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Utilization of Modified Lecithin to Control Lipid Oxidation in Bulk Oils

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UTILIZATION OF MODIFIED LECITHIN TO CONTROL LIPID OXIDATION IN BULK OILS

A Thesis Presented

By

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ABSTRACT

UTILIZATION OF MODIFIED LECITHIN TO CONTROL LIPID OXIDATION
IN BULK OILS

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Lipid oxidation is a major challenge faced by the food industry since it causes loss of quality in lipid containing foods which results in a decrease of shelf life. In order to delay the oxidation in lipids, food industries make use of antioxidants such as EDTA (ethylene diamine tetraacetic acid), BHA (t-butyl-4-hydroxyanisole), BHT (t-butyl-4-hydroxytoluene), and TBHQ (tert-butyl-hydroxyquinone). However, these antioxidants are chemically synthesized and consumers desire simpler and cleaner labels without artificially synthesized antioxidants. Also, artificially synthesized antioxidants such as t-butyl-4-hydroxyanisole (BHA) can cause cancer in humans.

Previous studies have shown that phospholipids such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC) affect the activity of nonpolar antioxidants such as α-tocopherol in bulk oils. PE when added to stripped soybean oil containing 100 μM of α-tocopherol was able to improve oxidative stability of the oil. However, PC decreased the activity of α-tocopherol or had no effect. HPLC demonstrated that tocopherols were regenerated by PE which explains its synergism with α-tocopherol. This study evaluated the effect of α-tocopherol with varying levels of PE in stripped soybean oil.

Additionally, antioxidant activity of α-tocopherol has been shown to increase with increasing PE/PC ratio in lecithin. This study also examined the possibility of converting PC to PE in egg lecithin which will can be further used with α-tocopherol. The enzyme phospholipase D was used for the conversion since it has been shown to have transphosphatidylation activity with phospholipids. The synergism of the modified lecithin with α-tocopherol was analyzed in stripped soybean oil as well as in commercially refined oils.

KEYWORDS: *phospholipid, antioxidant, lipid oxidation, tocopherols, lecithin.*

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CHAPTER 1

INTRODUCTION

Lipid oxidation can cause undesirable changes to the sensory properties of food. Additionally, there is also a decrease in the product shelf life which can be problematic for the industries. Lipids are naturally present in foods or are added as a part of the product formulation. Presently, food industries use various techniques such as addition of antioxidants, oxygen scavengers, and using nitrogen flushing in order to delay lipid oxidation in the food. Out of all these options, antioxidants are the most convenient since they are cheaper and more efficient. Antioxidants can be categorized as natural such as tocopherols or artificial like tertiary butylhydroquinone. Artificial antioxidants are less expensive and more convenient to use commercially[1, 2]. However, they are known to cause adverse health effects on a long term. Therefore, consumers nowadays are demanding a simpler label in their food products containing all natural ingredients and additives.

Phospholipids have been found to affect the antioxidant activity of tocopherols in bulk oils. Phosphatidylethanolamine (PE) works synergistically with alpha tocopherol in delaying the lipid oxidation in bulk oils. Whereas phosphatidylcholine (PC) is found to have little or no effect[3-5]. This research examined the possibility of using lecithin present in oils to improve the the activity of tocopherol. However, lecithins naturally contain high amounts of PC which as stated above is not desirable for delaying the oxidation using tocopherols. In order for lecithin to be used in synergism with α-tocopherol it must contain a high PE/PC ratio. This can be achieved by converting PC in the lecithin to PE by the enzyme phospholipase D. There are past studies which have shown phospholipase D to have transphosphatidylation activity[6-12]. This research study focuses on optimizing the action of phospholipase D on egg lecithin to produce a modified

lecithin containing high amount of PE. The effect of this lecithin on the antioxidative activity of α-tocopherol in soybean oil was analyzed further.

The first part of the study involved storage studies done with pure PE to examine how it influences the activity of α-tocopherol in delaying lipid oxidation. Samples with two concentrations of isolated PE (1000 and 2000 μmol/ kg of oil) added to stripped soybean oil (SSO) containing α-tocopherol were subjected to incubation at 55^0C and checked for hydroperoxides and hexanal formation. The results suggested that pure isolated PE at both levels was able to work synergistically with α-tocopherol in delaying lipid oxidation. However, 1000 μmol PE was more effective than 2000 μmol PE concentration. From this study it can be proposed that phospholipid PE is able to inhibit lipid oxidation in bulk oils in the presence of antioxidant such as tocopherol. This can potentially be due to their ability to regenerate α-tocopherol from its oxidized form α-tocopherylquinone.

The next study involved optimizing the transphosphatidylation action of phospholipase D to get high PE/PC ratio in egg lecithin. The results showed that phospholipase D at pH 10.3 can be optimally used to convert PC in lecithin to PE giving high yield. The modified lecithin as well as the unmodified lecithin were examined for its effect on the antioxidant activity of α-tocopherol in SSO. Finally, the impact of the modified lecithin at various concentrations were analyzed in commercial soybean oil which naturally contain tocopherols.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction to Lipid Oxidation

Oxidation of lipids is known to degrade the quality and shelf life of the oil as well as develop rancid flavors and cause texture modification in the food. The appearance, safety and the nutritional quality of the food is also affected. Lipid oxidation generates potentially toxic products that can cause cancer, atherosclerosis, aging, and several inflammatory diseases[13]. These toxic products can enter the body through diet and get absorbed into the blood. From the blood they can further be transported to tissues[14].

Unsaturated lipids get oxidized to form alkyl radicals when they come in contact with an initiator. In presence of oxygen, these alkyl radicals then form peroxyl radicals and the peroxyl radicals can react with more lipids to produce hydroperoxides[15]. With time, the lipid hydroperoxide degrades to forms numerous volatile compounds such as hexanal and propanal, which affect the sensory quality of foods.

Formation of repugnant aromas and flavors in oils is known as rancidity. Color change in the oil can be caused by both formation of melanoidins, free radical destruction of conjugated double bonds (e.g. carotenoid bleaching). Other sensory changes are a change in texture (e.g. protein cross linking), and the degradation of nutrients and pigments such as vitamin A, vitamin E, vitamin C, and flavonoids[14]. Also, food processing operations like cooking and frying increase lipid oxidation rates in foods. Lipids can undergo oxidation at substantial rates at such high temperatures[16].

As mentioned earlier, the consumption of oxidized lipids can have adverse health effects. This is a major point of concern since the consumption of oxidatively susceptible vegetable oils has increased 130% in the United States between 1909 and 1999[17].

2.2. Mechanism of Lipid Oxidation

Lipid oxidation occurs through three steps – initiation, propagation, and termination[18]. The first initiation step occurs when the hydrogen atom present on the double bond gets extracted in the presence of heat, light or metal ions. This leads to the formation of alkyl radical ($R^{\cdot}$). This is followed by the propogation step where the alkyl radical reacts with oxygen to form the peroxyl radical ($ROO^{\cdot}$). The alkyl and peroxyl radicals then further react with hydrogen atom from another unsaturated lipid to form hydroperoxides (ROOH), the primary lipid oxidation product. The duration of the propogation step mainly depends upon the degree of unsaturation of the lipid[19]. The hydroperoxides are unstable and breakdown into carbonyl compounds. This forms the final termination step. The carbonyl compounds are the secondary oxidation products.

This whole process is known as autooxidation which primarily means the degradation of lipids into various oxidation products[20].

Initiation: $RH \rightarrow R\bullet + H\bullet$

Propogation: $R\bullet + O_2 \rightarrow ROO\bullet$

$ROO\bullet + RH \rightarrow ROOH + R\bullet$

Termination: $ROO\bullet + ROO\bullet \rightarrow ROOR + O_2$

$ROO\bullet + R\bullet \rightarrow ROOR$

$R\bullet + R\bullet \rightarrow RR$

2.3. Control of Lipid Oxidation

There are various proposed ways through which lipid oxidation can be inhibited or delayed, such as the use of antioxidants, nitrogen flushing, and incorporating oxygen scavengers in the food package. Antioxidants can work through different mechanisms which are free radical scavenging, pro-oxidant metal chelators, reducing agents, and singlet oxygen quenchers[21].

Free radical scavengers are classified as type 1 or primary antioxidants[21]. They work by competing with the other oxidation prone compounds for the peroxyl radicals[18]. The free radical scavengers interfere in the initiation or propogation step. They react with the peroxyl radical or the alkoxy radical and thus preventing the free radicals from reacting with the unsaturated fatty acids[22]. The then formed free radical scavenger radical is less prone to react with the unsaturated fatty acid and gets inactivated in the termination stage. The most commonly used free radical scavenging antioxidants are Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT), Propyl gallate (PG), and Tertiary butylhydroquinone (TBHQ)[21]. But since consumer preference is moving more towards the natural sources instead of artificial ones, companies are trying to incorporate natural antioxidants into their products. These antioxidants include tocopherols, carotenoids and plant phenolics.

Lipid oxidation can also be delayed by controlling the activity of pro-oxidant metals in the oil. This can be done by using metal chelators which decrease the formation of free radicals produced by metals. Metal chelators inhibit lipid oxidation by either forming insoluble metal complexes or by providing stearic interference between the food and the metal[23]. EDTA and citric acid are the most commonly used metal chelators. Phospholipid also act as metal chelator in oils[24].

2.4. Use of Antioxidant Combinations in Food Systems

Free radical scavengers have been effectively used in oil and emulsion system alone as well as in combinations previously. Green tea is a good source of different phenolics such as catechin, epigallocatechin, and epicatechin. Yin[25] combined α-tocopherol with different green tea extracts in sunflower oil emulsions and conducted lipid oxidation studies. The combination of α-tocopherol with green tea extract showed a significant synergistic effect on the antioxidant activity of both. The oxygen depletion lag phase of α-tocopherol at 0.0625% of the oil concentration was 147 seconds and that of green tea extract at 0.03175% concentration was 100 seconds. The oxygen depletion lag phase of the combination of both was increased to 1138 seconds. Furthermore, the study results also showed that extracts containing α-tocopherol and epicatechin in the ratio of 1:1 (upto 0.0625% each) had a slight synergist effect whereas and catechin showed an additive effect when combined with α-tocopherol in the same ratio.

Panya[26] conducted a study on the stability of soybean oil-in-water emulsions which contained α-tocopherol with rosmarinic acid or its alkyl esters. Rosmarinic acid is a polyphenol found in perilla oil and rosemary. The lag phase of oxidation in the sample containing α-

tocopherol alone was 5 days in a 20 day study. Rosmarinic acid and its ester with a 20 carbon chain was not as effective as α-tocopherol, having a lag phase of 3 and 2 days respectively. However, rosmarinic acid esters with 4 and 12 carbon chains were better antioxidants than α-tocopherol, which was demonstrated by the lag phase. The lag phase of the 4 carbon and the 12 carbon esters were 14 and 12 days respectively. These esters had a different effect on the oxidation when they were combined with α-tocopherol in the emulsions. The ester with 12 carbon chain was the most effective having a lag phase of 25 days. This was followed by the 4 carbon chain ester and rosmarinic acid which showed a 24 days lag phase. Rosmarinic acid was also effective when combined with α-tocopherol giving a lag phase of 22 days. However, the same synergistic effect was not observed in the case of the ester with 20 carbon chain. The lag phase of the sample containing the 20 carbon ester with α-tocopherol had a lag phase of 3 days. The concentrations of the antioxidants used were 20μM.

At levels of 0.45, 2, and 20 μg/g in emulsion, β-carotene acted as pro-oxidant[27]. The oxidative activity of β-carotene depends upon the other antioxidants present in the system. Heinonen[27] checked the effectiveness of β-carotene with analogues of tocopherol, namely alpha and gamma. The data reports that while γ-tocopherol was an effective antioxidant in oil-in-water emulsions, it did not produce any synergistic effect when combined with β-carotene. On the other hand, α-tocopherol was effective in working together with β-carotene. Low levels of 1.5 μg/g α-tocopherol and 2 μg/g β-carotene produced significant increases in antioxidant activity.

Chlorogenic acid is phenolic compound which is majorly found in coffee. Chlorogenic acid is shown to have some synergism when combined with α-tocopherol[28]. The antioxidant

capacity was measured by $ORAC_E$ assay. $ORAC_E$ value is the estimate of the antioxidant activity of a compound. The $ORAC_E$ values for the individual antioxidants were 0.42 and 0.79 for chlorogenic acid and α-tocopherol respectively. A combination of chlorogenic acid and α-tocopherol gave a 44.8% increase in the $ORAC_E$ value of α-tocopherol. A combination of 200 ppm each of α-tocopherol and rosemary extract gave a slightly lesser shelf life to margarine as using TBHQ[29]. The control sample of margarine containing no antioxidant had an anisidine value lag phase of around 5 days. Whereas the margarine samples containing 200 ppm each of α-tocopherol and rosemary extract had a lag phase of 12 days and the sample containing 120 ppm TBHQ did not show any lag phase in a 25 day study. Furthermore, the time taken for the peroxide value to reach a value of 20 at 60^0C was calculated. The time required for the control, α-tocopherol and rosemary extract, and TBHQ were 4, 4.3, and 6.2 days respectively.

Metal chelators like free radical scavengers are quite effective in inactivating the free radicals which are a main cause of lipid oxidation. The chelators decrease the formation of the free radicals produced by metals. This in turn helps in an increase in the concentration of the free radical scavengers at a given time since their degradation is reduced[18]. Hence there are a lot of studies which have been done previously to check the antioxidant efficacy when a combination of both free radical scavengers and metal chelators is used.

In a study conducted by Let[30], the efficacy of γ-tocopherol was checked when used in combination with EDTA. The concentration of the volatiles released were significantly decreased in the combination as compared to the control having only tocopherol. The lag phase of the volatile production for 120 μL of γ-tocopherol in the 100 ml salad dressing was obtained as 4 weeks. Whereas the volatile production lag phase for 50 μg/g dressing of EDTA was around 6

weeks. For the sample containing 30 μL of γ-tocopherol and 10 μg/g dressing of EDTA, the lag phase was 6 weeks. Hence γ-tocopherol can be used as a combination in order to reduce the concentration of EDTA in the product and give a similar stability. In an another study conducted using a mixture of different tocopherols, Djordjevic[31] found that the antioxidant activity of mixed tocopherols and EDTA together can be a better antioxidant in O/W emulsions as compared to tocopherol alone. However, the efficacy of the combination was same as that of EDTA. Both these results were in accordance with the earlier study mentioned.

Also, amino acids and proteins have iron binding capacities which can help in delaying lipid oxidation. Almajano[32] showed that there was an increase in the stability of O/W emulsions when 0.5mM of Epigallocatechin gallate(EGCG) was used in combination with 0.5mM of albumin. The hydroperoxide lag phase of the emulsion containing epigallocatechin and albumin alone were 20 and 15 days respectively. A combination of both had a hydroperoxide lag phase of more than 30 days. This shows that EGCG can work synergistically with albumin. Similar results were observed when the study was done with Epicatechin and Bovine Serum Albumin as the phenolic and protein respectively. The change in the conjugated dienes were monitored. The conjugated dienes lag phase for epicatechin and bovine serum albumin were 10 and 5 days respectively. There was no lag phase detected in a 45 days study for a sample which contained a combination of both.

Almajano[33] used different tea infusions in combination with bovine serum albumin in sunflower oil emulsions. The variety of tea infusions used were red tea, black tea, green tea, white tea, and rooibos tea. 0.1% of tea extract with the protein worked effectively to inhibit lipid oxidation. Bovine serum albumin alone gave an increase in hydroperoxide lag phase by 3 days in

the emulsions. Green tea was the most efficient among all the combinations, with no detectable hydroperoxide lag phase in an 80 days study (45 days lag phase for green tea alone). The next most effective combination was black tea with a hydroperoxide lag phase increase from 25 days for black tea alone to 70 days when combined with bovine serum albumin. Rooibos tea did not have much effect when combined with bovine serum albumin with a minimum increase in lag phase. Albumin was able to work effectively with red tea and white tea as well. The hydroperoxide lag phase for the emulsions containing the white tea and rea tea were 12 and 25 days respectively. However on addition of bovine serum albumin, the lag phase was increased to 27 and 40 days for white and red tea respectively.

Additionally in water in oil emulsions, Zhu[34] used an antioxidant combination of α-tocopherol with whey protein isolate in walnut oil emulsions. There was a 3 fold increase in the lag phase as compared to the control in which whey protein isolate was used alone. When it was combined with α-tocopherol, the lag phase was increased by 10 fold. When trolox (water soluble analogue of tocopherol) was used in place of tocopherol, the lag phase of oxidation increased by 6 fold.

The shelf life of emulsions has also been improved by incorporating a combination of tocopherol (rich in delta derivative and low in alpha) with lecithin and ascorbyl palmitate. Trace metal chelation by the lecithin and ascorbyl palmitate works efficiently with tocopherols[35]. Phospholipids such as phosphatidylethanolamine and phosphatidylcholine have been shown to have metal chelating properties[35]. Leqi[5] showed that phosphatidylethanolamine can synergistically work with α-tocopherol in bulk oils. The lag phase of the oil was increased by more than twice when a combination of α-tocopherol and phosphatidylethanolamine was used

instead of α-tocopherol alone. Phosphatidylethanolamine proved to be pro-oxidative when used alone with a hexanal lag phase of 4 days whereas for the sample containing α-tocopherol, the lag phase was observed to be around 22 days. The combination of α-tocopherol and phosphatidylethanolamine produced a hexanal lag phase of 45 days which shows a significant synergism.

Oxygen scavengers are useful in products containing headspace oxygen or dissolved oxygen[21]. Ascorbic acid is a very commonly used oxygen scavenger in food systems. Other oxygen scavenging agents are ascorbyl palmitate, erythorbic acid and sulfites. Reducing agents work by donating hydrogen atom. However ascorbic acid can act as a prooxidant in O/W emulsions because of its ability to increase the reactivity of metals. Hence ascorbic acid and its lipid analogue ascorbyl palmitate function as antioxidants at very low levels. This was demonstrated by a study conducted by Azizkhani[29], in which a combination of a mixture of tocopherols and ascorbyl palmitate was not so effective in improving the shelf life of margarine.

Frankel[36] showed that ascorbyl palmitate when used alone, increases the amount of hydroperoxides and volatiles produced in corn oil emulsions. But when it was used in combination with α-tocopherol, there was an one day increase in the hydroperoxide as well as the hexanal lag phase.

2.5. Tocopherols

Tocopherols are fat soluble compounds which are known to have high antioxidant activity against lipids[37]. The structure of the tocopherols consists a 6 chromanol ring with a saturated side chain. Tocotrienols have a similar basic structure as tocopherols but with an unsaturated side chain with three double bonds[38]. The tocopherol and tocotrienol homologues are classified as α, β, δ, and γ depending on the position and number of the methyl substitutions on the chromanol ring[39]. Fig 2.1 shows the different structures of the tocopherols and tocotrienol homologues.

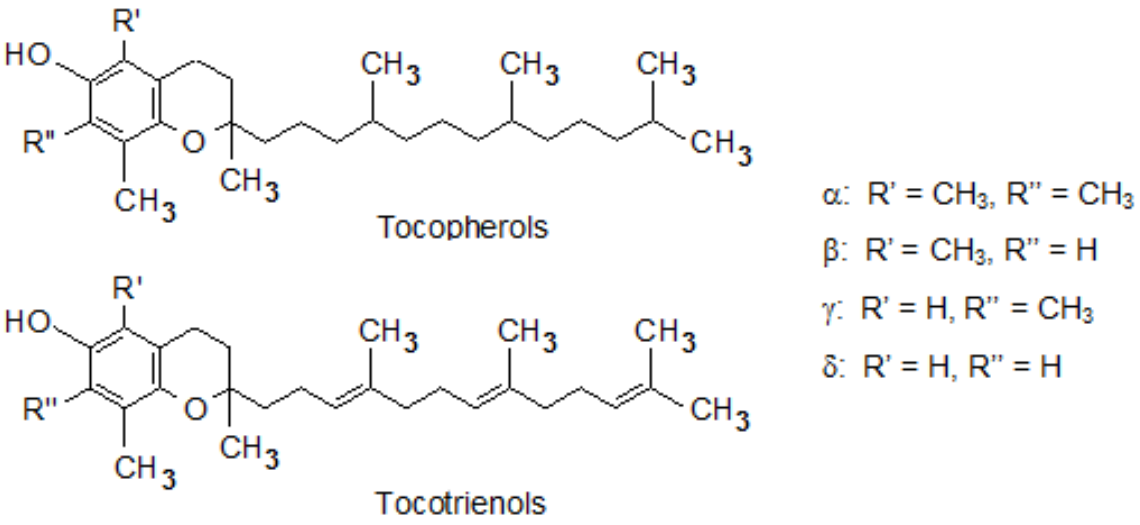

Fig 2.1. Structures of Tocopherol and Tocotrienols

Tocopherols are naturally present in vegetable oils. Table 2.1 shows the natural tocopherol content (mg/kg) of sunflower and soybean oils.

Tocopherols	Soybean oil	Sunflower oil
α- tocopherol	120	610
β- tocopherol	10	10
γ- tocopherol	610	30
δ- tocopherol	260	10

Table 2.1. Natural tocopherol content (mg/kg) of soybean and sunflower oils[40].

The antioxidant activity of tocopherol is based on its ability to donate its phenolic hydrogen to the free radicals and thus inhibiting the formation of hydroperoxides. The hydrogen donating capacity of the tocopherols varies with the model system and the oxidation conditions[39].

There have been studies done to compare the activity of the tocopherol homologues in fats and oils. Kanno[41] measured the hydroperoxides formed in milk fat samples containing the different tocopherol homologues at 50^0C. The results showed that γ-tocopherol was the most effective at 0.001, 0.01, and 0.05% concentrations. α- tocopherol was the least effective at all the three concentrations. However, when added at 0.003% it was the most effective. In another study conducted in fish oil, Kulas[42] found that α- tocopherol was the most effective at 30^0C followed by γ and then δ-tocopherol when added at 100 ppm. But at 1000 ppm concentrations, the order was reversed with δ-tocopherol being the most effective.

Jung[43] examined the effect of the tocopherol homologues in chlorophyll b-photosensitized soybean oil. The samples were prepared with 0.16 mol/L purified soybean oil added to methylene chloride containing 3.3×10^{-9} mol/L chlorophyll b and incubated at 25^0C. The tocopherols were added at 0, 0.001, 0.002, 0.004 mol/L concentrations. The results showed that the antioxidant effects were α- tocopherol> γ-tocopherol> δ-tocopherol at 0.001 mol/L, α-tocopherol $\cong$ γ-tocopherol> δ-tocopherol at 0.002 mol/L, and α- tocopherol $\cong$ γ-tocopherol $\cong$ δ-tocopherol at 0.004 mol/L. Kinen[44] studied the impact of α- and γ-tocopherol at different concentrations in methyl linoleate at 40^0C. The oxidation was measured by analyzing the hydroperoxide isomers produced in the samples by high performance liquid chromatography. At low concentrations (10 ppm), α- was a better antioxidant than γ-tocopherol. However, γ-tocopherol was more effective at higher concentrations (100-1000 ppm). All these studies show that α- tocopherol is a better antioxidant at low concentrations but γ-tocopherol is more effective at higher concentrations. Seppanen[39] suggests that γ-tocopherol being a less of a pro-oxidant at higher concentrations than α- tocopherol might be one reason for this.
The activity of tocopherols can vary with the oxidation system. Chaiyasit[45] conducted a study in which α- tocopherol was compared with δ-tocopherol in bulk oil as well as in emulsions. The results showed that α- tocopherol was a better antioxidant in bulk oil but δ-tocopherol was a more stronger antioxidant in the emulsion system.

There have been studies done which try to find the optimum concentrations of the tocopherol homologues in oil. In purified soybean oil, the optimum concentration for α, γ, and δ- tocopherol were found to be 100, 250 and 500 ppm[46]. The study was conducted in dark at 55^0C. The author also found that above these optimum concentrations, these antioxidants had prooxidant effects under the same experimental setup. Similarly, the optimum tocopherol

concentrations for soybean and sunflower oils have been found out to be 500-700 ppm and 350-550 ppm respectively[39]. Huang[47] studied the formation of hydroperoxides in vacuum distilled corn oil in the presence of α- and γ-tocopherols. The study showed that α-tocopherol worked best at 100 ppm whereas γ-tocopherol worked best at any concentration between 250-500 ppm. Similar results were obtained by Yoshida[48], where the optimum concentration for β- and γ-tocopherol was between 150-200 ppm in vacuum distilled oils. In the same experimental study, the δ-tocopherol worked best at 500 ppm.

2.6. Phospholipids

The phospholipid structure consists of a glycerol backbone with a phosphate head group present usually on the sn-3 position. They are present in all living species from which foods are obtained[3]. Depending upon the chemical group on the phosphate group, phospholipids can be classified into different classes. When the phosphate group has a choline on it, the phospholipid is categorized as phosphatidylcholine. Similarly, there is phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol where the substituted group is ethanolamine, serine and inositol respectively.

Soybean and egg lecithin are great source of phospholipids with a commercial production of 200 tonnes/year and 300 tonnes/year respectively[49].

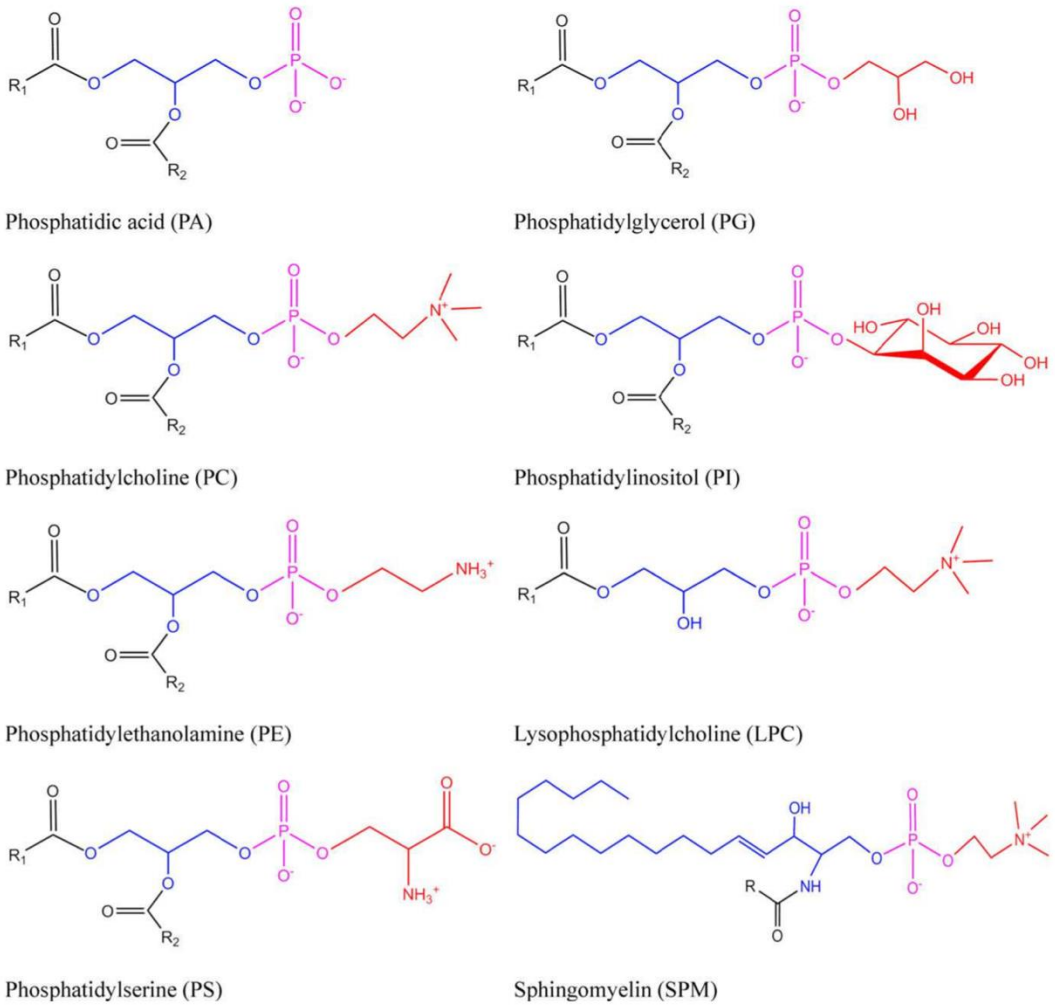

Fig 2.2. Phospholipid structures

Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) have been shown to have an impact on lipid oxidation in bulk oils. Phosphatidylethanolamine has been shown to act synergistically with α-tocopherol to delaying the onset of lipid oxidation in vegetable oils [50-53] whereas phosphatidylcholine does not increase the antioxidant activity of α-tocopherol[5]. PE increases the antioxidant activity of tocopherols by regenerating oxidized tocopherol quinones back to the original tocopherol so it can scavenge an additional free radical. PC has the ability to

form association colloids such as reverse micelles in oils since they are surface active and have an intermediate hydrophilic-lipophilic balance value [5]. These reverse micelles form lipid-water interfaces which generally accelerate the rate of lipid oxidation.

The different phospholipids have different susceptibility to oxidation because of the polar head groups and also the degree of unsaturation[3]. Yin[54] showed that PE liposomes oxidized faster than PC liposomes, both having the same fatty acids. One reason for this can be due to PE regenerating tocopherols whereas PC remains stable in system.

In order to study the effect of phospholipids on oil system, it is essential that the oil is free of all the minor components such as tocopherols and iron which might cause interference. Tocopherol already present in the system can delay the oxidation of the oil by itself or can work in synergism with the phospholipid and thus making it difficult to produce clear results. For example, in a study conducted by Sugino [55], the phospholipids in egg yolk was shown to inhibit lipid oxidation in DHA rich oil. However, the oil naturally contained 3000 ppm of tocopherols which might have interfered in the system. Similarly King[56] demonstrated the effect of individual phospholipids on the oxidative stability of salmon oil which contained 280 ppm of tocopherols.

Phospholipids have been shown to interact with metals in bulk oil and inhibit lipid oxidation. They can bind with the pro-oxidant metals through the negative charge present on their phosphate head group[3]. Yoon[57] showed that phospholipids (PC, PI, PA, PE) acted as antioxidants in soybean oil when 1 ppm of ferrous iron was added to it. Zago [58] also showed that ferrous was able to bind with PC/PS liposomes. The ability to bind with the metal increases with negative charge on the phospholipids. Dacaranhe[59] showed that the iron binding

capacity of the phospholipids in egg yolk PC unilamellar liposomes was in the order PA≥PS>PE=PC.

Another pathway through which the phospholipids can affect lipid oxidation is through Maillard reaction. Maillard reaction occurs in the presence of carbonyl and free amine groups. Phospholipids such as PE have amino group which react with the carbonyl groups produced during the lipid oxidation, and can lead to the formation of Maillard products[3]. This was shown by Hidalgo [60, 61] where PE having a primary amine group reacted with 4,5-epoxy-2-heptenal to produce antioxidant maillard products. Whereas PC which has a tertiary amine group showed no oxidative effect on the lipid oxidation[62]. These results where in accordance with Alaiz[63] where primary and secondary amines showed inhibitory effects in lipid oxidation of soybean oil whereas tertiary amines showed no effect.

Lecithin can be classified as a mixture of phospholipids obtained from plant or animal sources[64]. Lecithin can be used as an emulsifier as well as for its antioxidant activity. Apart from the food industry, it is also used in cosmetics and lubricants[3]. Table 2.2. shows the composition of phospholipids in various plant based and animal based lecithins.

	Soybean Lecithin	Rapeseed Lecithin	Sunflower Lecithin	Egg Lecithin
Phosphatidylcholine	24.1	21.9	31.0	83
Phosphatidylethanolamine	25.9	12.2	13.8	9
Phosphatidylinositol	18.2	14.1	23.7	1.2
Phosphatidic acid	6.1	4.8	5.6	4.2

Other/lyso-phospholipids	10.9	10.6	3.5	2.6

Table 2.2. Phospholipid composition of Commercial lecithins [50, 65]

Soybean lecithin was added to virgin oil in order to check its effect on oxidative stability of the oil. The oxidation was analyzed through Rancimat method[66]. The results showed that the lecithin was able to increase the tocopherol concentrations in the oil and thus increase its oxidative stability. Hamilton[67] studied the effect of α-, δ-tocopherol alone as well as in combination with soybean lecithin on soybean oil. The hydroperoxides and off flavors formed in the oil samples incubated at 20^0C were measured for checking stability. Both the tocopherol homologues were effective in inhibiting the oxidation of the oil. However, the oxidation of oil was further delayed in the presence of lecithin. However, not all the phospholipids present in lecithin work synergistically with tocopherols. Cui[5] showed that 1000 μM PE in presence of 100 μM of α-tocopherol was able to increase the lag phase for lipid oxidation by 10 days when compared with tocopherol alone. However, similar results were not observed with PC at the same concentrations and conditions. In the presence of α-tocopherol, PC had a pro-oxidant or no effect at all.

Judde[51] reported that the combination of γ- or δ- tocopherols with lecithin which contained high amounts of phosphatidylcholine and phosphatidylethanolamine was more effective at inhibiting lipid oxidation than the individual components. However, phosphatidylcholine was not always found to work synergistically with tocopherols. Kashima[68] reported that phosphatidylethanolamine was able to suppress lipid oxidation in perilla oil which contained mixed tocopherols, whereas phosphatidylcholine showed no effect. Also, Takenaka[52] found that only phosphatidylethanolamine increased the activity of α-tocopherol, but not phosphatidylcholine. The conclusion made from these reports is that

phoshphatidylethanolamine has a positive effect on the activity of α-tocopherol whereas phosphatidylcholine has no effect or can act as a prooxidant.

Lambelet[69] observed that in the presence of phospholipids that contain primary amines such as PS and PE, the tocopherol in methyl linolenate were degraded slowly. On the other hand, phospholipids containing tertiary amine groups such as PC, had no effect on the oxidation of the tocopherols. In another study, Doert[50] examined the interaction between the individual phospholipids with α-tocopherolquinone which is the oxidized product of α-tocopherol. They found that all the individual phospholipids were able to regenerate α-tocopherol from α-tocopherolquinone, except for PC.

2.7. Phospholipase D

Phospholipase D (PLD) is widely present in bacterial, plant and mammalian cells[8]. In the presence of alcohols, phospholipase D is able to facilitate head group exchange in phospholipids. This activity is known as transphosphatidylation[12, 70]. This activity is specific to phospholipase D since the other phospholipases act on different sites of the phospholipid compound. This is shown in Fig 2.3., the various phospholipase enzymes act on different action sites of the phospholipids. Phospholipases A_1 and A_2 cleave the ester bond. Whereas C and D cleave at the phosphodiester bonds.

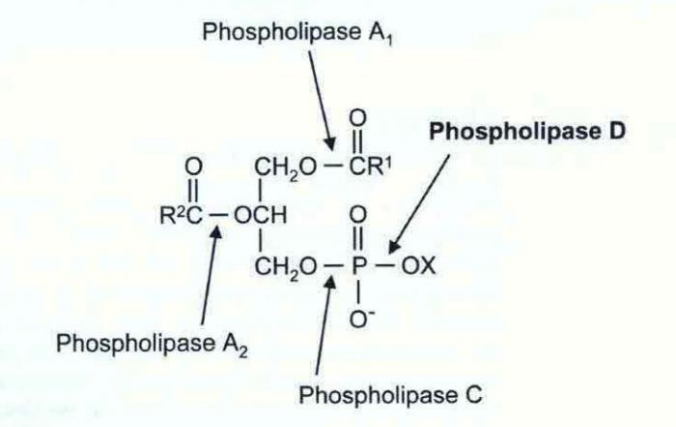

Fig 2.3. Schematic diagram of the cleavage sites of the phospholipases [71]

The transphosphatidylation activity of Phospholipase D on PC is schematically shown in Figure 2.4. In the presence of primary alcohols, the choline group is substituted by the alcohol. However there is a percentage of PC which gets hydrolyzed to phosphatidic acid, which happens simultaneous to the transphosphatidylation reaction[11]. In order to reduce the hydrolysis reaction, it is essential to avoid the presence of water in the system. Previous lab studies have been done using Phospholipase D from plant sources such as cabbage[6]. However recently *Streptomyces* species have been preferred as a source of Phospholipase D. The reason being that Phospholipase D from *Streptomyces* species have a better transphosphatidylation potential compared to its hydrolytic activity[70].

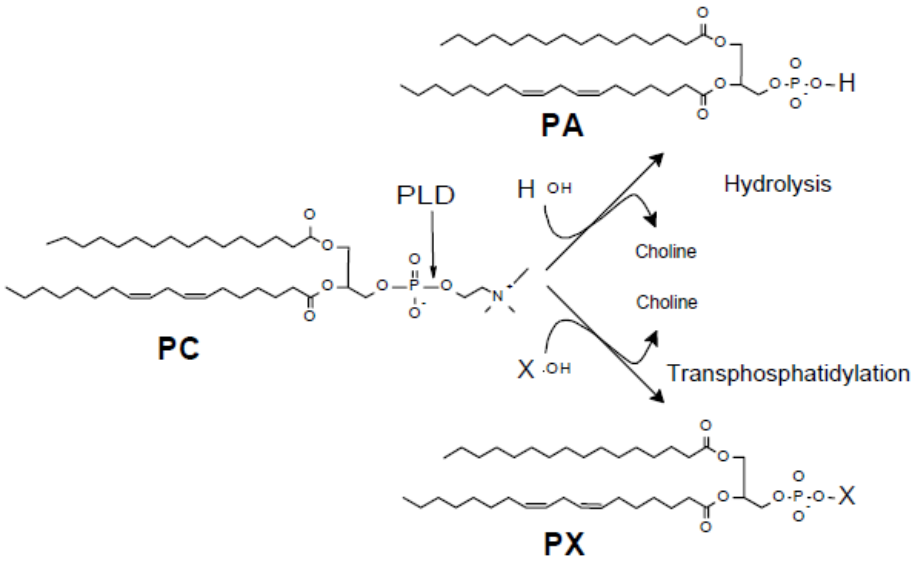

Fig 2.4. Schematic diagram showing the Transphosphatidylation activity of phospholipase D

Previous studies have looked into the enzymatic conversion of the phosphatidylcholine to phosphatidylethanolamine. However, these studies vary in the terms of the source of phospholipids, origin of the enzyme, and the conditions used for the conversion. For example, Juneja et al [10] showed the conversion of pure phosphatidylcholine using phospholipase D from microbial as well as vegetable sources. Enzymes from *Streptomyces antibioticus* (SaPLD), *Streptomyces chromofuscus* (SchPLD) and cabbage leaves were used for the study and the reaction was carried out at 30^0C. The transphophatidylation activity was highest with the SaPLD enzyme followed by the cabbage PLD and then the SchPLD. This shows that the transphosphatidylation activity of the Streptomyces enzymes were different irrespective of their same genus. Ogino et al [12] reported a conversion rate of more than 80% when the enzyme from the culture medium of *Streptoverticillium Cinnamoneum* was used for the transphosphatidylation reaction. These studies were carried out on pure isolated phospholipids. There is minimum research done on the phospholipids in lecithin.

The activity of enzymes varies with the reaction pH. Zhou[72] studied the effect of pH on the PC hydrolysis activity of PLD from *Streptomyces sp.* at 37^0C for 60 minutes. The optimum pH for PLD was obtained to be between 7 and 8. At pH 7.5, PLD was able to catalyze suitable conversion of PS from soybean lecithin.

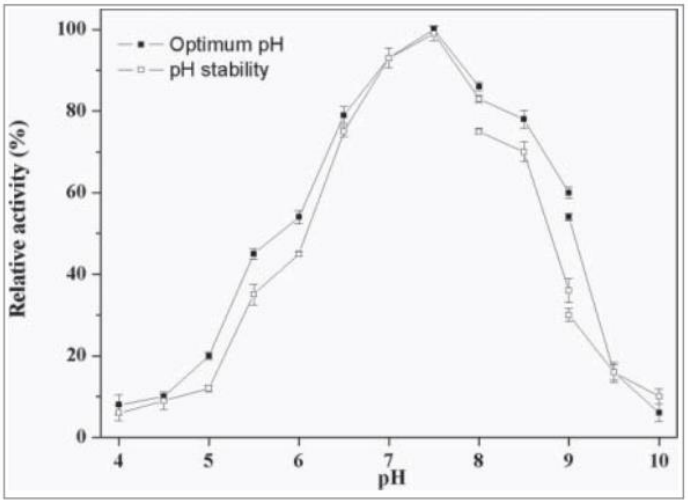

Fig 2.5. Effect of pH on the activity of phospholipase D[72]

Piazza[6] studied the conversion of phosphatidylcholine to phosphatidylglycerol using commercial phospholipase D preparations from cabbage and *Streptomyces Chromofuscus* in the presence of glycerol. The study found that the enzyme from *Streptomyces sp.* had a higher selectivity for glycerol than cabbage PLD. Other studies have also shown that *Streptomyces sp.* PLD have a better transphosphatidylation potential and less hydrolytic activity[70]. Khatoon[11] examined the head group selectivity of PLD for different phospholipids. The PLD was isolated from Indian mustard seeds. The study found that the head group selectivity of PLD was in the order PC > PE > PG. Additionally, the enzyme was able to substitute the choline group in PC by ethanolamine, glycerol and ethylene glycol with high efficiency. The transphosphatidylation reaction with serine had low efficiency.

CHAPTER 3

IMPACT OF MODIFIED LECITHIN ON OXIDATIVE STABILITY OF BULK OILS

3.1. Introduction

Lipid oxidation is a major challenge faced by the food industry since it causes loss of quality in lipid containing foods which results in a decrease of shelf life [15]. In order to delay the oxidation in lipids, food industries make use of antioxidants such as EDTA (ethylene diamine tetraacetic acid), BHA (t-butyl-4-hydroxyanisole), BHT (t-butyl-4-hydroxytoluene), and TBHQ (tert-butyl-hydroxyquinone). However, these antioxidants are chemically synthesized and consumers desire simpler and cleaner labels without artificially synthesized antioxidants. Also, artificially synthesized antioxidants such as t-butyl-4-hydroxyanisole (BHA) may pose health risks to humans[13].

Phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are the most abundant phospholipids present in foods. Conflicting research has been published on the impact of both these phospholipids on lipid oxidation with publications claiming both prooxidant and antioxidant activities. Phosphatidylethanolamine has been shown to act synergistically with α-tocopherol to delaying the onset of lipid oxidation in vegetable oils [50-53] whereas phosphatidylcholine does not increase the antioxidant activity of α-tocopherol[5]. PE increases the antioxidant activity of tocopherols by regenerating oxidized tocopherol quinones back to the original tocopherol so it can scavenge an additional free radical. PC has the ability to form association colloids such as reverse micelles in oils since they are surface active and have an intermediate hydrophilic-lipophilic balance value [7]. These reverse micelles form lipid-water interfaces which generally accelerate the rate of lipid oxidation.

Doert[50] studied the impact of soybean lecithin and soybean PC on α-tocopherol in ethyl linoleate. At room temperature, soybean lecithin at 1% was able to slow down the degradation of ethyl linoleate by 4 times (17 weeks as compared with 4 weeks for control). However, soybean PC acted as a pro-oxidant, fastening the degradation of ethyl linoleate by 1 week. Similar results were obtained at 110^0C. All the samples contained 0.05% of α-tocopherol. Similar results were obtained by Bandarra[73], soybean lecithin had a better synergistic activity with α-tocopherol when compared with soybean PC. Other researchers have also shown that among both the phospholipids, PE has better synergistic activity with α-tocopherol relative to PC [5, 53, 74, 75]. Furthermore, PC at higher levels such as 1000 μmol/ kg of oil can exhibit pro-oxidant effects [5] or has no effect at all [52, 68].

Damnjanovic[8], and Ogino[9] showed that phospholipase D from microbial sources was able to convert phosphatidylcholine to phosphatidylethanolamine in the presence of ethanolamine by transphosphatidylation. In a study conducted by Juneja [10], significant conversion of PC to PE was achieved using phospholipase D obtained from cabbage leaves. To our knowledge, there is no published research on using phospholipase D to convert PC to PE in lecithin.

The scope of this study is that phosphatidylcholine can be enzymatically converted to phosphatidylethanolamine using phospholipase D to increase the ability of lecithin to regenerate tocopherols and further inhibit lipid oxidation. Therefore, the objective of this study was to determine the conditions under which phospholipase D was able to convert

phosphatidylcholine present in lecithin to phosphatidylethanolamine. The enzyme modified high PE lecithin was then tested for its ability to inhibit lipid oxidation in vegetable oil.

3.2. Materials

Lecithin (~60 % L-α-phosphatidylcholine) from egg yolk, the enzyme phospholipase D from *Streptomyces chromofuscus* (>50,000 units per mL), silicic acid, activated charcoal, and α-tocopherol were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Soybean oil was bought from a local grocery store and kept at -20^{0}C until use. The solvent ethyl acetate was purchased from Fischer Scientific (Pittsburg, PA, USA). All other reagents were of HPLC grade or purer. Distilled and deionized water was used throughout.

3.3. Preparation of Stripped Soybean Oil

Stripped soybean oil (SSO) was prepared as per the protocol described in Cui[4]. This was done in order to avoid the interference of minor components such as phospholipids, tocopherols, and free fatty acids present in commercial soybean oil. Commercial soybean oil (30g) was mixed with 30 mL of hexane and passed through a chromatographic column (3.0 cm diameter, 35 cm long) using 270 mL hexane for elution. The column was packed with 3 layers sequentially. The bottom layer was 22.5 g of silicic acid (washed with distilled and deionized water). Activated charcoal (5.6 g) formed the middle layer which was followed by another 22.5 g

of silicic acid for the top layer. The packed column was used to isolate the soybean oil triacylglycerols. The solvent was removed by using a vacuum rotary evaporator (Brinkman, Flawil, Switzerland) at 25^0C followed by further evaporation by nitrogen flushing. SSO mentioned in the paper refers to a mixture of 25% stripped soybean oil and 75% MCT was used for following experiments unless specified.

3.4. Preparation of Sample for Lipid Oxidation

The experiment was divided into six parts. Part one included the addition of 1000 or 2000 μmol pure PE/ kg of SSO. Part two included the addition of of 1000 or 2000 μmol pure PE/ kg of SSO containing 100 μmol α-tocopherol/ kg of SSO. Part three included conversion of PC to PE using the enzyme phospholipase D and 30 mL ethanolamine as the substrate. Part four included the addition of 1000 or 2000 μmol unmodified lecithin or modified lecithin/ kg of SSO. Part five included the addition of of 1000 or 2000 μM unmodified lecithin or modified lecithin and 100 μM α-tocopherol/ kg of SSO. Part six included the addition of the modified lecithin to commercial soybean oil at 500, 1000, 2000, 3500, and 5000 μmol/ kg of oil concentrations. According to the different parts, the antioxidant α-tocopherol was dissolved in ethanol and pipetted into empty beakers. The solvent was evaporated by nitrogen flushing. SSO was added and the samples were magnetically stirred for 48 hours. Similarly, PE was dissolved in chloroform and pipetted into empty beakers. The chloroform was evaporated by nitrogen and the SSO containing antioxidant was added. The samples were magnetically stirred for 5 hours at room temperature. For lipid oxidation studies, 1 mL of oil sample was aliquoted into 10 mL headspace vials and sealed with aluminum caps with PTFE/Silica caps and stored in the dark for 55^0C.

3.5. Oxidation Measurements

3.5.1. Hydroperoxide Analysis

Lipid oxidation was analyzed by monitoring lipid hydroperoxides and headspace hexanal formation at various time intervals. Lipid hydroperoxides and hexanal formation were measured as the primary and secondary oxidation products respectively. Hydroperoxides were analyzed by the spectrophotometric method described in Shantha and Decker [76]. Oil samples were weighed and mixed with 2.8 mL of methanol/butanol (2:1, v/v). Then 15 μL of 3.94 M ammonium thiocyanate and 15 μL of 0.072 M Fe^{2+} (ferrous sulfate) were added. The ferrous sulfate solution was made by mixing 0.13 M $BaCl_2$ and 0.14 M $FeSO_4$. After a waiting period of 20 minutes, the absorbance was measured on a Genesys 20 spectrophotometer (ThermoSpectronic, Waltham, MA, USA). The calculations were made from a standard curve of cumene hydroperoxide.

3.5.2. Hexanal Headspace Analysis

Headspace hexanal was measured using a GC – 17A Shimadzu gas chromatograph which had an AOC-5000 autosampler (Shimadzu, Kyoto, Japan). According to Chen[77], a 30 m Å × 0.32 mm Equity DB-1 column with a 1 μm film thickness was used for separations. Oil samples (1 mL) in 10 mL headspace vials were heated at in the autosampler heating block for 8 min at 55 °C. A 50/30 μm DVB/Carboxen/PDMS solid-phase microextraction (SPME) fiber needle was used to adsorb volatiles, and then the fiber was placed into the 250 °C injector port at a split ratio of 1:5.

The GC separations were 10 min at 65 °C for each sample. Helium was used as a carrier gas, with a total flow rate of 15.0 mL/min. A flame ionization detector at 250 °C was used to detect hexanal, and concentrations were determined from peak areas using a standard curve made from SSO containing known hexanal concentrations from 2 to 800 µM.

3.6. Enzymatic Conversion of Lecithin

The phospholipase D enzyme solution was prepared immediately before the experiment. Phospholipase D (≅250 units) was diluted with 200µL of buffer solution. The buffer consisted of 0.2M sodium acetate (pH 8.0) with 0.01M calcium chloride. The enzyme solution was stored at 4^0C and used within 1 hour of dilution. 0.3 g of lecithin (stored at -20^0C) was dissolved in 30mL of ethyl acetate. Ethanolamine hydrochloric acid mixture was prepared by adding 1.0mL of hydrochloric acid slowly to 3.0mL of ethanolamine placed in an ice bath which was then added to the lecithin dissolved in ethyl acetate. The enzymatic reaction was started by adding 0.2mL of phospholipase D solution and was carried out at 37^0C for 40 minutes with continuous stirring.

After the enzymatic reaction, the organic phase was separated out using a separating funnel. The water phase was stored at -20^0C. The organic phase was evaporated with a rotary evaporator (Brinkman, Flawil, Switzerland) at 40^0C. The resulting residue was recombined with the water phase previously stored at -20^0C to further recover materials from the water phase. To this mixture, 80mL chloroform, 30mL methanol, and 20mL of water was added. The organic phase was separated from the water phases using a separating funnel. The water phase was further extracted using 50mL chloroform and this chloroform was combined with the organic

phase. The total organic phase was extracted with 30ml water to remove the remaining water soluble components. The organic phase was evaporated at 40^0C using the rotary evaporator and then vacuum dried by Savant™ centrifugal vacuum concentrator (Thermo Fischer, Waltham, MA, US) to get the lecithin solids.

Previous studies[9, 10, 78] done with phospholipase D helped in developing the above protocol.

3.7. Determination of Phospholipids by HPLC

The phospholipids in the lecithin were quantified using HPLC. The sample was dissolved in acetonitrile and 10μL of it was injected into the Shimadzu (Kyoto, Japan) HPLC system after passing it through a 0.2μm syringe filter (Merck Millipore Ltd., Darmstadt, Germany). HPLC was setup in HILIC mode with a Phenomenex Luna NH2 column (150 mm X 4.6 mm, 5μm). HILIC mode comprises of silica surface covered with cross-linked diol groups which helps in separating polar compounds. The mobile phase was set up in gradient mode going from 5% water and 95% acetonitrile to 50% water and 50% acetonitrile over 15 minutes at a flow rate of 1 mL/min. The phospholipids were detected using an evaporative light scattering detector operating at 30^0C. Standard 1,2-dioleylphosphatidylcholine, 1,2-dioleylphoshphatidylethanolamine, and 1,2-dioleylphosphatidic acid were used to prepare the standard curves. The phospholipids in the lecithin were identified and quantified by using the relative retention times and peak areas of the standards.

3.8. Interaction Index

Interaction Index [79-81] was calculated in order to find if interaction between antioxidants was synergistic. The formula used for the calculation was: {(observed lag phase of combination – lag phase of control) / [(lag phase of tocopherol alone – lag phase of control) + (lag phase of phospholipid alone – lag phase of control)]. An interaction index value of > 1 indicates a synergistic interaction between the antioxidants, a value =1 indicates additive effect and a value < 1 indicates antagonistic interaction between the antioxidants.

3.9. Statistical analysis

Results are presented as means and standard deviations of analyses conducted in triplicate. Oxidation lag phases were defined as the first data point statistically greater than day zero within each treatment tested using one-way analysis of variance (ANOVA) with comparison of the oxidation measurement means performed using Tukey's HSD post hoc test ($p = 0.05$). Calculations were performed using Minitab version 18 (State College, PA, USA).

CHAPTER 4

RESULTS

4.1. Impact of pure PE with or without α-tocopherol on SSO Oxidation

The SSO control had a lag phase for both lipid hydroperoxides and hexanal formation of 4 days. Addition of 1000 μmol and 2000 μmol pure PE/kg oil reduced the hydroperoxide lag phase to 2 days and 0 days, respectively. Both concentrations did not change hexanal lag phases compared to the control. Addition of 100 μmol α-tocopherol/kg oil extended the hydroperoxide and hexanal lag phase to 22 and 20 days respectively. When 1000 μmol PE/kg of oil was added with α-tocopherol to the oil it extended the hydroperoxide lag phase to 30 days and hexanal lag phase to 34 days which produced interaction indexes of 1.6 and 1.9, respectively, indicating that PE could increase the antioxidant activity of α-tocopherol synergistically. The combination of 2000 μmol PE/kg of oil and α-tocopherol extended the hydroperoxide lag phase to 26 days and hexanal lag phase to 24 days which produced interaction indexes of 1.8 and 1.3 respectively. Synergism exhibited between PE and α-tocopherol could be due to regeneration of α-tocopherol by PE [50] or could be due to other antioxidant properties of PE such as metal chelation [56].

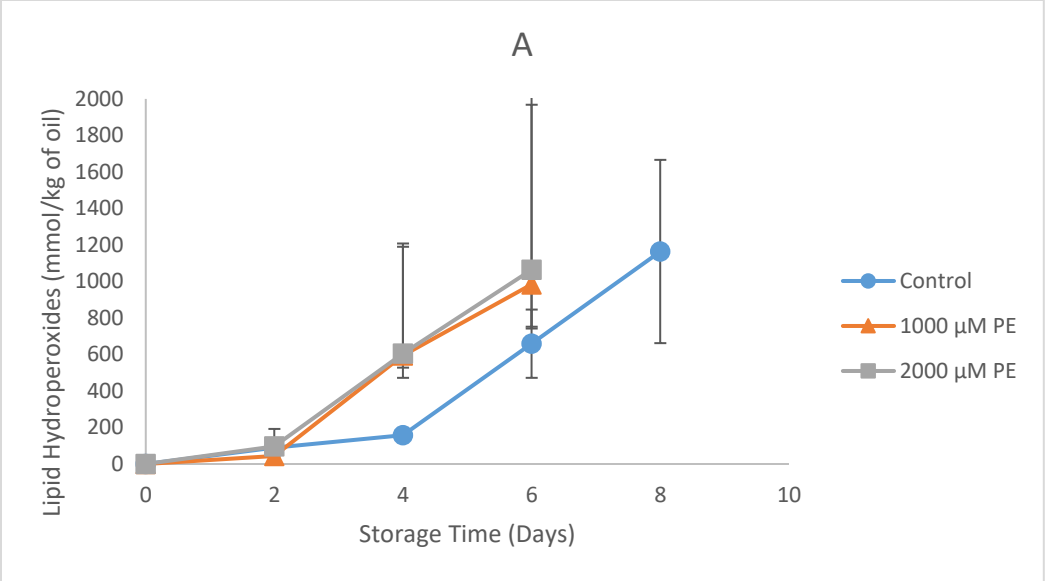

Figure 4.1 Lipid hydroperoxides of SSO with 1000 µM or 2000 µM pure PE concentrations at 55^{0}C

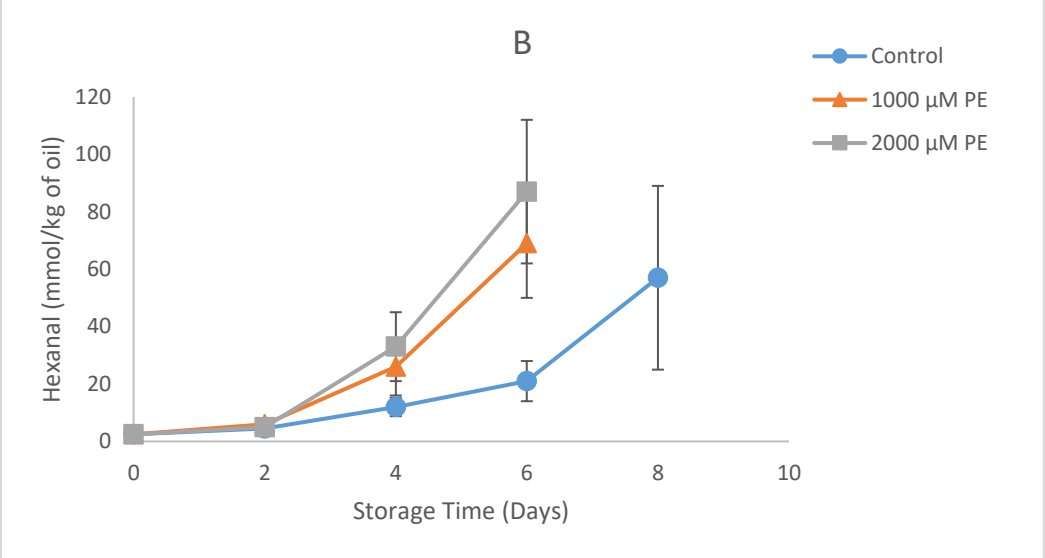

Figure 4.2 Hexanal of SSO with 1000 µM or 2000 µM pure PE concentrations at 55^{0}C

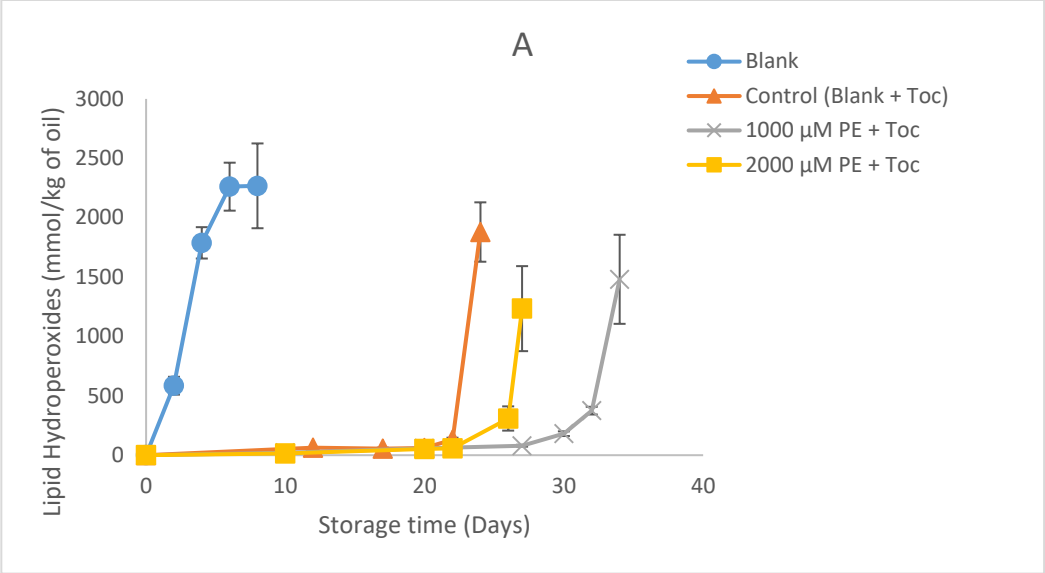

Figure 4.3: Lipid Hydroperoxides of SSO with 100 µM α-tocopherol with addition of 1000 µM or 2000 µM pure PE concentrations at 55^{0}C

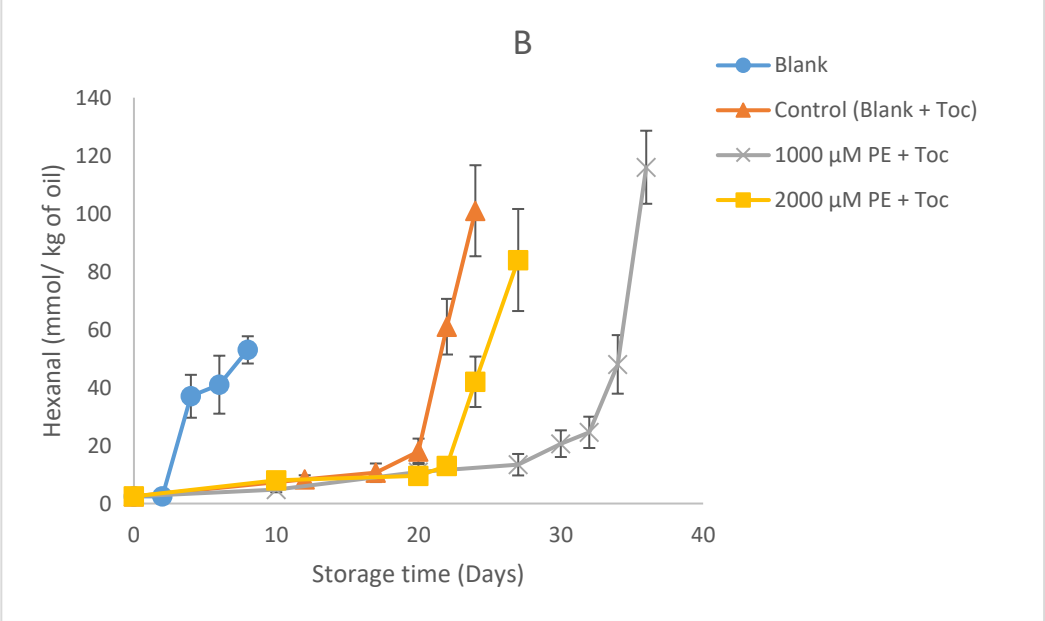

Figure 4.4: Hexanal of SSO at SSO with 100 µM α-tocopherol with addition of 1000 µM or 2000 µM pure PE concentrations at 55^{0}C

4.2. Enzymatically modifying lecithin to increase the PE/PC ratio

The PC, PE and PA composition of the egg lecithin used for the experiment was analyzed using reverse phase HPLC. The yield of the enzymatic reaction is dependent upon factors such as pH, substrate concentrations and temperature. This experiment was carried out to optimize pH to get maximum conversion to PE. The final conversion conditions were pH 10.3 obtained by adding 0.2 mL buffer and 1 mL of 1N hydrochloric acid to the ethanolamine. The enzymatic reaction was carried out at 37^0C for 40 minutes. The phospholipid composition of the egg lecithin after the enzymatic modification is shown in Table 4.1.

Phospholipids	Unmodified Lecithin (%wt)	Modified Lecithin(%wt)
PE	33.4	89.9
PC	61.7	3
PA	0.1	7.1

Table 4.1. Initial and final phospholipid composition of the egg lecithin before and after phospholipase D conversion.

4.3. Impact of Phospholipase D modified lecithin with or without α-tocopherol on SSO oxidation

The SSO control had a lag phase for both lipid hydroperoxides and hexanal formation of 2 days. Addition of 1000 μmol of unmodified lecithin/kg of oil did have any effect on the hydroperoxide and hexanal lag phase as compared to the control (Figure 4.5 and 4.6). However the addition of 1000 μmol of phospholipase D modified lecithin/kg of oil caused both the hydroperoxide and hexanal lag phases was extended to 4 days, respectively. Similar results were obtained with 2000 μmol unmodified lecithin PE/kg oil where the hydroperoxide and hexanal lag phases both were 2 days. Whereas on the addition of 2000 μmol phospholipase D modified lecithin PE/kg oil, the hexanal lag phase was extended to 4 days but the hydroperoxide lag phase was unchanged at 2 days.

Addition of 100 μmol α-tocopherol/kg oil to the SSO extended the hydroperoxide and hexanal lag phase to 27 and 25 days respectively (Figure 4.7 and 4.8). When 1000 μmol unmodified lecithin/kg of oil was added with α-tocopherol in the oil it reduced the hydroperoxide lag phase to 20 days and hexanal lag phase to 22 days (Figure 4.7 and 4.8) giving interaction indexes of 0.7 and 0.9, respectively, indicating an antagonistic interaction. The combination of 1000 μmol phospholipase D modified lecithin/kg of oil and α-tocopherol extended the hydroperoxide lag phase to 34 days and hexanal lag phase to 32 days producing interaction indexes of 1.2 for both. When 2000 μmol unmodified lecithin/kg of oil was added with α-tocopherol in the oil it reduced the hydroperoxide lag phase to 20 days and hexanal lag phase to 20 days producing interaction indexes of 0.7 and 0.8 respectively, resulting in a

decreased in oxidative stability compared to tocopherol alone (Figure 4.9 and 4.10). The combination of 2000 μmol phospholipase D modified lecithin/kg of oil and α-tocopherol did not change the hydroperoxide lag phase compared to α-tocopherol alone but the hexanal lag phase was extended by 4 days compared to tocopherol alone which produced interaction index of 1.1.

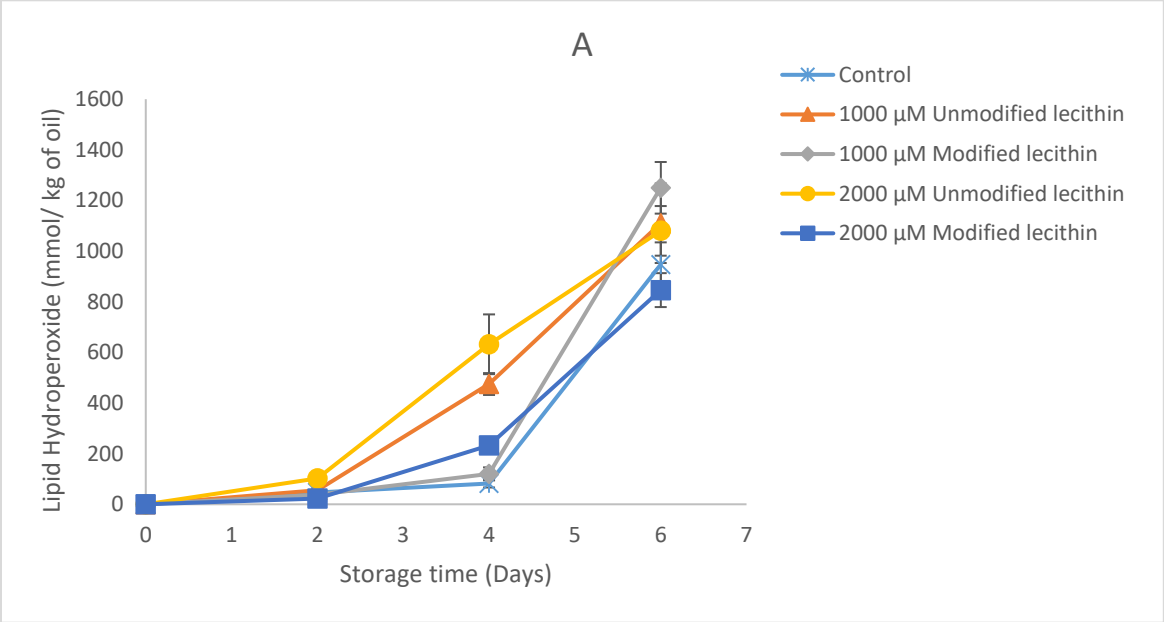

Figure 4.5: Lipid Hydroperoxides of SSO with 1000 μM or 2000 μM Modified or Unmodified Lecithin concentrations at 55^{0}C

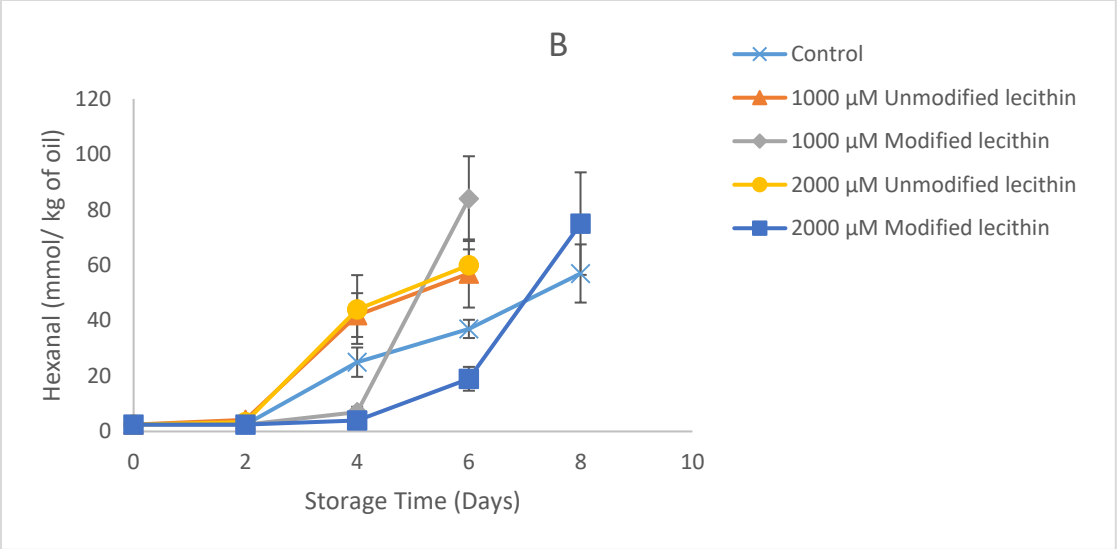

Figure 4.6: Hexanal of SSO with 1000 µM or 2000 µM Modified or Unmodified Lecithin concentrations at 55^{0}C

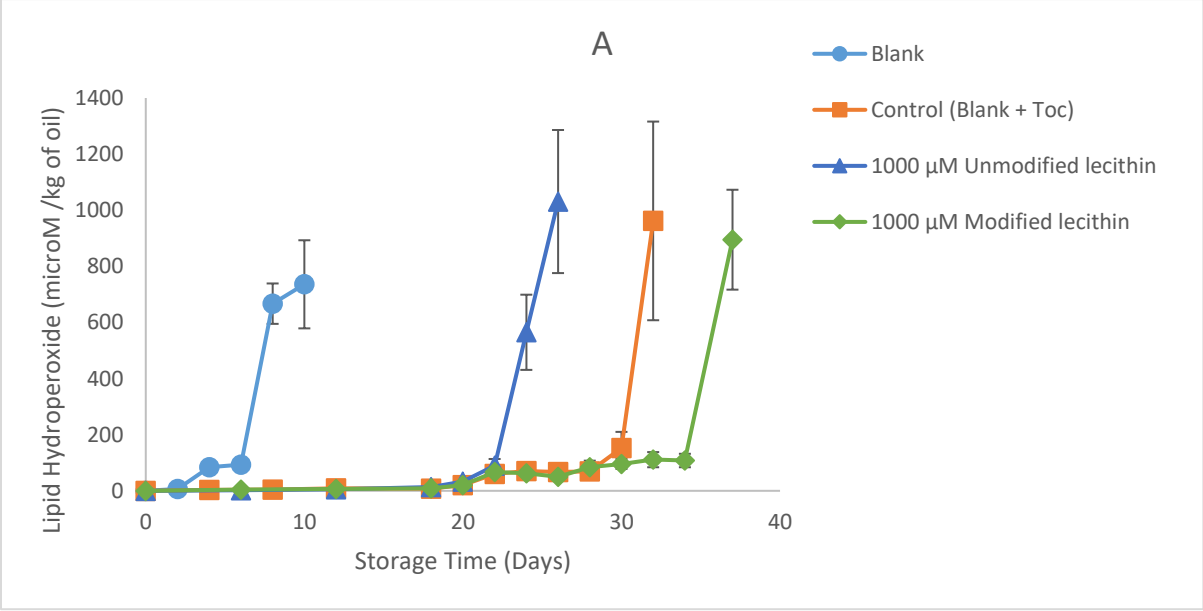

Figure 4.7: Lipid hydroperoxides of SSO with 100 µM α-tocopherol with addition of 1000 µM modified or unmodified lecithin concentrations at 55^{0}C

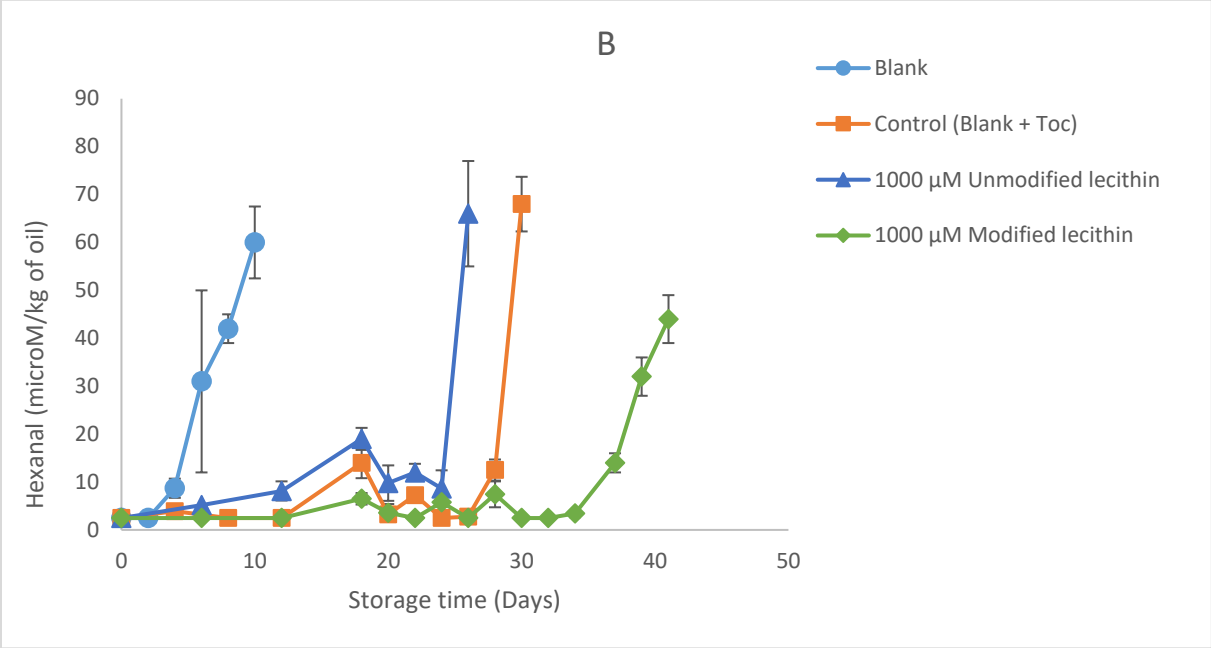

Figure 4.8: Hexanal of SSO with 100 µM α-tocopherol with addition of 1000 µM modified or unmodified lecithin concentrations at 55^{0}C

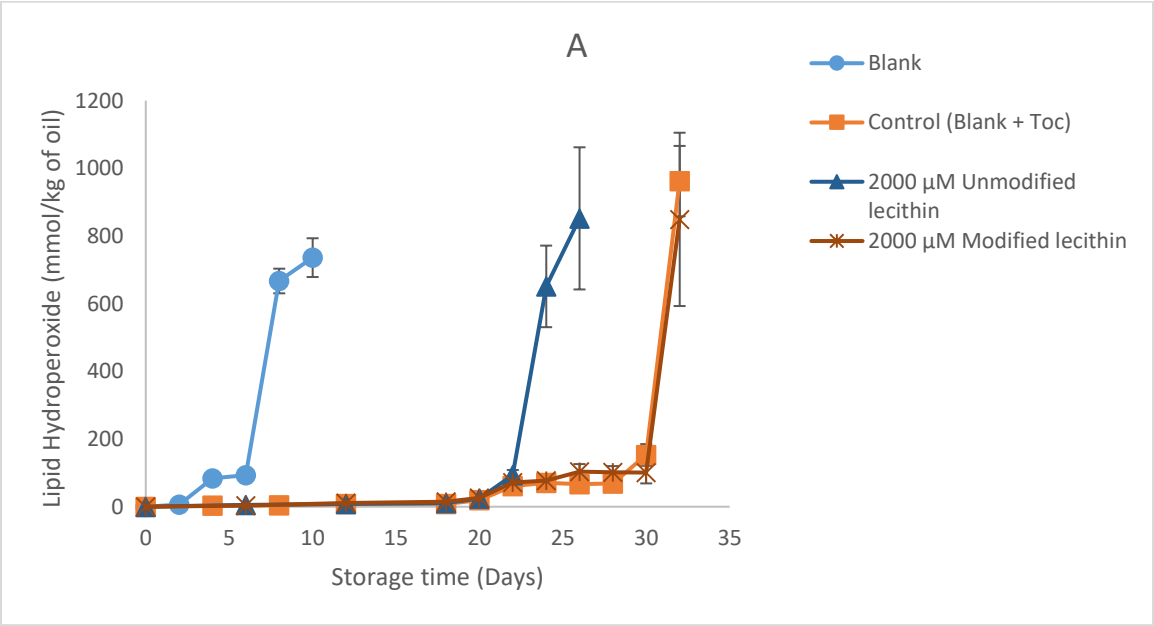

Figure 4.9: Lipid Hydroperoxides of SSO with 100 µM α-tocopherol with addition of 2000 µM modified or unmodified lecithin concentrations at 55^{0}C

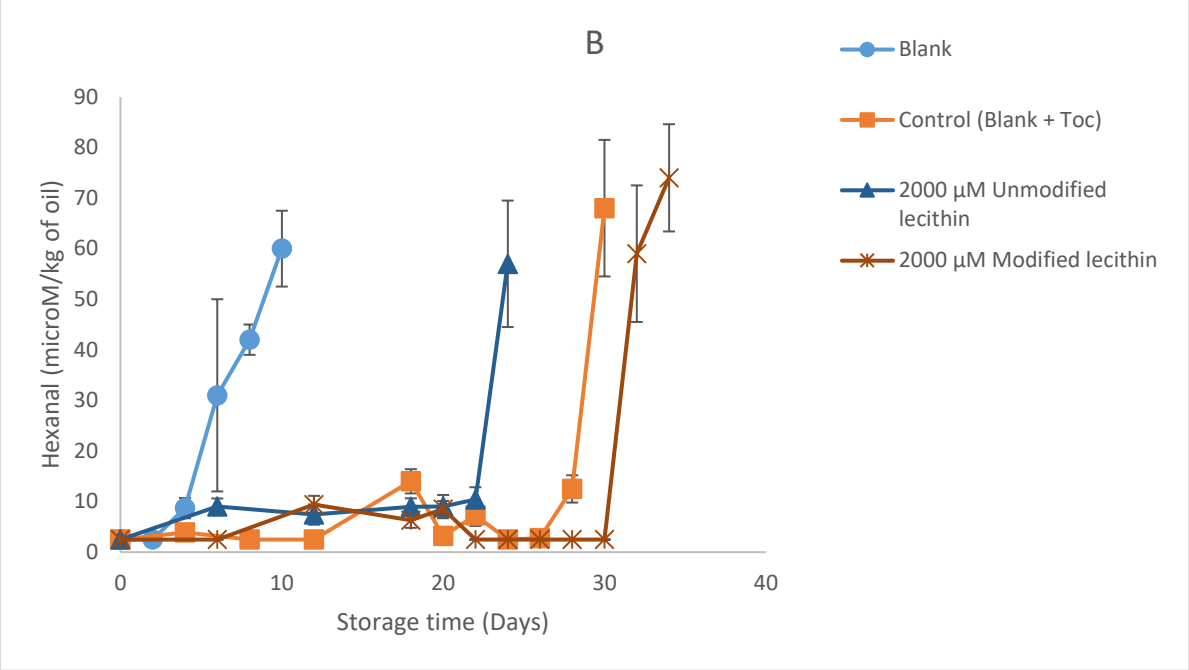

Figure 4.10: Hexanal of SSO with 100 µM α-tocopherol with addition of 2000 µM modified or unmodified lecithin concentrations at 55^{0}C

4.4. Effect of Phopholipase D modified lecithin on the oxidative stability of commercial vegetable oil

The effect of the phospholipase D modified lecithin on the oxidative stability of refined oil was analyzed. Commercial soybean oil used generally contains natural tocopherols in it (≅800 ppm)[5]. This study determined the ability of phospholipase D modified lecithin at 500, 1000, 2000, 3500, and 5000 µmole/ kg of oil concentrations to increase oxidative stability. The phospholipase D modified lecithin had little impact on hydroperoxide lag phases compared to the oil only control with most treatment having a lag phase of 4-6 days. The hexanal results showed that the control had a lag phase of 16 days. The samples containing 500 and 1000 µmole lecithin/kg of oil exited the hexanal lag phase at the same time as the control. Increasing

the phospholipase D modified lecithin to 2000, 3500 and 5000 µmole modified lecithin/kg increased the hexanal lag phase extension to 20, 22 and 22 days respectively.

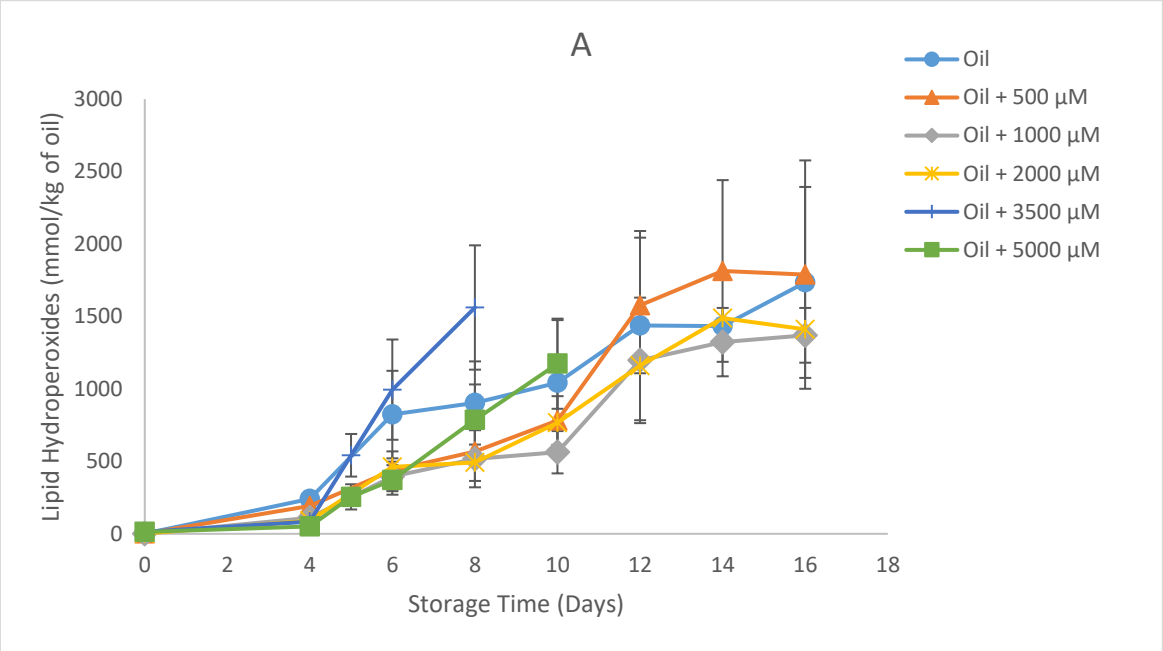

Figure 4.11: Lipid hydroperoxides of commercial vegetable oil with addition of 500, 1000, 2000, 3500, and 5000 µM modified lecithin concentrations at 55^{0}C.

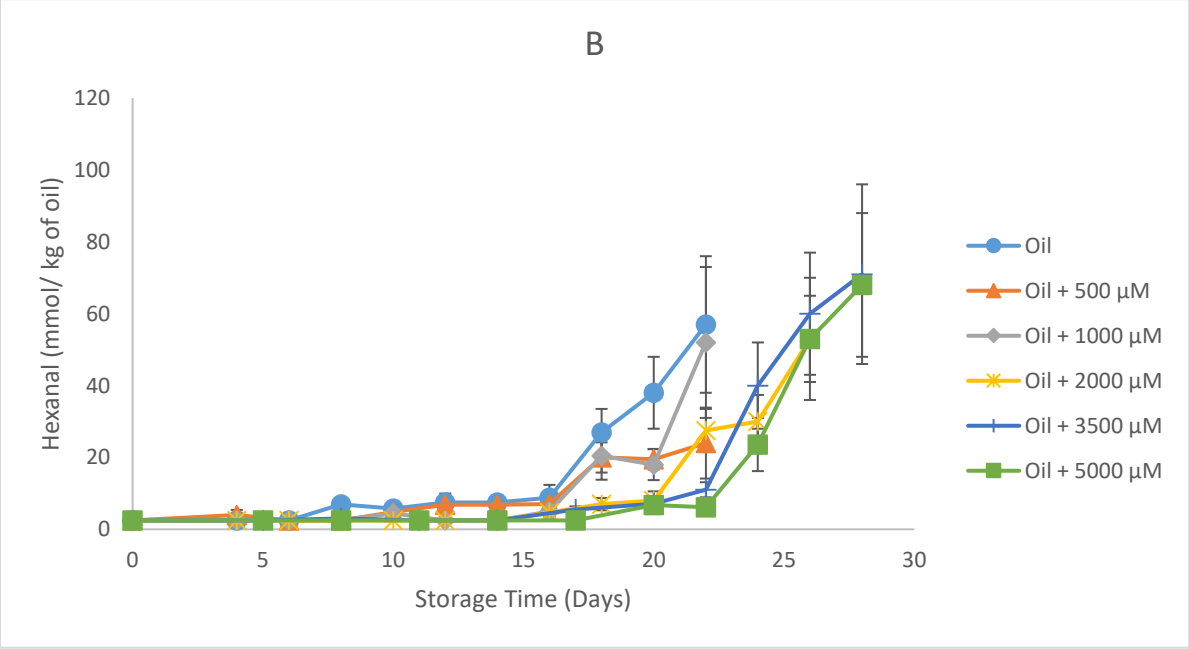

Figure 4.11: Hexanal of commercial vegetable oil with addition of 500, 1000, 2000, 3500, and 5000 µM modified lecithin concentrations at 55^{0}C

CHAPTER 5

DISCUSSION AND CONCLUSION

In the presence of 1000 and 2000 μmol PE/kg oil concentrations in the absence of tocopherols (Figure 4.1 and 4.2), the rates of lipid hydroperoxide and hexanal formation increased compared to the control. This pro-oxidant activity of PE could be due to the formation of prooxidative association colloids since the PE concentrations were above their CMC values[5, 77].

In the presence of tocopherols, 1000 μmol of isolated PE/kg of oil synergistically increased the oxidative stability of the SSO. This synergistic activity could be due to the ability of PE to regenerate oxidized α-tocopherol since in the absence of tocopherols, pure PE was prooxidative. Previous studies have reported the regeneration of α-tocopherylquinone to α-tocopherol by phospholipids containing amino groups [5, 50]. However, increasing PE to 2000 μmol of isolated PE/kg of oil decreased oxidative stability compared to 1000 μmol of isolated PE/kg of oil. It unclear why this prooxidant activity occurred but the higher PE level could increase the number of proxidative association colloids which might decrease the positive effect of tocopherol regeneration.

Using pure PE as an antioxidant is not commercially viable due to its high cost. Lecithin, is an inexpensive by-product of oil refining [64] but it's low levels of PE might not allow it to promote the regeneration α-tocopherol to synergistically inhibit lipid oxidation. Phospholipase D has transphosphatidylation activity which can convert PC in the presence of primary alcohols such as ethanoamine to PE [7-11, 71, 82, 83]. However, phospholipase D can also remove

choline from PC in the presence of water to form phosphatidic acid (PA) [6]. In order to get maximum conversion to PE, it was essential to avoid water in the system and maintain the pH in the alkaline range. In this study we were able to increase the PE/PC ratio in egg lecithin using phospholipase and ethanolamine. Egg lecithin was used for the experiment since it contains the highest amount of PC amongst the commercial lecithins [65].

When unmodified lecithin was added to SSO in the absence of tocopherol, a prooxidative effect was observed (Figure 4.6 and 4.7). This again could be due to the formation of prooxidative association colloids since the level of PE and PC in the unmodified lecithin is above their CMCs[5]. Unlike isolated PE, phospholipase D modified lecithin by itself was slightly antioxidative in the absence of α-tocopherol. It is unclear why this occurred in phospholipase modified lecithin but not in isolated PE but it's possible that other components in the lecithin could also inhibit lipid oxidation. Previous studies show that lecithin exhibits antioxidant activity depending upon its phospholipid concentration as well as the dose [51, 55]. Sugino[55] showed that egg yolk lecithin containing high amounts of PE was able to exhibit antioxidant activity in DHA rich oil.

The combination of unmodified lecithin and α-tocopherol resulted in lower oxidative stability than α-tocopherol itself (Figure 4.5 and 4.6). This could be due to the high level of prooxidative PC (≅60%) in the unmodified lecithin would overcome any positive effect due to PE regeneration of α-tocopherol. The phospholipase D modified lecithin at 1000 μmol levels was able to synergistically increase the antioxidant activity of α-tocopherol increasing the hydroperoxide and hexanal lag phase by 4 and 5 days, respectively. This agrees with the studies with the isolated PE and since isolated PE and phospholipase D modified lecithin were not

strong antioxidants by themselves this again suggests that the enhanced antioxidant activity of PE and α-tocopherol combinations is due to α-tocopherol regeneration by PE. However, when phospholipase D modified lecithin was increased to 2000 μmol, enhanced antioxidant activity with α-tocopherol was decreased. This also occurred with isolated PE. It's again unclear why higher levels of PE would decrease antioxidant activity but it's possible that at high PE levels, new types of association colloids could be formed or association colloid numbers would increase which could that inhibit the ability of PE and α-tocopherol to interact. Finally, addition of the phospholipase D modified lecithin to commercial soybean oil showed an increase in the oil stability in a dose dependent manner. Unlike isolated PE or phospholipase D modified lecithin added to SSO with α-tocopherol, increasing PE concentrations did not decrease the ability of phospholipase D modified lecithin to increase hexanal formation in the refined oil. Since the refined oil contains multiple components that could impact lipid oxidation reactions (e.g. FFA, mono- and diglycerols, carotenoids, etc.) it's difficult to know why antioxidant activity continued to increase with increasing PE concentrations. However, the ability of the phospholipase D modified lecithin to increase the lag phase of hexanal formation by 5 days suggests that phospholipase D modified lecithin could be a viable strategy to increase the antioxidant activity of commercial oils.

This research shows good conversion of PE from PC in egg lecithin using phospholipase D, which has not been done previously. The combination of this phospholipase D modified lecithin with α-tocopherol was able to give good oxidative stability in bulk oils increasing the lag phase 1.2 fold. Phospholipase D modified lecithin could therefore be used as a good clean label antioxidant strategy however the biggest challenge here is identifying a plant origin lecithin with naturally high PC that can be converted to PE. Most lecithins of plant origin are commercially

available but they contain low amounts of PC and PE which make them less viable for the enzymatic conversion.

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