88±13.85	116.35±0.56
5	160	200	191	86.41±7.93	117.69±0.97
6	200	200	191	117.13±11.27	116.05±0.94
7	160	400	191	86.29±9.41	115.71±0.88
8	200	400	191	121.71±12.85	113.31±0.63
9	146	300	120	87.99±11.47	121.67±0.94
10	214	300	120	63.24±10.90	111.26±0.80
11	180	100	120	111.09±3.17	118.81±0.60
12	180	500	120	117.52±14.48	118.47±0.97
13	180	300	0	2.33±0.30	135.86±1.10
14	180	300	240	105.73±12.81	111.17±0.47
15	180	300	120	134.32±8.25	115.82±0.34

As it can be seen from Table 3.3 and Fig. 3.3A, formation of CLA during isomerization was greatly dependent on reaction temperature and time. Stirring speed only slightly affected the isomerization. Increasing reaction temperature from 146^{o}C (treatment 9) to 180^{o}C (treatment 15) resulted in an increase of total CLA contents (Fig. 3.3A). This was due to the isomerization reaction of linoleic acid which decreased as reaction temperatures increased (Fig. 3.3B). Another source for CLA might have been

linolenic acid as it decreased from 67.70 mg/g of oil in the starting soybean oil to 12.10 mg/g of oil in treatment 2.

The maximum yield of total CLA was 202.42 mg/g oil obtained at 200^{o}C with stirring speed 200 rpm for 49 min (treatment 2, table 3.3). At this temperature, Jung, M.O., Yoon, and Jung, M.Y. (2001) also found high CLA contents in partially hydrogenated soybean oil using a Nickel catalyst. They reported a total CLA content of 162.82 mg/g oil at a hydrogenation temperature of 210ºC; however, the *trans,trans* isomers were very high (51.17% of total CLA).

When the temperature increased to 214ºC (treatment 10), the total CLA significantly ($p<0.05$) decreased due to hydrogenation, which was determined by a decrease in iodine value from 115.53 in treatment 2 to 111.26 in treatment 10 ($P<0.05$). Supporting information is shown by an increase in C18:1 and C18:0 as a result of loss of double bonds from C18:2 and C18:3 (Fig. 3.3C and 3.3D). In addition, when the reaction time was long (4 h, treatment 14), total CLA decreased to 105.73 mg/g oil. It was due to hydrogenation being favored over isomerization as indicated by an increase of monounsaturated and saturated fatty acids (Fig. 3.2C and 3.2 D) and a decrease in iodine value to 111.17. Jung et al. (2001) also found the hydrogenation of soybean oil significantly increased when a nickel catalyst was used in the reaction for 3-4 h.

The proportions of individual CLA isomers affected by the isomerization conditions are shown in Table 3.4 Only reaction temperatures and times affected the alteration of CLA configuration. Increasing temperature to 180ºC resulted in an increase in *cis,trans*/*trans,cis* isomers. Further increasing of reaction temperatures caused a decrease in *cis,trans*/*trans,cis*-CLA as a result of bond shifting of *cis,trans*/*trans,cis*- to *trans,trans*-CLA. Reaction time also affected *cis,trans*/*trans,cis*-

CLA proportions. Long reaction time (4 h) caused a decrease in *cis,trans*/*trans,cis* isomer but *trans,trans*-CLA increased.

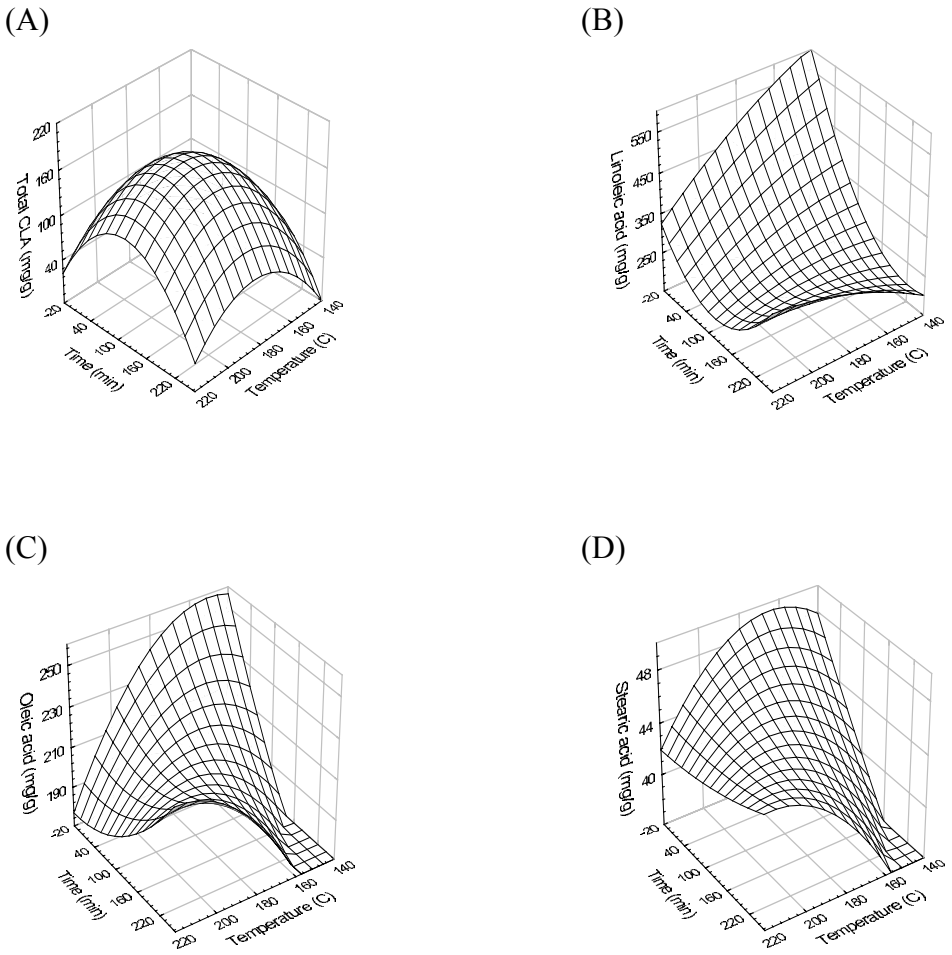


Figure 3.3 Response surface graphs showing effect of temperature and isomerization time at stirring speed 200 rpm on total CLA (A), linoleic acid (B), oleic acid (C) and stearic acid (D).

Table 3.4. Distribution of individual CLA isomers (% of total CLAs) affected by isomerization conditions

CLA isomer	Treatment														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
*t*13,*t*15	0.16	0.41	0.35	0.36	0.48	0.33	0.29	0.42	0.13	1.06	0.34	0.17	0.00	0.34	0.29
*t*12,*t*14	2.37	2.61	1.68	2.22	1.70	2.04	1.94	2.41	1.82	3.63	2.06	2.19	0.00	2.20	2.38
*t*11,*t*13	6.28	5.76	6.63	6.95	7.09	7.06	7.43	7.75	6.75	9.81	7.06	6.85	8.86	7.92	7.27
*t*10,*t*12	6.27	7.65	6.61	6.87	7.08	6.97	7.40	7.69	6.76	10.14	7.06	6.82	12.15	7.91	7.23
*t*9,*t*11	2.62	3.98	2.96	3.35	2.78	2.94	2.76	3.46	2.35	5.28	3.14	2.88	4.81	3.47	3.13
*t*8,*t*10	0.17	0.15	0.36	0.46	0.08	0.29	0.08	0.19	0.03	0.22	0.30	0.11	0.90	0.11	0.15
*c*12,*t*14/*t*12,*c*14	0.97	0.13	0.94	0.62	0.28	0.67	0.21	0.37	0.24	1.64	0.48	0.26	0.94	0.12	0.29
*c*11,*t*13/*t*11,*c*13	1.19	4.40	1.42	4.30	2.05	9.60	2.04	8.25	1.18	13.16	4.20	3.80	4.78	5.61	3.67
*c*10,*t*12/*t*10,*c*12	37.97	34.84	38.89	34.79	38.14	29.08	38.16	29.35	40.84	17.25	34.83	36.20	17.82	32.56	36.20
*c*9,*t*11/*t*9,*c*11	32.08	28.78	31.76	28.75	30.96	23.97	31.71	24.76	33.22	16.22	29.82	30.47	20.92	27.05	30.20
*c*8,*t*10/*t*8,*c*10	1.56	4.31	1.47	4.26	2.38	9.50	2.32	8.12	1.62	12.65	4.34	3.71	2.46	5.83	3.66
*c*11,*c*13	0.68	0.41	0.30	0.49	0.27	0.47	0.21	0.50	0.17	0.00	0.46	0.34	0.79	0.50	0.30
*c*10,*c*12	3.76	3.08	2.99	3.04	3.32	3.20	2.74	3.31	2.46	2.16	2.80	3.11	15.45	3.04	2.54
*c*9,*c*11	3.39	3.01	2.98	2.84	3.09	3.27	2.41	3.02	2.11	3.47	2.61	2.71	7.16	3.08	2.33
*c*8,*c*10	0.52	0.50	0.67	0.70	0.31	0.61	0.32	0.42	0.32	3.31	0.50	0.37	2.96	0.26	0.36
Total *trans,trans*	17.87	20.56	18.59	20.21	19.21	19.63	19.90	21.92	17.84	30.14	19.96	19.02	26.72	21.95	20.45
Total *Cis,trans/trans,cis*	73.77	72.46	74.48	72.72	73.81	72.82	74.44	70.85	77.1	60.92	73.67	74.44	46.92	71.17	74.02
Total *cis,cis*	8.35	7.00	6.94	7.07	6.99	7.55	5.68	7.25	5.06	8.94	6.37	6.53	26.36	6.88	5.53

The maximum CLA yield was obtained from treatment 2. This isomerization condition provided 20.56, 72.46 and 7.00% of *trans*/*trans*, *cis,trans*/*trans,cis* and *cis,cis* CLA, respectively. The amounts of isomers *c*10,*t*12/*t*10,*c*12 (34.84%) and *c*9,*t*11/*t*9,*c*11 (28.78%) indicated that the double bond at Δ9, which was closer to the carboxyl group, had a greater absorption of Rh and was more reactive than the double bond at Δ12, which is closer to the methyl end of the carbon chain. Therefore, it might be concluded that the isomerization condition at 200ºC, 200 rpm for 49 min provided CLA contents high in *cis,trans*/*tran,cis* isomers, of which *c*9,*t*11 and *t*10,*c*12 have been reported to have anticarcinogen and body fat reduction properties. In addition, this isomerization condition exhibited rather low concentrations of *trans*,*trans* and *cis*,*cis*-CLA isomers. Compared to a Ni catalyst (Jung et al., 2001), Rh provided much higher proportions of *cis,trans/tran,cis* and lower amounts of *trans,trans*-CLA. However, alkaline isomerization (Adlof et al., 2001) has greater activity and selectivity than using Rh catalyst because it provided higher proportions of *cis,trans/tran,cis* and lower amounts of *trans,trans*-CLA. The catalytic pathway for double bond migration of linoleic acid by heterogeneous catalysts was described clearly by Bernas et al. (2002).

3.3.3 Analysis of CLA position

Investigation of acyl selection during isomerization was done by partially hydrolyzing the triacylglycerol structure of the isomerized oil using enzyme pancreatic lipase. This enzyme has the ability to cleave the fatty acids at *sn1-* and *sn-3* positions. The 4 fractions of FFAs, MAG, DAG and TAG shown on the TLC plate (Fig. 3.4) were reported by Valle et al. (2000) to be FFAs and *sn-2* MAG bands representing the position of *sn-1, -3* and *sn-2* of TAG, respectively. Individual fractions were

methylated by BF_3/methanol and analyzed with HPLC to estimate total CLA content (Table 3.5) and for monitoring the effect of Rh heterogeneous catalyst on the conversion of linoleic acid to CLA at the various *sn*-TAG positions.

Fig. 3.4 Hydrolysate from partially hydrolyzed isomerized soybean oil with pancreatic lipase on TLC.

Table 3.5 Proportion of total CLA (%) of individual fraction

Fraction	Total CLA (%)
TAG	11.99
FFA	46.96
DAG	10.96
MAG	30.09

The HPLC chromatogram showed each fraction of hydrolysate contained high amounts of CLA. However, the intensity of the FFA fraction was much higher than the MAG fraction, which indicates that fatty acids in both, sn-*1* and -*3* positions can be isomerized to CLA. However, the results do not reveal the % conversion differences between *sn-1* and -*3* but according to Valle et al. (2000) it is possible that linoleic acid at any position in triacyl-*sn*-glycerol could be isomerized to CLA.

3.4 Conclusions

Conjugation of unsaturated fatty acids in vegetable oil can be done by using Rhodium heterogeneous catalyst at high temperatures. Isomerization conditions set up in this experiment definitely provided high CLA-TAG contents in soybean oil up to 202.42 mg/g which contained 63.62 % of beneficial *cis9*,*trans11* and *trans10*,*cis12* isomers. Only 15 grams of isomerized oil would have a CLA content that is high enough to match the daily recommended (Pariza and Hargraves, 1985) dose of 3 g/day.

References

Adlof, R.O. (1999). The lindlar-catalyzed reduction of methyl santalbate: A facile preparation of methyl 9-cis,11-trans-octadecadienoate-9,10-d_2. **J. Am. Oil Chem. Soc.** 76: 301-304.

American Oil Chemists Society. (1999). Official Method and Recommended Practices of the American Oil Chemists Society, 4th ed.; Tirestone, D., Champaign, IL

Bernas, A., Kumar, N., MäkArevela, P., Kul'kova, N.V., Holmbom, B., Salmi, T., and Murzin, D.Y. (2003). Isomerization of linoleic acid over supported metal catalysts. **Appl. Catal.** 6483: 1-19.

Brodie, A.E., Manning, V.A., Ferguson, K.R., Jewell, D.E., and Hu, C.Y. (1999). Conjugated linoleic acid inhibits differentiation of pre- and postconfluent 3T3-L1 preadipocytes but inhibits cell proliferation only in preconfluent cells. **J. Nutr.** 129: 602-606.

Cochran, W.G., and Cox, G.M. (1992). Experimental Design. 2nd ed.; John Wiley & Sons Inc. New York

Cook, M. E., Miller C.C., Park, Y., and Pariza, M.W. (1993). Immune modulation by altered nutrient metabolism; Nutritional control of immune-induced growth depression. **Poultry Sci.** 72: 1301-1305.

Cross, R.F., Ostrowska, E., Muralitharan, M., and Dunshea, F.R. (2000). Mixed mode retention and the use of competing acid for the Ag+-HPLC analysis of

underivatized conjugated linoleic acids. **J. High Resol. Chromatogr.** 23 (4): 317-323.

Ertl, G. (1997). Handbook of Heterogeneous Catalyst. VCH Verlagsgesellschaft mbH. Weinheim, Germany

Fritsche, J., Yurawecz, M.P., Pawlosky, R., Flanagan, V.P., Steinhart, H., and Ku, Y. (2001). Spectroscopic characterization of unusual conjugated linoleic acid (CLA) isomers. **J. Sep. Sci.** 24: 59-61.

Gavino, V.C., Gavino, G., Lablanc, M.J., and Tuchweber, B. (2000). An isomeric mixture of conjugated linoleic acids but not pure cis-9,trans-11-octadecadienoic acid affects body weight gain and plasma lipids in hamsters. **J. Nutr.** 130: 27-29.

Ha, Y.L., Storkson, J., and Pariza, M.W. (1990). Inhibition of benzo(a)pyrene-induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. **Cancer Res.** 50: 1097-1101.

Ip, C., Chin, S.F., Scimeca, A., and Pariza, M.W. (1991). Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. **Cancer Res.** 51: 6118-6124.

Jung, M.O., Yoon, S.H., and Jung, M.Y. (2001). Effect of temperature and agitation rate on the formation of conjugated linoleic acids in soybean oil during hydrogenation process. **J. Agri. Food Chem.** 49 (6): 3010-3016.

Kepler, C.R., and Tove, S.B. (1967). Biohydrogenation of unsaturated fatty acids. **J. Biol. Chem.** 242 (24): 5686-5691.

Larock, R.C., Dong, X., Chung, S., Reddy, C.K., and Ehlers, E. (2001). Preparation of conjugated soybean oil and other natural oils and fatty acids by homogeneous transition metal catalysis. **J. Am. Oil Chem. Soc.** 78 (5): 447-453.

Mossoba, M.M., McDonald, R.E., Armstrong, D.J., and Page, S.W. (1991). Identification of minor C18 triene and conjugated diene isomers in hydrogenated soybean oil and margarine by GC-MI-IR spectroscopy. **J. Chromatogr. Sci.** 29: 324-330.

Nichols, P.L., Herb, Jr.S.F., and Riemenschneider, R.W. (1951). Isomers of conjugated fatty acids. I. Alkaline-isomerized linoleic acid. **J. Chem. Soc.** 73: 247-252.

Pariza, M.W., and Hargraves, W.A. (1985). A beef-derived mutagenesis modulator inhibits initation of mouse epidermal tumors by 7, 12-dimethylbenz[a]anthracene. **Carcinogenesis** 8: 591-593.

Park, Y., Storkson, J.M., Albright, K.J., Liu, W., and Pariza, M.W. (1999). Evidence that the trans-10, cis-12 isomer of conjugated linoleic acid induces body composition changes in mice. **Lipids** 34: 235-241.

Roach, J.A.G., Mossoba, M.M., Yurawecz, M.P., andKramer, J.K.G. (2002). Chromatographic separation and identification of conjugated linoleic acid isomers. **Analytica Chimica Acta.** 465: 207-226.

SAS Institute (1989). SAS users guide, SAS Institute, Inc. Cary, NC.

Schultz, T.D., Chew, B.P., and Seaman, W.R. (1992). Differential stimulatory and inhibitory responses of human MCF-7 breast cancer cells to linoleic acid and conjugated linoleic acid in culture. **Anticancer Res.** 12: 2143-2146.

Sehat, N., Kramer, J.K.G., Mossoba, M.M., Yurawecz, M.P., Roach, J.A.G., Eulitz, K., Morehouse, K.M., and Ku, Y. (1998). Identification of conjugated linoleic acid isomers in cheese by gas chromatography, Silver ion high performance liquid chromatography and Mass spectral reconstructed ion profiles. Comparison of chromatographic elution sequences. **Lipids** 33 (10): 963-970.

Spitzer, V., Marx, F., and Pfeilsticker, K. (1994). Electron impact mass spectra of the oxazoline derivatives of some conjugated diene and triene C18 fatty acids. **J. Am. Oil Chem. Soc.** 71: 873-876.

Valle, F.M., Enrique, M.F., and Rafael, G. (2000). Identification of triacylglycerol species from high-saturated sunflower (*Helianthus annuus*) mutants. **J. Agric Food Chem**. 48: 764-769.

Yang, L. Leung, L.K. Huang, Y., and Chen, Z. (2000). Oxidative stability of conjugated linoleic acid isomers. **J. Agric. Food Chem.** 48: 3072-3076.

CHAPTER IV

EFFECT OF EXTRUSION PARAMETERS ON CONJUGATED LINOLEIC ACIDS OF CORN EXTRUDATES

Abstract

The effects of extrusion temperature, 150-190^{o}C and torque, 50-70 % on the content and configuration of CLA(s) in corn extrudates were analyzed by GC and HPLC. Extrusion at a product temperature of 150^{o}C increased CLA content from 1.17 mg/g of oil in feeds to 7.75 mg/g of oil in corn extrudates. Further increasing product temperature to 190^{o}C showed a significant ($p<0.05$) decrease in total CLA contents. Alteration of CLA geometrical configuration was observed at higher extrusion temperatures. *Trans,trans* CLA significantly increased ($p<0.05$) from 10.19% in feed to 11.88% of CLA at the extrusion condition of 190^{o}C and 70 % torque. The highest expansion of extrudate was founded at the product temperature of 150^{o}C and 70 % torque. This extrusion condition also resulted in a maximum total CLA content and in minimum *trans,trans*-CLA formation.

4.1 Introduction

Conjugated linoleic acid (CLA) was first identified in grilled ground beef (Ha, Grimm, and Pariza, 1989). A number of studies have reported its anticarcinogenic property in several animal organs, such as forestomach, lung, large intestine and colon (Ha, Storkson, and Pariza, 1990; Ip, Chin, Scimeca, and Pariza, 1991; Kim, and Liu, 1999; Yamasaki, Kishihara, Ikeda, Sugano, and Yamada, 1999). Moreover, CLA also has been shown to have antioxidant and antiatherosclerotic properties as well as reduce body fat (Gavino, Gavino, Lablanc, and Tuchweber, 2000; Brodie, Manning, Ferguson, Jewell, and Hu, 1999; Yamasaki, Kishihara, Ikeda, Sugano, and Yamada, 1999; Park, Storkson, Albright, Liu, and Pariza, 1999). CLA has gained considerable attention after being found to have these physiological benefits and as a result the enhancement of CLA in natural food has been widely studied.

Ha, Grimm, and Pariza (1989) reported that CLA formation in food involved the oxidation of linoleic acid. It was explained that radicals from the oxidation reacted with protons from hydrogen donors, such as proteins, and then rearranged to form conjugated diene structures. Therefore, lipid and protein contents are important for CLA formation. Shantha, Decker, and Ustunol (1992), and Lin, Boylston, Chang, Luedecke, and Shultz (1995) also reported these results in dairy products.

Beside food components, the influence of food processing, such as temperature, air and starter cultures, which were observed in cheddar-type cheese processing, on CLA content was also reported (Lin, Boylston, Luedecke, and Shultz, 1999). However, there are no published reports in terms of thermomechanical processing. Mechanical and thermal treatment in extrusion probably modulates the conversion of linoleic acid into CLA. The objective of this study was to investigate the

effect of extrusion conditions on content and configurations of CLA in corn extrudates. Total CLA contents and fatty acids profiles were analyzed by a gas chromatographic (GC) method, whereas silver high performance liquid chromatography (Ag^+-HPLC) was used to determine the proportion of *cis,cis*; *cis,trans*/*trans,cis* and *trans,trans*-isomers of CLA.

4.2 Materials and methods

4.2.1 Materials

Corn meal was obtained from Thai Maize Industrial Co., Ltd. (Bangkok, Thailand). Sunflower oil was purchased from Tanakorn Oil Product Ltd. (Samutprakran, Thailand). Three standard CLA methyl ester isomers; *cis*9,*cis*11, *cis*9,*trans*11 *and trans*9,*trans*11, 98% purity, were purchased from Matreya, Inc. (Pleasant Gap, PA) and stored at –20^{o}C. Standard fatty acid methyl ester mixtures were purchased from Sigma, Inc. and also stored at –20^{o}C. All chemicals and solvents were reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific, Inc (Pittsburgh, PA).

4.2.2CLA synthesis by alkaline isomerization method

Two separate reactions used to synthesize a mixture of CLA methyl ester isomers from sunflower oil. Sunflower oil was methylated to fatty acid methyl ester (FAME) by sodium methoxide and extracted by hexane. After the evaporation of hexane using a rotary evaporator, linoleic acid methyl ester was isomerized into conjugated linoleic acid methyl esters (CLAME) according to the method of Berdeaux,

Voinot, Angioni, Juanédaand, and Sébédio (1998) with slight modifications (see Chapter 7). The isomerized CLA oil was used in corn extrusion.

4.2.3 Viscosity measurement

Viscosity of sunflower oil and isomerized oil were measured at 25 ^{O}C with a Brookfield, model RVTD rotational viscometer (Brookfield Engineering Laboratories, Inc., Stoughton, MA) with a No.2 spindle.

4.2.4 Extrusion and sample preparation

The extrusion was carried out using a MPF19:25 twin screw extruder (APV Baker, Peterborough, England) with a L:D (barrel length:barrel diameter) ratio of 15:1 and equipped with a 3 mm diameter die. Screw configuration was set for direct expanded snack, which was a combination of mixing and conveying elements. Details of the screw elements and configuration are shown in Table 7.1 (Chapter 7). The barrel temperatures were set up to accommodate the product temperature of 150, 170, and 190^{o}C (see Table 7.2, Chapter 7). The water feed rate was controlled to acquire the torque of 50, 60, and 70%. The extrudates were collected and dried at ambient temperatures.

Corn meal mixed with 2% sunflower oil was used as starting feed for assessing how total CLA contents is affected by extrusion conditions, whereas, 2% of the isomerized oil mixed was applied to estimate the alteration of CLA configuration during extrusion. The process parameters are shown in Table 4.1. All experiments

were operated at constant feed rate and screw speed. Changing of % torque was done by operating the water feed (see Table 7.3, Chapter 7).

Table 4.1 Processing parameters for assessing effect of extrusion on CLA contents

Parameter	Run-A*	Run-B*
Feed rate (kg/h)	9.08	9.24
Screw speed (rpm)	232	232

* Run-A: containing 2% sunflower oil

Run-B: containing 2% isomerized oil mix

4.2.5 Specific mechanical energy

The specific mechanical energy (SME) was calculated according to Choudhury and Gautam (2003) as follows;

$$\text{SME (kJ/kg)} = \frac{n(actual)}{n(rated)} \times \frac{\%T}{100} \times \frac{P(rated)}{m}$$

where n is the screw speed (rpm), T the net torque, P the motor power (kJ/s), and m the feed rate (kg/s)

4.2.6 Expansion ratio

The diameter of five corn extrudates was measured with a vernier caliper. The average diameter was used for determining expansion ratio, which was defined as the ratio between the diameter of extrudates and the diameter of the die.

4.2.7 Oil extraction

Thirty gram of corn extrudates from each treatment was mixed with 100 ml of solvent (chloroform/methanol, 2:1) and homogenized at 7000 rpm for 3 min and then rinsed with 50 mL of the solvent. The mixture was centrifuged at 2000 x g for 20 min. and filtered through filter paper. Fifty mL of distilled water was added to a 150 mL aliquot of the filtrate and the solvent layer was separated in a separatory funnel. Finally, solvent was evaporated by a rotary evaporator at 40-45^{o}C.

4.2.8 FAME analysis

4.2.8.1 Preparation of fatty acid methyl ester (FAME)

Approximately 30 mg of the extracted oil was placed into a 15-mL reaction tube fitted with a teflon-lined screw cap. One and a half mL of 0.5 M sodium hydroxide in methanol was added. The tubes were flushed with nitrogen, capped, heated at 100^{o}C for 5 min with occasional shaking and then cooled to room temperature. One mL of C_{17} internal standard (2.00 mg/mL in hexane) and 2 mL of boron trifluoride in methanol were added and heated at 100^{o}C for 5 min with occasional shaking. After methylation was completed, 10 mL of deionized water was added. The solution was transferred to a 40-mL centrifuged tube and 6 mL of hexane was added for FAME and CLAME extraction. The solution was centrifuged at 2000 x g, at 10^{o}C for 20 min and then the hexane layer was dried over sodium sulfate and analyzed by gas chromatography (GC) and high performance liquid chromatography (HPLC).

4.2.8.2 FAME and CLA methyl ester (CLAME) analysis by GC

FAME and CLAME were analyzed by GC (HP 1100, Hewlett-Packard Inc, Palo Alto, CA) equipped with a 100 m x 0.25 mm fused silica capillary column SP2560 (Supelco Inc, Bellefonte, PA). Injector and detector temperatures were 240^{o}C. The column temperature was kept at 75^{o}C for 1 min, then increased at 20^{o}C/min to 185^{o}C and held at 185^{o}C for 15 min, then increased at 4^{o}C/min to 220^{o}C and held at 220^{o}C for 35 min.

4.2.8.3 CLAME analysis by HPLC

The CLAME were analyzed by HPLC (HP 6890, Hewlett-Packard Inc, Palo Alto, CA), equipped with a 20-μL rheodye injection loop, and a UV detector set at 233 nm. Two ChromSpher 5 lipids analytical (4.6 mm i.d. x 250 mm stainless steel, 5 μm particle size) silver-impregnated columns with guard column were used in series. HPLC separation was performed isocratically with the mobile phase of 0.1% acetonitrile in hexane freshly prepared and at the flow rate of 1.0 mL/min.

4.2.9 Experimental design and statistical analysis

A 3x3 factorial design with three replications was employed. The data were analyzed by ANOVA, and a t-test was conducted to identify differences between means.

4.3 Results and Discussions

4.3.1 Effect of extrusion parameters on total CLA using sunflower oil

The oil extracted from the starting feed contained 9.28, 3.40, 30.51, 55.30, 0.96 and 0.55 % of oleic acid, stearic acid, palmitic acid, linoleic acid, linolenic acid and CLA, respectively. It was shown that the formation of CLA during extrusion was dependent on product temperatures and torques ($p<0.05$) (Fig. 4.1) due to thermal and mechanical energy as a result of extrusion processing. Increasing product temperature from 150 to 170 and 190^{O}C resulted in a decrease in total CLA content ($p<0.05$) (Table 7.5, Chapter 7). At the lowest % torque of 50, total CLA decreased from 4.28 to 0.20 mg/g of oil whereas at 70% torque, a decrease in total CLA from 7.75 to 1.48 mg/g of oil was exhibited when product temperature increased to 190^{O}C. A similar effect of temperature on the formation of CLA by chemical reaction using homogeneous $RhCl(PPh_3)_3$ catalyst was also reported (Larock, Dong, Chung, Reddy, & Ehlers, 2001). The isomerization of ethyl linoleate to CLA was found to yield the maximum CLA amount when the reaction was carried out at 80^{o}C and decreased when the reaction temperature was increased to 120^{o}C.

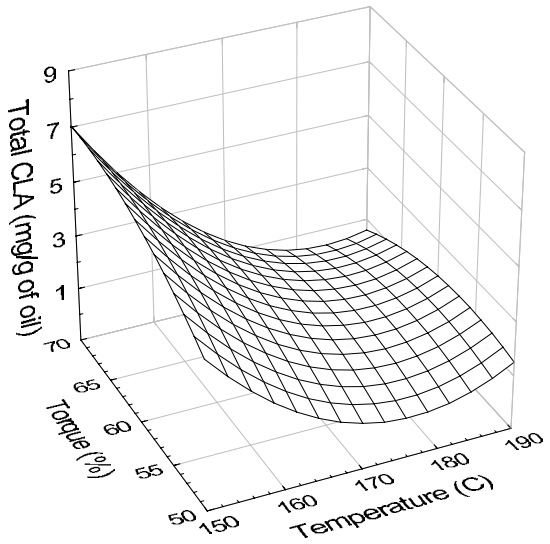


Fig. 4.1 Response Surface plot of total CLA of corn extrudates (sunflower oil mix) affected by extrusion conditions.

In addition, product temperature also had an influence on the proportion of *trans,trans*, *cis,trans/trans,cis* and *cis,cis*-CLA. It was observed that at the lowest % torque of 50, an increase in product temperature resulted in an increase in *trans,trans* and *cis,cis*-CLA (Fig 4.2). Thus, the highest amount of *trans,trans* and *cis,cis*-CLA were found at the product temperature of 190^{o}C (Fig. 4.2A and 4.2C). In contrast, this product temperature showed a decrease in *cis,trans/trans,cis* isomers. Therefore, the extrusion at product temperature of 150-170^{o}C was likely to provide the highest formation of *cis,trans/trans,cis* isomers (Fig. 4.2B).

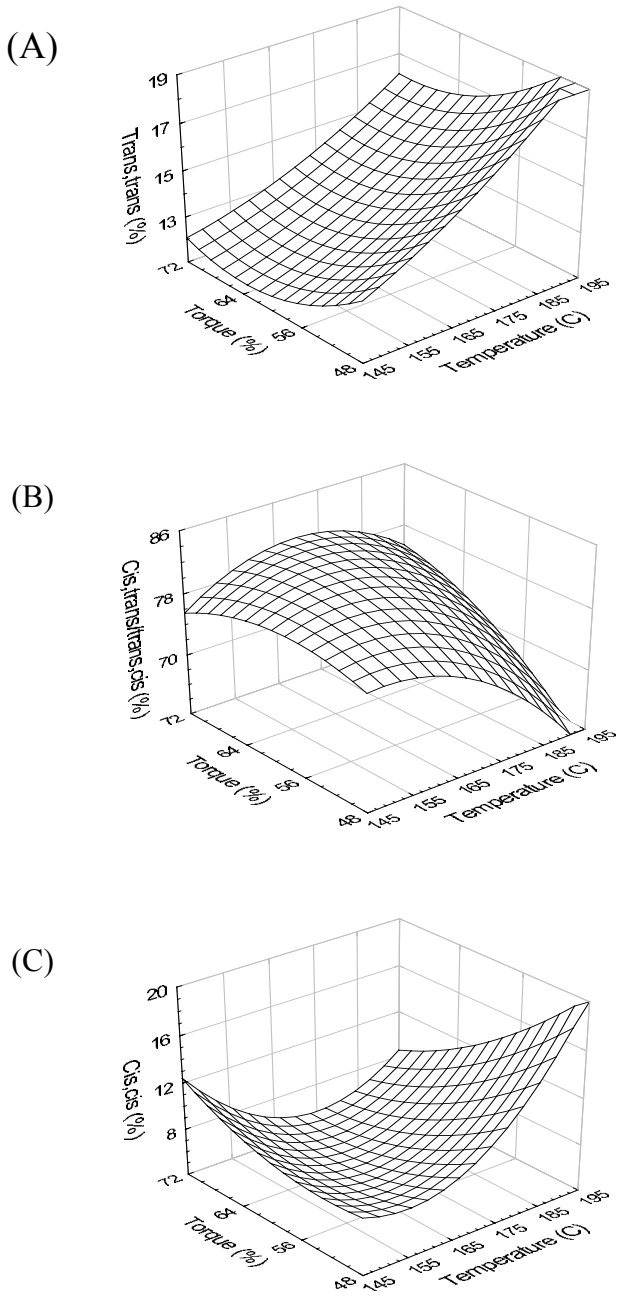


Fig. 4.2 Response Surface plot of *trans,trans*-CLA (A); *cis,trans*/*trans,cis*–CLA (B) and *cis,cis*-CLA (C) of corn extrudates (sunflower oil mix) as a result of extrusion conditions.

Increasing % torques from 50 to 70% led to an increase in SME from 184 to 258 kJ/kg (Table 7.4, Chapter 7). Alteration of % torque directly affected the mechanical energy whereas product temperature is a result of the temperature of the barrel plus the frictional heat from the mechanical energy. At the product temperature of 150^{o}C, a significantly greater yield ($p<0.05$) of total CLA content was obtained at a higher % torque. However, a slight increase ($p>0.05$) in total CLA was noticed due to as increase in % torque even at the product temperature of 190^{o}C.

Proportion of CLA isomers was also affected by changing of % torque. At the product temperature of 190^{o}C, *cis,trans*/*trans,cis* increased from 63.83 to 75.93% (Table 7.6, Chapter 7) as % torque increased from 50 to 70%. On the contrary, *trans,trans* and *cis,cis*-CLA had a tendency to decrease with an increase in at % torque at high product temperature (Fig 4.2).

The interaction between product temperatures and % torques had an influence on total CLA content ($p<0.05$). The highest yield of CLA (7.75 mg/g of oil) was obtained by extrusion at a product temperature of 150^{o}C and % torque of 70. With respect to Ag^{+}HPLC results, the CLA configurations of corn extrudates extruded at this condition was 79.02, 12.09 and 8.77% of *cis,trans*/*trans,cis*; *trans,trans* and *cis,cis*-CLA, respectively.

It was assumed that the combination of mechanical and thermal energies affected the double bonds of linoleic acid and influenced the formation of conjugated diene. In general, the double bonds at *cis*9 and *cis*12 of linoleic acid are shifted to *cis*9,*trans*11 and *trans*10,*cis*12 in the first step of bond shifting, resulting in the predominant *cis,trans*/*trans,cis*-CLA isomers. Since only *cis,trans*/*trans,cis*-CLA showed health benefit properties such as anticancer, antiatherosclerosis and body fat

reduction (Ha, Storkson, and Pariza, 1990; Gavino, Gavino, Lablanc, and Tuchweber, 2000), further studies to enhance these isomers and total CLA content under other extrusion conditions or with other processing methods should be carried out.

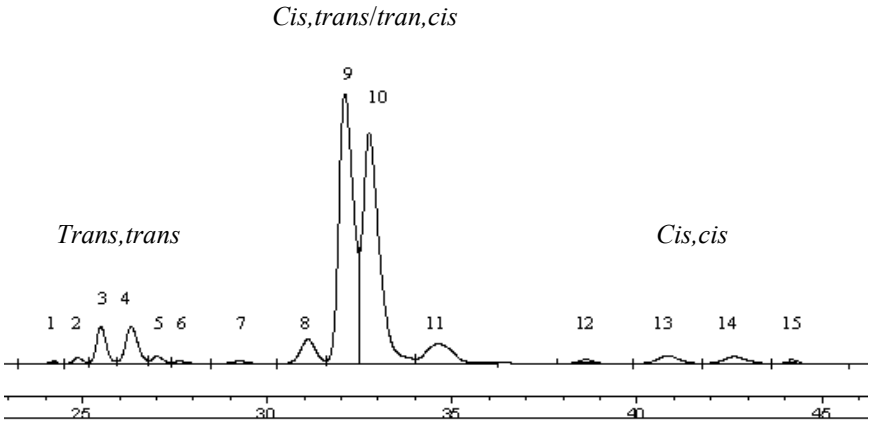
An advantage of the extrusion process is that both heat and shear enhanced CLA content of the starting feed (1.17 mg/g of oil) up to 562.39 % by selecting optimum extrusion conditions. The combination of heat and shear effects has not previously been reported but there are several articles stating an increase of CLA content due to processing which was mainly found in dairy products. An increasing of CLA content was presented up to 11.24-32.08 % from raw materials that contained 3.38 to 6.14 mg of CLA/g fat in cheese (Lin, Boylston, Luedecke, and Shultz, 1999) and butter (Shantha, Ram, O'leary, Hicks, and Decker, 1995). However, the articles also reported that processing did not alter the ratio of *cis*9,*trans*11 to the total CLA.

High energies could cause bond shifting from *cis,trans*/*trans,cis* isomers to *trans,trans* isomers, which was observed when extrusion was carried out at product temperature of 190^{o}C. Furthermore, very high extrusion temperature may cause the transformation of conjugated diene to non-conjugated dienes, and a breaking down of the C18 structure to some short chain fatty acids as well as oxidation of the CLA to furan fatty acids (Yurawecz, Hood, Mossoba, Roach, and Ku, 1995).

4.3.2 Effect of extrusion parameters on the alteration of CLA configurations using isomerized oil

As analyzed by Ag^{+}HPLC, alkaline isomerized sunflower oil used in corn extrusion contained 15 peaks in 3 groups of isomers; *trans,trans*; *cis,trans*/*trans,cis* and *cis,cis*-CLA (Fig. 4.3A). Identification and elution order of each isomer were as follows (1) *t*12,*t*14; (2) *t*11,*t*13; (3) *t*10,*t*12; (4) *t*9,*t*11; (5) *t*8,*t*10; (6) *t*7,*t*9; (7) *c*12,*t*14/*t*12,*c*14 ; (8) *c*11,*t*13/*t*11,*c*13 ; (9) *c*10,*t*12/*t*10,*c*12 ; (10) *c*9,*t*11/*t*9,*c*11 ; (11) *c*8,*t*10/*t*8,*c*10 ; (12) *c*11,*c*13 ; (13) *c*10,*c*12 ; (14) *c*9,*c*11 ; and (15) *c*8,*c*10. However, only 8 peaks were observed on the GC chromatogram (Fig. 4.3B), due to co-elution of isomers within positional and geometrical groups (Mossoba et al., 1999). The GC elution order of CLA isomer groups were *cis,trans/trans,cis* followed by *cis,cis* and finally *trans,trans*. Quantification by GC using C17 fatty acid as the internal standard showed that the isomerized oil contained a total of 509.52 mg CLA per g oil of which 81.82% were *cis,trans*/*trans,cis* isomers. In addition, 73.92% of high beneficial isomers, *cis9,trans11* and *trans10,cis12* were obtained. The Fatty acids profile of the isomerized oil, presented in % of total fatty acids, is shown in Table 4.2.

(A)



(B)

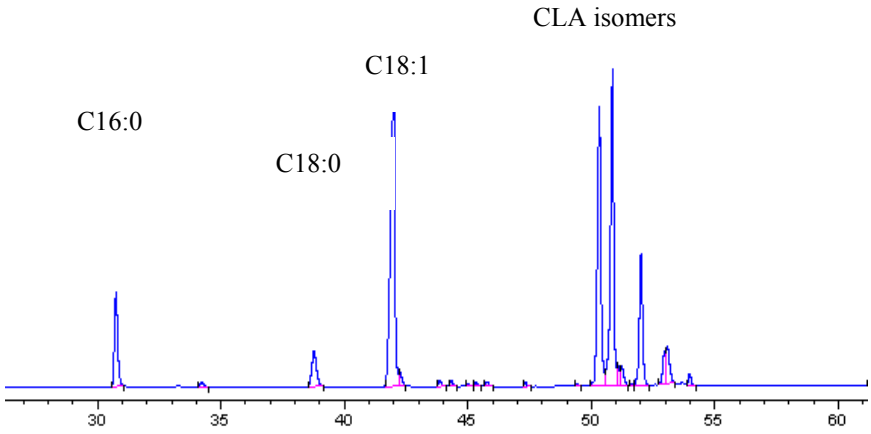


Fig. 4.3 (A) Partial HPLC chromatogram of isomerized oil used as starting feed, peak 1-15 represent (1) *t*12,*t*14; (2) *t*11,*t*13; (3) *t*10,*t*12; (4) *t*9,*t*11; (5) *t*8,*t*10; (6) *t*7,*t*9; (7) *c*12,*t*14/*t*12,*c*14; (8) *c*11,*t*13/*t*11,*c*13; (9) *c*10,*t*12/*t*10,*c*12; (10) *c*9,*t*11/*t*9,*c*11; (11) *c*8,*t*10/*t*8,*c*10; (12) *c*11,*c*13; (13) *c*10,*c*12; (14) *c*9,*c*11; and (15) *c*8,*c*10.

(B) Partial GC chromatogram of the isomerized oil

Table 4.2 Fatty acids profile of isomerized oil synthesized by alkaline isomerization

Fatty acids	Percent of total fatty acids
C16:0	6.74±0.05
C18:0	3.69±0.02
C20:0	0.27±0.00
C22:0	0.67±0.01
C18:1	29.07±0.17
C18:2n6t	0.47±0.00
C18:2n6c	1.28±0.19
CLA	56.53±0.64
C18:3n3	0.21±0.00
C20:2	1.06±0.38
C20:5n3	0.00±0.00

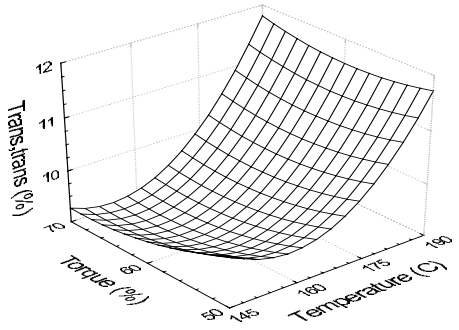
The starting feed contained 85.82 and 10.19% of *cis,trans*/*tran,cis* and *trans,trans* isomers, respectively (Table 7.8, Chapter 7). Extrusion at the lowest product temperature of 150^{o}C and highest torque of 70 increased *cis,trans*/*trans,cis*-CLA to 87.44% while *trans,trans* decreased to 9.13%. This could be due to the conversion of linoleic acid in corn meal into CLA as previously mentioned. Thus, the proportion of *cis,trans*/*trans,cis*-CLA was higher and resulted in lower proportion of *trans,trans*-isomers.

Variation of extrusion conditions caused alteration of CLA geometrical configurations as shown in Fig. 4.4A-4.4C. Increasing product temperature was an important factor inducing higher *trans,trans*-CLA. At 70 % torque, an increase in

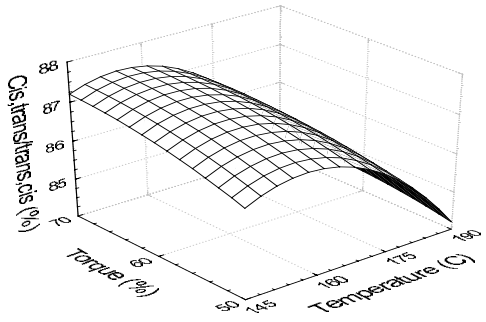
product temperature from 150 to 190^{o}C resulted in significantly increased ($p<0.05$) *trans,trans* isomers from 9.13 to 11.88% while *cis,trans*/*trans,cis* CLAs significantly decreased ($p<0.05$) from 87.44 to 84.56% (Table 7.8, Chapter 7). This seems to confirm the previous experiment with sunflower oil in which *cis,trans*/*tran,cis* conjugated bonds were formed at the low product temperature. When the energy was increased, the *cis,trans*/*trans,cis* isomers stated shifting and the configuration became a *trans,trans* one as shown in Fig. 4.4A.

The proportion of CLAs in the *cis,cis* configurations only slightly changed during extrusion, and it was in almost similar proportions compared to the starting feed (Table 7.8, Chapter 7). However, *cis,cis*-CLA have neither been shown to have any harmful nor any beneficial properties.

(A)



(B)



(C)

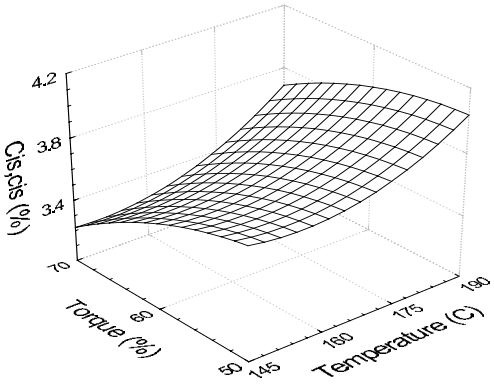


Fig. 4.4. Response surface plot of *trans,trans*-CLA (A); *cis,trans*/*trans,cis*-CLA (B) and *cis,cis*-CLA (C) of corn extrudate (isomerized oil mixed) as a result of extrusion conditions

Changing torques also affected CLA configurations. The mechanical energy in this study was slightly lower than in the previous study because of a higher dry feed rate (Table 4.1). Obviously, results from both studies, using 2% sunflower oil and 2% isomerized oil, were similar. Increasing SME from 181 to 253 kJ/kg (Table 7.4, Chapter 7) as % torque increased from 50 to 70 %, affected an increase in *cis,trans*/*trans,cis* isomers whereas most *trans,tran* isomers decreased although not significantly ($p>0.05$). These results indicate that a combination of mechanical and thermal energies influence the transformation of *cis,trans*/*trans,cis* and *trans,trans* configuration. However, it is obvious that product temperature was the variable with greater influence in this study.

All processing parameters resulted in different die pressures and expansion ratios (Table 4.3). At the same extrusion conditions, sunflower oil mix treatments had lower dry feed rates and lower water feed rates (data not shown), higher SMEs, higher die pressures and consequently higher expansion ratios. This was due to the viscosity of the sunflower oil (86 mPa.s) being greater than that of the isomerized oil (64 mPa.s), which contributed to the differences in lubrication of starting feed and in dough viscosity, resulting in higher expansion ratios.

Table 4.3 Die pressures and expansion ratios of corn extrudates

Treatment (temperature (^{O}C) / torque (%))	Experiment-A*		Experiment-B*	
	Die pressure (psi)	Expansion ratio	Die pressure (psi)	Expansion ratio
190/70	180	2.22 ± 0.06^{b}	215	1.51 ± 0.06^{e}
190/60	150	1.81 ± 0.02^{c}	185	1.49 ± 0.06^{e}
190/50	130	1.41 ± 0.05^{d}	140	1.36 ± 0.03^{f}
170/70	280	2.79 ± 0.05^{a}	190	1.76 ± 0.05^{c}
170/60	210	2.51 ± 0.03^{a}	170	1.61 ± 0.06^{d}
170/50	180	1.96 ± 0.06^{c}	145	1.32 ± 0.04^{f}
150/70	290	2.89 ± 0.08^{a}	255	2.21 ± 0.06^{a}
150/60	230	2.67 ± 0.03^{a}	210	1.97 ± 0.05^{b}
150/50	185	2.25 ± 0.10^{b}	150	1.77 ± 0.02^{c}

* Experiment-A: containing 2% sunflower oil

Experiment-B: containing 2% isomerized oil

$^{a-f}$ Mean with different letters in a column is significantly different ($p<0.05$)

Table 4.3 showes that the higher the product temperature, the lower the expansion ratio as a result of decreased melt viscosity (Valley, Vergnes, Colonna, & Patria, 1997). In contrast, increasing % torque increased die pressure, resulting in higher expansion ratios. Therefore, the extrusion condition at 150^{o}C and 70% torque provided the highest expansion ratio in this study. In addition, this extrusion condition also fabricated bright yellow, homogeneously aerated, high puffing extrudates which gave the product a good appearance (Fig 4.5).

Fig. 4.5 Corn extrudates of sunflower oil mix (S) and isomerized oil (CLA) mix (C), 190/70 represents the extrusion condition at product temperature of 190°C and 70% torque

4.4 Conclusion

Extrusion conditions obviously affected CLA contents and also altered CLA configurations of corn extrudates**.** Thermal and mechanical energies carried out at product temperature of 150^{o}C and 70% torque provided the maximum total CLA content, maximum *cis,trans*/*trans,cis* isomers and minimum *trans,trans*-CLA. In addition, corn extrudates from this condition had a good appearance with the highest expansion. The conditions obtained would be suitable for making extruded snacks.

References

Berdeaux, O., Voinot, L., Angioni, E., Juanéda, P., and Sébédio, J.L. (1998). A simple method of preparation of methyl *trans-10,cis-12-* and *cis-9,trans-11-*Octadecadienoates from methyl linoleate. **J. Am. Oil Chem. Soc.** 75 (12): 1749-1755.

Brodie, A.E., Manning, V.A. Ferguson, K.R. Jewell, D.E., and Hu, C.Y. (1999). Conjugated linoleic acid inhibits differentiation of pre- and postconfluent 3T3-L1 preadipocytes but inhibits cellproliferation only in preconfluent cells. **J. Nutr**. 129: 602-606.

Gavino, V.C., Gavino, G., Lablanc, M.J., and Tuchweber, B. (2000). An isomeric mixture of conjugated linoleic acids but not pure cis-9,trans-11-octadecadienoic acid affects body weight gain and plasma lipids in hamsters. **J. Nutr.** 130: 27-29.

Govindasamy, S. Campancella, O.H., and Oates, C.G. (1997). High moisture twin screw extrusion of sago starch. II. Saccharification as influenced by thermomechanical history. **Carbohydrate Polymers** 32: 267-374.

Ha, Y.L., Grimm, N.K., and Pariza, M.W. (1989). Newly recognized anticarcinogenic fatty acids: Identification and quantification in natural and processed cheese. **J. Agric. Food Chem.** 37(1): 75-81.

Ha, Y.L., Storkson, J., and Pariza, M.W. (1990). Inhibition of benzo(a)pyrene-induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. **Cancer Res.** 50: 1097-1101.

Ip, C., Chin, S.F., Scimeca, A., and Pariza, M.W. (1991). Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. **Cancer Res.** 51: 6118-6124.

Kim, Y.J., and Liu, R.H. (1999). Selective increase in conjugated linoleic acid in milk fat by crystallization. **J. Food Sci**. 64 (5): 792-795.

Larock, R.C., Dong, X., Chung, S., Reddy, C.K., and Ehlers, E. (2001). Preparation of conjugated soybean oil and other natural oils and fatty acids by homogeneous transition metal catalysis. **J. Am. Oil Chem. Soc.** 78 (5): 447-453.

Lin, H., Boylston, T.D., Chang, M.J., Luedecke, L.O. and Shultz, T.D. (1995). Survey of the conjugated linoleic acid contents of dairy products. **J. Dairy Sci.** 78: 2358-2365.

Lin, H., Boylston, T.D., Luedecke, L.O., and Shultz, T.D. (1999). Conjugated linoleic acid content of cheddar-type cheeses as affected by processing. **J. Food Sci.** 64 (5): 874-878.

Mossoba, M.M., Kramer, J.K.G., Yurawecz, M.P., Sehat, N., Roach, J.A.G., Eulitz, K., Fretsche, J., Dugan, M.E.R., and Ku, Y. (1999). Impact of novel methodologies on the analysis of conjugated linoleic acid (CLA). Implication of CLA feeding studies. **Fett/Lipid** 101: 235-243.

Park, Y., Storkson, J.M., Albright, K.J., Liu, W. and Pariza, M.W. (1999). Evidence that the trans-10, cis-12 isomer of conjugated linoleic acid induces body composition changes in mice. **Lipids** 34: 235-241.

Shantha, N.C., Ram, L.N., O'Leary, J., Hicks, C.L., and Decker, E.A. (1995). Conjugated linoleic acid concentrations in dairy products as affected by processing and storage. **J. Food Sci.** 60 (4): 695-720.

Shantha, N.C., Decker, E.A. & Ustunol, Z. (1992). Conjugated linoleic acid concentration in processed cheese. **J. Am. Oil Chem. Soc**. 69 (5): 425-428.

Valley, D.G., Vergnes, B., Colonna, P., and Patria, A. (1997). Relations between rheological properties of molten starches and their expansion behaviour in extrusion. **J. Food Eng.** 31: 277-296.

Yamasaki, M., Kishihara, K., Ikeda, I., Sugano, M., and Yamada, K. (1999). A reccommended esterification method for gas chromatographic measurement of conjugated linoleic acid. **J. Am. Oil Chem. Soc.** 76 (8): 933-938.

CHAPTER V

SUMMARY

1. A heterogeneous catalyst was used to catalyze isomerizations and double bond migrations of Δ9 and Δ12 of linoleic acid. Rhodium metal was much more active for isomerization than palladium and platinum, while palladium was preferential for hydrogenation. The isomerization of soybean oil by rhodium heterogeneous catalyst was obtained at high temperature in cooperation with ethylene glycol as a good medium in the reaction.

2. The isomerized oil contained 15 isomers that were identified as (1) *t*12,*t*14; (2) *t*11,*t*13; (3) *t*10,*t*12; (4) *t*9,*t*11; (5) *t*8,*t*10; (6) *t*7,*t*9 ; (7) *c*12,*t*14/*t*12,*c*14; (8) *c*11,*t*13/*t*11,*c*13; (9) *c*10,*t*12/*t*10,*c*12; (10) *c*9,*t*11/*t*9,*c*11; (11) *c*8,*t*10/*t*8,*c*10; (12) *c*11,*c*13; (13) *c*10,*c*12; (14) *c*9,*c*11 and (15) *c*8,*c*10.

3. Reaction temperature and time greatly affected the formation of CLA during isomerization ($p<0.05$). Maximum content of CLA (202.42 mg/g oil) in isomerized soybean oil was found when the isomerization was done at 200^{o}C, with stirring speed of 400 rpm for 49 min. Under these reaction conditions, isomerization provided CLA contents high in *cis,trans*/*tran,cis* isomers (72.46%), and rather low in *trans,trans* (20.56%) and *cis,cis*-CLA (7.00%). It can be assumed that only 15 grams of isomerized oil would have a CLA content that is high enough to match the daily recommended (Pariza and Hargraves, 1985) dose of 3 g. Further increasing reaction temperature or time resulted in a decrease ($p<0.05$) of total CLA content due to

hydrogenation reaction, which was determined by an increase in saturated fatty acids and a decrease in iodine value.

4. Partial hydrolysis of triacylglycerol of isomerized oil by enzyme lipase showed 4 fractions of free fatty acid, monoacyl glycerol, diacyl glycerol and triacyl glycerol on TLC plate. Investigation by HPLC exhibited that linoleic acid at any position in triacylglycerol could possibly be isomerized to CLA.

5. Thermal and mechanical energy of extrusion obviously affected CLA contents and configurations. Total CLA was increased from 1.17 mg/g of oil in starting feed to 7.75 mg/g of oil in corn extrudates when extruded at a product temperature of 150^{o}C and 70% torque. A further increase of product temperature to 190^{o}C showed a significant decrease ($p<0.05$) of total CLA contents, which might be due to an oxidation and breaking down of the CLA conformation.

6. Alteration of CLA configuration was observed at high extrusion temperature. At 70% torque, an increase of product temperature from 150 to 190^{o}C resulted in a significant increase ($p<0.05$) *trans,trans* isomers from 9.13% to 11.88% and a decrease ($p<0.05$) in *cis,trans/trans,cis* isomers from 87.44% to 84.56%.

7. An optimum extrusion condition in the experiment was shown to be at a product temperature of 150^{o}C and 70 % torque. Extrudates from this condition had the highest expansion, good appearance and contained a maximum total CLA content with a minimum of *trans,trans*-CLAs.

CHAPTER VI

APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER III

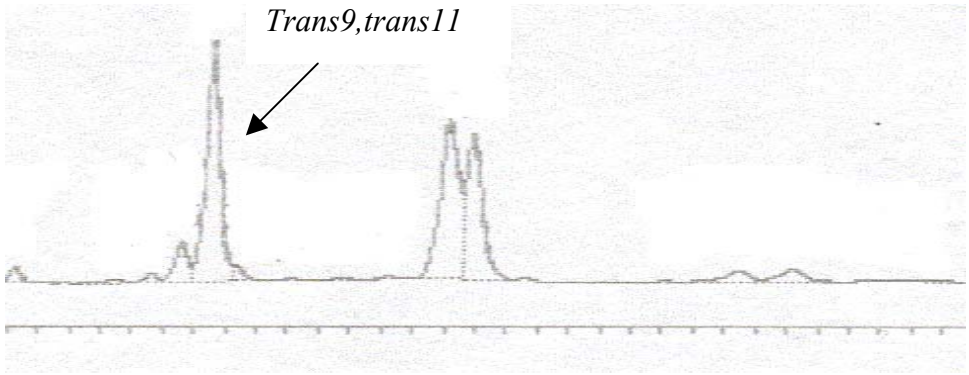
Central composite rotatable design (CCRD)

This design is one of the response surface method designs. It is used to predict optimal conditions with minimizing the number of necessary analytical runs. A model with three variables (reaction temperature, reaction time and stirring speed) can described as follows:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_{11}X_1^2 + B_{22}X_2^2 + B_{33}X_3^2 + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{23}X_2X_3 + B_{111}X_1^3 + B_{222}X_2^3 + B_{333}X_3^3 + B_{112}X_1^2X_2 + B_{113}X_1^2X_3 + B_{122}X_1X_2^2 + B_{223}X_2^2X_3 + B_{133}X_1X_3^2 + B_{233}X_2X_3^2 + B_{123}X_1X_2X_3$$

where B_0, B_1, B_2, B_3, …, and B_{123} are constant regression coefficients of the model, and X_1, X_2 and X_3 are the independent variables in coded values.

(A)



(B)

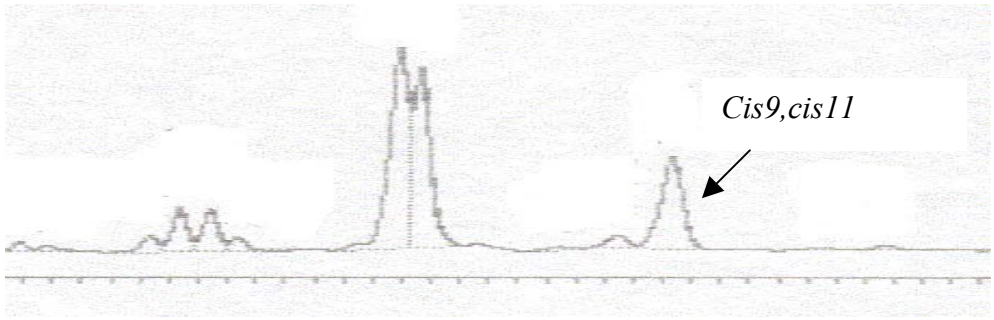


Fig. 6.1 Partial HPLC chromatogram of spiked standard *trans9,trans11* (A) and *cis9cis11*-CLA (B)

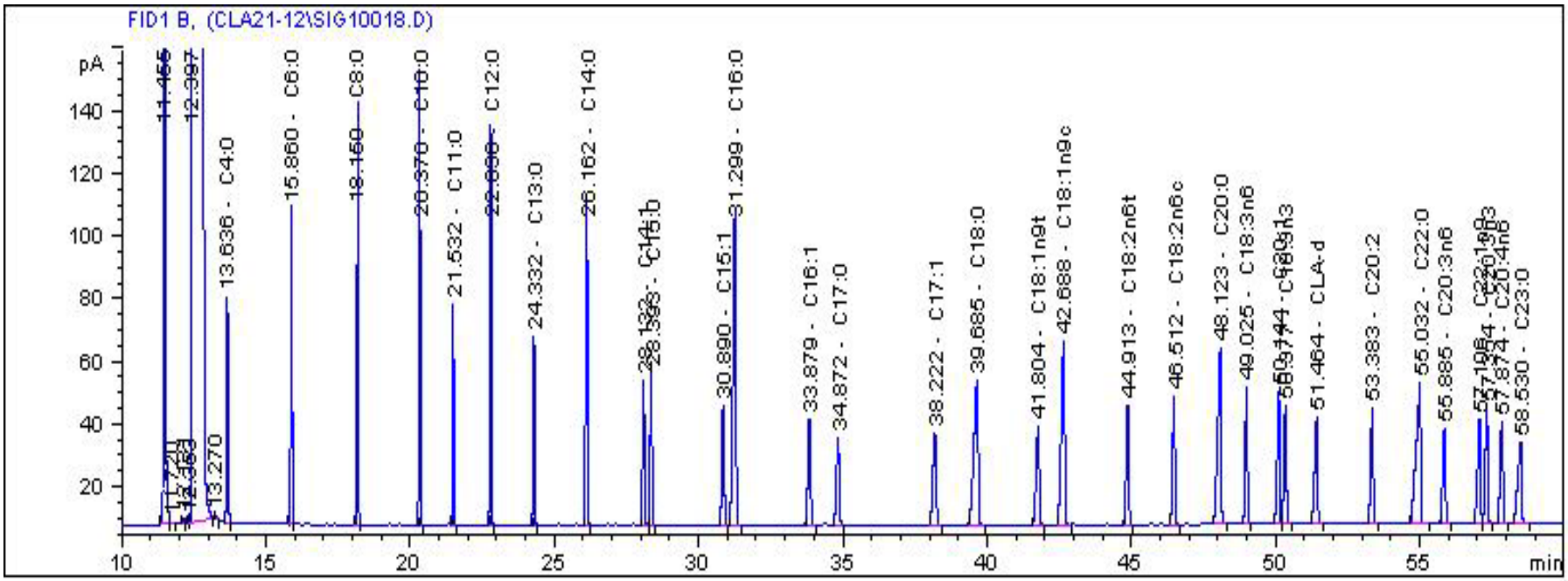


Fig. 6.2 GC chromatogram of standard fatty acid methyl esters

Table 6.1 Percent of fatty acids and total CLA resulted in isomerization condition of T1-T3*

Type	T1	T2	T3
C16:0	14.20±0.05	16.05±1.59	14.80±0.17
C16:1	0.13±0.02	0.12±0.01	0.11±0.01
C18:0	5.32±0.10	5.83±0.56	5.46±0.21
C18:1	24.86±2.15	25.80±2.59	25.87±2.47
C18:2n6t	1.60±0.02	1.81±0.07	1.80±0.04
C18:2n6c	37.74±1.70	26.55±8.10	36.00±2.88
C20:0	0.42±0.01	0.43±0.03	0.42±0.03
C18:3n3	2.58±0.41	1.39±0.91	2.36±0.57
CLA	12.53±0.14	21.39±2.40	12.60±1.00
C22:0	0.61±0.06	0.62±0.16	0.59±0.04

*T1 = isomerization at 160^{O}C, 49 min, 200 rpm

T2 = isomerization at 200^{O}C, 49 min, 200 rpm

T3 = isomerization at 160^{O}C, 49 min, 400 rpm

Table 6.2 Percent of fatty acids and total CLA resulted in isomerization condition of T4-T6*

Type	T4	T5	T6
C16:0	15.33±0.90	15.18±0.51	14.65±0.30
C16:1	0.11±0.01	0.12±0.02	0.09±0.02
C18:0	5.53±0.21	5.97±0.85	5.26±0.05
C18:1	25.69±2.32	26.29±2.58	26.43±0.23
C18:2n6t	1.75±0.06	1.62±0.03	1.55±0.01
C18:2n6c	30.05±4.16	35.72±2.25	36.76±0.34
C20:0	0.38±0.03	0.44±0.05	0.41±0.03
C18:3n3	1.63±0.59	2.28±0.74	2.32±0.00
CLA	18.84±1.78	11.92±1.51	12.08±0.85
C22:0	0.67±0.08	0.44±0.08	0.42±0.05

*T4 = isomerization at 200^{O}C, 49 min, 400 rpm

T5 = isomerization at 160^{O}C, 191 min, 200 rpm

T6 = isomerization at 200^{O}C, 191 min, 200 rpm

Table 6.3 Percent of fatty acids and total CLA resulted in isomerization condition of T7-T9*

Type	T7	T8	T9
C16:0	15.66±0.39	15.58±0.73	14.42±0.30
C16:1	0.14±0.02	0.13±0.02	0.13±0.01
C18:0	5.89±0.05	5.75±0.10	5.17±0.40
C18:1	28.72±0.10	27.73±0.30	24.73±1.88
C18:2n6t	1.86±0.42	1.99±0.02	1.35±0.15
C18:2n6c	34.90±0.19	31.68±0.00	41.10±2.94
C20:0	0.45±0.04	0.44±0.02	0.37±0.03
C18:3n3	1.93±0.02	1.63±0.05	3.17±0.90
CLA	10.08±0.24	14.60±0.36	9.12±1.17
C22:0	0.36±0.07	0.46±0.04	0.43±0.01

*T7 = isomerization at 160^{O}C, 191 min, 400 rpm

T8 = isomerization at 200^{O}C, 191 min, 400 rpm

T9 = isomerization at 146^{O}C, 120 min, 300 rpm

Table 6.4 Percent of fatty acids and total CLA resulted in isomerization condition of T10-T12*

Type	T10	T11	T12
C16:0	16.60±0.43	15.46±0.40	16.03±1.15
C16:1	0.14±0.00	0.11±0.00	0.13±0.01
C18:0	5.93±0.02	5.44±0.13	5.19±0.57
C18:1	25.14±0.26	25.83±2.63	24.88±0.39
C18:2n6t	2.34±0.17	1.56±0.23	1.62±0.34
C18:2n6c	39.31±0.10	35.93±2.50	36.52±2.59
C20:0	0.44±0.01	0.35±0.00	0.35±0.05
C18:3n3	2.23±0.05	2.54±0.61	2.57±1.09
CLA	7.57±0.29	12.24±1.40	12.25±2.55
C22:0	0.32±0.18	0.53±0.12	0.46±0.15

*T10 = isomerization at 214^{O}C, 191 min, 400 rpm

T11 = isomerization at 180^{O}C, 120 min, 100 rpm

T12 = isomerization at 180^{O}C, 120 min, 500 rpm

Table 6.5 Percent of fatty acids and total CLA resulted in isomerization condition of T13-T15*

Type	T13	T14	T15
C16:0	12.57±0.07	17.67±0.99	16.85±1.58
C16:1	0.11±0.01	0.14±0.00	0.13±0.03
C18:0	4.15±0.07	5.94±0.08	5.72±0.21
C18:1	21.84±1.76	28.55±0.03	26.48±2.42
C18:2n6t	0.29±0.08	1.74±0.06	1.84±0.02
C18:2n6c	53.66±1.05	32.19±0.30	30.72±0.05
C20:0	0.29±0.01	0.41±0.06	0.40±0.08
C18:3n3	6.60±0.78	1.61±0.05	1.55±0.05
CLA	0.23±0.04	11.36±0.30	15.79±0.05
C22:0	0.26±0.04	0.38±0.09	0.52±0.10

*T13 = isomerization at 180^{O}C, 0 min, 300 rpm

T14 = isomerization at 200^{O}C, 240 min, 300 rpm

T15 = isomerization at 200^{O}C, 120 min, 300 rpm

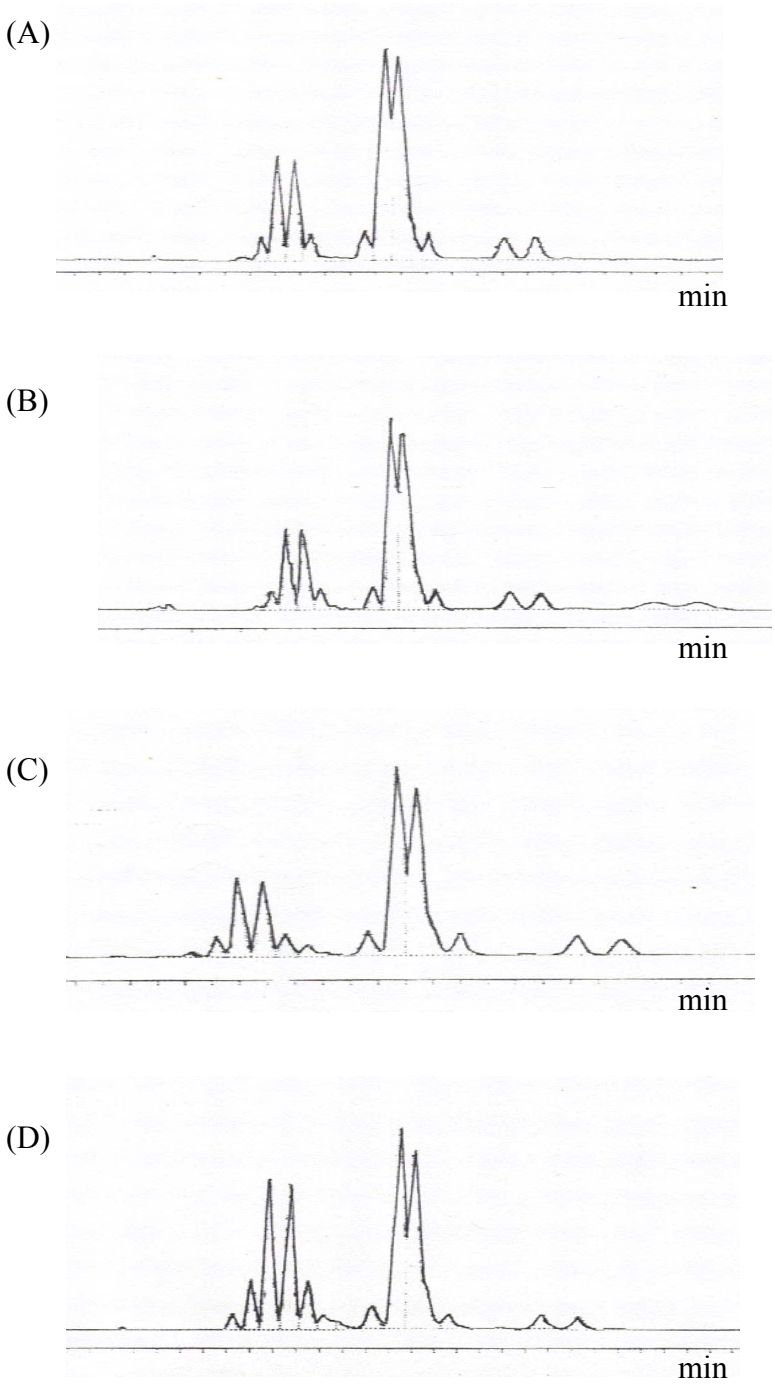


Fig. 6.3 HPLC chromatogram shows CLA of TLC fractions; free fatty acids (A); mononacyl glycerol (B), diacyl glycerol (C); and triacyl glycerol (D)

CHAPTER VII

APPENDIX B

SUPPORTING INFORMATION FOR CHAPTER IV

Isomerization of linoleic acid (modified from Berdeaux et al., 1998)

Methyl linoleate (110 g) was added to 216 mL of dry ethylene glycol containing 62.4 g of potassium hydroxide. The mixture was heated at 180^{O}C for 2 h while a slow stream of nitrogen was passed through the reaction mixture. After cooling the mixture to room temperature, 480 ml of distilled water and 68 mL of concentrated HCl were added and the isomerized fatty acids were extracted with hexane (3 x 400 mL). The combined hexane layers were washed with distilled water, dried over anhydrous sodium sulfate, and hexane-evaporated under N_2.

Table 7.1 Screw configuration for direct expanded snack

Screw element type	amount	Length (mm)
Feed screw	11D	209.00
60 forward paddles	4	19.00
feed screw	3D	57.00
60 forward paddle	4	19.00
single lead screw	2D	38.00
30 forward paddle	5	23.75
30 forward paddles	4	19.00
single lead screw	1D	19.00
60 forward paddle	6	28.50
60 forward paddle	5	23.75
single lead screw	1D	19.00
Total lengths (mm)		475.00

Table 7.2 Setting of barrel temperature at zone 1-4 to accommodate the product temperature of 150, 170 and 190^{o}C

Product temperature (^{o}C)	Barrel temperature (^{o}C)			
	Zone 1	Zone 2	Zone 3	Zone 4
150	90	115	120	140
160	90	115	140	160
170	90	115	165	180

Table 7.3 Setting of water feed at product temperatures of 150, 170 and 190°C to acquire the torque of 50, 60 and 70%

		Water feed (kg/h)		
		At product temperature		
		150 (°C)	170 (°C)	190 (°C)
Run A*	At 50% torque	2.06	1.82	1.58
	At 60 % torque	1.70	1.46	1.40
	At 70 % torque	1.46	1.17	1.05
Run B*	At 50% torque	2.12	1.88	1.52
	At 60 % torque	1.88	1.58	1.40
	At 70 % torque	1.52	1.35	1.05

* Run-A: containing 2% sunflower oil

Run-B: containing 2% isomerized oil

Table 7.4 Specific mechanical energy at % torque of 50, 60 and 70

	Specific mechanical energy (kJ/kg)		
	50 % torque	60 % torque	70 % torque
Run A*	184	221	258
Run B*	181	217	253

* Run-A: containing 2% sunflower oil

Run-B: containing 2% isomerized oil

Table 7.5 Total CLA content of sunflower oil added corn extrudates as a result of extrusion conditions

Experiment (temperature (^{o}C)/torque (%))	Total CLA (mg/g of oil)
Starting feed	1.17 ± 0.18^{e}
190/70	1.48 ± 0.75^{de}
190/60	1.15 ± 0.42^{e}
190/50	0.20 ± 0.32^{e}
170/70	3.80 ± 0.52^{c}
170/60	2.48 ± 0.75^{d}
170/50	1.31 ± 0.78^{de}
150/70	7.75 ± 0.41^{a}
150/60	5.99 ± 0.89^{b}
150/50	4.28 ± 1.72^{c}

[a-e] Mean with different letters in a column was significantly different ($p<0.05$)

Table 7.6 Isomeric mixtures of CLA in sunflower oil added corn extrudates

Experiment	CLA isomers (%)		
	trans, trans	*cis, trans/trans,cis*	*cis, cis*
190/70	15.85±1.53^{bc}	75.93±2.54^{ab}	8.22±1.02^{b}
190/60	15.98±1.96^{bc}	75.12±4.83^{ab}	8.90±2.99^{b}
190/50	18.51±1.78^{a}	63.83±1.31^{c}	17.66±3.03^{a}
170/70	12.85±1.30^{d}	81.14±3.06^{a}	6.01±2.03^{b}
170/60	13.71±1.08^{dc}	78.93±5.17^{ab}	7.36±2.44^{b}
170/50	16.34±1.48^{ab}	75.88±3.55^{ab}	7.78±2.07^{b}
150/70	12.09±1.42^{d}	79.02±2.01^{ab}	8.77±1.91^{b}
150/60	12.21±0.71^{d}	78.86±2.20^{ab}	8.93±2.88^{b}
150/50	13.19±1.32^{d}	78.81±2.66^{ab}	8.00±1.07^{b}

$^{a-d}$ Mean with different letters in a column is significantly different ($p<0.05$)

Table 7.7 Comparison of fatty acids profile (% of total fatty acids) of sunflower oil and isomerized oil

Fatty acids	Sunflower oil	isomerized oil
C16:0	8.29±0.01	6.74±0.05
C18:0	3.37±0.01	3.69±0.02
C20:0	0.03±0.00	0.27±0.00
C22:0	0.02±0.00	0.67±0.01
C18:1	30.25±0.02	29.07±0.17
C18:2n6t	0.22±0.01	0.47±0.00
C18:2n6c	56.32±0.21	1.28±0.19
CLA	0.45±0.17	56.53±0.64
C18:3n3	0.95±0.00	0.21±0.00
C20:2	0.09±0.01	1.06±0.38
C20:5n3	0.00±0.00	0.00±0.00

Table 7.8 Isomeric mixtures of CLA in isomerized oil added corn extrudates

Experiment	CLA isomers (%)		
	trans, trans	*cis, trans/trans,cis*	*cis, cis*
Starting feed.	10.19±0.19^{b}	85.82±1.70^{bc}	3.99±0.12^{a}
190/70	11.88±0.16^{a}	84.56±0.10^{c}	3.56±0.17^{b}
190/60	11.47±0.14^{a}	84.46±0.17^{c}	4.07±0.04^{a}
190/50	11.73±0.06^{a}	84.24±0.08^{c}	4.03±0.13^{a}
170/70	9.56±0.40^{bc}	86.87±0.47^{ab}	3.56±0.07^{b}
170/60	9.56±0.43^{bc}	87.01±0.64^{ab}	3.42±0.20^{b}
170/50	9.99±0.60^{b}	86.24±0.60^{bc}	3.75±0.31^{ab}
150/70	9.13±0.54^{c}	87.44±0.89^{a}	3.12±0.37^{b}
150/60	9.33±0.03^{c}	87.04±0.89^{ab}	3.62±0.06^{b}
150/50	9.68±0.44^{BC}	86.66±0.18^{AB}	3.73±0.27^{AB}

$^{a-c}$ Mean with different letters in a column is significantly different ($p<0.05$)

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PRESENTATIONS

1. Pakdeechanuan, P., Intarapichet, K., Fernando, L. and Grün, I. 2003. Effects of rhodium heterogeneous catalyst and isomerization conditions on linoleic acid conjugation of soybean oil. 36th Annual Australian Institute of Food Science and Technology Convention, August 24-27, 2003, Melbourne Convention Centre, Melbourne, AUSTRALIA.

2. Pakdeechanuan, P., Intarapichet, K. and Tongta, S. 2004. Effect of extrusion conditions on conjugated linoleic Acid of corn extrudates. 10th World Congress on Clinical Nutrition, November 30-December 3, 2004, Pearl Village, Phuket, THAILAND.