SYNTHESIS OF STRUCTURED PHOSPHOLIPIDS WITH CONJUGATED LINOLENIC ACID, AND EVALUATION OF THEIR PHYSICAL PROPERTIES

A Thesis

by

NATHALIE QUEZADA ARBOLEDA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2007

Major Subject: Food Science and Technology

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Approved by:

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ABSTRACT

Synthesis of Structured Phospholipids with Conjugated Linolenic Acid, and Evaluation of Their Physical Properties. (August 2007)

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Structured phospholipids with conjugated linolenic acid were produced for potential applications in nutraceuticals and functional foods. Structured phospholipids were synthesized with conjugated linolenic acid (CLnA) from natural sources by catalytic enzymatic reaction. Pomegranate seed oil, as a natural source of CLnA, and an isomerized-concentrated mixture (ICM) of CLnA from flaxseed oil were used for the enzymatic reaction with phosphotidylcholine (PC) using Liposyme TL IM for fatty acid modification at 57 °C for 96 h. The enzymatic process was an effective way to produce structured phospholipids with CLnA. The maximum incorporation of CLnA from pomegranate seed oil and ICM from flaxseed oil into PC was 11.3% and 4.9% after 72 h, respectively. Structured lysophospholipids were also obtained as a result of the enzymatic reaction. The maximum incorporation of CLnA from pomegranate oil and ICM from flaxseed oil into lysophospholipids was 17.2% and 13.5% after 72h, respectively.

Physical properties such as dropping point and viscosity at 40 and 50 °C of the structured phospholipids produced were measured when they were added to a chocolate mixture (unsweetened chocolate 94.6%, coconut oil 5% and 0.4 % phospholipids). Two controls were used for comparison: the chocolate mixture without phospholipids and the chocolate mixture with Lipoid S100 (phosphatidylcholine 94%). Structured phospholipids with CLnA showed lower dropping point and viscosities than the controls.

Oil-in-water emulsions were prepared with whey protein (1%), soy bean oil (10%) and phospholipids (0.5%) in a high pressure homogenizer at 20MPa. The emulsion stability of the emulsions prepared, control (without phospholipids), Lipoid S 100 and structured phospholipids with CLnA were determined by visual observation of phase separation. The structured phospholipids emulsion showed higher emulsion stability than the controls. This emulsion was stable up to 108 h while the emulsion without phospholipid and Lipoid S100 were 48 h and 96 h stable, respectively.

Oxidative stability of the emulsions prepared was determined by measuring the peroxide value and *p*-anisidine value after 1, 3 and 7 days at 50 °C. Oil was extracted from the emulsions using isooctane:isopropanol (3:2 v/v). The structured phospholipid emulsions showed lower oxidative stability than the controls.

DEDICATION

To life, which has always encouraged me to continue fighting, learning from my errors and making me grow….

ACKNOWLEDGEMENTS

I want to thank Dr. Ernesto Hernandez, for making this happen and for his support and encouragement during this project. Thanks to Dr. Rooney for his great support and good advice. Thanks to my good friend Hema, for the useful discussions about this research, and for her support, help and care.

I want to thank Dr. Cisneros and Dr. Murano for their comments, suggestions and time as members of my committee.

I want to thank my family, especially my father for all the support, love and care during all my life. I want to thank Fernando, my witness, for his love, support and advice.

I want to thank to all the new friends I have made during this journey for their friendship, company and support.

I want to thank to all the staff of the Food Protein R&D Center, Dr. Alam, Dr. Riaz, Carl, Rich, Chris M., Asif, Stacey, Elizabeth, Leslie, Travis, Bryan, Andy, Allen, Nick, Kellie, and Chris K for their assistance when I needed it.

I want to thank Dr. Castillo and Dr. Castell for their kind help in this research. Finally, I want to thank Lipoid GmBH for kindly supplying me with phospholipid samples.

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CHAPTER I

INTRODUCTION

Lifestyle-related diseases, such as obesity, hyperlipidemia, arteriosclerosis, diabetes mellitus, cancer and hypertension are increasing in industrialized countries (Nagao and Yanagita 2005). It has been reported that one-third of human cancers is associated with dietary habits and life style (Doll 1992). Modulation of dietary fat quality such as the balance omega-6/omega-3 has been related to immunologic functions diseases (Harbige 2003) and with the presence and development of insuline resistance and the metabolic syndrome. Quality of dietary lipids could decrease the mortality of the life-style diseases (Vessby 2003).

Polyunsaturated fatty acids such as docosahexaenoic acid (DHA, 22:6, n-3), eicosapentaenoic acid (EPA, 20:5, n-3), α-linolenic acid (LnA, 18:3, n-3), linoleic acid (18:2, n-6), and arachidonic acid (ARA, 20:4, n-6) are vital for maintaining biofunctions in mammalians and may be responsible for alleviating certain human health disorders related to heart, circulation problems and inflammation (Kris-Etherton and others 2004). On the other hand, special attention has been given to conjugated fatty acids because they have shown beneficial biologic effects of attenuation lifestyle-related diseases (Ha and others 1989; Ip and others 1991; Cook and others 1993; Lee and others 1994; Park and others 1997).

"Conjugated fatty acids" is a collective term for the positional and geometric isomers of polyunsaturated fatty acids containing conjugated double bonds. Conjugated fatty acids can be obtained after alkali isomerization of linoleic, linolenic and arachidonic acid (Nichols and others 1950). However, they can be also found in natural sources such beef tallow and milk fat (Ha and others 1987) in which conjugated linoleic acid (CLA, 18:2) is present in aprox. 1%. Other sources of these fatty acids are some

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seed oils which contain conjugated linolenic acid (CLnA, 18:3) in levels between 30-70% such as pomegranate seed oil (72%; 9cis, 11trans, 13cis) bitter gourd seed oil (60-70%; 9cis, 11trans, 13trans), tung seed oil (60-70%; 9cis, 11trans, 13cis), catalpa seed oil (31%; 9trans, 11trans, 13cis), and pot marigold seed oil (33%; 8trans, 10trans, 12cis) (Nagao and Yanagita 2005).

Conjugated linolenic acids have shown interesting biological effects such as: anticarcinogenic (Igarashi and Miyazawa 2000; Hora and others 2003; Albrecht and others 2004; Kohno and others 2004; Mehta and Lansky 2004; Tsuzuki and others 2004b), improving immune function and lipid metabolism (Yamasaki and others 2006), prevention and treatment of inflammation (Lansky and Newman 2007), decrease body fat content (Koba and others 2002), hypoglycemic (Akhtar and others 1981; Chao and others 2003; Chuang and others 2006), hypolipidemic (Jayasooriya and others 2000; Noguchi and others 2001; Koba and others 2002) and antioxidant (Dhar and others 1999; Dhar and others 2006).

Other lipids of great interest are phospholipids. Phospholipids have shown important biological functions and health benefits, and are currently used at several food products (Schneider 2001). These compounds play an important role in biological functions such as maintaining cell membranes integrity, prevention of neurological diseases and regulation of basic biological processes such as cell to cell signaling (Guo and others 2005). Phospholipids have shown health benefits such as reducing blood cholesterol and triglycerides (Knuiman and others 1989), liver detoxification and repair of damaged liver tissue (Lieber and others 1990), improvement of cognitive functions (Pepeu and others 1996), and visual acuity (Koletzko and others 1995). Besides, phospholipids display amphipathic character, as components of bilayers or micelles and liposomes and have been applied in food, cosmetic and pharmaceutical products. Phospholipids have been used as natural emulsifiers, wetting and dispersing agents (Szuhaj 1983) and as liposome formers. These unique characteristics have made phospholipids the target of product development where specific structures are able to meet particular nutritional and, pharmaceutical requirements (Guo and others 2005).

Moreover, phospholipids may be considered an effective and versatile delivery system of functional compounds because they may increase their bioavailability and chemopreventive effect (Hossen and Hernandez 2005).

Structured phospholipids are phospholipids in which the head group and/or fatty acid composition have been changed. Structured phospholipids have been synthesized with diverse fatty acids such as oleic acid (Yagi and others 1990), heptadecanoic acid (Svensson and others 1992), caprylic acid and eicosapentaenoic acid (Peng and others 2002), docosahexaenoic acid (Peng and others 2002; Hosokawa and others 1998) conjugated linoleic acid (Hossen and Hernandez 2005; Peng and others 2002) and phytosterols (Hossen and Hernandez 2004). Structured phospholipids with conjugated linolenic acid (CLnA) may be desirable for the development of "nutraceutical lipids" which may improve health, and prevent or treat disease (Hossen and Hernandez 2005).

This research is focused on the synthesis of structured phospholipids with CLnA from pomegranate seed oil and flaxseed oil.

Specific objectives were:

1. Isomerization of fatty acids of flaxseed oil to obtain conjugated linolenic acids.
2. Synthesis of structured phospholipids with CLnA from flaxseed oil.
3. Synthesis of structured phospholipids with CLnA from pomegranate seed oil.
4. Examine four physicochemical properties of the structured phospholipids produced from pomegranate seed oil: dropping point, viscosity, emulsion stability, and oxidative stability.

CHAPTER II

LITERATURE REVIEW: SYNTHESIS OF STRUCTURED PHOSPHOLIPIDS WITH CONJUGATED LINOLENIC ACID (CLnA)

2.1. Phospholipids

There is a great diversity of phospholipids in nature. The most known phospholipid is lecithin which is a mixture of several phospholipids present in cells and plant membranes. Common sources of lecithin are soybean oil or egg yolk. Lecithin is usually defined as a mixture of acetone insoluble polar lipids and triglyceride oil together with other minor components produced by degumming of crude vegetable oils and animal fats (Szuhaj 1988).

Phospholipids generally contain a glycerol or a sphingosyl backbone and are classified into main four classes: glycerophospholipids, sphingolipids, ether phospholipids and phosphonopholipids, depending on their backbones and bonding types (Akoh and Min 1998). Glycerophospholipids are built up from glycerol, fatty acids, phosphoric acid, and a second hydroxyl compound which commonly contains nitrogen. The hydroxyl group can be choline, ethanolamine, serine, inositol and glycerol (Figure 1).

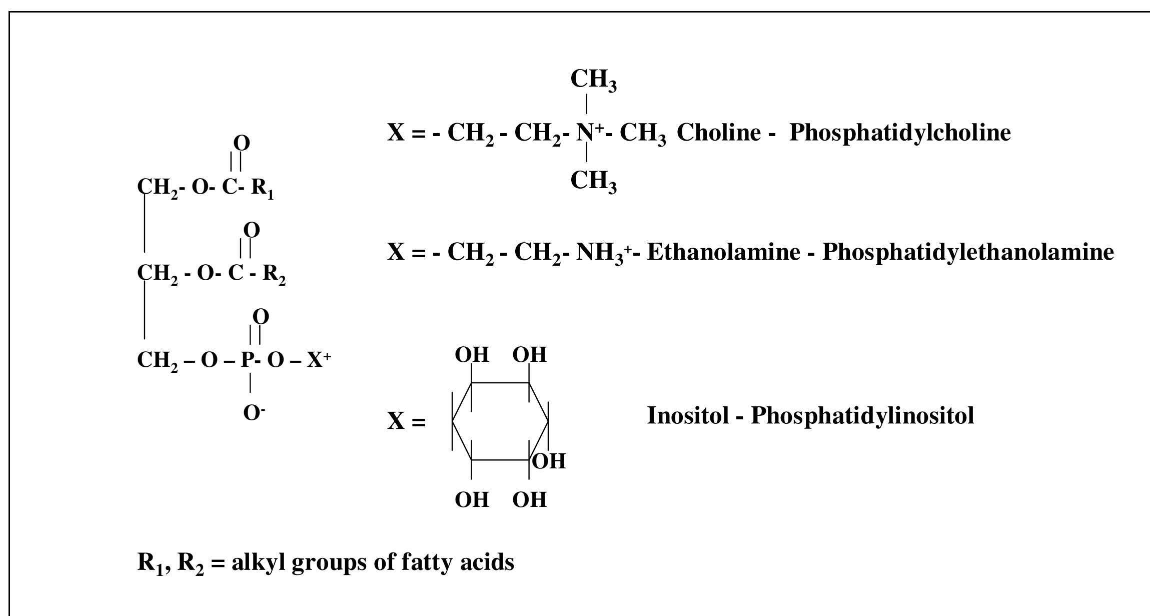

Figure 1. Structure of glycerophospholipids

2.1.1. Functional properties of phospholipids and application in foods

Phospholipids have multiple applications due to their surface-active properties. Phospholipids have a bipolar, amphiphilic molecular structure due to a lipophilic part in the form of two fatty acids and a hydrophilic group in the form of a phosphoric acid ester. Because of this amphipathic character, phospholipids tend to form bilayer structures or micelles which make them possible to encapsulate specific reactive agents into so called liposomes (Figure 2) (Bretscher 1985, van Nieuwenhuyzen and Szuhaj 1998).

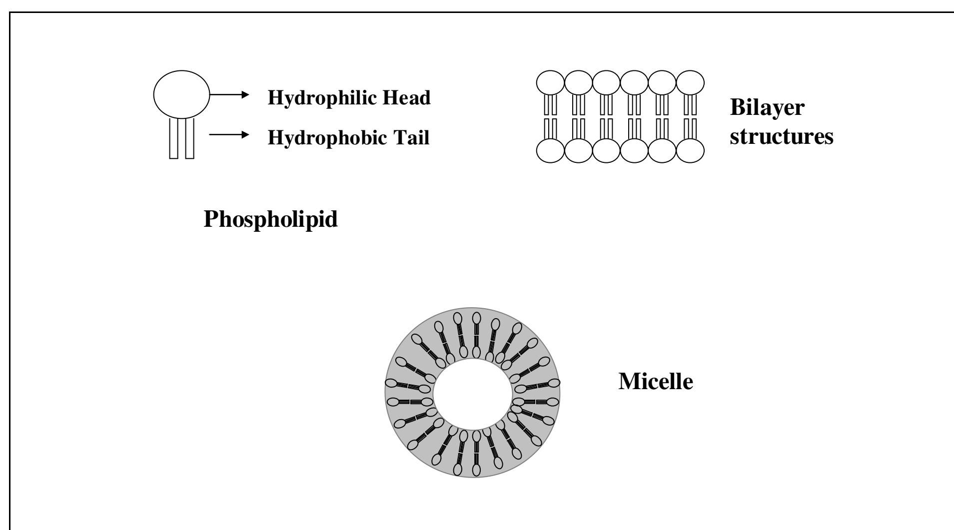

Figure 2. Functional properties of phospholipids

Phospholipids have been used in a range of food products as emulsifiers, stabilizers, control crystallization agents, viscosity modifiers, antioxidants and reducers or replacers of fat (Silva 1990; Sinram 1991). Table 1 shows some food applications of phospholipids.

Table 1. Functional properties of phospholipids

APPLICATION	FUNCTIONALITY
Baked goods	Volume improvement
	Dough conditioner
	Fat dispersion
	Anti-staling
	Egg Yolk replacer
Chocolate	Viscosity modification
	Water uptake Ice coatings
Instant drinks	Wetting
Dairy	Dispersability
Cocoa/Chocolate	Dispersabilty
	Fat reducer
	Reduce viscosity
	Inhibit bloom
Margarine	Anti-spattering
	Emulsification
	Improve spreadability
Baking-pan release products	Antisticking
Flavours	Encapsulation
Release agents	Separation
	Wetting
Confectionary	Antisticking
Vegetable Oils	Antioxidant

2.1.2. Biological properties and health benefits of phospholipids

Phospholipids along with proteins are the major structural components of membranes of all kinds of cells, from bacteria (with few exceptions) to mammalian tissues. The key of phospholipids biological role is the ability of the phosphate group to combine with water and hydrophilic molecules such as proteins, sugars, oxygen while the fatty acids will combine with hydrophobic substances such as hormones and non-esterified fatty acids (Bretscher 1985).

Phospholipids are vital in some essential biological functions in live organisms. They are important in maintaining cell membranes integrity and play a significant role in functions such as: membrane permeability, membrane fluidity, membrane interactions (lipid-protein) and membrane deterioration (Bezrukov 2000; White and others 2001). Phospholipids are involved in metabolism-related and neurological diseases (Lohmeyer and Bittmann, 1994; Lee 1998) and in the regulation of basic biological processes as signaling compounds (Izumi and Shimizu 1995; Hannun and others 2001). Furthermore, phospholipids act as emulsifiers in the bile digestive fluid, ensuring fine dispersion of fatty food molecules in the water phase, thus improving digestion and absorption (Kidd 2002). Phospholipids combined with bioactive substances can enhance their bioavailability such as in the case long-chain polyunsaturated fatty acids (Carnielli and others 1998), n-3 polyunsaturated fatty acids (Cansell and others 2003, Cansell and others 2006) and tocopherol (Nacka and others 2001). Phospholipids act also as surface-active wetting agents in the lungs (Robertson and others 1990), intestines and kidneys, phospholipids ensure miscibility at air-liquid and liquid-solid interfaces (Kidd 2002).

Phospholipids have been used as nutritional supplements because they have shown beneficial physiological effects. Soy phospholipids mixtures have shown to reduce elevated blood cholesterol and triglycerides (Wilson and others 1998), soy phosphatidyl choline helps in liver detoxification and repair of damaged liver tissue (Lieber and others 1990) and phosphatidyl serine from soybean, brain and other sources improves cognitive functions (Pepeu and others 1996). Egg phospholipids have been used in infant formula to improve visual acuity (Koletzko and others 1995).

2.2 Modification of lipids

Functional, physical and quality properties of lipids are directly related with the fatty acid composition and position in the triacylglycerol molecule and, the nature of the fatty acid constituents such as degree of unsaturation, chain length and geometric isomers. Lipids either in the form of triacylglycerols or as phospholipids have been subject of study for their modification to attain specific physical, functional or nutritional characteristics.

2.2.1 Structured lipids

Structured lipids are tailor-made fats and oils with improved nutritional or physical properties through the incorporation of new fatty acids or the change of the position of existing fatty acids on the glycerol backbone (Akoh 1995). Structured lipids may be developed with specific fatty acids that offer health or functional benefits for the structured lipid. Besides, the specific fatty acid and its position in the triacylglyceride molecule determine the functional and physical properties, the metabolic fate, and the health benefits of a structured lipid (Akoh and Min 1998). Structured lipids have been developed for nutrition and medical applications such as infant formulas, low calorie fats, enteral and parenteral nutrition as well as food applications with specific functionalities such as plastic fats, cocoa butter alternatives, frying oils, etc, (Osborn and Akoh 2002).

A great variety of fatty acids have been used in the synthesis of structured lipids taking advantage of the functions and properties of each to maximize the benefits of a given structured lipid. Short and medium chain fatty acids have been used for the production of structured lipids due to the advantages associated with their digestability, absorption, and metabolism (Fomuso and Akon 1997; Mu and Hoy 2000; Fomuso 2002). Structured lipids have been synthesized with long polyunsaturated fatty acids such as conjugated linoleic acid (Lee and others 2004; Lumor and Akoh 2005; Villeneuve and others 2007; Guo and Sun 2007), conjugated linolenic acid (Lumor and Akoh 2005), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)

(Halldorsson and others 2001; Hamam and Shahidi 2005; Hamam and Shahidi 2006) to take advantage of the health benefits associated with these compounds.

Two main processes have been used for the production of structured lipids: chemical interesterification and enzymatic synthesis. Chemical synthesis involves hydrolysis of a mixture of medium chain triacylglycerides and long chain triacylglycerides and then re-esterification after random mixing of the medium chain fatty acids and long chain fatty acids (transesterification). The reaction is catalyzed by alkali metals or alkali metal alkylates (Akoh 1995). This process requires high temperature and anhydrous conditions. This process is inexpensive and easy to scale up but the reaction lacks of specificity and offers little or no control over the positional distribution of fatty acids in the final product (Willis and others 1998).

The enzymatic synthesis uses lipases which occur widely in nature and can hydrolyze triacylglycerols to diacylglycerols, monoacylglycerols, free fatty acids and glycerol. Lipases can catalyze the hydrolysis of triacylglycerols and the transesterification of triacylglycerols with fatty acids (acidolysis), direct esterification of free fatty acids with glycerol and reaction of a triacylglycerol with an alcohol (alcoholysis) (Akoh and Min 1998). Lipase-catalyzed reactions are a combination of esterification and hydrolysis reactions. A continuously removal of water is important during the reaction in order to increase the esterification reactions, minimizing hydrolysis and obtaining high yield products. However, some water is necessary in the system to maintain enzyme dynamics during noncovalent interactions. Thus, a balance between hydrolysis and esterification is necessary. Lipases offer a great advantage in catalyzed interesterification reactions because they can control the positional distribution of fatty acids in the final product, due to their selectivity and regiospecificity (Akon and Min 1998; Haman and Shahidi 2005).

The production of structured lipids can be carried out in organic solvents, where substrates are soluble, hydrolysis can be minimized and they are less likely to cause enzyme inactivation in esterification reactions. However, the type of solvent for the reaction should be considered carefully as it can dramatically affect the reaction kinetics

and catalytic efficiency as well as the stability of the enzyme. Hydrophilic or polar solvents can penetrate into the hydrophilic core of the proteins and alter their functional structure (Senanayake and Shahidi 1999).

Optimum temperatures for active lipases are 30 to 40 ºC (Shahani 1975). When the temperature increases, enzyme molecules unfold by destruction of the disulfide bonds, hydrolysis of peptide bonds and deamidation of aspargine and glutamine residues. These processes can be avoided in a water-free environment or by immobilization of the enzyme which result in great thermostability.

Other factors that affect enzymatic activity and product yield include pH, substrate molar ratio, enzyme activity and load, incubation time, specificity of enzyme to substrate type and chain length, and regiospecificity (Akoh and Min 1998). The advantages of the enzymatic reactions over the chemical reactions are energy saving and minimization of thermal degradation.

Genetic techniques have also been used to modify the composition of oils and fats in order to improve their stability and quality such as to increase short chain fatty acid content, low linoleic, low erucic acid and high oleic acid (Broun and others 1999). The presence of *trans* fatty acids in the diet has recently become a major health concern for consumers because of the negative effects of *trans* fat in high density lipoprotein (HDL). Thus, several companies are actively pursing the development of seeds oils that contain levels of saturated fatty acids high enough to permit the elimination of the need for hydrogenation, and, subsequently, the production of *trans* fats (Knauf and Del Vecchio 1998).

2.2.2 Structured phospholipids

Little phospholipid variety exists in native lecithins. However, phospholipids can be also modified as well as lipids to improve their properties or meet particular functional requirements. For this purpose, the fatty acids or head group of phospholipid structure can be changed or modified. These modifications can be performed by physical, chemical, and enzymatic modification (Doig and Dick 2003). Physical

processes such as solvent fractionation using acetone and alcohol and chromatography have been used to obtain deoiled and high purity phospholipids and individual from commercial sources by (Doig and Dicks 2003). The chemical modification of phospholipids usually allows little or no control over the positional distribution of fatty acids in the final product (Willis and others 1998) and require high temperatures, solvents and anhydrous conditions making this process difficult to use in food or nutritional applications (Osborn and Akon 2002). Enzymatic modification offers a great advantage in catalyzed interesterification reactions because they can control the positional distribution of fatty acids in the final product, due to their selectivity and regiospecificity (Akoh and Min 1998). Guo and others 2005 published a review of enzymatic modification of phospholipids for functional applications and human nutrition. Figure 3 shows the enzymes most used for phospholipid modification. Phospholipases and lipase1- specific selectively recognize each of the four individual ester bonds in a phospholipids molecule (Servi 1999).

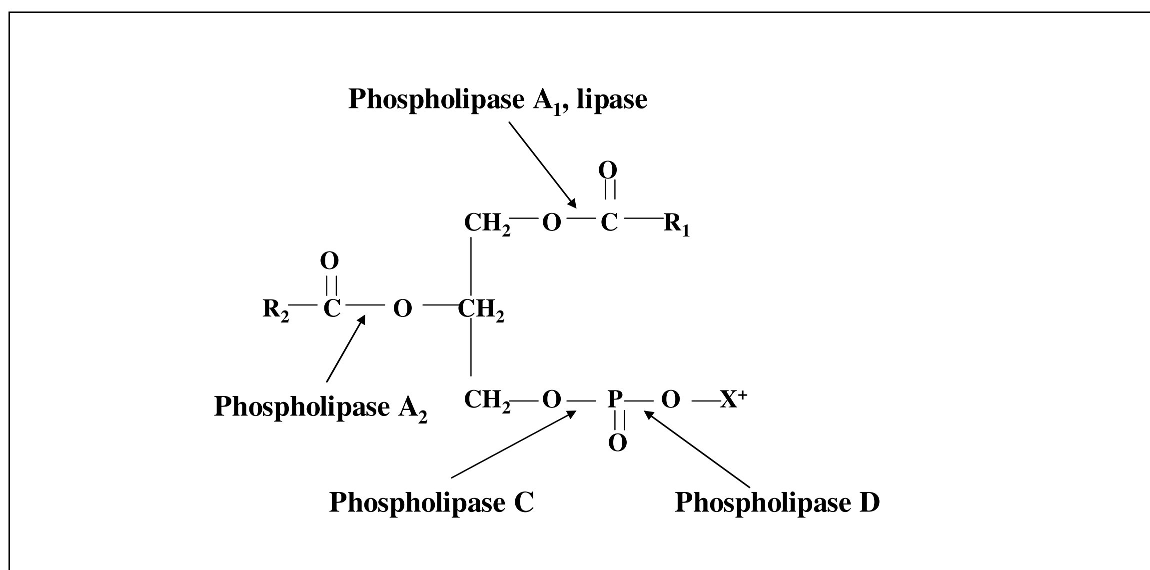

Figure 3. Phospholipases and lipase in phospholipid transformation

Table 2 summarizes the main factors that affect enzymatic modification of phospholipids (Guo and others 2005).

Table 2. Factors affecting the enzymatic modification of phospholipids

Factors	Remarks
Enzyme	• Phospholipase A_1 and A_2 or lipases can be used. • Increasing enzyme dosage may result in higher incorporation. • Increasing of enzyme may result in increased hydrolysis. • Enzyme can have different effect on phospholipids with different Head groups.
Water	• For some enzymes, low water activity is recommended for high yields. • Reaction time to reach equilibrium increases with decreasing water activity. • Water activity influences the molecular organization of phospholipids substrate. The packing density of phospholipids molecules increases with decreasing water activity.
Acyl donor	• By using a large excess of free fatty acids, hydrolysis reaction is Inhibited • Very high concentration of acyl donors slight decrease of in reaction rate. • Generally free fatty acids are more efficient acyl donors than their esters. • Reactivity relates to chain length and degree of saturation.
Solvent	• Solvent reduces the viscosity of the system as a consequence the reaction rate is increased through mass transfer increase of substrates. • Reaction is solvent type dependent, the rate being inversely proportional to solvent polarity. • Polar solvents should be avoided as they compete with the enzyme on available water. • Solubility of the substrate depends on solvent type.
Reaction time	• The longer the reaction time the higher the incorporation of acyl donors into phospholipids can be expected. • Long reaction time however may also result in increased acyl migration.
Temperature	• Optimal temperature changes with enzyme source and type. • Increased temperature may result in higher acyl migration. • Higher temperature lowers viscosity of reaction medium. • Enzyme stability has reverse relationship with temperature.

Enzymatic fatty acid modification of phospholipids has been attained using lipases 1-specific and phospholipase A_2 through hydrolysis and then direct interesterification or re-esterification in the *sn-1* and *sn-2* position (Aura and others 1995). Structured phospholipids have been synthesized with diverse fatty acids such as caproic acid (Adlercreutz and others 2002) using a lipase from *Rhizopus oryzae* in a water activity-controlled organic medium; oleic acid (Yagi and others 1990) using a lipase from *Rhizopus delemar* with a final content of 25% of oleic acid into phosphatidylcholine; heptadecanoic acid (Svensson and others 1992) using *Rhizopus arrhizus* lipase with an incorporation nearly of 50% into phospholipid, caprylic acid and eicosapentaenoic acid were incorporated into phosphatidylcholine using *Thermomyces lanuginosa* lipase (Peng and others 2002) in a solvent free-system, docosahexaenoic acid using *Thermomyces lanuginosa* lipase (Peng and others 2002) and using porcine pancreatic phospholipase A_2 (Hosokawa and others 1998), and conjugated linoleic acid using *Thermomyces lanuginosa* lipase (Hossen and Hernandez 2005; Peng and others 2002).

The enzymatic modification of the head group has been attained by phospholipase D which catalyzes the transphosphatidyl reaction and hydrolysis of the phosphoester bond (Frohman and Morris 1999; Servi 1999). Phospholipase D has been obtained from plants such as cabbage and bacterial sources such as *Stretomyces* sp. Low molecular weight primary alcohols to large secondary ones have been shown to be substrates for phosphalipase D with different yields and selectivity (Takami and others 1994; D'Arrigo and others 1997; Servi 1999; Iwasaki and Yamane 2004). Ulbrich-Hoffman (2000) published a review of phospholipids used in lipid transformation and included a table with the head groups introduced by phospholipase D into glycerophospholipids. Hossen and Hernandez (2004) synthesized a phosphatidyl-sterol by successfully incorporating β-sitosterol into phosphatidylcholine using phospholipase D.

2. 3 Conjugated Linolenic Acid (CLnA)

Conjugated Linolenic Acids (CLnAs) is a generic term for geometric and positional isomers of linolenic acid (C18:3) which contain conjugated double bonds. Figure 4 shows the structures of the conjugated fatty acid punicic acid (C18:3, 9cis,11trans,13cis) and the non-conjugated fatty acid alpha linolenic acid (C18:3, 9cis,12cis,15cis).

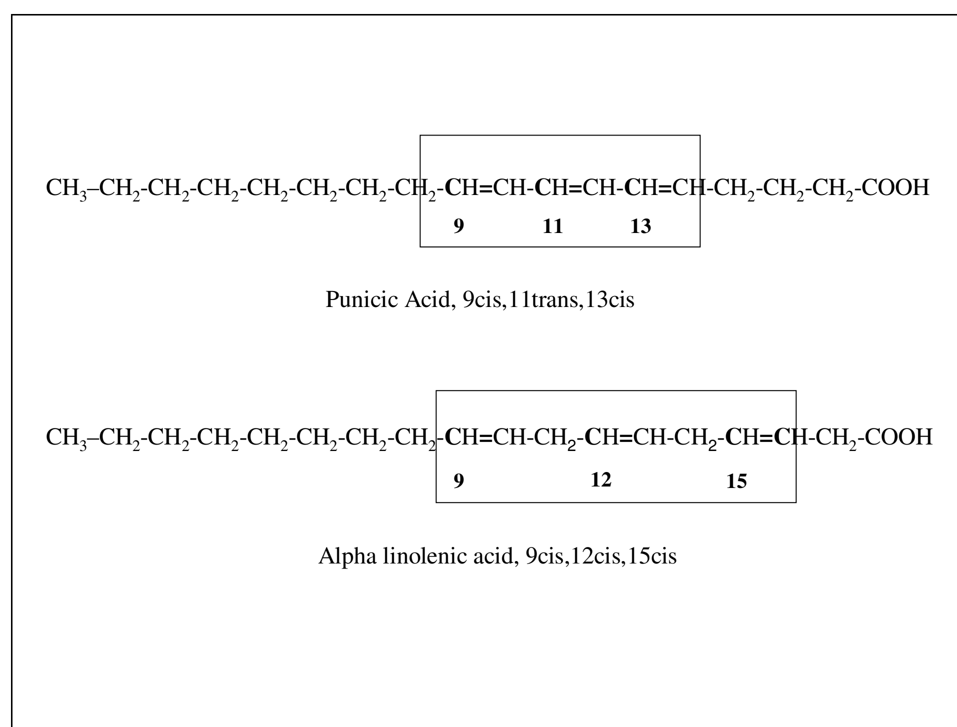

Figure 4. Punicic acid and alpha linolenic acid structure

Due to the three double bonds of conjugated linolenic acids, they show multiple configurations. Table 3 shows the most important configurations of CLnAs.

Table 3. Most common isomers of CLnA

Name	Configuration
Punicic Acid	9*cis*,11*trans*,13*cis*
α-Eleostearic Acid	9*cis*,11*trans*,13*trans*
Catalpic Acid	9*trans*,11*trans*,13*cis*
Calendic Acid	8*trans*, 10*trans*, 12*cis*

2.3.1 Sources of CLnA

Conjugated linolenic acids are present in some plant seed oils. Table 4 shows the principal sources of CLnAs, content and principal isomer.

Table 4. Natural sources of CLnAs

Source	Content (%)	CLnA Isomer
Pomegranate (*Punica granatum*) seed oil	72	Punicic acid
Bitter gourd (*Momordica Charantia* L.) oil	60	α-Eleostearic acid
Tung seed (*Aleurites moluccana)* oil	70	α-Eleostearic acid
Catalpa (*Catalpa ovata*) seed oil	31	Catalpic acid
Pot marigold (*Calendula officinalis*) seed oil	33	Calendic Acid

Pomegranate (*Punica granatum*) has been of special attention of scientists in the last 7 years because each part of the plant (seeds, juice, peel, leaf, flower, bark and root) has shown interesting pharmacologic activities (Lansky and Newman 2007). This fact is reflected by the number of scientific publications about the bioactivities of this plant presented in the last 7 years (7 times) compared with the number of publications presented between 1950 -1999. Lansky and Newman (2007) published a review in

pomegranate and its potential for prevention and treatment of inflammation and cancer. There is a lot of scientific interest in pomegranate seed oil because this oil has shown anticancer properties (Toi 2003; Mehta and Lansky 2004; Hora 2003; 2004; Kohno 2004).

CLnAs can be synthetically produced by alkali isomerization which has been used for the commercial production of conjugated linoleic acid (CLA). Natural sources with a high content of linolenic acid such as flaxseed oil may be used for this purpose.

2.3.2 Metabolism of CLnAs

Studies of the metabolism of α-eleostearic acid and punicic acid in rats have demonstrated that these acids are metabolized to conjugated linoleic acid (CLA) by enzymatic hydrogenation. Tsuzuki and others (2004a) determined that α-eleostearic acid is converted to conjugated linoleic acid (CLA; 9cis,11trans–18:2) in the liver and plasma of rats when these animals were fed with diets including 1% α-eleostearic acid for 4 weeks. The concentrations of conjugated linolenic acid and CLA were determined after 0, 3, 6, and 24 h oral administration of α-eleostearic acid to rats. Furthermore, these authors determined also that enteric bacteria are not involved in the conversion of α-eleostearic acid to CLA using germ-free rats but they demonstrated that the conversion of α-eleostearic acid into CLA was enzyme-mediated. α-Eleostearic acid was reacted with tissue homogenates (liver, kidney, and small intestine mucous) and coenzymes (NADH, NAD+, NADPH, and NADP+), and the enzyme activities were estimated from the amount of CLA produced. CLA was detected when α-eleostearic acid was reacted with liver, kidney, and small intestine mucous homogenates and coenzyme NADPH. These results indicated that α-eleostearic acid is converted to 9cis, 11trans-CLA in rats by a Δ13-saturation reaction carried out by an NADPH-dependent enzymatic reaction that occurred rapidly in rat liver and small intestine. In a later article, these authors (Tsuzuki and others 2006) studied the characteristics of absorption and metabolism of α-eleostearic acid in the small intestine of rats such as the ratio of conversion of α-eleostearic to CLA and absorption of this fatty acid and compared with the unconjugated

fatty acid linolenic acid (LnA) and CLA. Tsuzuki and others 2006 studied also whether structural differences of CLnAs affect absorption in the small intestine as well as whether the saturation reaction occurs regardless of the configuration (*cis* or *trans*) of the double bonds comparing α-eleostearic (9*cis*, 11*trans*, 13*trans*) with punicic acid (9*cis*, 11*trans*, 13*cis*). The structure and configuration of metabolites produced after the digestion of both fatty acids were determined by GC-EI-MS and ^{13}C-NMR. The results of this study showed that most of the LnA, CLA, α-eleostearic acid and punicic acid fed to the rats were absorbed within 24h. The absorption rate was LnA>CLA>α-Eleostearic>punicic acid. α-Eleostearic as well as punicic acid were converted in CLA (9*cis*, 11*trans* -18:2) in the rat intestine. Approximately 21% and 12% of the α-eleostearic and punicic acid were absorbed by the rats and converted to 9*cis*, 11*trans*-CLA. The double bond that underwent saturation in α-eleostearic was the 13 *trans* while in punicic acid was the 13 *cis* double bond.

In another study (Noguchi and others 2001), the formation of the CLA isomer in the liver lipids of rats fed with bitter gourd oil diets could be explained by either of the following two metabolic pathways, namely, enzymatic biohydrogenation of 9*cis*,11*trans*,13*trans*- 18:3 or enzymatic isomerization of 9*cis*,12*cis*-18:2.

Most of the commercial conjugated linoleic acid (CLA) is obtained by alkali isomerization of safflower oil. Conjugated linoleic acid (CLA) is present in foodstuffs in just 1% in comparison with CLnAs which can be found in natural sources at high concentrations (30-70%). As mentioned above, Tsuzuki and others 2004a demonstrated that CLnAs are slowly metabolized in the rat intestine to 9*cis*, 11*trans*-CLA. Thus, CLnAs may be used as a source of 9*cis*, 11*trans*-CLA after metabolism providing the health benefits associated with this isomer.

2.3.3 Health benefits of CLnA

Conjugated linolenic acids have shown important health benefits such as anticarcinogenic (Igarashi and Miyasawa, 2000; Tsuzuki and others 2004b; Yashui and others 2005), hypoglycemic (Akthar and others 1981; Chuang and others 2006),

hypolipidemic (Jayasooriya and others 2000, Noguchi and others 2001, Koba and others 2002), reduce body fat content (Koba and others 2002), antioxidant effects (Dhar and others 1999; Dhar and others 2006), immuno function (Yamazaki 2006) and prevent and treat inflammation (Lansky and Newman 2007).

2.3.3.1 Anticancer properties

Conjugated linolenic acids have shown selective cytotoxic activity on several tumors cell lines (Table 5). Some studies have suggested that citotoxic activity of these fatty acids involves lipid peroxidation and the alteration in fatty acid composition of membrane phospholipids, eicosanoid synthesis and membrane fluidity (Igarashi 2000). α-Eleostearic acid have shown stronger antitumor effect than CLA (conjugated linoleic acid) both in vivo and in vitro. The proposed mechanism for this activity of CLnAs is apoptosis (cell death) induction via lipoperoxidation (Tsuzuki 2004b).

Pomegranate seed oil rich in punicic acid has demonstrated to have anti-angiogenic activity (Toi and others 2003). Angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing vessels. Angiogenesis is a critical process for the development and progression of cancer. The anti-angiogenic effect was evaluated by measuring the vascular endothelial growth factor (VEGF), interleukin-4 (IL-4) and migration inhibitory factor (MIF) in the conditioned media of estrogen sensitive (MCF-7) or estrogen resistant (MDA-MB-231) human breast cancer cells or immortalized normal human breast epithelial cells (MCF-10A) grown in the presence or absence of pomegranate seed oil or fermented juice polyphenols.

Table 5. Anticancer properties of CLnAs

Oil/Acid	Compound	Cells Type	Reference
Pomegranate seed oil	Punicic acid	Breast	Toi 2003; Mehta and Lansky, 2004
		Skin	Hora 2003
		Prostate	Mehta and Lansky, 2004
		Colon	Kohno 2004
Isomerized of linolenic acid, α-linolenic acid and tung oil		Colorectal, hepatoma, lung, breast, stomach	Igarashi and Miyasawa, 2000
Bitter gourd oil	A-Eleostearic acid	Colon	Igarashi and Miyasawa, 2000, Yasui et al. (2005)

2.3.3.2 Hypoglycemic effects

Bitter gourd is a common vegetable used in Asia and has been use in traditional medicine for the treatment of Diabetes. The hypoglycemic effect of α-eleostearic was demonstrated by Akhtar and others (1981). The peroxisome proliferator activated receptors (PPARs: PPARα, PPARγ, PPARδ) are ligand-dependent transcription factors that belong to the steroid hormone nuclear receptor family and control lipid and glucose homeostasis in the body (Desvergne and Wahli 1999). Chao and Huang (2003) demonstrated that an ethyl acetate extract from bitter gourd activated not only PPARα but also PPARγ and regulated the expression of PPARγ target genes in H4IIEC3 cells. Later these authors found that 9cis, 11trans, 13trans-conjugated linolenic acid was responsible for the effect on PPARs (Chuang and others 2006).

2.3.3.3 Hypolipidemic effects

Noguchi and others (2001) studied the effects of bitter gourd oil (BGO) on blood and liver lipids of rats. In this study, three groups of rats were given a basal diet containing 7% fat by weight. The dietary fat consisted of soybean oil (control), soybean oil + BGO (6.5:0.5, w/w; 0.5% BGO), or soybean oil + BGO (5:2, w/w; 2.0% BGO). This fat treatment gave 3.4 and 15.4% of 9*cis*, 11*trans*, 13*trans* -18:3 in the dietary fat of 0.5 and 2.0% BGO, respectively. Fatty acid analysis showed the occurrence of 9*cis*,11*trans*-18:2 in the liver of rats fed BGO diets, whereas this conjugated linoleic acid (CLA) isomer was not detected in the liver of rats fed the control diet. Furthermore, dietary BGO decreased the concentration of 18:2n-6 and increased the concentration of 22:6n-3. The BGO diets significantly reduced free cholesterol levels with a trend toward an increase in HDL cholesterol, but there was no significant change in the total cholesterol. The dietary BGO also affected the plasma level of hydroperoxides, a slight but significant increase in hydroperoxides.

Arao and others (2004) studied the effect of 9cis, 11trans, 13cis conjugated linolenic acid on Apolipoprotein B100 secretion and triacylglycerol synthesis in HepG2 cells. ApoB100 is an essential component in very low density lipoprotein and its blood level is positively correlated with the incidence of coronary heart disease and atherosclerosis. 9cis, 11trans, 13cis Conjugated linolenic acid significantly decreased apolipoprotein B100 secretion compared with α-linolenic acid. The uptake of 14C-oleate into newly synthesized cellular triacylglycerol decreased more when 9cis, 11trans, 13*cis* conjugated linolenic acid was supplied in the diet than when α-linolenic acid was provided.

2.3.3.4 Reducing body fat content

Koba and others (2002) studied how dietary CLnAs affected body fat mass as well as serum and liver lipids in rats and compared with those of CLA, LnA and LA. Feeding CLnAs reduced the weight of perirenal adipose tissue significantly more than

LA and LnA. The body fat-reducing effect of CLnAs was comparable or even stronger than that of CLA under the experimental conditions of this study. On the other hand, serum triacylglycerides (TG) was 2.5 fold higher in rats fed with CLnAs than those fed with LA, LnA and CLA. It is expected from this fact that serum cholesterol should be higher too. However, serum cholesterol concentration was significantly lower in rats fed with CLnAs than those with LA. The elevated level of TG may be explained by an inability to clear very low density lipoprotein (VLDL) possibly by inhibition of lipoprotein lipase activity as the rats were fasted 16 h prior to collection of blood. CLnAs may reduce more efficiently fat deposition than CLA, as CLA contained 33% of 10*trans*, 12*cis*-18:2 (which has shown to inhibit lipoprotein lipase activity) and CLnAs just contained 15% of this isomer and 32% and 17% of conjugated 18:3. Furthermore, rats fed with CLnAs showed increased concentration of free fatty acids (FFA) than those fed with LA and LnA suggesting that CLnAs may increase fatty acid mobilization from adipose tissues.

This research also investigated the effect of adipocytokines such as leptin and TNF-α as these compounds may modulate the amount of adipose tissue. TNF-α has shown to decrease the synthesis of lipoprotein lipase in adipose tissue but increase the activity of lipoprotein lipase in the liver. TNF-α was reported to decrease PPARγ mRNA level in 3T3-L1 preadipocytes. Recent studies have shown that PPARγ mRNA levels in adipose tissue of obese women were positively correlated with serum concentrations of HDL-cholesterol. In this study, serum leptin level was found to exhibit a significant correlation with the weigth of perirenal and epididymal adipose tissues and with serum HDL-cholesterol. Serum leptin and HDL cholesterol levels were the lowest in the CLnAs group which could explain why CLnAs (more than CLA) decreased the weight of adipose tissues more than the corresponding non conjugated fatty acids. Besides, in the liver, CLnAs significantly enhanced the activity of mitocondria and peroxisomal β-oxidation as it was 3-3.5 fold in rats fed with CLnA as compared with the other diets. This may be explained by the fact that CLnA activated PPARα which regulates hepatic β-oxidation. The feeding of CLnA affected hepatic lipid metabolism more potently than

CLA and resulted in a net reduction in adipose tissue weight which may be explained by a decrease in TG deposition from circulation and/or lipolysis in adipose tissues.

2.3.3.5 Antioxidant activity

Degenerative diseases such as Diabetes mellitus (Type I), cardiovascular disease, arthritis and aging require antioxidative defense systems against the oxidative stress generated by them (Dhalla and others 2000; Sayre and others 2001). CLnAs have shown scavenging action against lipid peroxidation. Dhar and others (1999) demostrated that CLnAs significantly lowered plasma lipid peroxidation, erythrocyte membrane lipid peroxidation and liver tissue lipid peroxidation in comparison with the sunflower oil group at a 0.5% level. The antioxidant activity decreased as the concentration of CLnAs increased.

In another study Dhar and others (2006) investigated the antioxidant effect of CLnAs in the absence and presence of α-tocopherol. CLnAs + α-tocopherol diet decreased liver lipid peroxidation significantly more than α-tocopherol alone. A possible explanation of the antioxidant activity of CLnAs is based on the bioconversion reactions of CLnAs. Under in vivo conditions, CLnAs can undergo isomerization, hydrogenation, and oxidation reactions to form PUFA of both nonconjugated acids (LA and LnA) and conjugated fatty acids. It is known that CLA posses antioxidant activity (Ip and others 1991). This fact may explain the antioxidant activity of CLnAs even at reduced doses with a fixed dose of α-tocopherol. The results of this study showed also a synergestic antioxidant effect of CLnAs with α-tocopherol in vivo on lipid peroxidation in diabetic rats.

2.3.3.6 Immune function

Modulation of dietary fat quality such as the balance omega-6/omega-3 is related to immunologic functions (Harbige 2003). On the other hand, conjugated fatty acids such as conjugated linoleic acid (CLA) has shown to promote strong immune function through the production of immunoglobulin (Ig) and modulate the production of some

cytokines (signaling compounds important in immune response) in rat and mouse splenocytes (characteristic white blood cells of splenic tissue) (Sugano and others 1998).

Yamasaki and others (2006) studied the dietary effect of pomegranate seed oil on immune function and lipid metabolism in mice. In this study, mice were fed with experimental diets containing 0%, 0.12% or 1.2% of pomegranate seed oil for 3 weeks. Splenocytes isolated from mice fed with 0.12% and 1.2% pomegranate seed oil diet produced larger amounts of immunoglobulins G and M. These results suggested that pomegranate seed oil may enhance immune response in vivo.

2.3.3.7 Inflammation

Prostaglandins are a complex group of oxygenated fatty acids that is present in most mammalian tissues. It has been reported that there is a positive correlation between prostanglandins and inflammation (Kuehl and Egan 1980). Nugteren and Christ-Hazelhof (1987) reported the anti-inflammatory activity of naturally occurring conjugated trienoic acids such as jacarandic acid, calendic acid, catalpic acid, punicic acid and α- eleostearic acid. In this study, the anti-inflammatory activity was determined by measuring the 50% inhibitory concentration (IC_{50}) (Table 6) of these fatty acids to cyclooxygenase, enzyme that catalyzes prostaglandin's biosynthesis.

Table 6. Inhibition of cyclooxygenase by naturally conjugated linolenic acids

Fatty acid	Source	Configuration	$[I]_{50}$ µM
Jacarandic	Jacaranda seeds	8*cis*,10*trans*,12*cis* (n-6)	2.4
Calendic	Pot marigold seed oil	8*trans*,10*trans*,12*cis* (n-6)	31
Catalpic	Catalpa seed oil	9*trans*,11*trans*,13*cis* (n-5)	58
Punicic	Pomegranate seed oil	9*cis*,11*trans*,13*cis* (n-5)	68
α-eleostearic	Bitter gourd, Tung seed oil	9*cis*,11*trans*,13*trans* (n-5)	200

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Lipoid S100 (phosphatidylcholine 94%) was donated by Lipoid GmBH (Ludwigshafen, Germany). Unsweetened chocolate baking bars and commercial soy bean oil were purchased from a local super market. Coconut oil was purchased from Spectrum Organic Products, Inc. (Petaluma, CA). Sweet dairy whey protein was purchased from Foremost Farms USA (Baraboo, WI).

3.1.1. Chemicals

Soybean phosphotidylcholine (aprox. 99%), a mixture of 37 fatty acid methyl esters to identify the different fatty acids, and other chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA). Flaxseed oil used for the synthesis of CLnA was supplied by a Polar Food Inc. (Manitoba, Canada). Pomegranate seed oil was donated by Botanic Innovations (Spooner, WI, USA). All solvents used were of analytical or HPLC grade and purchased from VWR-Scientific Products (Batavia, IL, USA). Immobilized lipase from Thermomyces lanuginose (Lipozyme TL IM) was supplied by Novozymes North America (Franklinton, NC, USA).

3.2 Synthesis and concentration of CLnAs from flaxseed oil

3.2.1 Synthesis of CLnA from flaxseed oil

CLnA as free fatty acids were produced by alkali isomerization of flaxseed oil with 7% NaOH in propylene glycol at 180 °C for 2 h, according to Rocha-Uribe and Hernandez (2004). Flaxseed oil (500 g) was added to a 5-liter, 3-neck flask containing 203 g of NaOH dissolved in 2900 g of propylene glycol (7% NaOH in propylene

glycol). The reaction mixture was flushed with nitrogen and heated to 180 °C for 2 h. This mixture was cooled to room temperature, concentrated hydrochloric acid (500 ml) was added and it was stirred for 15 min. The pH of the reaction mixture was adjusted to 3 with additional concentrated hydrochloric acid. The mixture was transferred to a separator funnel and 200 ml of water was added and extracted with 500x2 mL of hexane. The hexane solution was washed with 250x3 mL of deionized water, dried overnight with magnesium sulfate, filtered through filter paper (Watman No. 41) and placed in a rotary evaporator at 60 °C. The isomerized mixture was placed in a plastic bottle in a nitrogen atmosphere and stored at – 20 °C.

3.2.2 Urea treatment of CLnA mixture from flaxseed oil

The isomerized mixture (IM) obtained after flaxseed oil isomerization was treated with urea to concentrate the mixture in unsaturated fatty acids according to Yang and Liu (2004). CLnA mixture (5 g) was added to urea (20 g) dissolved in warm methanol (80 mL). The mixture was heated till complete dissolution, and then it was allowed to cool slowly to room temperature and stored in the refrigerator overnight at 5 °C. The mixture was filtered under vacuum, washed with water and HCL (6 N) and extracted with hexane. The hexane phase was dried over anhydrous sodium sulfate and hexane was removed using a rotary evaporator and the remanent was stored at -20 °C. UV/VIS spectrophotometric analysis of the isomerized-concentrated mixture (ICM) was performed with a spectrophotometer Beckman DU 520 (Beckman Instrument Inc., Fullerton, CA, USA) at 233 nm for conjugated diene and at 262, 268 and 274 nm for conjugated triene fatty acids (AOAC, 1990).

3.3. Synthesis of structured phospholipids with CLnA

3.3.1 Lipase catalyzed acidolysis/interesterification reaction

Figure 5 shows the reaction scheme for the synthesis of structured phospholipids with conjugated linolenic acid from pomegranate seed oil or isomerized concentrated mixture from flaxseed oil. Phosphatidylcholine (100 mg) was mixed with the isomerized

concentrated mixture (ICM) from flaxseed oil (185 mg) or pomegranate seed oil (555 mg) in 1 mL of hexane in screw cap vial. Lipase enzyme (20 wt.% of phospholipid) was then added to the reaction mixture. The reaction was carried out at 57 °C in a water bath agitated with magnetic stirring at 250 rpm. After 24, 48, 72 and 96 h an aliquot (50 µL) was collected from the reaction mixture. The solvent was evaporated using N_2 and 300 µL of chloroform:methanol (2:1 vol/vol) was added to the aliquot and centrifuged for 5 min using a mini-centrifuge (Phoenix Research System, Hayward, CA, USA). The upper layer was carefully withdrawn and kept in the freezer (<-20°C) for analysis.

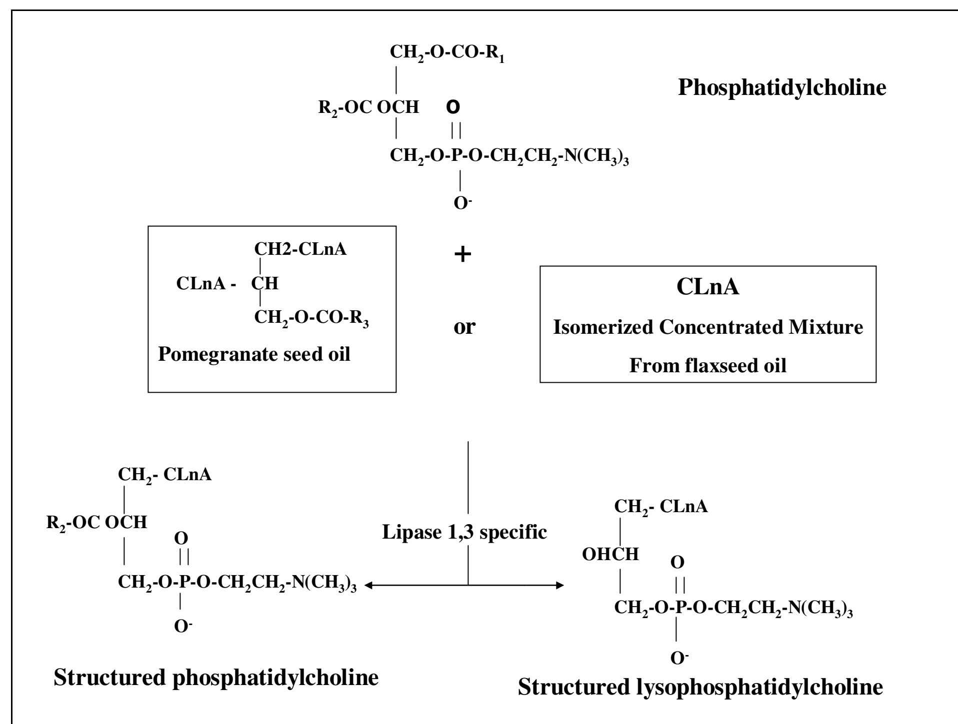

Figure 5. Reaction scheme of the interesterification or acidolysis enzymatic reaction for the production of structured phospholipids with CLnA

3.3.2 Thin Layer Chromatography (TLC) analysis

TLC is a well known analytical technique used to separate chemical products. The reaction products (phospholipid of the enzymatic reaction of structured

phospholipids with conjugated linolenic acid were separated using TLC according to Hossen and Hernandez (2005). The mobile phase was a mixture of chloroform/methanol/water (65:25:4, vol/vol/vol). The sample aliquots collected in chloroform:methanol (3:2 v/v) at different time intervals were applied to TLC plates (silica gel 60 F254; E. Merck Co., Darmstadt, Germany). Eluted compounds were visualized by spraying 5% phosphomolybdic acid in ethanol followed by heating.

3.3.3 HPLC analysis

The enzymatic synthesis of structured phospholipids involves interesterification or acidolysis reactions. However, hydrolysis or interesterification of the triacylglyceride or phospholipids molecule regarding the reaction conditions could occur in lipase catalyzed reactions (Akoh and Min 1998). The hydrolysis degree of phospholipids during acidolysis reaction was determined by HPLC according to Hossen and Hernandez (2005). The HPLC system consisted of a Beckman Gold Module (Beckman Instrument Inc., Fullerton, CA) with 508 auto sampler module (20 μL injection loop), 126 pump module and 168 UV detector module was used. A normal-phase silica column (Lichrosorb, Phenomenex) was used at room temperature for this analysis. The mobile phase was acetonitrile/methanol/phosphoric acid (130:5:1.5 vol/vol/vol) and run isocratically at a flow rate of 1.5 mL/min. Phospholipids were detected at 205 nm.

3.3.4 Fatty acid analysis of structured phospholipids

The percent of incorporation of conjugated linolenic acid into phosphatidylcholine was determined by analyzing the fatty acid composition of the reaction products (structured phospholipids and lysophospholipids) using preparative TLC followed by gas chromatography (GC) (Hossen and Hernandez 2005). Reaction products were first separated on preparative TLC plates (20 cm × 10 cm) using chloroform/methanol/water (65:25:4, vol/vol/vol) as mobile phase. The portion of the plate cut and revealed with 5% phosphomolybdic acid was used as a template to identify the phospholipid compounds which were then scraped off from the TLC plates and put

into a test tube for fatty acid methyl ester preparation. Fatty acid methyl esters were prepared by adding 1.0 mL of 0.25 M sodium methoxide in methanol to the scrapings in test tubes from the TLC plates. This mixture was incubated for 10 min in a water bath shaker at 45 °C, and 1 mL of hexane was added followed by 3 mL of saturated NaCl solution. Then the mixture was vortexed and centrifuged, and methyl esters extracted in hexane were collected from the upper layer for GC analysis. The GC system consisted of a Varian Model 3400 equipped with a split injector, a flame ionization detector, and a fused silica capillary column Supelco SP 2560 (100 m, 0.25 mm I.D., 0.20 µm film, Bellefonte, PA). The initial column oven temperature was 150 °C for 3 min and raised to 200 °C at 10 °C/min, finally it was raised to 230 °C at 3 °C/min and held for 10 min. The injector and detector temperatures were 250 °C and 300 °C, respectively. Fatty acids were identified by comparing their retention times with the ones of the standards.

3.4 Physical properties of structured phospholipids

Pomegranate seed oil (31 g), Lipoid S100 (6 g) Lipozyme TL IM (1.2 g) and 30 mL of hexane were used for the production of structured phospholipids with CLnA in higher scale for the study of their physical properties. The reaction mixture was placed in a water bath agitated with magnetic stirring at 250 rpm at 55 – 57 °C for 72 h. After this time, the reaction mixture was filtered and water (8 g) was added to it. This mixture was placed in a silicone oil bath at 50 - 55 °C with stirring for 1 h, then placed in the freezer for 1 h and finally centrifuged in Sorvall RC 5C Plus (Sorvall Inc., Newtown, CT) centrifuge at 10000 RPM for 10 min. Structured phospholipids were freeze dried in a Labconco Freeze Dry 5 (Labconco Corporation, Kansas City, MI) for 16 h.

Phospholipids have been used in chocolate as a viscosity reducer and cocoa butter replacer (van Nieuwenhuyzen 1995; Sinram 1991). The effects of structured phospholipids with CLnA in the physical properties of a food product were evaluated through their incorporation in a chocolate system and the dropping and viscosity were determined. The chocolate system consisted in a mixture of unsweetened chocolate

(94.6%) and coconut oil (5%) and phospholipids (0.4%). Two controls were prepared for comparison: one without phospholipids and the other with Lipoid S100.

3.4.1 Dropping point

The dropping point is the temperature at which the sample becomes fluid enough to flow. Physical properties such as the dropping point (equivalent to the melting point) is of great significance in chocolate's quality as this property is involved in chocolate's gloss, snap and texture (Loisel and others 1998).

The dropping point of the structured phospholipids chocolate mixture and controls were determined following the AOCS method Cc 18-80 using a Mettler processor (Mettler FP80) and a dropping furnace (Mettler FP83, Mettler Instruments Corp., Highstown, NJ). The sample cup was previously keep in the freezer (< °0 C) and then filled with approximately 15 drops of the melted chocolate mixture and placed in the freezer for 20 minutes. The solidified sample was placed in the furnace and warmed in the temperature-programmed furnace.

3.4.2 Viscosity

The viscosities of the structured phospholipids chocolate mixture and controls were measured in a Brookfield Rheometer HB (model DV-III, Brookfield Co., Stoughton, MA) using a HB2 and HB3 spindles (Brookfield Engineering Laboratories Inc., Stoughton, MA) at 40 and 50 °C. The temperature was controlled using a water bath at a constant temperature. Viscosities were reported in centipoises (cP).

3.5 Emulsion stability

Phospholipids have been used mainly as natural emusifiers. The emulsion stability of the structured phospholipids produced was tested through the production of oil-in-water emulsions which contained whey protein (1%), soy bean oil (10%) and phospholipids (0.5%) according to the method of Hernandez (2001). The emulsions were prepared in an APV Rannie Lab 2000 High Pressure Homogenizer (Albertslund,

Denmark) at 20 MPa. Two controls were prepared for comparison: one without phospholipids and the other with Lipoid S100. The emulsions prepared were placed at room temperature for visual observation of their stability till phase separation.

3.6 Oxidative stability

Structured phospholipids emulsion and controls were placed in a water bath at 50 °C for 1, 3 and 7 days. After these periods, oil was extracted from the prepared emulsions according to Osborn and Akoh (2003). The peroxide value was evaluated following the AOCS official method Cd 8-53. 50 mL of acetic acid:isooctane (300:200 v/v) were added to the oil extracted (5 g) and then 0.5 mL of saturated KI were added to the mixture and shaked for 1 min. After this time, water (30 mL) and starch (0.5 mL) was added to the mixture. This mixture was titrated with sodium thiosulfate 0.01 N till colorless. The *p*-anisidine value of the oil extracted was evaluated following the AOCS official method Cd 18-90. Oil extracted from the emulsion (0.4 g) was placed in a 25 mL volumetric flask and the volume was completed with isooctane. The absorbance of this solution was measured at 350 nm using isooctane as blank. 5 mL of this solution were placed in a glass tube and 1 ml of *p*-anisidine was added to the tube, and then capped and shaked. After 10 min the absorbance of this solution was measured. An isooctane - *p*-anisidine mixture was used as blank.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Incorporation of CLnA from Isomerized Concentrated Mixture from flaxseed oil into phospholipids (PC)

Table 7 shows the fatty acid composition of flaxseed oil, isomerized mixture (IM) and isomerized concentrated mixture (ICM) from flaxseed oil after urea treatment. After isomerization, the concentration of CLnAs increased from 0 to 31.0% while the concentration of linolenic acid decreased from 50.3% to 20.3%. After the urea treatment the concentration of CLnAs increased from 31.0% to 43.5 %, the concentration of palmitic, stearic and oleic acids decreased to less than 1% while the concentration of linoleic and linolenic aids increased to 18.4%, 35.6% respectively. The GC analysis of this mixture showed one big CLnA peak and five small CLnA peaks. The big CLnA peak accounted for nearly 68% of the total CLnA isomers. Spectrophotometric readings confirmed the formation of conjugated diene and triene fatty acids. The ICM contained 34.4% of conjugated diene and 2.5% of conjugated triene fatty acids. More research is required to establish the configuration of these CLnA isomers.

The incorporation of CLnAs isomers into PC was monitored for 96 h (Fig. 6). After 72 h, 4.9 % CLnAs were incorporated and decreased to 4.8 % after 96 h. Fatty acid composition of the ICM, PC and the structured phospholipids with CLnAs are shown in Table 8. CLnA isomers were incorporated into PC mainly by the exchange of linoleic and palmitic acid. On the other hand, the percent of incorporation of oleic, linolenic acid and CLnAs into PC increased after 72 h. Lysophospholipids (LPC) are formed during the interesterification reaction (Hossen and Hernandez 2005). TLC and HPLC analysis of reaction products confirmed qualitative and quantitatively the formation of LPC with time. Percent of hydrolysis was calculated based on the relative amount of LPC present in the reaction products (Hossen and Hernandez 2005).

The hydrolysis rate for the ICM from flaxseed oil (Fig. 7) was faster during the first 72 h reaching 44.2%, and increased slowly after this time, reaching 49.4 % after 96 h. The hydrolysis rates of PC were higher when the ICM from flaxseed oil was used as CLnAs source than the pomegranate seed oil. The pretreatments such as isomerization and urea concentration carried out on the flaxseed oil might favor the hydrolysis reaction of PC.

Table 7. Fatty acid composition (%) of flaxseed oil, isomerized mixture (IM) and isomerized concentrated mixture (ICM) from flaxseed oil

Fatty acid	Flaxseed oil	IM	ICM
C16:0	5.1± 0.62	4.8 ± 0.04	0.1 ± 0.002
C18:0	4.1± 0.02	4.1 ± 0.15	0.2 ± 0.01
C18:1	23.8 ± 0.23	24.7 ± 0.28	0.5 ± 0.05
C18:2	14.2 ± 0.11	13.6 ± 0.13	18.4 ± 0.09
C18:3	50.3 ± 0.18	20.3 ± 0.20	35.6 ± 0.14
CLnAs	ND	31.0 ± 0.75	43.5 ± 0.31
Others	2.5 ± 0.03	1.5 ± 0.03	1.7 ± 0.01

†Average of two analyses.

ND – not detected

Phospholipids have been used in a range of food products as emulsifiers, stabilizers, control crystallization agents, viscosity modifiers, antioxidants and reducers or replacers of fat (Silva 1990; Sinram 1991). Table 1 shows some food applications of phospholipids.

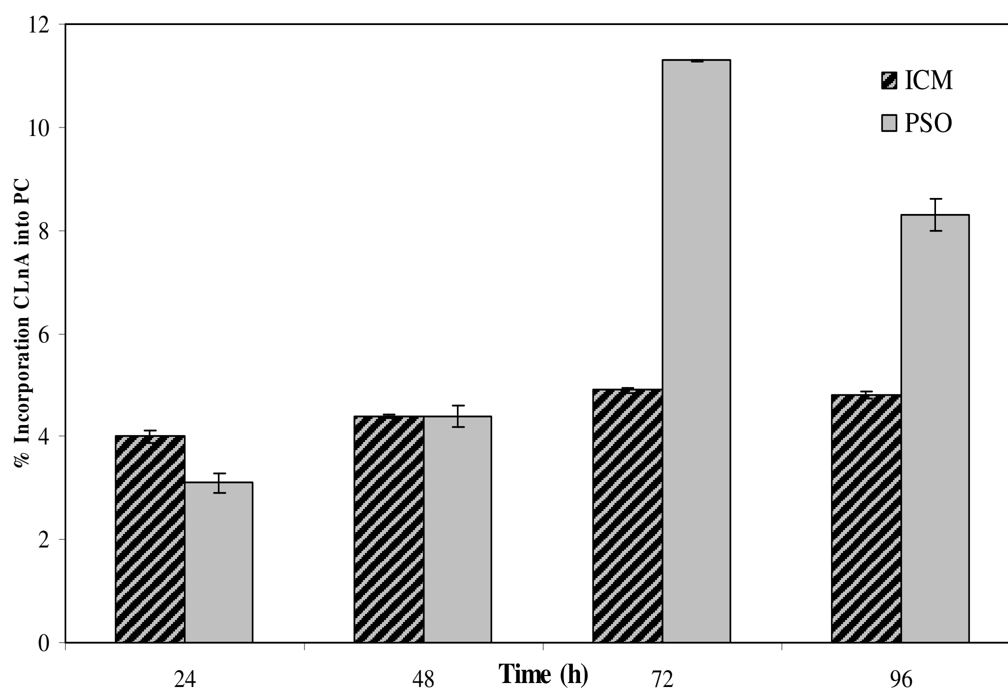

Figure 6. % Incorporation of CLnA into phospholipids (PC) from isomerized concentrated mixture (ICM) from flaxseed oil and pomegranate seed oil (PSO)

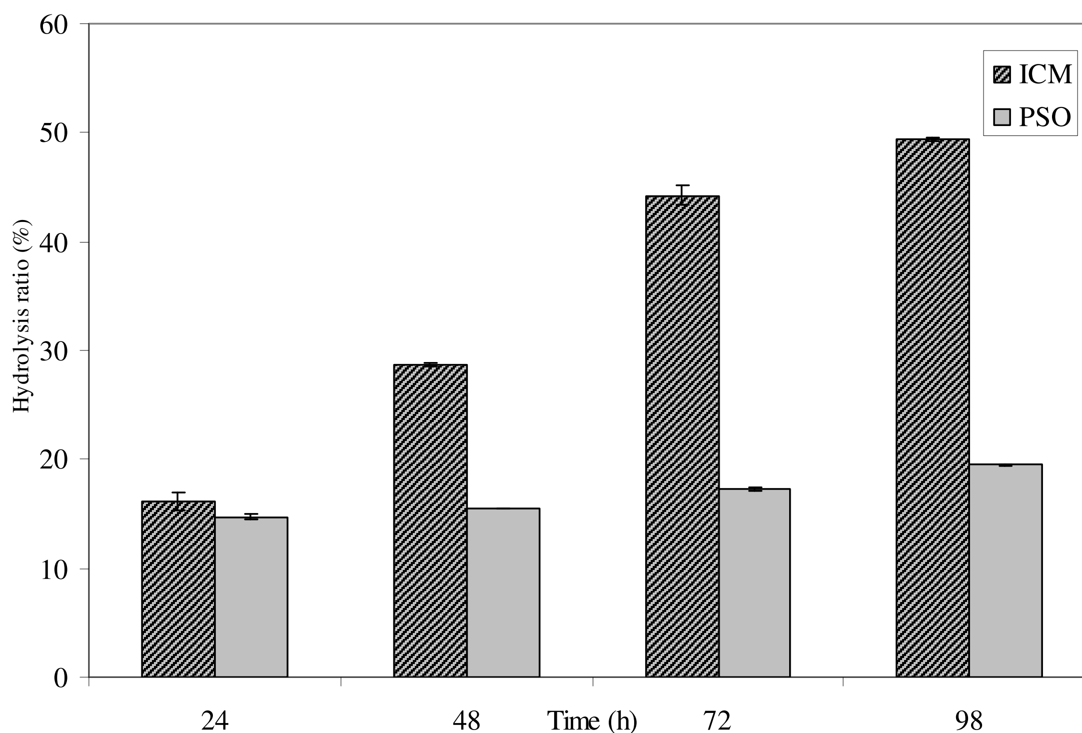

Figure 7. Degree of hydrolysis of phospholipids during enzymatic reaction of phospholipids with isomerized concentrated mixture from flaxseed oil and pomegranate seed oil

The fatty acid composition of lysophospholipids (LPC) showed that the incorporation of palmitic, stearic, oleic and linoleic was random. The concentration of linolenic acid and CLnAs increased after 96 h reaction time. About 13.5 % of CLnAs from flaxseed oil were incorporated into LPC after 72 h of reaction, which then decreased to 13.0 % after 96 h (Fig. 8). The higher incorporation of CLnAs into the LPC than the PC can be explained by the high hydrolysis rate obtained (nearly 50% after 96 h, Fig. 6) when the ICM from flaxseed was used. The reaction has only been described as a transesterification reaction, but a combination hydrolysis and reesterification step could be possible as well. The intermediate product, 2-acyl LPC is prone to acyl migration, yielding the more stable 1-lyso acyl LPC (Adlercreutz and others 2002).

Higher incorporation rates obtained when ICM (4.9%) was used for the acidolysis reaction compared to IM (1.2%). This may be due to an increase in CLnAs concentration after the urea treatment. For production operations, further concentration or purification techniques of the isomers of interest prior to enzymatic synthesis may be required for increased incorporation levels of these compounds into PC.

4.2 Incorporation of CLnA from pomegranate seed oil into phospholipid (PC)

Pomegranate seed oil contained 75.4% of conjugated linolenic acid as punicic acid (C18:3, 9cis, 11trans, 13cis) which makes it a natural source of CLnA. The incorporation of punicic acid into PC was monitored for 96 h. After 72 h, 11.3% CLnA was incorporated and decreased to 8.8% after 96 h. Fatty acid composition of pomegranate seed oil, PC and structured phospholipids are shown in Table 9. Punicic acid was incorporated into PC mainly by the exchange of linoleic, linolenic and palmitic acids. Other fatty acids incorporated randomly with time. Fig. 6 shows the hydrolysis ratio of the acidolysis and esterification reaction for the CLnA from ICM from flaxseed oil and pomegranate seed oil. The hydrolysis rate was fast during the first 24 h, was almost constant at 15% between 24 h and 48 h and reached 20 % after 96 h. Higher

Table 8. Fatty acid composition of structured phospholipids (phospholipid + isomerized concentrated mixture from flaxseed oil) at different reaction times

	Fatty acid composition [% area][†]					
	Substrate		Modified Phospholipid			
Fatty acid	Phospholipid	ICM	24 h	48 h	72 h	96 h
C16:0	14.1 ± 0.20	0.1 ± 0.002	10.6 ± 0.01	9.5 ± 0.03	9.0 ± 0.12	9.2 ± 0.04
C18:0	4.0 ± 0.28	0.2 ± 0.01	3.8 ± 0.07	3.8 ± 0.02	3.6 ± 0.12	3.8 ± 0.06
C18:1	11.8 ± 1.27	0.5 ± 0.05	10.5 ± 0.14	10.5 ± 0.06	11.2 ± 1.08	10.5 ± 0.16
C18:2	64.0 ± 0.76	18.4 ± 0.09	60.5 ± 0.12	60.0 ± 0.03	59.1 ± 0.79	59.2 ± 0.13
C18:3	6.1 ± 0.03	35.6 ± 0.14	10.5 ± 0.06	11.8 ± 0.05	12.3 ± 0.12	12.4 ± 0.07
CLnA isomers	ND	43.5 ± 0.31	4.0 ± 0.13	4.4 ± 0.03	4.9 ± 0.06	4.8 ± 0.06
Others	ND	1.7 ± 0.01	ND	ND	ND	ND

[†]Average of two analyses
ND – not detected

hydrolysis rates were reported by Hossen and Hernandez (2005) such as 18.5% and 19.5% after 72 h for the incorporation of conjugated linoleic acid (CLA) into PC using Lipozyme TL IM and Lipozyme RM IM, respectively.

The fatty acid composition of LPC was determined by GC. Incorporation of linoleic acid (C18:2) decreased with reaction time while palmitic acid (C16:0) increased. Fig. 7 shows the percent of incorporation of CLnA as punicic acid in the LPC. About 17.2% of punicic acid was incorporated into LPC after 72 h of reaction, which then decreased to 9.6% after 96 h. The higher incorporation of punicic acid into the LPC than into the PC after 72 h may be explained by the complex reaction kinetics consisting of interesterification as well as hydrolysis of PC and by the loss of specificity of the enzyme.

Lypozyme TL IM effectively incorporated two isomers of CLnA, one from pomegranate seed oil (punicic acid) and one from ICM from flaxseed oil into PC and LPC. This enzyme showed higher incorporation of CLnA as conjugated triene (punicic acid) than as conjugated diene from ICM from flaxseed oil. Besides, the high concentration of CLnA as punicic acid in the pomegranate seed oil (75.4%) could also favor higher incorporation of this isomer into PC.

Table 9. Fatty acid composition of structured phospholipid (phospholipid+ pomegranate seed oil) at different reaction times

	Fatty acid composition [% area][†]					
	Substrate		Modified Phospholipid			
Fatty acid	Phospholipid	Pomegranate seed oil	24 h	48 h	72 h	96 h
C16:0	14.1 ± 0.20	3.8 ± 0.09	14.4 ± 0.05	14.4 ± 0.67	10.6 ± 0.11	10.5 ± 0.36
C18:0	4.0 ± 0.28	2.3 ± 0.21	4.6 ± 0.05	5.1 ± 0.02	4.0 ± 0.06	4.4 ± 0.07
C18:1	11.8 ± 1.27	6.6 ± 0.12	12.4 ± 0.04	13.2 ± 0.01	11.0 ± 0.04	12.4 ± 0.22
C18:2	64.0 ± 0.76	11.3 ± 0.28	60.4 ± 0.08	58.3 ± 0.53	57.5 ± 0.06	57.8 ± 1.80
C18:3	6.1 ± 0.03	0.7 ± 0.01	5.1 ± 0.04	4.7 ± 0.03	4.7 ± 0.04	4.6 ± 0.01
CLnA (Punicic Acid)	ND	75.4 ± 0.70	3.1 ± 0.18	4.4 ± 0.20	11.3 ± 0.02	8.8 ± 0.32

[†]Average of two analyses.

ND – not detected

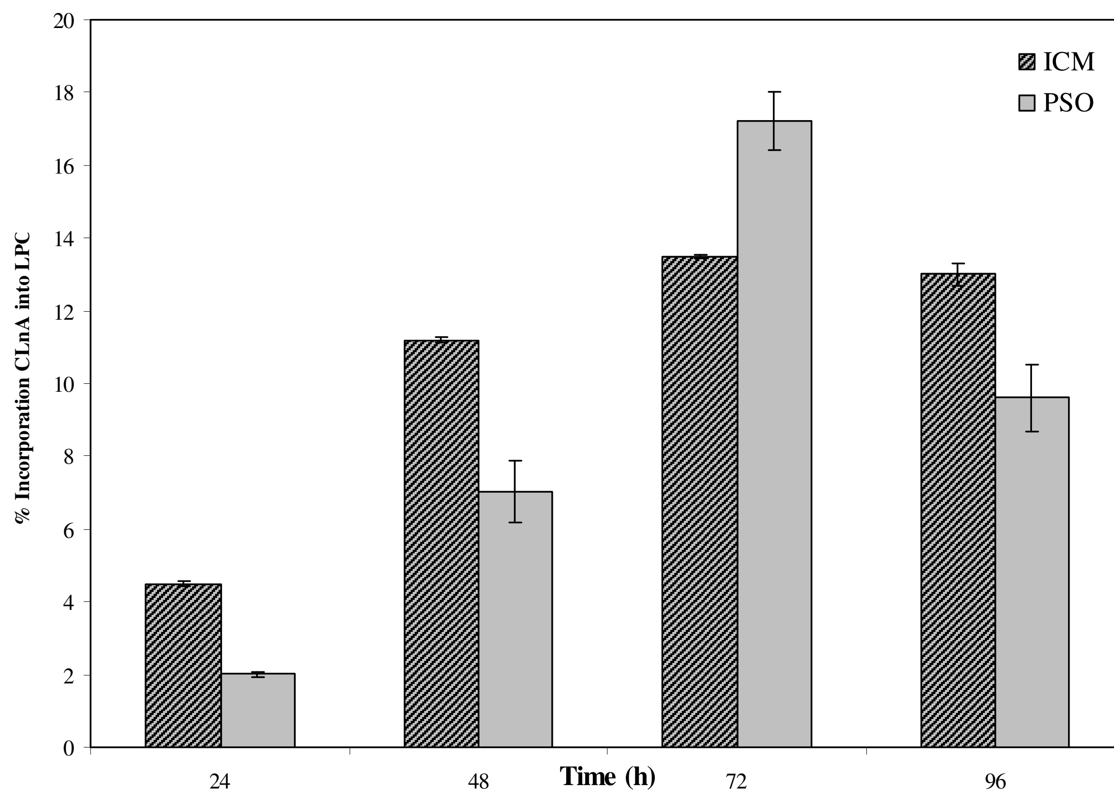

Figure 8. % Incorporation of CLnA into lysophospholipids (LPC) from isomerized concentrated mixture (ICM) and from pomegranate seed oil (PSO)

4.3 Physical properties of structured phospholipids produced

Structured phospholipids with CLnA from pomegranate seed oil were produced in a larger scale (g amounts) to evaluate two physical properties: dropping point and viscosity. The structured phospholipids produced were incorporated in a chocolate system, where lecithin is commonly used (unsweetened chocolate 94.6%, coconut oil 5%, phospholipids 0.4%) for the evaluation of these physical properties.

4.3.1 Dropping point

The PC-CLnA chocolate system showed lower dropping point (Figure 9) than the controls ($p<0.05$). A chocolate system has a crystalline nature in which physical properties such as the dropping point or viscosity could be affected by ingredients like fat or phospholipids and their structural characteristics, chain length, unsaturation degree and distribution of fatty acids (deMan 2000). The modification of phosphatidylcholine through the substitution of palmitic, oleic, or linoleic acid for a three double bond conjugated fatty acid (CLnA) could explain this observation as well as the presence of lysophospholipids in which one of the fatty acids has been hydrolyzed during the enzymatic reaction.

From a sensory point of view, the differences in the dropping points between the structured chocolate system and controls are not significant (< 0.7 °C) as fats can show a wide range of melting point which is related with their fatty acid composition. In the case of cocoa butter, its melting point goes from 30 to 35 °C.

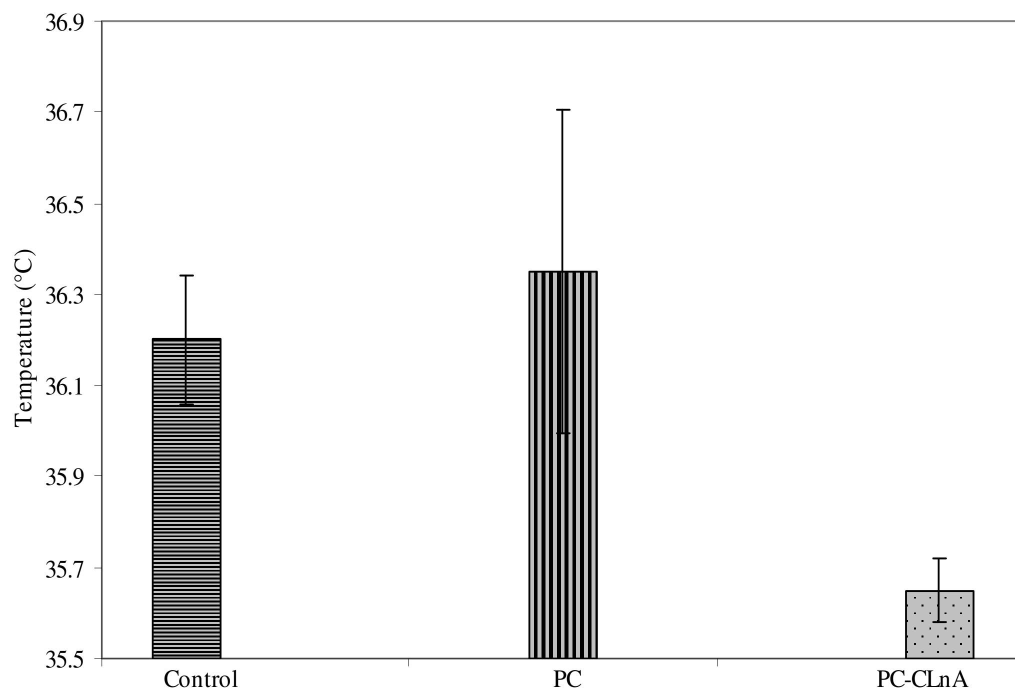

Figure 9. Dropping point PC-CLnA chocolate system and controls ($p<0.05$)

4.3.2 Viscosity

Lecithin is an important chocolate ingredient as it has been used to decrease viscosity and blooming. Viscosity is reduced due to a drop in internal friction and a rise in lipophilicity of hydrophilic chocolate ingredients (sugars) which in consequence facilitates the formation and stabilization of the emulsion. Furthermore, the reduction of viscosity depends on lecithin content and chocolate formulation (van Nieuwenhuyzen 1995; Nebesny and Żyżelewicz 2005). From the manufacturer point of view, the viscosity strongly affects costs and efficiency of chocolate making process (Nebesny and Żyżelewicz 2005).

The chocolate system used for this study didn't contain sugar however, the PC-CLnA chocolate system showed lower viscosities than the controls at 40 and 50 °C ($p<0.05$) (Figure 10). The dropping of viscosity of the PC-CLnA chocolate system at 50 °C was higher than the one obtained at 40 °C when compared with the controls. The modification of the phospholipid through the incorporation of a conjugated fatty acid and the presence of lysophospholipids (better emulsifier properties) produced during the interesterification reaction could explain these observations.

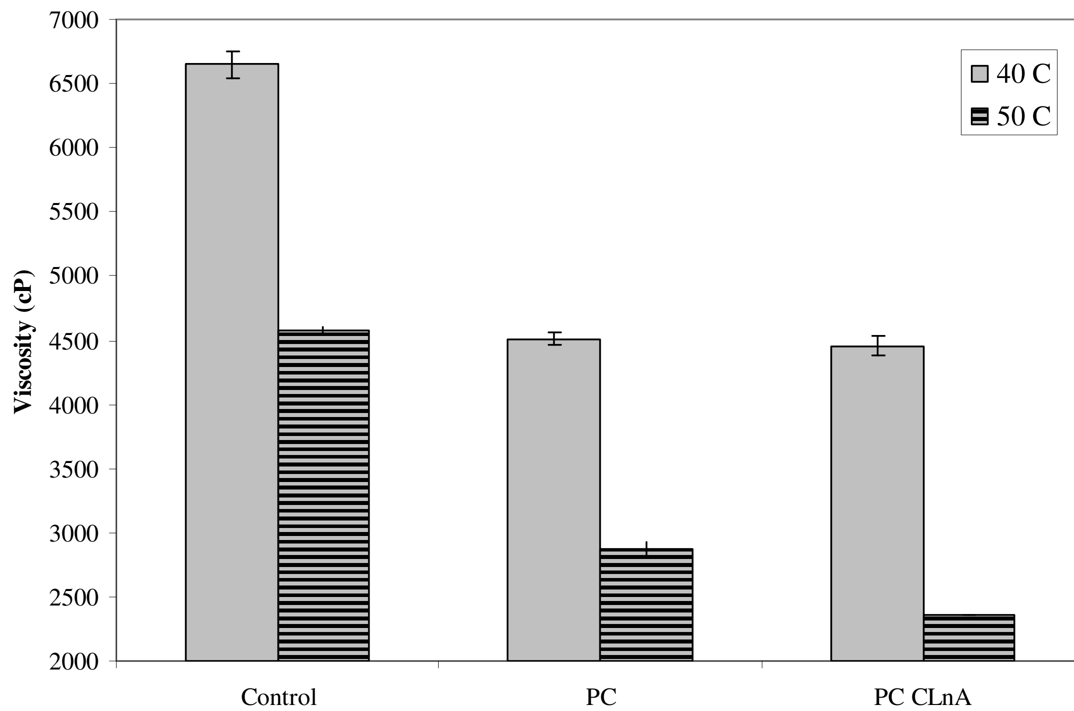

Figure 10. Viscosities of PC-CLnA chocolate system and controls at 40 and 50 °C (p<0.05)

4.4 Emulsion stability

Emulsion stability is a kinetic concept. An emulsion is stable when it doesn't have a distinguishable change in the size distribution of the droplets, in their state of aggregation, or in their spatial arrangement within the sample vessel, over the time-scale of observation (Dickinson and Stainsby 1982). Emulsion stability was evaluated by monitoring phase separation by visual observation during storage time. PC-CLnA emulsion showed higher emulsion stability than the controls. This emulsion was stable up to 108 h while the emulsions without phospholipid and with Lipoid S100 were 48 h and 96 h stable, respectively. This observation could be explained due to the presence of lysophospholipids in the PC-CLnA emulsion which have better emulsifier properties than the phospholipids (Schneider 1997).

4.5 Oxidative stability

Oxidative stability is an important parameter for the quality assessment of animal and vegetable fats and oils and lipid-containing foods (Läubli and Bruttel 1986). PC-CLnA emulsions showed lower oxidative stability, higher peroxide values and *p*-anisidine values (Figures 11 and 12) than controls after 1, 3 and 5 days of incubation at 50 °C. Antioxidant activity has been reported to phospholipids when added to vegetable and fish oils (List and Friedrich 1989; King and others 1992). However, because of the phosphorous group and polyunsaturated fatty acids that they contain, their role as pro- or antioxidant is complex (King and others 1992). The incorporation of a three double bond fatty acid (more susceptible to oxidation) into the phospholipid could explain the lower oxidative stability of the PC-CLnA emulsions than controls. These results contrast with the ones obtained by Hossen (2005). This author observed lower peroxide values and *p*-anisidine values (high oxidative stability) of the emulsions prepared with structured phospholipids with conjugated linoleic acid at the same conditions and formulation used in this study.

Oxidation during the production of structured phospholipids with CLnA could also occur due to reaction temperature, air oxidation, and purification steps. The

incorporation of an antioxidant such as tocopherol during the synthesis of the structured phospholipids should be considered as this antioxidant has shown to be effective to prevent lipid oxidation of conjugated fatty acids (Tsuzuki and others 2004c) and has also shown an antioxidant synergistic effect with phospholipids (Ohshima and others 1993).

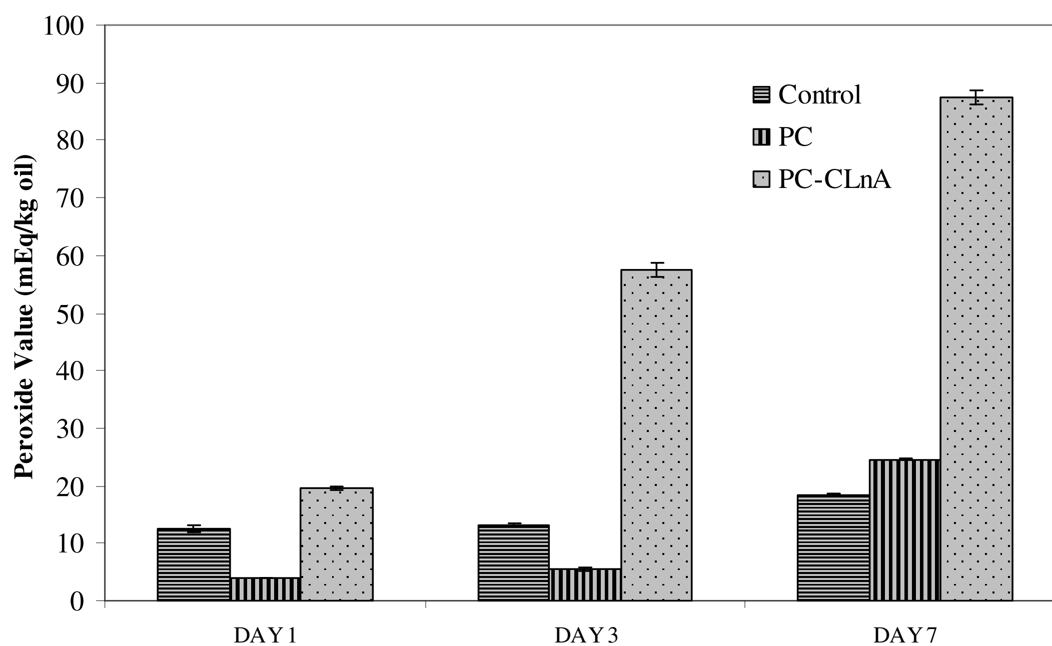

Figure 11. Oxidative stability (peroxide value) of PC-CLnA emulsions and controls ($p<0.05$)

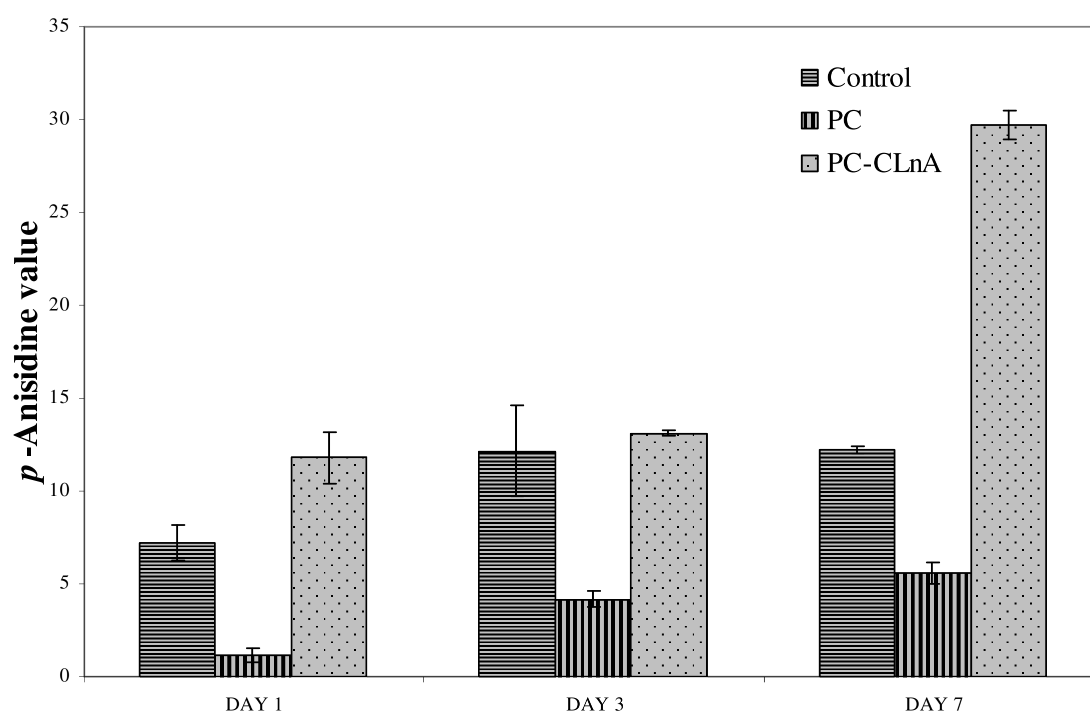

Figure 12. Oxidative stability(*p*-anisidine value) of PC-CLnA emulsions and controls (p<0.05)

CHAPTER V

CONCLUSIONS

Conjugated linolenic acids from pomegranate seed oil and an isomerized concentrated mixture from flaxseed oil were successfully incorporated into phospholipids by an enzymatic reaction. Higher incorporation of conjugated linolenic acids from pomegranate seed oil into phospholipids were obtained than for isomerized concentrated mixture from flaxseed oil. Higher percents of incorporation of conjugated linolenic acids into lysophospholipids were obtained than into phospholipids for both sources of these fatty acids. The maximum incorporation of CLnA was 17.2% into lysophospholipid after 72 h when pomegranate seed oil was used as a source of the conjugated fatty acids. Higher hydrolysis ratio was obtained (around 20% after 96 h) for the acidolysis reaction with the isomerized concentrated mixture from flaxseed than when the pomegranate seed oil was used.

Physical properties such as dropping point and viscosity of the structured phospholipids produced from pomegranate seed oil were studied. Structured phospholipids with CLnA incorporated in a chocolate system showed lower dropping point and viscosities (at 40 and 50 °C) than the controls. Emulsion stability of the structured phospholipids produced was higher than the controls when they were incorporated in whey protein based oil-in-water emulsion after 1, 3, and 7 days at 50 °C. Oxidative stabilities of the PC-CLnA emulsions were lower than the controls (higher peroxide values and p-anisidine values than controls).

Phospholipids are involved in vital biological functions and show interesting health benefits and technological properties. Furthermore, they are naturally found in living organisms and can be obtained in bulk from natural sources. These characteristics make products synthesized from them such as structured phospholipids advantageous compounds for the development of nutraceuticals and functional foods.

However, to validate structured phospholipids with CLnA as potential nutraceuticals and functional foods further research is needed. The testing of bioactivities such as anticancer, hypolipidemic, hypoglycemic, etc, of these structured phospholids as well as the study of other physical properties and sensory evaluation of food systems in which these compounds have been incorporated are required.

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