UNIVERSITI PUTRA MALAYSIA

PRODUCTION AND CHARACTERIZATION OF HYDROPHILIC CONJUGATED LINOLEIC ACID WITH IMPROVED OXIDATIVE STABILITY USING SUNFLOWER OIL

SARA KOOHIKAMALI

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for Degree of Doctor of Philosophy

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Chairman: Ling Tau Chuan, PhD

Faculty: Engineering

Conjugated linoleic acids (CLAs) as isomers of linoleic acid (LA) with two conjugated double bonds are regarded as functional lipids. CLA is commercially produced by alkali isomerization of LA rich oils (e.g., sunflower and safflower oils). However, the water insolubility and oxidative instability of commercial CLA are barriers which limit the applications of CLA for fortification of aqueous based foods.

The main target of this research was to produce a hydrophilic derivative of CLA with an improved oxidative stability. In order to achieve this goal, sunflower oil was subjected to the transesterification. The optimum conditions resulting in a maximum yield (100 %) were at reaction time of 60 min, 6.5:1 molar ratio of methanol to oil and 0.5 % sodium methoxide ($NaOCH_3$). The fatty acid methyl esters (FAMEs) were mainly the linoleic acid methyl esters (LAMEs) which then proceeded to the

isomerization. An optimum point of 5 % $NaOCH_3$, 1.06 % polyethylene glycol and 140 °C reaction temperature lead to an isomerization degree of 96.6 % and 72.90 % of the total conjugated linoleic acid methyl esters (CLAMEs). The CLA obtained from the purification of the hydrolyzed CLAMEs soap was then reacted with both Lysine (Lys) and Arginine (Arg). The formation of Lys-CLA and Arg-CLA was confirmed by FT-IR spectroscopy at 1650 and 1550 cm^{-1}.

The measurement of the peroxide values (PVs) of the samples as an indicator of the oxidative stability showed that the PVs of the Lys-CLA and Arg-CLA only increased by 1.4 and 1.5-fold, respectively, whereas the PVs of the CLA increased by 16-fold (12 h of storage at 60 °C). Furthermore, the mean of the Lys-CLA PVs (0.32) was significantly lower ($p < 0.05$) compared to the Arg-CLA (0.49). The Lys-CLA, Arg-CLA, Lys, Arg and CLA showed some antioxidative potential in the DPPH and ABTS assays. Lys-CLA with the smallest half maximal inhibitory concentration (IC_{50}) exhibiting the greatest antioxidant activity ($p < 0.05$) among the samples.

A visual inspection test on the stability of the Lys-CLA and Arg-CLA solutions indicated that the stability of Arg-CLA in neutral water is greater than that of Lys-CLA at concentrations > 0.2 % (two weeks of storage at 25 ± 1 °C). Moreover, the pH, concentration and type of salt (NaCl and $CaCl_2$) and Lys-CLA concentrations as well as the interaction between pH and the salt concentration significantly affected the solubility of the Lys-CLA ($p < 0.05$). The highest water solubility was at pH 2 and when the concentration of Lys-CLA was ≤ 1.75% with no added salt (0 mM). In contrast, the solubility of Lys-CLA was the least at pH 5-7, 200 mM salt concentration and 2.50 % Lys-CLA concentration. This study confirmed that Lys can be a substitute for Arg in Arg-CLA as suggested in earlier literature due to the inherent polarity and antioxidative potential. Thus, the incorporation of Lys into the CLA molecule can improve the oxidative stability, antioxidant capacity and water miscibility of CLA, and expand the applications of CLA into the enrichment of the nutraceutical beverages.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGHASILAN DAN PENCIRIAN HIDROFILIK ASID LINOLEIK TERKONJUGAT DENGAN PENINGKATAN KESTABILAN PENGOKSIDAAN MENGGUNAKAN MINYAK BUNGA MATAHARI

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Asid linoleik terkonjugat (CLA) sebagai isomer asid linoleik (LA) dengan dua ikatan kembar terkonjugatnya dianggap sebagai lipid fungsian. CLA secara komersilnya dihasilkan oleh pengisomeran alkali LA di dalam minyak (contohnya, minyak bunga matahari dan safflower). Walau bagaimanapun, sifat ketidaklarutan air dan ketidakstabilan oksidatif CLA komersial mengehadkan aplikasi CLA sebagai pengukuh makanan berasaskan berair.

Sasaran utama kajian ini adalah untuk menghasilkan terbitan hidrofilik CLA dengan kestabilan oksidatif yang lebih baik. Dalam usaha untuk mencapai matlamat ini, minyak bunga matahari telah melalui proses transesterifikasi. Keadaan optimum yang memberi hasilan maksimum (100 %) adalah pada tempoh tindakbalas selama 60 minit, 6.5:1 nisbah molar metanol terhadap minyak dan 0.5 % natrium metoksida ($NaOCH_3$). Asid lemak metil ester (FAME) yang sebahagian besar adalah asid

linoleik metil ester (LAME) dimana kemudiannya telah melalui proses pengisomeran. Pada titik optimum 5 % $NaOCH_3$, 1.06 % polietilena glikol dan 140 °C suhu tindak balas memberikan tahap pengisomeran sebanyak 96.6 % dan 72.90 % daripada jumlah asid linoleik metil ester terkonjugat (CLAME). CLA yang diperolehi daripada penulinan terhidrolisis sabun CLAME kemudiannya bertindak balas dengan kedua-dua Lisin (Lys) dan Arginin (Arg). Pembentukan Lys-CLA dan Arg-CLA telah disahkan oleh spektroskopi FT-IR pada 1650 dan 1550 cm^{-1}.

Pengukuran nilai peroksida (PV) sampel sebagai penunjuk kepada kestabilan oksidatif menunjukkan bahawa PV Lys-CLA dan Arg-CLA hanya meningkat sebanyak 1.4 dan 1.5 kali ganda, setiap satu, manakala PV CLA meningkat sebanyak 16 kali ganda (12 jam penyimpanan pada 60 °C). Tambahan pula, purata PV Lys-CLA (0.32) adalah secara signifikannya lebih rendah ($p < 0.05$) berbanding dengan Arg-CLA (0.49). Lys-CLA, Arg-CLA, Lys, Arg dan CLA menunjukkan potensi antioksida dalam assai DPPH dan ABTS. Lys-CLA dengan separuh terkecil maksimum kepekatan perencatan (IC_{50}) menunjukkan aktiviti antioksidan terbesar ($p < 0.05$) di antara sampel.

Ujian pemeriksaan visual terhadap kestabilan larutan Lys-CLA dan Arg-CLA menunjukkan bahawa kestabilan Arg-CLA di dalam air neutral adalah lebih tinggi daripada Lys-CLA pada kepekatan > 0.2 % (dua minggu penyimpanan pada 25 ± 1 °C). Selain itu, pH, garam (NaCl dan $CaCl_2$) dan kepekatan Lys-CLA serta interaksi antara pH dan kepekatan garam secara signifikannya mempengaruhi kebolehlarutan Lys-CLA ($p < 0.05$). Kebolehlarutan air tertinggi adalah pada pH 2 dan pada kepekatan Lys-CLA ≤ 1.75 % dengan tanpa penambahan garam (0 mM). Sebaliknya pula, kebolehlarutan Lys-CLA adalah paling rendah pada pH 5-7, 200 mM kepekatan garam dan 2.50 % kepekatan Lys-CLA. Kajian ini mengesahkan bahawa Lys boleh menjadi pengganti untuk Arg di dalam Arg-CLA seperti yang dicadangkan di dalam kajian awal kerana kekutuban yang wujud dan potensi antioksida. Oleh itu, penambahan Lys ke dalam molekul CLA boleh meningkatkan kestabilan oksidatif, kapasiti antioksidan dan kebolehcampuran air, dan mengembangkan aplikasi CLA untuk penambahbaikan minuman nutraseutikal.

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I certify that a Thesis Examination Committee has met on 4th of February 2013 to conduct the final examination of Sara KoohiKamali on her thesis entitled “Production and Characterization of Hydrophilic Conjugated Linoleic Acid with Improved Oxidative Stability using Sunflower Oil” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the University Putra Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the Degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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SARA KOOHIKAMALI

Date: 4 February 2013

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LIST OF ABBREVIATIONS

ABTS	2,2'-Azino-bis (3-ethylbenzothi sulphonic acid) diammonium sa
ANOVA	analysis of the variance
AOCS	American oil chemists' society
bar	unit of pressure measurement and equal to the atmospheric pressure on earth at sea level.
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
Arg	arginine
Arg-CLA	complex of arginine and CLA
C	carbon
^{o}C	degree Celsius
$CaCl_2$	calcium chloride
CVD	cardiovascular disease
C16:0	palmitic acid
C17:0	heptadecanoic acid
C18:0	stearic acid
C18:1	oleic acid

C18:2	linoleic acid
C18:3	linolenic acid
C20:1	gadoleic acid
C22:0	behenic acid
CCD	central composite design
CDs	Cyclodextrins
CFAMEs	conjugated fatty acid methyl esters
CLA	conjugated linoleic acid
CLA-UIC	urea crystals including the CLA
CLAME	conjugated linoleic acid methyl ester
COOH	carboxyl group
cm	centimeter (10^{-2} m)
D	diameter
dH_2O	deionized water
DHA	docosahexaenoic acid
DOE	design of experiment
DPPH	2, 2'-diphenyl-1picrylhydrazyl
EFAs	essential fatty acids

e.g	Meaning "for example."
FA	fatty acid
FAME	fatty acid methyl ester
FFA	free fatty acid
FID	flame ionization detector
FT-IR	Fourier transform infrared
g	gram
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GLM	general linear model
h	hour
H	height
HDL	high density lipoprotein
HZ	hertz
HPLC	high performance liquid chromatography
HCl	hydro chloride acid
H_3PO_4	phosphoric acid
IC_{50}	half maximal inhibitory concentration

i.e	Meaning "that is."
IR	infrared
KBr	potassium bromide
KCl	potassium chloride
KOH	potassium hydroxide
kg	killogram
L	litre
LA	linoleic acid
LAME	linoleic acid methyl ester
LDL	low density lipoprotein
LNA	α-linolenic acid
Lys	lysine
Lys-CLA	complex of lysine and CLA
M	molar concentration
m	meter
meq/kg	milli equivalent per kilogram
mg	milligram (10^{-3} g)
$MgCl_2$	magnesium chloride

min	minute
mL	millilitre (10^{-3} L)
µL	microlitre (10^{-6} L)
mM	millimolar (10^{-3} M)
µM	micromolar (10^{-6} M)
mm	millimeter
mmol/L (mM)	millimol per litre (millimolar or 10^{-3} M)
N	normality
N_2	nitrogen
Na_2HPO_4	di-sodium hydrogen phosphate
NaCl	sodium chloride
$NaOCH_3$	sodium methoxide (methylate)
NaOH	sodium hydroxide
NH_2	amine group
NMR	nuclear magnetic resonance
nm	nanometer (10^{-9} m)
PEG 400	polyethylene glycol with molar mass of 400
pH	Puissance hydrogen

pI	isoelectric point
pKa	acid ionization constant
PTC	phase transfer catalyst
PV	peroxide value
p-value (p)	probability value
RH	lipid
$R^{\bullet}$	lipid radical
ROOH	lipid hydroperoxide
$ROO^{\bullet}$	peroxy or peroxyl radical
R^2	coefficient of determination
rpm	revolutions per minute
RSM	response surface methodology
s	second
SD	standard deviation
TAG	Triacylglycerols
TBHQ	tertiary butyl hydroquinone
TEAC	Trolox equivalent antioxidant capacity

TFAs	trans fatty acids
Trolox	6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid
UIC	urea inclusion crystallization
UICs	urea inclusion compounds
US FDA	United states food and drug administration
UV	ultra violet
V	volt
W	width
w/w	weight per weight
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

1.1 Research Background

There is an increasing need to develop functional and nutraceutical foods with specific characteristics that improve the health, treat or prevent disease. The term ' conjugated linoleic acids ' (CLAs), commonly refers to the positional and geometrical isomers of linoleic acid (LA) containing conjugated double bonds rather than isolated bonds (Ha et al., 1987). CLA has been found to be an extraordinary essential fatty acid (EFA) with miscellaneous interesting functional effects on the human body such as: anti-carcinogenic (McGuire and McGuire, 2000; Bell, 2003; Lee et al., 2005; Bhattacharya et al., 2006; Kelley et al., 2007; Park, 2009), anti-atherogenic (Gavino et al., 2000; Kritchevsky et al., 2004; McLeod et al., 2004), anti-diabetic (Belury and Vanden Heuvel, 1999; Ryder et al., 2001) and anti-adipogenic (body fat reduction) effects (Smedman and Vessby, 2001; Park and Pariza, 2007). The most dominant and beneficial CLA isomers that have been recognized to participate in the abovementioned biological tasks are 9c11t-C18:2 and 10t12c-C18:2 (Brownbill et al., 2005; Park, 2009). CLA is naturally produced in meat and dairy products derived from ruminant animals and through the bio-hydrogenation of C18-unsaturated fatty acids by anaerobic gram-positive bacteria in the rumen (Harfoot and Hazlewood, 1997; Parodi, 2004; Nam and Garnsworthy, 2007). Therefore, the main natural sources of CLA are the beef tallow and milk fat of the ruminant animals in which the CLA content is less than 1 % (Ha et al., 1987). The recommended daily intake of CLA, that is necessary for good health and with regard to safety issues, is 3-3.4 g/day for a human with a 70 kg body weight (Berven et al., 2000). As the amount of CLA in natural sources is never higher than 12 mg/g from fat, these sources cannot provide a sufficient amount of CLA. Therefore, consumption of CLA through enriched foods or dietary supplements is highly recommended (Goodhart and Shils, 1980; Pakdeechanuan, 2004). To date, many studies have been performed on the production of CLA on a large scale. However, the alkali isomerization of linoleic acid has been found to be the principle approach for commercial CLA production (Reaney et al., 2002; Saebo, 2003; Rongione et al., 2007; Westfechtel et al., 2007).

1.2 Problem Statement

Although commercial CLA is rich in total CLA content, it is in fact a mixture of positional isomers. The problem with most of the commercial CLA produced by non-optimized conventional approaches is the heterogeneity of their isomers which occurs batch to batch together with the formation of undesirable and toxic by-products (Rongione et al., 2005). Moreover, accumulation of *trans, trans*-CLA isomers (unwanted or unusual CLA isomers) in the liver have been reported to interfere with the normal metabolism of fatty acids (Holman et al., 1991). It has already been reported that the reason for the formation of *trans, trans* isomers and the re-distribution of the double bonds is the harsh conditions applied during the isomerization process (e.g. high temperature, pressure, solvents and large amounts of alkalis). Furthermore, by application of such severe conditions the theoretical expected yields of the isomerization cannot be achieved (Saebo, 2003; Wenk and Haeser, 2005; Saebo and Saebo, 2006). Another weakness of commercial CLA is its insolubility in water, which limits its application for enrichment of liquid foods and drinks. This means that the addition of CLA into an aqueous phase results in the formation of unstable emulsions (Kim et al., 2003). A further concern with commercial CLA is its oxidative instability. Conjugated fatty acids have been reported to be more sensitive and more exposed to free radical attack than non-conjugated fatty acids (Van den Berg, 1994). Kim et al. (2003) did some ground-breaking research and discovered that the incorporation of Arginine (Arg) as a highly polar and antioxidant amino acid into the CLA structure can improve the oxidative stability and solubility of CLA in pure water. However, no further information is available showing the influence of various factors on the solubility of the Arg-CLA complex in the aqueous phase. In addition, the possibility of using other amino acids that may have similar effects on CLA as an alternative for Arg is still obscure.

1.3 Problem Solving Approaches and Research Hypothesis

In order to produce commercial CLA with less unwanted isomers that mostly contain two predominant isomers, an optimized transesterification of sunflower oil was used to obtain linoleic acid methyl esters (LAMEs) as the substrate of isomerization (Abney and Anderson, 2002). The reason for choosing such compounds was that the application of LAMEs (i.e., methyl linoleate) as the feedstock of isomerization was found to result in the production of a high yield of conjugated linoleic acid methyl esters (CLAMEs) (Zander et al., 2006; Westfechtel et al., 2007). Since the fatty acid methyl esters resulting from oils with high phosphatides and sterol content (e.g., soybean and corn oil) are not suitable for human and animal consumption (Saebo, 2006), sunflower oil was chosen as a perfect and accessible source of linoleic acid (about 65 % on average). This oil contains a low content of phosphatides and sterols as well (Saebo, 2003; 2006). It is anticipated that by optimization of the transesterification conditions, less basic catalyst would be used; whereas the process yield and production of LAMEs would be improved. Therefore, LAMEs were first provided by transesterification of sunflower oil under optimum conditions. These compounds directly precede the isomerization process using sodium methoxide (alkali catalyst) and polyethylene glycol 400 as a phase transfer catalyst (PTC). By utilization of a PTC in the reactions with two immiscible phases, not only can the need for the use of toxic solvents be avoided, but also the overall yield can be increased (Metzger, 1998; Makosza, 2000; Wenk and Haeser, 2005). Thus, a mixture of conjugated linoleic acid methyl esters with less toxic by-products and comparatively high levels of preferred isomers can be produced. Despite the application of PTC in the isomerization process was previously conducted by earlier researchers (Wenk and Haeser, 2005), the maximal degree of isomerization was only 93 %. Yet, no further studies have been made available regarding further optimization of the approach proposed by Wenk and Haeser (2005). In the present research, optimization of the isomerization conditions hypothesized to improve the degree of isomerization or conversion of linoleic acid methyl esters (LAME) into conjugated linoleic acid methyl esters (CLAME). Furthermore, lysine (Lys) is introduced into the CLA structure to form the Lys-CLA complex and in order to improve the oxidative stability and water miscibility of the commercial CLA. The reason for choosing Lys is that, similar to Arg, Lys is a highly polar and hydrophile amino acid which may also have some antioxidative effect on the oxidation of CLA (Marcuse, 1961; Elias et al., 2008). Moreover, Lys is found to be the second amino acid after Arg which can enhance insulin secretion which helps the blood glucose to be transferred into cells (Fajans et al., 1967). Therefore, the formation of the Lys-CLA complex can also alleviate the possible side effects that may result from a CLA overdose such as the insulin resistance effect as previously reported (Tsuboyama-Kasaoka et al., 2000; Clement et al., 2002). It is hypothesized that Lys can serve as an alternative for Arg in the complex of an amino acid-CLA. It is supposed that by coupling the Lys with CLA, the water solubility and stability of CLA against oxidation can be increased.

1.4 Objectives

The main target of this research is to produce a derivative of CLA that is soluble in the aqueous phase with an improved oxidative stability compared to CLA alone. Below is a list of objectives to achieve the overall aim:

- To enhance the production yield of fatty acid methyl esters (FAMEs) through optimization on transesterification conditions of sunflower oil using sodium methoxide as an alkaline catalyst.
- To optimize the synthesis of conjugated fatty acid methyl esters (CFAMEs) via isomerization of sunflower oil FAMEs using sodium methoxide an alkaline catalyst and poly ethylene glycol 400 (PEG 400) as a phase transfer catalyst and its subsequent recovery as CLA using two-step urea crystallization.

- To investigate and characterize the complex of conjugated linoleic acid (CLA) with lysine (Lys) and arginine (Arg).

1.5 Thesis Outline

The dissertation has been organized in six chapters including three working chapters from Chapter 3 to Chapter 5. This chapter (the introduction) presents the background, problem statement, approaches, hypothesis and objectives pursued in this research. **Chapter 2** comprises the preliminary studies of the physicochemical properties of CLA and the functional effects of CLA on the human body. In the chapter, the effect of the reaction parameters on transesterification is evaluated. Likewise, the common methods of CLA production are introduced and compared based on their advantages and drawbacks. In **Chapter 3**, the possibility of increasing the transesterification yield of sunflower oil by process optimization is studied. **Chapter 4** examines the possibility of improvement of the isomerization yield by the optimization of isomerization condition using PEG 400 as a phase transfer catalyst, the conversion of conjugated methyl esters (conjugated methyl linoleate) into free fatty acids and further purification procedures. **Chapter 5** investigates the possibility of hydrophilic CLA production and the evaluation of the characteristics of the final product. Finally, **Chapter 6** includes a general conclusion and recommendations for future studies. A flow diagram of the experimental work of this dissertation is depicted in Figure 1.1.

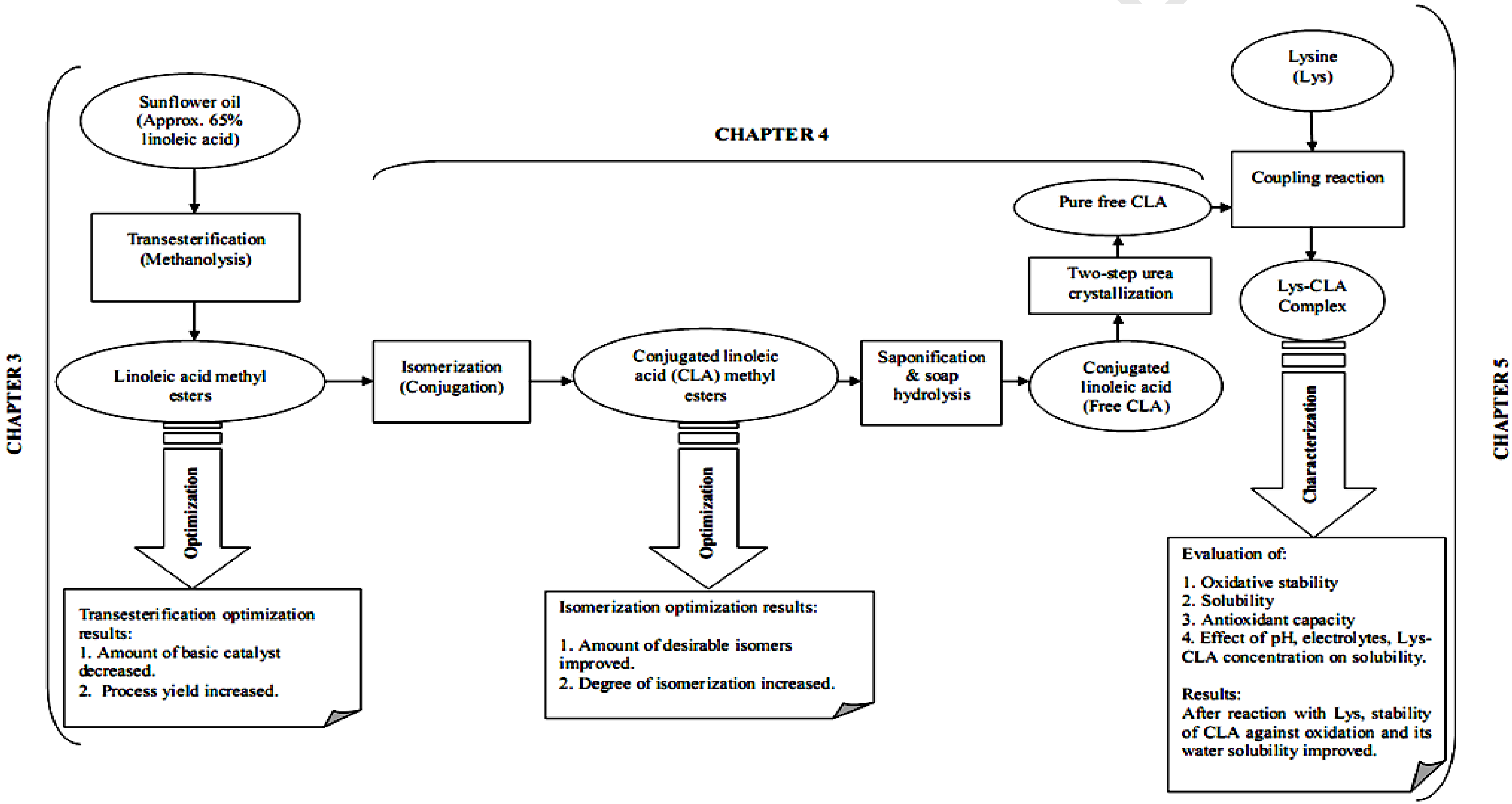

Figure 1.1: The flow diagram of the research experimental works showing the outstanding results and contributions.

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