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## **Novel Approaches for Preventing Lipid Oxidation in Emulsion-Based Food Systems**

Mitchell D. Culler  
*University of Massachusetts Amherst*

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**NOVEL APPROACHES FOR PREVENTING LIPID OXIDATION IN EMULSION-  
BASED FOOD SYSTEMS**

A Dissertation Presented

by

MITCHELL DEWITT CULLER

Submitted to the Graduate School of the  
University of Massachusetts Amherst in partial fulfillment  
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

September 2022

Food Science

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BASED FOOD SYSTEMS**

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MITCHELL D. CULLER

Approved as to style and content by:

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Eric A. Decker, Chair

---

David J. McClements, Member

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Lynmarie K. Thompson, Member

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Jaikai Liu, Member

---

Lynne A. McLandsborough, Department Head  
Food Science

## DEDICATION

This work is dedicated to my late chameleon, Mittens. He and I first met in a Pennsylvania pet store a few months into my master's degree, and I was immediately won over by his exuberant arm waving, color, and, of course, mitten-like hands. Taking care of Mittens became a daily ritual while I completed my master's degree, moved to UMass and pursued my Ph.D. until he passed away just a few months before I finished the lab work for this dissertation. I am deeply grateful to have had the emotional support and friendship of this tiny dinosaur throughout the course of my graduate school career.

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I would first like to thank my advisor, Dr. Eric Decker, for his constant guidance, advice, patience, and support over the past several years. Getting the opportunity to work and learn in his lab has had a profoundly positive impact on my development as a scientist and my personal growth as an individual.

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Lastly, I'm deeply grateful to have been aided by the support of my friends, the love from family, and the comradery of my labmates.

## **ABSTRACT**

### **NOVEL APPROACHES FOR PREVENTING LIPID OXIDATION IN EMULSION-BASED FOOD SYSTEMS**

SEPTEMBER 2022

MITCHELL D. CULLER, B.A., THE PENNSYLVANIA STATE UNIVERSITY

B.S., THE PENNSYLVANIA STATE UNIVERSITY

M.S., THE PENNSYLVANIA STATE UNIVERSITY

Ph.D. UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Eric A. Decker

Consumer interest in “clean” labels has continued to be a key driver of consumer behavior and purchasing habits over the past decade. Food manufacturers are therefore eager to replace synthetic antioxidants such as ethylenediaminetetraacetic acid (EDTA) with natural alternatives, however alternative chelators that will bind iron at low pH remain elusive. Coupled with the current oil shortages and supply chain challenges that have arisen recently, there is an urgent need for innovative solutions to increase the oxidative stability of edible oils. One available strategy is diluting oils high in unsaturated fatty acids into more stable, more saturated oils, thus delaying lipid oxidation by decreasing free-radical propagation reactions between oxidized fatty acids and unsaturated lipids. The effect of diluting fish oil into medium-chain triglycerides (MCTs) on oxidative stability was investigated using lipid hydroperoxides and gas chromatography headspace analysis. Dilutions up to 1 in 20 of fish oil in MCT extended propanal formation from 1 to 6 days in Tween-80-stabilized oil-in-water emulsions. This protective effect was not observed in emulsions wherein the two oils were in separate droplets. Fish oil blended with high oleic sunflower oil (HOSO) also demonstrated a protective effect

when the oils were in the same emulsion droplets but not in separate emulsion droplets. The present study indicates that dilution can be used to increase the oxidative stability of polyunsaturated fatty acids in oil-in-water emulsions.

Another potential strategy relies on oxidized  $\alpha$ -tocopherol being regenerated by phosphatidylethanolamine (PE). Although current commercial sources of PE are too expensive for use as a food additive, the present study aims to determine the optimal reaction conditions for generating high PE lecithin (MHPEL) enzymatically and to validate the MHPEL's synergism with tocopherol in delaying lipid oxidation in an oil-in-water emulsion systems at pH 7, and 4, and in bulk oil. Under optimal conditions of pH 9.0, 37°C and 4h, a MHPEL with ~71.6% PE was obtained from 96% phosphatidylcholine lecithin using phospholipase D from *Streptomyces chromofuscus*. Compared to mixed tocopherols alone, the addition of MHPEL synergistically increased the both the hydroperoxide and hexanal lag phase of lipid oxidation in oil-in-water emulsions by 3 days in o/w emulsions at pH 7 and 3 and 2 days, respectively, at pH 4. In combination with 50  $\mu\text{mol/kg}$  oil of  $\alpha$ -tocopherol, the addition of 1000  $\mu\text{mol/kg}$  oil MHPEL synergistically increased the lag phases by 5 and 4 days in bulk oil compared to tocopherol alone. The novel approaches contained herein represent potential clean-label strategies for increasing the oxidative stability of food systems and have strong potential for commercial applications to decrease food waste.



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## LIST OF COMMON ABBREVIATIONS

DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine)

GC (Gas Chromatography)

HOSO (High Oleic Sunflower Oil)

MCT (Medium Chain Triglycerides)

MHPEL (Modified High PE Lecithin)

PA (Phosphatidic Acid)

PC (Phosphatidylcholine)

PLD (Phospholipase D)

PLD<sub>sc</sub> (Phospholipase D from *Streptomyces chromofuscus*)

PE (Phosphatidylethanolamine)

SHOSO (Stripped High Oleic Sunflower Oil)

toc (tocopherol)

## Chapter 1

### INTRODUCTION

Currently, a problem exists in the food industry where consumers are looking to increase their consumption of unsaturated fats while simultaneously decreasing their consumption of ingredients perceived to be “synthetic.” This creates a unique challenge for food manufacturers because polyunsaturated fats are less stable than saturated fats and are typically stabilized using synthetic antioxidants such as butylated hydroxytoluene (BHT) or tert-butylhydroquinone (TBHQ). In some cases, evidence has shown that these antioxidants may pose health risks to consumers<sup>1</sup>. This challenge is augmented in food emulsion systems since the multiple phases in the system as well as the presence of emulsifiers can impact the partitioning of the antioxidants and thus their efficacy. In the following body of research, two promising methods for naturally retarding the rate of lipid oxidation are examined.

The first relies on diluting an oil containing primarily polyunsaturated fatty acids with a more oxidatively stable oil. It has been suggested that when diluting oils rich in polyunsaturated fatty acids in oils high in saturated fatty acids, the rate of lipid oxidation becomes slower because it takes longer for lipid radicals to diffuse to and to oxidize unsaturated fatty acids. So far, the literature in this area leaves it unclear if the inhibition of oxidation in oil-in-water emulsions was due to dilution or was due to other antioxidant factors originating in the specific food systems used. Therefore, the purpose of this study will be to determine if fish oil stability could be increased by dilution into a more oxidatively stable oil and if increased stability was due to dilution or components of the diluting oil.

The second strategy investigated here relies on using a modified lecithin to increase the efficacy of tocopherol. Commercial lecithins are made up of phospholipids which are

amphiphilic molecules that are often used to stabilize emulsions. Although phosphatidylcholine (PC) from soybean has been shown to have pro-oxidant activity in bulk oil at high levels<sup>2</sup>, phosphatidylethanolamine (PE) and phosphatidylethanolamine (PS) can convert the oxidized form of  $\alpha$ -tocopherol ( $\alpha$ -tocopherol quinone) back to  $\alpha$ -tocopherol, thus extending the tocopherol's ability to delay lipid oxidation. PC is unable to participate in this reaction because it is a non-amine phospholipid, and plant sources of lecithin are typically low in PE and PS which poses a further challenge for vegan or vegetarian foods. Furthermore, purified phospholipids are typically too expensive for usage in food, and are not "label friendly" to consumers.

Phospholipase D (PLD) is an enzyme that cleaves off the polar head group on the phospholipid and can then catalyze the transphosphatidylation of a different headgroup. For instance, it could convert the PC in the lecithin to PE or PS with PA as an intermediate. This could allow for the creation of a new lecithin to work synergistically with tocopherol to further delay lipid oxidation in emulsified products. The amount of PC and PA in the lecithin will need to be kept at a minimum, which is why it is important to have an understanding of how the reaction parameters affect the product. So far, most of the research in this area has used hydrolysis assays as an indicator of enzyme activity instead of measuring the actual transphosphatidylation reactions. Since transphosphatidylation has the extra step of adding a headgroup, it seems likely that there would be differences in the optimizations of these two reactions. Therefore, the purpose of this research will be to evaluate the optimal conditions for converting high PC lecithin to high PE lecithin and testing how this modified lecithin can work synergistically with tocopherol in modified emulsion systems.

## Chapter 2

### LITERATURE REVIEW

#### Lipid Oxidation

##### Overview

Lipid oxidation consists of a series of chemical reactions that affect the chemical makeup of fats and oils during processing and storage and lead to off flavors and aromas in food products eventually causing food waste. The three-part lipid oxidation reaction consists of initiation, propagation, and termination. The initiation step occurs when a fatty acid radical is produced, the alkyl radical. The alkyl radical then reacts with oxygen, a biradical, to form a peroxy radical. The peroxy radical is very reactive and can abstract a hydrogen from another unsaturated fatty acid. This produces a lipid hydroperoxide on the original fatty acids and an alkyl radical on a second fatty acid thus propagating the free radical chain reaction. This reaction pathway is terminated when two lipid radicals combine to form non-radical species. An addition step, the  $\beta$ -scission reaction, occurs via the decomposition of lipid hydroperoxides to form an alkoxy radical which in turn can abstract electrons from the alkyl chain to break the fatty acid into smaller molecules. These breakdown products are what produce off-flavors or aroma that are classified as rancidity. The reaction typically proceeds very quickly after an initial lag phase, the length of which is determined by numerous factors which are discussed below.

##### Significant Factors Affecting Lipid Oxidation

Degree of Fatty Acid Saturation: Having a double-bond in the fatty acid chain significantly lowers the energy needed to remove a hydrogen to form an alkyl radical (the initiation and propagation steps). A significant increase in oxidation rates occurs between a

mono- and di-unsaturated fatty acid due to two double bonds of the pentadiene system decreasing the carbon-hydrogen bond disassociation energy of the methylene interrupted carbon thus making hydrogen abstraction to form an alkyl radical more thermodynamically favorable. As more double bonds are added, the fatty acids are more prone to oxidation due to additional reaction sites for hydrogen abstraction.

Presence of Prooxidant Substances: Many substances such as metal ions, enzymes and compounds that can produce reactive oxygen species (e.g. riboflavin produced singlet oxygen) are capable of decomposing hydroperoxides to free radical (metals) or forming fatty acid hydroperoxides (e.g. lipoxygenase and singlet oxygen).

Environmental Factors: High temperatures, light, water activity, free fatty acids and the presence of oxygen all have the potential to increase the rate of the lipid oxidation reaction.

Lipid oxidation causes the formation of a wide array of products. Analyses of these byproducts are typically used to assess the state of lipid oxidation in a food. The products can be categorized into two main groups: primary and secondary lipid oxidation products.

### **Primary Oxidation Products**

Primary lipid oxidation products include conjugated dienes and trienes as well as lipid hydroperoxides. As shown in Figure 1, the primary lipid oxidation products are formed in the initial stages of lipid oxidation.

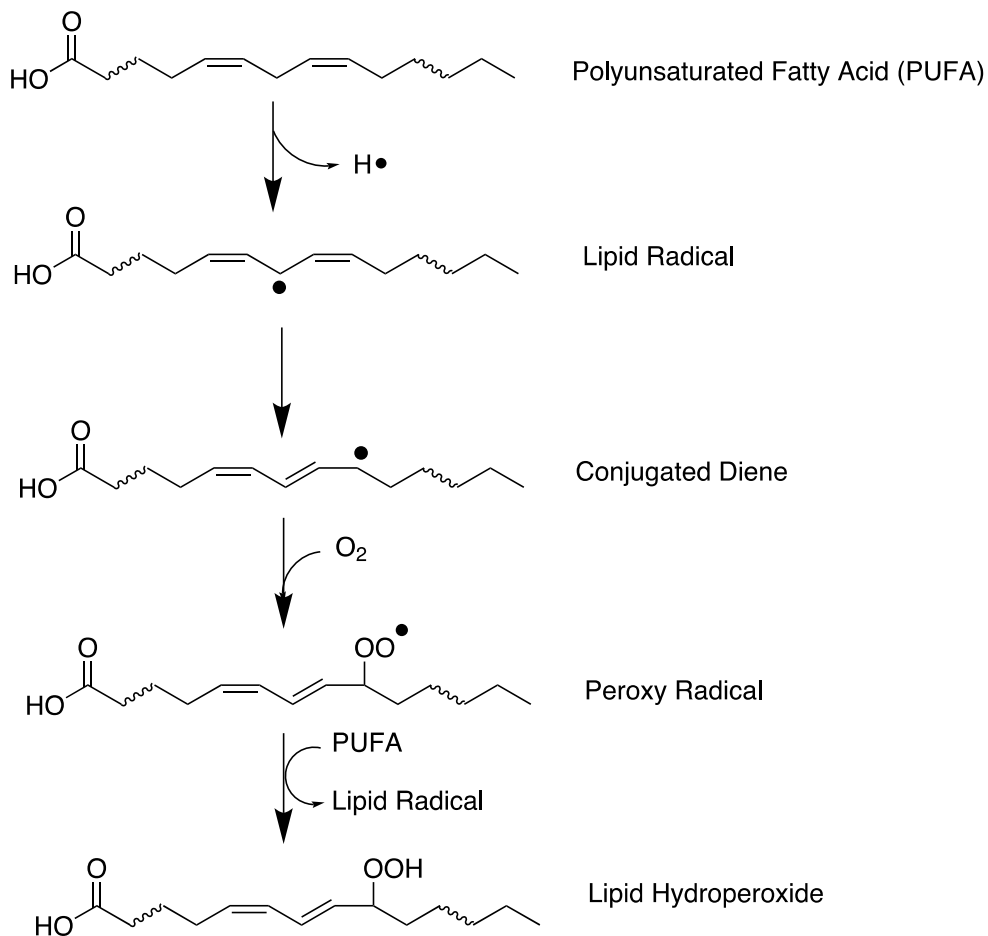


Figure 2.1. Schematic of primary lipid oxidation product formation. Figure created using ChemDraw JS.

### Conjugated Dienes and Trienes

Conjugated double bonds are formed when the alkyl free radical formed between the methylene interrupted double bonds of a polyunsaturated fatty acid shifts to produce the conjugation configuration. In the case of a fatty acid with three or more double bonds, a conjugated triene can be formed. It should be noted that conjugated trienes are sometimes not considered primary products as they are created by two oxidation events, e.g. the formation of 2 alkyl radicals and 2 double bond shifts. Since conjugated double bonds absorb light, it is possible to measure their existence at 233 and 268 nm for conjugated dienes and trienes respectively. This technique is not very sensitive and must be performed on isolated lipids since

many other compounds with conjugated double bonds will also absorb light at these wavelengths.

### **Lipid Hydroperoxide Formation**

After the formation of the alkyl radical, oxygen is added to the fatty acids by a diffusion limited radical-radical reaction to form the peroxy radical. Since the peroxy radical has higher energy than the alkyl radical, it can abstract a hydrogen from another unsaturated fatty acid or an antioxidant to form a lipid hydroperoxide. The determination of lipid hydroperoxides is one of the most widely used methods for detection of lipid oxidation in industrial food applications and fundamental research. Most lipid hydroperoxides methods are based on the ability of the hydroperoxide to oxidize an indicator molecule. For example, in bulk oils, hydroperoxides are often measured by the oxidation of iodine to iodide. The iodide formed (which is directly related to the lipid hydroperoxide content) is measured by titrating with a sodium thiosulfate to reduce it back to iodine which can be detected by forming a colorimetric complex with starch.

Spectrophotometric methods which rely on hydroperoxide's ability to oxidize  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  are also frequently employed for determining lipid hydroperoxides.  $\text{Fe}^{3+}$  specific indicators such as ammonium thiocyanate or xylenol orange are used to form colorimetric complexes which can be quantified with a spectrophotometer. As with conjugated dienes, this assay must be run with isolated lipids since foods can contain non-lipid oxidizing compounds. Thus, these assays are easily conducted in bulk lipids while in other foods the lipids must be isolated prior to analysis.

Primary products are unstable as they can be further oxidized by free radicals (conjugated dienes) or they can be decomposed by prooxidants (e.g. iron promoted hydroperoxide decomposition). Therefore, as lipid oxidation proceeds, primary oxidation products will initially increase and then subsequently decrease in the later stages of oxidation as the rate of primary

product formation becomes slower than the rate of primary product decomposition. An example of this phenomenon is shown in Figure 2. The phenomenon of hydroperoxide formation and decomposition has been modeled successfully using a decay factor superimposed on an accumulation term [1]. This can lead to a misinterpretation of results as low concentration of primary products can be seen in highly oxidized lipids. When the primary products decompose, they form secondary lipid oxidation products. By measuring both primary and secondary lipid oxidation products, one can determine if low primary product concentration are due to their decomposition as this would produce high secondary lipid oxidation product concentrations.

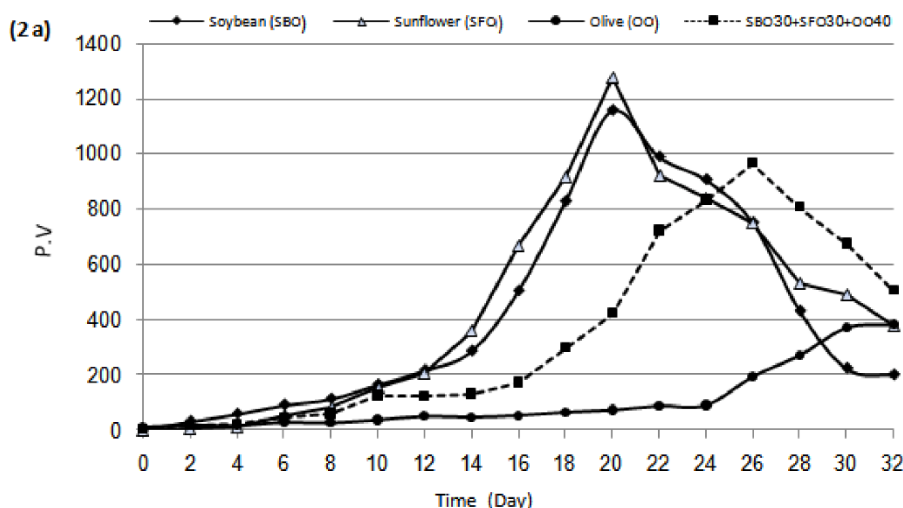


Figure 2.2. Peroxide values data showing the formation of lipid hydroperoxides and the subsequent decrease, presumably as more and more of the primary lipid oxidation products are converted to secondary products <sup>3</sup>.

### Secondary Oxidation Products

Although hydroperoxides are frequently used in industry to evaluate oil quality, it is the secondary, volatile products that are responsible for the off-flavors and aromas associated with oil rancidity<sup>4,5</sup>. As the lipid oxidation reaction progresses, lipid hydroperoxide decompose into alkoxy radicals, which in turn can breakdown the fatty acid chain via  $\beta$ -scission. These secondary breakdown products can vary depending on the location of the hydroperoxide, the



molecular size and composition of the fatty acid and the pathway of the  $\beta$ -scission reaction resulting in the formation of numerous products of different chain length such as aldehydes, ketones, epoxides and alcohols<sup>6</sup>. When  $\beta$ -scission occurs, two different products are formed, one on the methyl end of the fatty acid and the other on the acid end. The products from the methyl end are most often associated with rancidity since they are more volatile than products from the acid end which is typically bonded to glycerol meaning that they have high molecular weights and are not volatile. Since hydroperoxides form at different locations along the fatty acid chain, multiple products can be formed. Oleic acid most commonly forms hydroperoxides at carbons 8, 9, 10 and 11. If oleic acid forms a hydroperoxide at carbon 11 the methyl end breakdown product is octanal; a hydroperoxide at carbon 10 forms nonanal; a hydroperoxide at carbon 9 forms decanal and so on. The breakdown product from the methyl end can also include the double bond. For example, a hydroperoxide on carbon 9 can form 2-decenal and carbon 8 can form 2-undecanal. Linoleic acid most commonly forms hydroperoxides at carbons 9 and 13. These produce volatiles such as hexanal and 2,4-decadienal. Hexanal is a good marker for the oxidation of n-6 fatty acids while propanal is a good marker for n-3 fatty acids and is frequently used in studies examining fish oil. Because volatile products are dictated by the type of fatty acid and the location of hydroperoxides, rancidity is not the same in all foods. For example, in dry milk powder, hexanal is the major contributor to rancidity since linoleic acid is the main fatty acid oxidized<sup>7-10</sup>. Conversely, rancidity in fish oil has different sensory properties as the decomposition products of fish oils contain many unsaturated aldehydes.

### **Measurement of Lipid Oxidation**

Gas chromatography is an effective technique to monitor secondary lipid oxidation products. This technique commonly analyzes the headspace of food samples stored in a sealed

vials and can utilize static direct injection, solid phase microextraction and dynamic trapping systems<sup>11</sup>. Results can be reported on individual compounds or in some cases as all volatiles (e.g. total carbonyls)<sup>11</sup>. Spectrophotometric techniques are also used such as anisidine value, dinitrophenylhydrazine and thiobarbituric acid reactive substances where detector compounds react with secondary lipid oxidation products to form chromophores<sup>11</sup>.

One of the most common spectrophotometrically monitored secondary products methods is the reaction between aldehydes and thiobarbituric acid, which forms a pink adduct<sup>12</sup>. This assay is sometime suggested to measure malondialdehyde but thiobarbituric acid can react with other lipid oxidation aldehydes as well, so it's commonly referred to as "thiobarbituric acid reactive substances" (TBARS). It is also important to consider the food matrix conditions as thiobarbituric acid will also react with non-lipid carbonyls such as ascorbic acid, sugars and the products of non-enzymatic browning. Malondialdehyde has been found to only be formed by fatty acids with 3 or more double bonds so fatty acid composition will impact the yield of thiobarbituric acid reactive substances formed. Therefore, the TBARS assay should not be used to measure lipid oxidation in samples with different fatty acid compositions.

The anisidine value assay can also measures the aldehyde content of lipids, and reacts strongly with 1-alkenals and 2,4-dienals. These secondary oxidation products react with p-anisidine to form a chromogen which can be measured spectrophotometrically<sup>11</sup>.

The Totox ("total oxidation") value is determined by combining the anisidine value (secondary oxidation products) with the peroxide value (primary oxidation products) using Equation 1 and is often used to describe the overall oxidation state of the oil since peroxide values will eventually decrease over time (as they are converted into secondary oxidation products).

$$\text{Anisidine Value} + 2(\text{Peroxide Value}) = \text{Totox Value}$$

Equation 1. Calculation of Totox value from anisidine value and peroxide value.

## Phospholipids and Lecithin

### Structure of Phospholipids

Naturally, phospholipids are significant components of cell membranes and are found arranged in bilayers (with the hydrophobic tails on the inside and the hydrophilic heads on the outside). This arrangement is depicted on the left side of Figure 2.3. Naturally occurring phospholipids have a phosphate group esterified in the sn-3 position of glycerol with fatty acids at positions 1 and 2. The simplest phospholipid is phosphatidic acid, which simply has an -OH group attached to the phosphate group. Figure 2.3 shows where various molecules could attach to form the structure of some of the most common phospholipids.

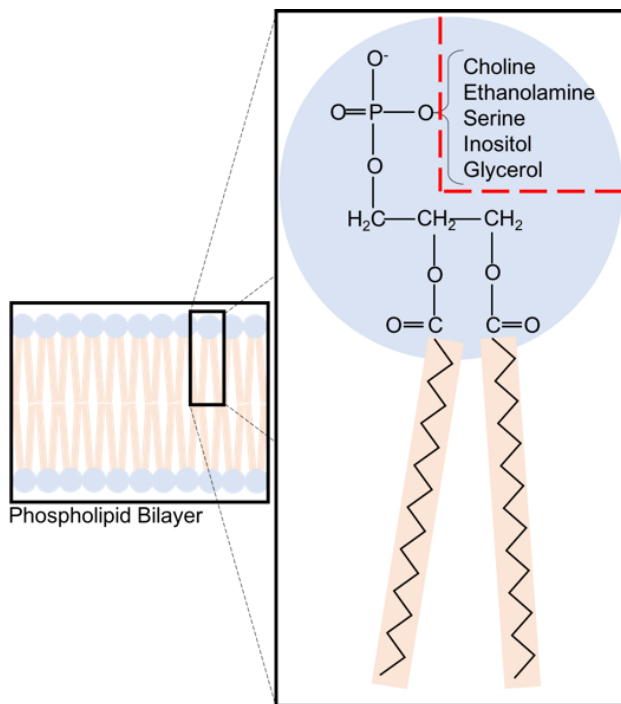


Figure 2.3. Shown is the structure of a phospholipid.

The polar headgroup combined with the fatty acid tails causes phospholipids to be amphiphilic, and thus, surface active. This gives them significant biological relevance as they form cell membranes where they align themselves in a bilayer with the phosphoric acid facing the water surface and the fatty tails a lipid layer.

## **Lecithin**

### **Introduction**

Lecithin is a catch-all term that refers to concentrated phospholipids, although it is sometimes used interchangeably to mean phosphatidylcholine. In foods, “lecithins” are typically blends of phospholipids used as emulsifiers due to their amphiphilic structure. Among other uses, they may also be used as wetting agents, for viscosity reduction, as release agents, or for control of crystallization<sup>13</sup>. The specific composition of which phospholipids are present and in what ratio is dependent on the source of the phospholipids. The composition of the attached head of the molecule, as well as the length and degree of saturation of the fatty acid tail will affect the specific emulsifying properties of that lecithin molecule. For example, PC is typically used more effectively in o/w emulsions, while PE and PI are more effective emulsifiers in w/o emulsions<sup>14-17</sup>, which is why commercial lecithins are typically a specific blend produced to function in a given food system depending on the pH, salt concentration, and the oil/water ratio<sup>17-19</sup>.

Phospholipids naturally occur in foods and they are also added as lecithin. The effect of phospholipids on the oxidative stability of foods varies greatly based on the phospholipid headgroup, the fatty acid composition and the food matrix. Due to this complexity, there is much conflicting evidence regarding the role of phospholipids on oxidative stability. Since phospholipids themselves contain unsaturated fatty acids, they are prone to lipid oxidation as any

other fat. This is especially true in muscle tissue where phospholipids are the first lipids to oxidize because of their highly unsaturated fatty acid content and the close proximity of the membrane phospholipids to prooxidants such as metal ions, enzymes, heme proteins and reactive oxygen species<sup>20</sup>.

### Sources of Lecithin

As the fundamental component of cell membranes, lecithin could be extracted from a wide array of plant or animal sources.. However, most commercial lecithin's are obtained from the degumming step of oil refining. Degumming is important for oil quality as the phospholipids can emulsify water into the oil and will causing browning when the oil is heated. Isolation of the phospholipids from the degumming step creates a value added product that benefits the economics of oil production. The composition of lecithin is heavily dependent upon the oil source (Table 1). In addition, the PC content in lecithin obtained from sunflower seeds from the Peredovik variety of sunflower has been reported to range from 12.7 to 64.2% <sup>21</sup>. This suggests that factors such as growing conditions or location may have a significant impact on the composition of the lecithin within a single oil seed variety.

Table 2.1. A large variation exists in the concentrations (%) of the phospholipids extracted between different plants and plants of the same variety from different locations.

	Corn	Soybean	Cottonseed	Sunflower Seeds		
				Peredovik	Peredovik	Peredovik
Phosphatidylethanolamine (PE)	3.2	14.1	13.46	46.6	15.7	19.5
Phosphatidylcholine (PC)	30.4	33	23.16	12.7	42.3	64.2
Phosphatidylinositol (PI)	16.3	16.8	13.41	3.7	36.6	15.2
Phosphatidic acid (PA)	9.4	6.4	8.76	32.6	5.2	1.2

## **Impact on Lipid Oxidation**

Although bulk oils are often thought of as homogeneous mixtures, it has been shown that the minor components that remain in the oil after refining are able to form physical structures known as association colloids<sup>22</sup>. These minor components can include monoacylglycerols, diacylglycerols, phospholipids, sterols, free fatty acids, and products of lipid oxidation such as fatty acid hydroperoxides, aldehydes, ketones, and epoxides<sup>23-25</sup>. Since phospholipids are amphiphilic, they have the ability to form physical structures in bulk oils assuming that they are present in sufficient quantities to exceed their critical micelle concentration (CMC). In bulk oil which typically contains small amounts of water after refining, the structures formed are both reverse micelles and lamellar structures. The CMC concentration of dioleoylphosphatidylcholine was estimated to be 550  $\mu\text{M}$  in stripped soybean oil using a spectrophotometric technique which relied on the formation of association colloids to solubilize 7,7,8,8-tetracyanoquinodimethane and increase its absorbance<sup>23</sup>.

## **Synergism with tocopherol**

Several papers have found that phospholipids, particularly phosphatidylethanolamine and phosphatidylcholine can interact with tocopherols to inhibit lipid oxidation in a variety of model food systems. In an early study, Hildebrand et. al. examined the ability of PC, PE, and PI plus mixed soybean tocopherols to alter the time to reach a peroxide value of 100 mEq/kg<sup>26</sup> at 110°C in commercially refined soybean oil. Authors concluded that PI and PE in combination with tocopherol were the most effective at increasing the oxidative stability of the oil compared to the control and samples with only added tocopherol. They further suggested that the amine group of PE and PC as well as the reducing sugar on PI can facilitate hydrogen donation to regenerate tocopherols. Similar results were obtained by Kashima et. al who measured the hydroperoxide

formation in refined perilla oil, perilla oils that had been stripped of tocopherol, and perilla oils that had been enriched with tocopherol<sup>27</sup>. Addition of PC, PS, or PE had no impact on the formation of hydroperoxides compared to the control in tocopherol-free perilla oil. However, in perilla oil samples enriched with 500 ppm mixed tocopherols, PS and PE increased the lag phase of hydroperoxide formation to 30 days at 37°C compared to the control and PC only samples which had lag phases of 10 days. This provided evidence that while PE and PS are able to increase the activity of tocopherols, PC does not.

Bandarra et. al. examined the effects of PC, PE and cardiolipin in refined sardine oil stored at 40°C for 33 days, and concluded that PE showed the strongest ability to increase the activity of tocopherol of the phospholipids tested<sup>28</sup>. This was calculated using the peroxide values of samples with and without added tocopherol plus the given phospholipid. Cardiolipin, a phospholipid without an amino group, had a very low antioxidant effect in the presence of tocopherols. Judde et. al. examined the effects of various commercially available lecithin blends on the oxidative stability of commercially available oils or in model systems to determine the commercial practicality of using combination of lecithin and tocopherols as an antioxidant strategy<sup>29</sup>. It was found that the main increases in antioxidant activity were between lecithins that were high in PC and PE and  $\gamma$ - and  $\delta$ -tocopherols, but increased antioxidant activity was not observed with  $\alpha$ -tocopherols in this experiment. In a separate study, however, Takenaka et. al. reported that  $\alpha$ -tocopherol could be regenerated using isolated PC and PE (with known fatty acid composition) in combination with  $\alpha$ -tocopherol in stripped bonito oil<sup>30</sup>. They found PE esterified to unsaturated fatty acids such as oleic acid (OA), linolenic acid (LA), arachidonic acid (AA), and docosahexaenoic acid (DHA) increased the activity of  $\alpha$ -tocopherol. In their study,

DHA had the strongest synergistic effect, increasing the lag phase of control bonito oil from less than 50 hours at 40°C to more than 200 hr.

Cui et. al. examined the oxidation rate of bulk oils enriched with tocopherols in addition to association colloids produced by phospholipids<sup>31</sup>. This study used stripped soybean oils as the oxidizable lipids and found that dioleoylphosphatidylcholine was unable to increase the activity of  $\alpha$ -tocopherol, while dioleoylphosphatidylethanolamine did. Dioleoylphosphatidylethanolamine was also able to decrease the rate of  $\alpha$ -tocopherol degradation during the storage of the stripped soybean oil. Finally, they also showed that dioleoylphosphatidylethanolamine and ethanolamine (just the headgroup) were able to regenerate  $\alpha$ -tocopherolquinone to  $\alpha$ -tocopherol.

Samdani et. al. examined the interaction between phospholipids and tocopherol homologs in oil-in-water emulsions. They showed that PE and  $\delta$ -tocopherol was a stronger antioxidant combination than  $\alpha$ -tocopherol and PE. The synergistic activity of PS and tocopherol was 1.5–3 times greater than PE and  $\alpha$ -tocopherol when Tween 20 was the emulsifier whereas there were not large difference in PE and PS in the presence of  $\alpha$ -tocopherol when bovine serum albumin was used as the emulsifier<sup>32</sup>. The increased activity of  $\delta$ -tocopherol was postulated to be due to its higher surface activity, allowing it to partition more at the emulsion interface where it could come in contact more with the amphiphilic phospholipids. Mixed tocopherols also showed synergistic activity with PE and PS in oil-in-water emulsions stabilized by both Tween 20 and bovine serum albumin. This suggests that factors that affect the partitioning of tocopherols and phospholipids in oil-in-water emulsions an important consideration when designing emulsion systems where phospholipids will be regenerating tocopherols. It should be noted that the increases in lipid oxidation lag phases by PE or PS in combination with tocopherols in the



emulsions was very impressive with some combination more than doubling the oxidation lag phase compared to tocopherol alone.

Xu and coworkers studied interactions of different tocopherol homologs with PE or PS in bulk oil<sup>33</sup>. Unlike oil-in-water emulsions, PE was much more effective than PS at increasing the activity of the tocopherol homologues in bulk oil. Of the different tocopherol sources, the combination of mixed tocopherols and PE resulted in the strongest synergistic activity increasing hydroperoxide and hexanal lag phases over 50 days compared to the mixed tocopherols alone. Phospholipase-D was used to increase the PE content of a commercial lecithin since isolated PE is too expensive for commercial uses. This modified lecithin was able to increase the antioxidant activity of  $\alpha$ -tocopherol add to stripped oil and increased hydroperoxide and hexanal lag phases in commercially refined oil which contained naturally occurring tocopherols. The study further indicates that the combination of PE and PS with tocopherols can be powerful tools to increase the oxidative stability of lipids in foods.

Doert et. al. examined the regeneration of tocopherols by individual phospholipids and soy lecithin, and also characterized the reaction mechanisms<sup>2</sup>. Their results for regeneration are in general agreement with previous literature. For example, authors prepared  $\alpha$ - or  $\gamma$ - tocopherol and soybean lecithin with ethyl linoleate in chloroform/methanol (3/1). They allowed the components to react at 110°C and used HPLC analysis to measure the amount of  $\alpha$ - or  $\gamma$ - tocopherol for 240 minutes, finding that the amount of tocopherol remained higher in their system for longer periods in the presence of PE and PS compared to a no phospholipid control.

Much of the early literature suggests that ascorbic acid regenerates tocopherol by the reduction of the tocopherol radical. However, Doert and coworkers found that PE and PS (but not PC) were able to regenerate  $\alpha$ -tocopheryl quinone back to  $\alpha$ -tocopherol<sup>2</sup>. Using mass

spectroscopy to analyze the intermediates of the regeneration reaction, authors were able to postulate the reaction scheme shown in Figure 2.4. They found that both PS and PE could form adducts with tocopherone and through different mechanisms regenerated the oxidized tocopherol. They also found that acetic acid catalyzed the regeneration reaction, and postulated that the carboxylic group present in phosphatidylserine could be responsible for its faster regeneration compared to PE<sup>2</sup>.

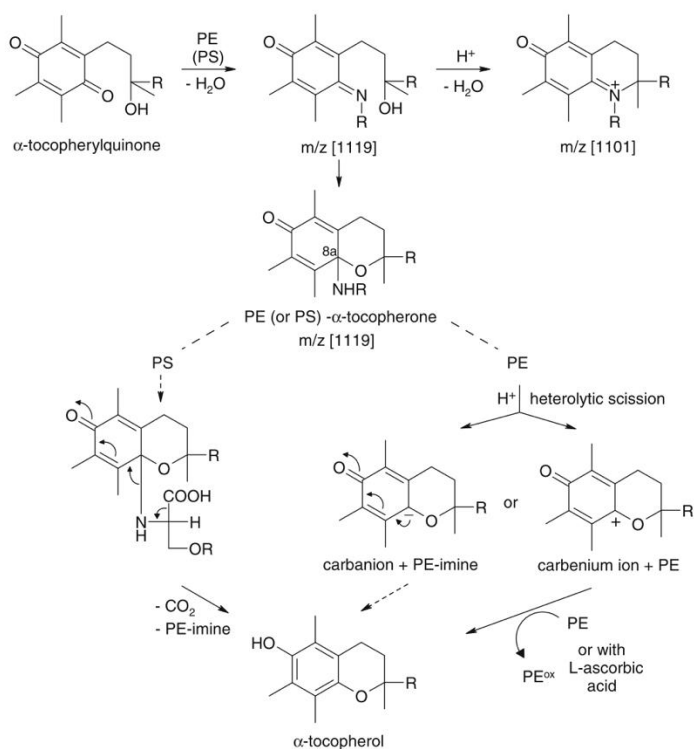


Figure 2.4. Postulated mechanism for the regeneration of  $\alpha$ -tocopherol quinone to  $\alpha$ -tocopherol by phosphatidylethanolamine (PE) and phosphatidylserine (PS). Figure reproduced from <sup>2</sup>.

Doert et. al. found that purified fractions of PE and PS, but not PC and PI, were able to regenerate  $\alpha$ -tocopherol quinone ( $\alpha$ -TQ) to  $\alpha$ -tocopherol<sup>2</sup>. The solvent used was found to have a significant impact on the rate of the regeneration by PE as the time required to regenerate 35% of the  $\alpha$ -tocopherol decreased from 11 to 3 days when the solvent was changed from isooctane

alone to a mixture of isooctane and acetic acid, showing that the surrounding environment's composition plays a significant role in lecithin's ability to regenerate tocopherol.

The same researchers also used a combination of chiral HPLC and electrospray ionization mass spectrometry (MS-ESI) to examine the intermediates of the reaction between PE or PS and  $\alpha$ -TQ and postulated the reaction mechanism in Figure 2.4, which starts with the reaction of PE (or PS) to form PE (or PS)-  $\alpha$ -tocopherone<sup>2</sup>. In this mechanism, PS-  $\alpha$ -tocopherone can convert directly into  $\alpha$ -tocopherol, while PE-tocopherone first requires the intermediate step of a carbenium ion which can be reduced to  $\alpha$ -tocopherol. Since regeneration by PS has fewer steps in the reaction, its regenerative action may be a more efficient process, and thus a more successful antioxidant in real-world applications. Although these researchers conclude "the reaction [with PS] is not of interest" because "PS only occurs in plant lecithins in small amounts," this statement underscores the importance of modifying lecithin to have higher PE and PS concentrations to recharge tocopherol for preventing lipid oxidation in food products. There is additional evidence that PE and PS may have antioxidant properties of their own. For instance, the  $-NH_2$  group present in PE has the potential to chelate trace metals found in the oil which would otherwise be prooxidants in an oil-based system, however phosphatidylcholine and phosphatidic acid are not able to chelate metals in a manner that decreased lipid oxidation<sup>34,35</sup>.

## **Phospholipase D**

### **Overview**

Phospholipase D was first described in 1947 by Hanahan and Chaikoff<sup>36</sup> when it was found in carrots. Since then, it has been isolated from numerous plant, animal and bacterial sources. PLD is part of a family of enzymes which cleave phospholipids at various positions. As shown in Figure 2.5, PLD is responsible for cleaving off the headgroup found on

phospholipids, thus converting a given phospholipid into phosphatidic acid. It has also been shown to facilitate transphosphatidyl transfer activity, thus converting one phospholipid into another.

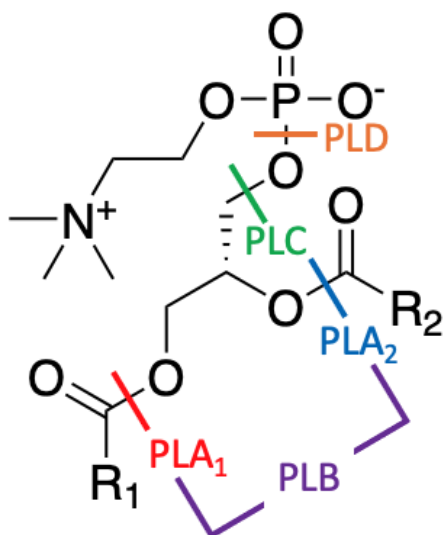


Figure 2.5. Diagram showing the action sites for Phospholipase A-D on a molecule of phosphatidylcholine. An enzyme is classified as PLB if it has the ability to cleave fatty acids in both the sn-1 and sn-2 position. Figure drawn using ChemDraw JS.

### General Structure

Numerous isoforms of PLD have been found, even within the same species. In pineapples alone, for example, ten different PLD genes coding for ten different phospholipase D proteins have been found, with calculated pI values ranging from 5.8 to 8 and molecular weights ranging from 57.3 to 127.8 kDa<sup>37</sup>. Although there are different isoforms of the enzyme, numerous studies have examined the structure of PLD and have found a specific region in the genome to be conserved throughout all enzymes in the PLD superfamily of enzymes. This is true for the myriad plant, animal or bacterial sources from which PLD has been isolated<sup>38-41</sup>.

This specific region, referred to as the HKD region due to the histidine (H), lysine (K), and aspartic acid (D) residues, is involved in the hydrolytic and transphosphatidylations reactions that the enzyme is responsible for catalyzing. The sequence of these amino acids is HxKxxxxD, where “x” represents different amino acids depending on the specific isoform of the enzyme. Although certain bacterial and viral strains of PLD only have one HKD motif, PLD enzymes more frequently have two HKD regions (referred to as the N- and C- terminals) and are folded such that the two HKD regions are in close proximity and form a single active site which interacts with the phosphate group on the phospholipid<sup>40</sup>. Figure 2.6 shows the active site of PLD from *Streptomyces* species made up of two HKD regions (shown in orange and white) with the phosphate group from a phospholipid depicted in the active site.

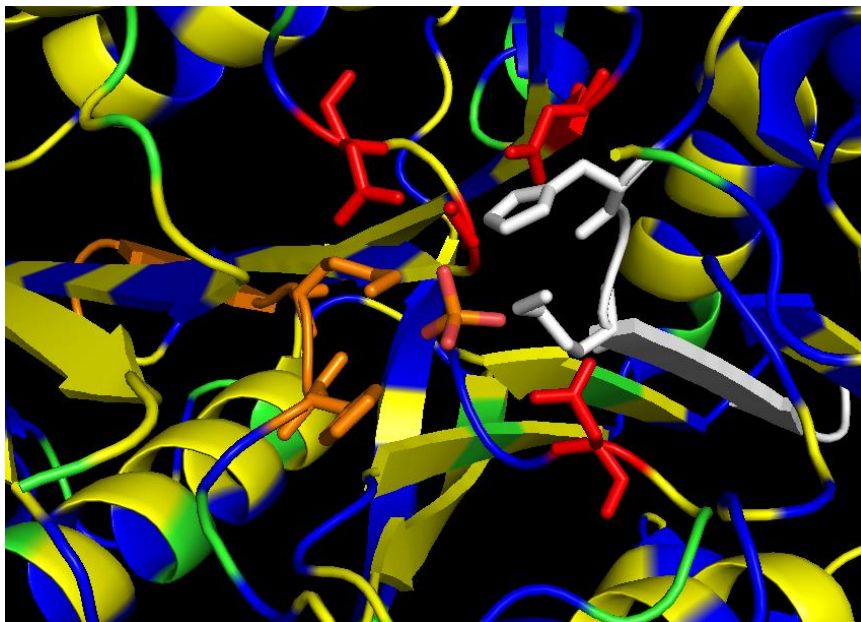


Figure 2.6. Depiction of the HKD regions (shown in orange and in white) interacting with the phosphate group (center). Figure generated using Pymol based on the crystalized structure found by Leiro et. al.<sup>40</sup>.

### **Methods of Phospholipase D Purification**

There are numerous methods described in the literature for purifying PLD. For plant sources, these may vary due to factors such as the intended usage of the enzyme extract as well

as the source of the enzyme. There are, however, similarities between the methods described. What follows is a general overview of the steps frequently included for extracting PLD from plant sources.

### 1. Overnight Soak

As an initial step, the plant material to be extracted is often soaked overnight in an aqueous solvent. The solvent may be pure water or water with buffer at a specific pH. This step is often skipped when the plant material already has a high water content. The overnight soak is typically carried out at low temperatures. For example, List et. al. soaked soybeans overnight in acetate buffer at pH 5.6 at 0°C<sup>42</sup>.

As an alternative to the low-temperature overnight soak, Heller et. al. rinsed peanuts (*Arachis hypogea*) with a commercial detergent containing sodium dodecylsulfate as a sterilization step before soaking the seeds overnight at 26°C<sup>43</sup>. In an attempt to remove lipids from the surface of rice bran, Ueki et. al. soaked rice bran (*Oryza sativa* L.) in n-hexane overnight<sup>44</sup>.

### 2. Homogenization

The plant matter is next homogenized. Authors report several methods using a mortar and pestle<sup>45</sup> with acid washed sand<sup>46</sup>, blender<sup>47,48</sup>, coffee grinder<sup>49</sup> in combination or separately. This step is performed at low temperature to slow reactions and minimize potential damage to the enzymes as cellular compartments are ruptured.

### 3. Filtration

The plant matter is next filtered to remove large particles and debris. This is often performed using cheese cloth<sup>49-51</sup>.

#### 4. Centrifugation

The resulting filtrate is further purified by centrifugation performed at low temperature. Centripetal forces used are typically around 13,000g to 30,000g<sup>50,52</sup>, however 105,000g for 1 hour was used as part of the purification of rapeseed<sup>49</sup>.

#### 5. Concentration

This step appears to have the most variation between methodologies, with some authors skipping it all together and others performing much more demanding purification protocols.

Often, chromatographic methods such as octyl-sepharose<sup>53,54</sup> or DEAE-cellulose columns<sup>55</sup> are used. Novotná et. al. extracted the supernatant of rapeseeds with hexane to remove fat and then precipitated the protein with ammonium sulfate.

### **Phospholipid Modification by Phospholipase D**

Although lipase-catalyzed transesterification reactions typically require care to ensure that there is minimal water present in the system, transphosphatidylations reactions catalyzed by PLD occur at the phospholipid-water interface, and are therefore minimally affected by the content of water in the system (assuming other conditions are optimal)<sup>56</sup>. Therefore, reaction systems described in the literature are typically biphasic<sup>33,57-59</sup>, consisting of an aqueous and organic phase. The organic phase contains the phospholipid substrate and the aqueous phase contains the PLD as well as the polar headgroups if transphosphatidylation is being performed. One potential concern for scaling up this type of reaction system for food production is the safety of these organic solvents which, in addition to be highly flammable, pose both human safety as well as environmental concerns. To this end, Duan and Hu used  $\gamma$ -valerolactone (GVL) as a solvent for the transphosphatidylation protocol for PS<sup>60</sup>. An anhydrous organic solvent system using powdered PLD in chloroform has also been described, wherein a cation-exchange resin

was used to remove choline from the area around the enzyme for increased efficiency<sup>61</sup>.

Furthermore, PLD is able to convert PC to PA at 65°C in water saturated hexane<sup>62</sup>.

The mechanism for PLD hydrolysis of phosphatidylcholine into phosphatidic acid and choline is shown in Figure 2.7. At the start of the reaction, the  $\epsilon_2$  nitrogen in the C-terminal is protonated, while the  $\epsilon_2$  nitrogen in the N-terminal is not. The first step in this reaction is a nucleophilic attack on phosphorous by His 170 (from the HKD region)<sup>56</sup>. The Lys residues stabilize the transition state. Next, His 448 (C-terminal) donates a hydrogen, forming an alcohol with the leaving group as well as a phosphatidyl-enzyme intermediate. In this case, the leaving group is choline. Next, His 448 deprotonates the incoming molecule (water or other -OH). In turn, the deprotonated molecule attacks the phosphorus on the intermediate, resulting in PA or phosphatidylalcohol<sup>56</sup>.



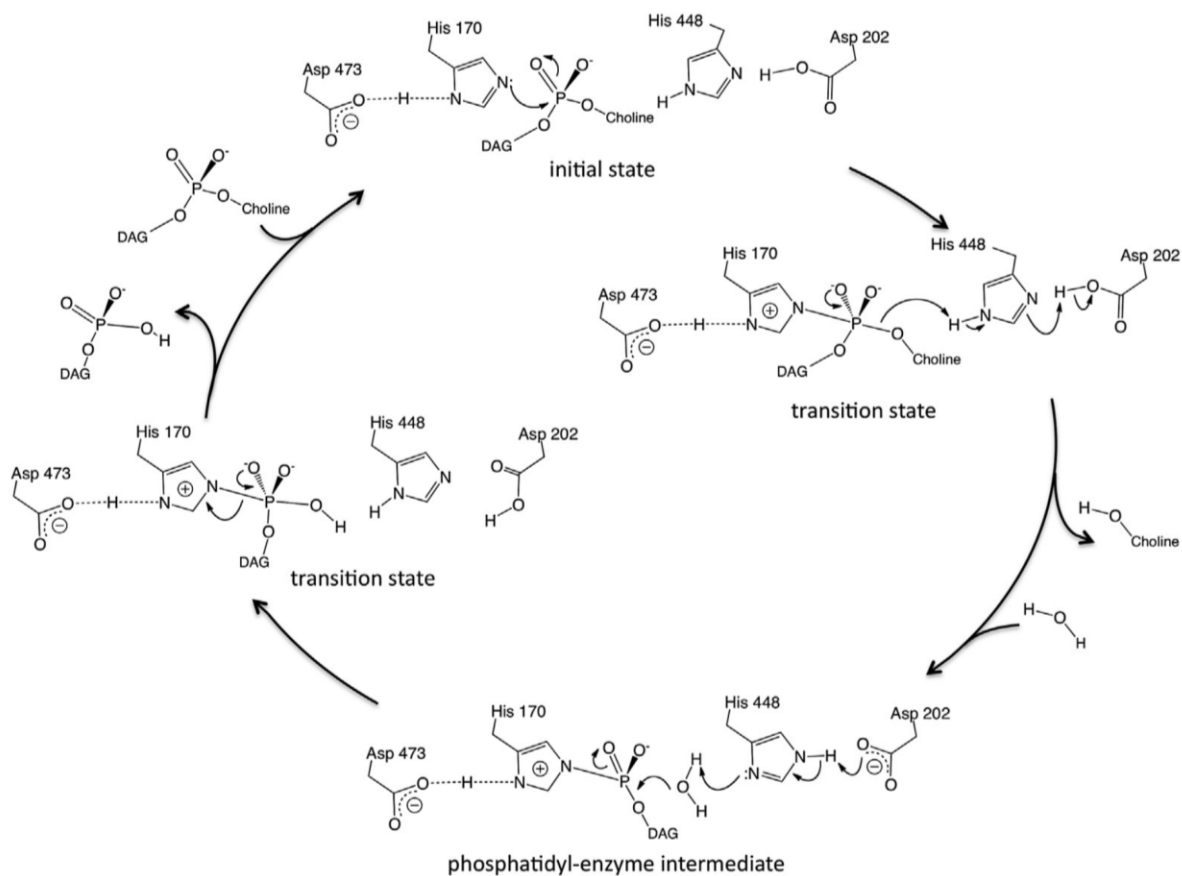


Figure 2.7. Postulated mechanism for PLD hydrolysis, reproduced from Damnjanovic and Iwasaki<sup>56</sup>.

Numerous factors have been found to affect the rate of this reaction. For example, divalent cations such as calcium and magnesium chloride have been found to stimulate PLD isolates from corn while monovalent cations such as sodium or potassium chloride did not affect PLD activity<sup>63</sup>. Other isoforms of PLD such as several bacterial sources are active regardless of the ions present in the system<sup>64</sup>.

Naturally occurring C6 aldehydes such as hexanal combined with an alcohol such as hexanol were found to inhibit PLD activity<sup>71</sup>. Additionally, acetaldehyde was found to be a potent inhibitor of PLD activity isolated from corn<sup>63</sup>. PLD's ability to completely convert PC to PS has also been found to be inhibited when choline concentrations are above 50 mM (in ethyl acetate) which means that only a partial conversion may occur<sup>57</sup>. One potential solution that has

been identified is to enzymatically remove choline (using a combination of choline oxidase and catalase) to convert the choline so that it cannot react with PLD<sup>65</sup>.

### **Phospholipase D from *Streptomyces chromofuscus***

Although some studies<sup>66,67</sup> have found that PLD from *S. Chromofuscus* (PLD<sub>SC</sub>) has a much higher hydrolysis rate than transphosphatidylation rate and thus concluded PLD<sub>SC</sub> has limited potential for usage in reactions where the goal is transphosphatidylation of head groups. Other studies (described below) have successfully used this enzyme for transphosphatidylation reactions<sup>68,69</sup>. Most studies which examine PLD transphosphatidylation activity focus on the conversion of PC to PS for its potential health benefits such as revitalizing brain cell membranes and improving memory functions as well as decreasing exercise induced increase in cortisol levels<sup>70</sup>.

For the present study, PLD from *S. chromofuscus* was chosen in large part because of its commercial availability. Furthermore, PLD<sub>SC</sub> was found to retain 81% of its initial enzyme activity after being used to transphosphatidylate PC to PS for 10 batches<sup>68</sup>. A separate study also examining the conversion of PC to PS using PLD<sub>SC</sub> found the enzyme retained a conversion rate above 50% after 7 repeated batch cycles<sup>70</sup>.

PLD<sub>SC</sub> was also found to have a much higher hydrolysis affinity for PC (specifically 1,2-Di-n-hexanoyl phosphatidylcholine) than for C<sub>6</sub>PE or C<sub>6</sub>PS<sup>71</sup>. This is important because as the transphosphatidylation reaction progresses and the concentration of the product increases in the system, the efficiency of the conversion could be diminished if the enzyme is hydrolyzing or transphosphatidylating the PE that has already been produced. Based on the results of their assay, the catalytic efficiency (kcat/Km) of PLD<sub>SC</sub> catalysis on PC was 202, but 38 for PE,

where  $k_{cat}$  describes how many substrate molecules are transformed into products and  $K_m$  describes the affinity of the substrate to the active site of the enzyme<sup>71</sup>.

PLD<sub>SC</sub> exhibits significantly different sequence homology from PLD extracted from other *Streptomyces* bacteria. For example, the widely conserved HKD motif displayed by a large number of PLD enzymes is not conserved, indicating a new class of PLD enzyme<sup>72</sup>.

Furthermore, PLD<sub>SC</sub> requires the presence of calcium to function, while other *Streptomyces* PLD are Ca<sup>2+</sup> independent<sup>73-75</sup>.

### **Effect of pH on PLD<sub>sc</sub> activity**

For PLD isolated from *Streptomyces chromofuscus*, there is general agreement in the literature about the activity maximum<sup>76,77</sup>. In order to find the optimal pH for PLD activity, authors typically react PLD with PC at varying pH levels and use some form of colorimetric assay which measures either the amount of choline liberated in the system or the amount of PA generated. While this approach allows for rapid collection of information about the hydrolysis rate from PLD, there may be differences between the optimal conditions for hydrolysis vs. transphosphatidylations reactions. These studies also use purified PC with short chain fatty acids. These are short chain phospholipid that are not typically present in lecithin and lecithin contains other lipids (e.g. triacylglycerols) that could impact the reaction.

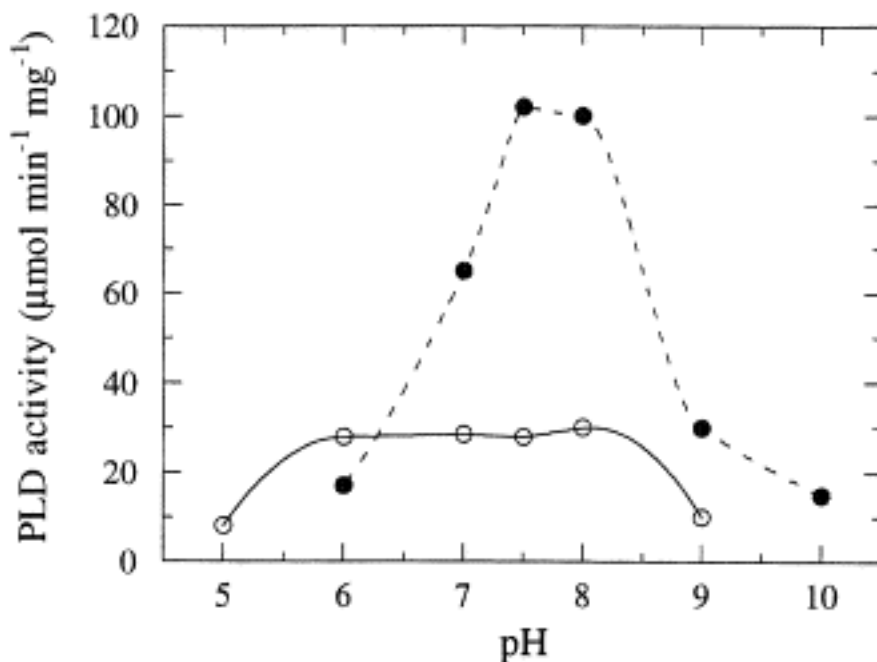


Figure 2.8. Shown is the specific activity of PLD<sub>SC</sub> as a function of pH using diC<sub>4</sub>PC as a substrate. Authors extracted two PLD enzymes from *Streptomyces chromofuscus*. Closed circles represent PLD<sub>42/20</sub>, a tightly associated complex of two peptides (42kDa and 20kDa, respectively), and open circles represent PLD<sub>57</sub>, a peptide of 57 kDa<sup>78</sup>.

Figure 2.8 shows the hydrolysis activity of two fractions of PLD<sub>SC</sub> on diC<sub>4</sub>PC. Authors isolated two fractions of PLD<sub>SC</sub>, one that was comprised of a complex of two peptides (42 and 20 kDa in weight) and another that was 57 kDa in weight<sup>78</sup>. PLD<sub>42/20</sub> was found to be more responsive to changes in pH, however both enzyme fractions displayed optimal pH around 7.5-8. PLD<sub>57</sub> was found to be ~4 times less active. Authors used the same methodology (pH-stat assay in 100 mM NaCl) to determine the affinity of PLD<sub>SC</sub> for hydrolyzing diC<sub>6</sub>PS and diC<sub>6</sub>PE. PLD<sub>57</sub> showed little headgroup specificity, while PLD<sub>42/20</sub> displayed a strong preference for diC<sub>6</sub>PS, with a 2-fold increase in  $V_{max}$ <sup>78</sup>.

Although changes in optimal pH in response to changing Ca<sup>2+</sup> concentrations have been reported in sources of plant PLD such as rape seed<sup>49</sup>, these changes were not observed in the optimum pH for PLD<sub>SC</sub> when the Ca<sup>2+</sup> concentration was varied from 0.5 mM, 5 mM, and 10 mM<sup>78</sup>.

Two additional studies, both with the goal of converting PC to PS using PLD<sub>SC</sub>, found the pH optimum to be 8.0 and 7.5, respectively<sup>69,79</sup>. Qin et. al. used a colorimetric assay of PC hydrolysis to find an optimum pH activity for PLD<sub>SC</sub> of 8.0. Similar results (pH maximum between 7.5-8) were obtained using a hydrolysis assay on PLD from *Streptomyces* PMF<sup>80</sup>.

Instead of using hydrolysis of choline, Li et. al. determined their optimum pH finding of 7.5 using the transphosphatidylation of PC to PS<sup>79</sup>. After cloning and sequencing the gene for PLD<sub>SC</sub> enzyme, they expressed the recombinant protein in *Escherichia coli*, and purified it. Figure 2.9 shows the rate of transformation of PC to PS by PLD<sub>SC</sub> in response to increasing pH, with a maximum rate obtained at pH 7.5.

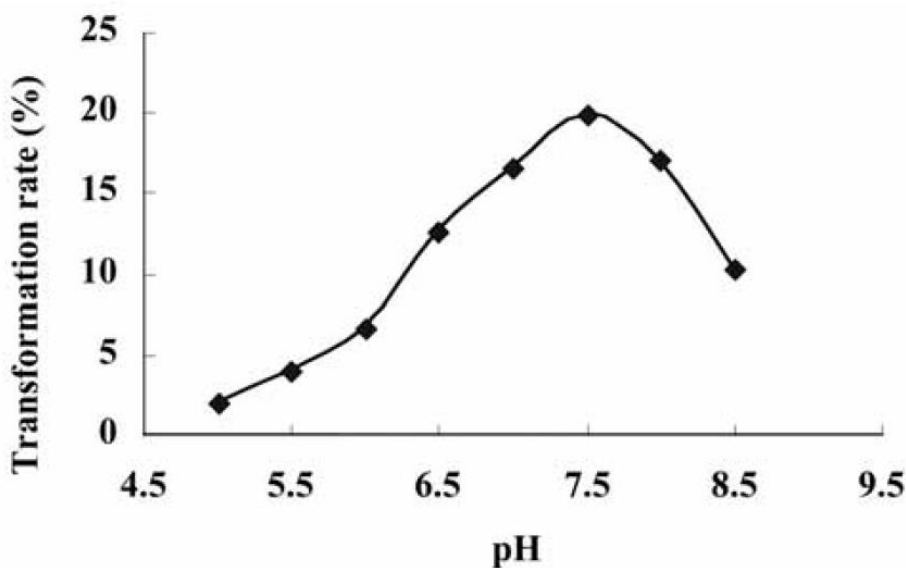


Figure 2.9. Shown is the effect of pH on the transformation rate of PC to PS by PLD<sub>SC</sub><sup>79</sup>.

Liu et. al. used cell-surface display, a technique which immobilizes cloned enzymes onto the surface of microorganisms to affix PLD<sub>SC</sub> to the surface of *Pichia pastoris* GS115/pKFS-pldh<sup>70</sup>. This allowed them to use an immobilized version of PLD<sub>SC</sub> instead of allowing it to move freely in the transphosphatidylation reaction system. Using a hydrolysis activity assay, authors found an activity maximum at pH 7.0 for non-displayed PLD<sub>SC</sub>, which is similar to other

reported results, however the optimum pH shifted to 6.0 when the PLD<sub>SC</sub> was displayed. This study also examined the pH stability of PLD<sub>SC</sub> by incubating the enzyme at a given pH for 1 hour before measuring the activity. Results for the pH stability assay are shown in Figure 2.10. Displayed PLD<sub>SC</sub> was more pH-stable at the extremes, but the secreted PLD<sub>SC</sub> was still stable from pH 6-10.

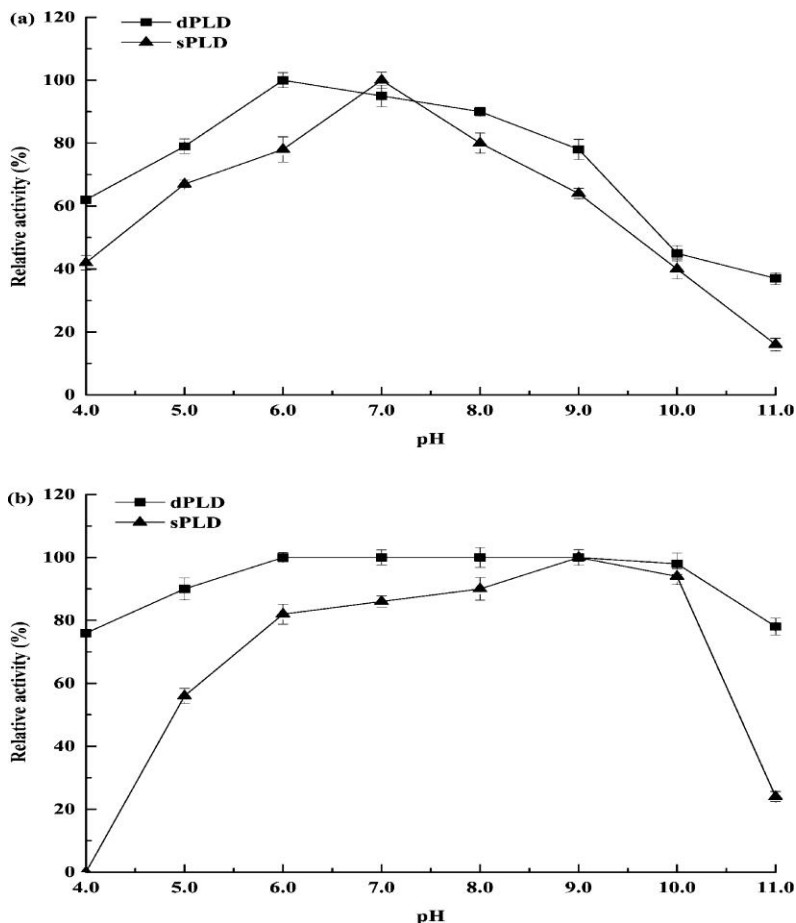


Figure 2.10. Influence of pH on the stability of displayed (dPLD) and secreted (sPLD) phospholipase D from *S. chromofuscus*<sup>70</sup>.

A study comparing the hydrolysis rates of PLD<sub>SC</sub> found similar activities between PS and PE at pH 8<sup>71</sup>, however this study's two-step hydrolysis assay used a second step where alkaline phosphatase was added to the mix. Authors were originally hoping to halt the reaction by alkalizing the pH with the addition of the alkaline phosphatase, but noted "PLD<sub>SC</sub> retained

sufficient activity at pH 11.5 such that there was significant background hydrolysis of the phospholipid during the time required.” Authors instead added EDTA to quench the reaction (since tightly bound iron is a critical component of PLD<sub>SC</sub> activity<sup>81</sup>), however, once the pH was shifted to alkaline conditions, the complexes formed by EDTA would weaken as metal hydroxide formation is more energetically favorable, which could have allowed background PLD<sub>SC</sub> hydrolysis of PE to occur. Additionally, since this assay only measures hydrolysis, it would not account for catalysis of products.

### **Effect of Temperature on PLD<sub>SC</sub> Activity**

As with studies on pH, research on the effect of temperature typically used assays which measure the hydrolysis caused by the PLD enzyme and use this as a basis for determining the optimal transphosphatidylase activity. Since the phosphatidylase reaction first requires a hydrolysis step and a second transfer of headgroups, it is possible that the optimum temperature for hydrolysis might cause the reaction to move too quickly to favor transphosphatidylase.

The reported optimal temperatures for PLD<sub>SC</sub> reported in the literature vary more than the reported pH optimums. Qin et. al. examined a high-yield process for producing PS from PC using *S. chromofuscus* and found the optimal temperature to be 70°C using a hydrolysis activity assay<sup>69</sup>. By contrast, Li et. al. reported an optimum at 30°C<sup>79</sup>.

A potential explanation for the reported differences in optimal temperature is due to the temperature stability of the enzyme. Although reaction rates typically increase with higher temperature, a temperature that is too high will affect enzyme structure and functioning. Zhou et. al. examined the optimum temperature of a PLD gene extracted from *Streptomyces* species mined from Genbank<sup>77</sup>. In their study, which focused on using PLD for converting PC to PS, authors examined both the optimal temperature for enzyme activity and the temperature stability

of the enzyme, as shown in Figure 2.11. The optimal temperature was examined by reacting 100 uL of 0.5% (w/v) soybean lecithin, 0.1% (v/v) Triton X-100, 40 mM Tris-HCl (pH 7.5), 10 mM CaCl<sub>2</sub> and 40 μL of an enzyme sample for 20 minutes at a given temperature, stopping the reaction and measuring the amount of choline liberated. Under these conditions, the enzyme displayed its highest activity at 60°C. When the enzyme was treated with the given temperature for 1 hour before being assayed at 37°C, however, the enzyme was not stable above ~55°C and displayed very little activity<sup>77</sup>.

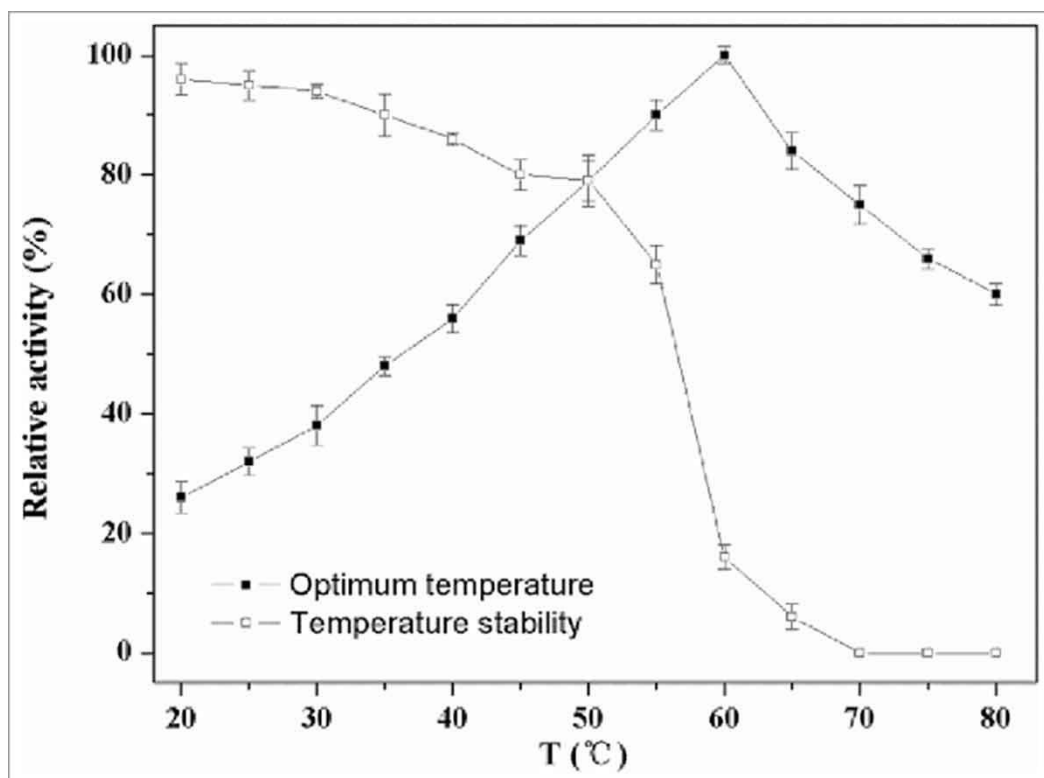


Figure 2.11. Shown is the effect of temperature on PLD activity<sup>77</sup>.

Similar results were also obtained by Liu et. al.<sup>70</sup> who reported an activity maximum for PLD<sub>SC</sub> of 50°C. In their thermostability study, which also involved exposing the enzyme to a given temperature for one hour prior to performing the hydrolysis assay, the PLD<sub>SC</sub> activity dropped significantly at 55°C, and no activity was observed at 65°C. When the enzyme was



displayed on a the surface of *Pichia pastoris* GS115/pKFS-pldh, the activity maximum shifted to 55°C, and the enzyme maintained 72.3% of its activity after 1 hour at 65°C<sup>70</sup>.

Hatanaka et. al. examined the thermal stability of three recombinant PLD enzymes from *Streptomyces* species<sup>75</sup>. Instead of the hour temperature treatment used by Zhou et. al.<sup>77</sup> and Liu et. al.<sup>70</sup>, Hatanaka et. al. performed their stability assay treating the PLD for just 10 minutes at a given temperature before measuring transphosphatidylation activity in a biphasic system of benzene and water<sup>66,75</sup>. At approximately 55°C, enzyme activity was observed to decrease to zero, further indicating this is the denaturation point of PLD<sub>SC</sub>. By contrast, Zambonelli et. al. used a 3 hour temperature incubation for their hydrolysis assay and reported 100% residual activity at 50°C, but 0% at 60°C<sup>80</sup>.

Instead of using a hydrolysis assay, Li et. al. measured their results by measuring the transphosphatidylation of PC to PS by PLD<sub>SC</sub><sup>79</sup>. The maximum conversion of PC to PS reported in this study occurred at 30°C, which is much lower than the activity maximums reported in other studies. One key difference, however, is that instead of using a hydrolysis assay, this study used a biphasic system testing various organic phases (dichloromethane, chloroform, ethyl acetate, and hexane) and acetate buffer to directly test the transphosphatidylation activity of PLD<sub>SC</sub> on converting PC to PS using L-serine.

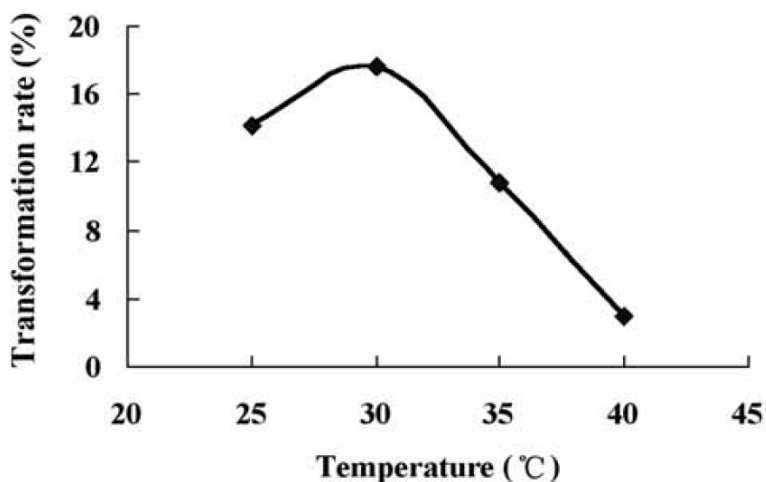


Figure 2.12. Effect of temperature on the transphosphatidylation rate of PC to PS by PLD<sub>sc</sub><sup>79</sup>.

Since temperature typically increases the reaction speed for enzymatic processes, it seems likely based on the reported optimal temperatures for transphosphatidylation and hydrolysis that the reaction must occur more slowly to favor transphosphatidylation. For PLD to catalyze hydrolysis, it must contact 1) a donor molecule and 2) a water molecule. For transphosphatidylation, however, it must contact 1) a donor molecule, 2) a water molecule, and 3) an alternate headgroup. Since the focus of the present work is to optimize the transphosphatidylation reaction of PC to PE, it is likely that a lower temperature will be needed to optimize the experiment.

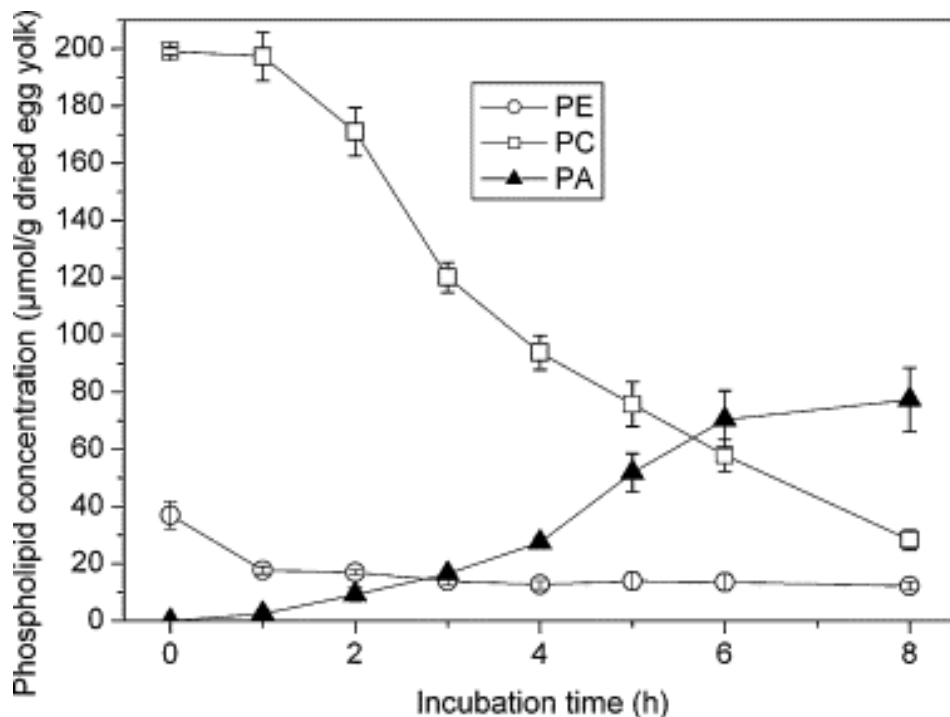


Figure 2.13. Shown are the phospholipid concentrations resulting from treating diluted egg yolk (70/30, w/w) during incubation with 750U/g of phospholipase D at 50°C<sup>82</sup>.

Figure 2.13 shows the results of the rate of the hydrolysis reaction of PLD from *S. chromofuscus* with egg yolk lecithin<sup>82</sup> at 50°C. For this research, egg yolk was diluted with deionized water (70/30, w/w) and reacted with 750 units of enzyme per gram of phospholipids contained in the diluted egg yolk. The rate of hydrolysis of PC increased for approximately the first three hours before reaching an inflection point and starting to decrease. Hydrolysis of PE slowed during the course of the experiment, probably due to the increasing scarcity of the substrate in the system. While the rate of phosphatidic acid production in the system was inversely related to the amount of PC in the system, the total amount of PA generated did not equal the starting amount of PC and PE. Authors gave the potential explanation that the PC was being converted into another phospholipid which their HPTLC method was not able to detect.

An explanation for the initially slow reaction rate of hydrolysis comes from Geng et. al. who found that the addition of 10 mol% of PA to a system containing *S. chromofuscus* and Ca<sup>2+</sup>

increased the rate of enzyme activity by seven fold<sup>78,83</sup>. This finding was attributed to an allosteric binding site where a PA and Ca<sup>2+</sup> complex bind to the enzyme and increase activity. This finding has since been corroborated in several additional studies<sup>78,84,85</sup>.

For the proposed work, it is likely that the rate of PLD<sub>SC</sub> activity will follow the trend shown in Figure 2.13 for the rate of PC degradation. Since this is a transphosphatidylation reaction, however, the amount of PE in the system should increase as the PC is converted. Some formation of PA will likely occur as well, and this amount will vary based on the reaction conditions. The amount of PA in the system should stay much lower, however, since the majority of the PC will be converted to PE. It is possible that the transphosphatidylation rate will be slower if a very efficient conversion of PC to PE occurs.

It is also important to note that the amount of PA in the modified lecithin has the potential to impact its effectiveness as an antioxidant. In meat systems, for example, PA has been found to effectively prevent iron-related oxidation where other phospholipids weren't successful. Dacharanhe and Terao investigated the effect of PA and PS in cooked beef homogenate and sardine oil-in-water emulsions and found that PA (but not PS) can suppress nonheme iron ion-induced lipid oxidation in this system<sup>86</sup>. In a system comprised of PC liposomes with arachidonic acid, the addition of PA was found to significantly slow iron-induced oxidation<sup>87</sup>. Cui et. al. used a commercial lecithin containing 5.0% PA, and found that increasing amounts of lecithin lead to increased iron chelation<sup>88</sup>.

### **High Phosphatidylethanolamine Lecithin Effect on Oxidation**

Previous work by Xu et. al. has examined the effect of PLD<sub>SC</sub> modified, high-PE lecithin in combination with  $\alpha$ -tocopherol in bulk oil<sup>33</sup>. Authors used a biphasic system with ethyl acetate as the organic phase to react PLD<sub>SC</sub> with ethanolamine and ~60% L- $\alpha$ -

phosphatidylcholine egg yolk lecithin to create a high PE egg lecithin. As shown in Figure 2.14, the addition of modified lecithin in combination with tocopherol increased the lag phase by four days over tocopherol alone. The unmodified lecithin had a shorter lag phase, confirming that PC has a prooxidative effect in this system. For a food system that needed to incorporate lecithin and tocopherol, an increase in lag phase of approximately 10 days could be achieved by using modified instead of unmodified lecithin. Addition of increasing amounts of modified lecithin in the absence of tocopherol had little to no effect on the lag phase of the oil. This finding is in agreement with other work comparing sunflower lecithin with either 59% or 90% PC found that the lower PC content lecithin had increased oxidative stability<sup>89</sup>.

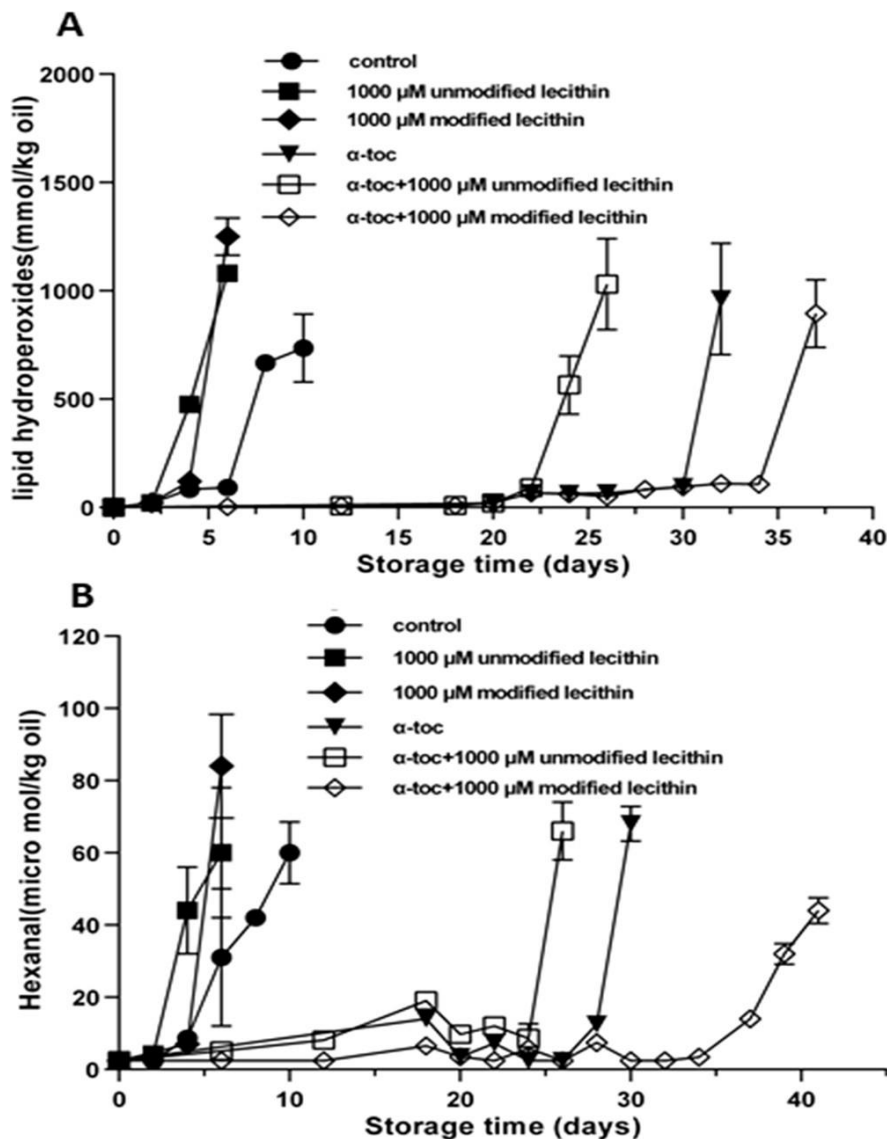


Figure 2.14. Formation of (A) lipid hydroperoxides and (B) hexanal in stripped soybean oil in response to additions of modified or unmodified lecithin and tocopherol<sup>33</sup>.

One of the potential problems with using modified lecithin in emulsions is the different areas in which the tocopherol, lipid, and lecithin can partition. For example, if one is completely partitioned into the core of the emulsion droplet, while another is in the aqueous phase, they might not interact to work synergistically. In a study examining the effect of phospholipid standards on soybean oil-in-water emulsions, Samdani et. al. found that PE in combination with α-tocopherol resulted in a minimal (1 day for hydroperoxides and 2 days for hexanal) increase in

lag phase<sup>32</sup>, however when combined with  $\delta$ -tocopherol and mixed tocopherols, the lag phases increased significantly. As shown in Figure 2.15, the addition of PE and mixed tocopherols increased the lag phase over tocopherols alone by two days for both hydroperoxides and hexanal formation.

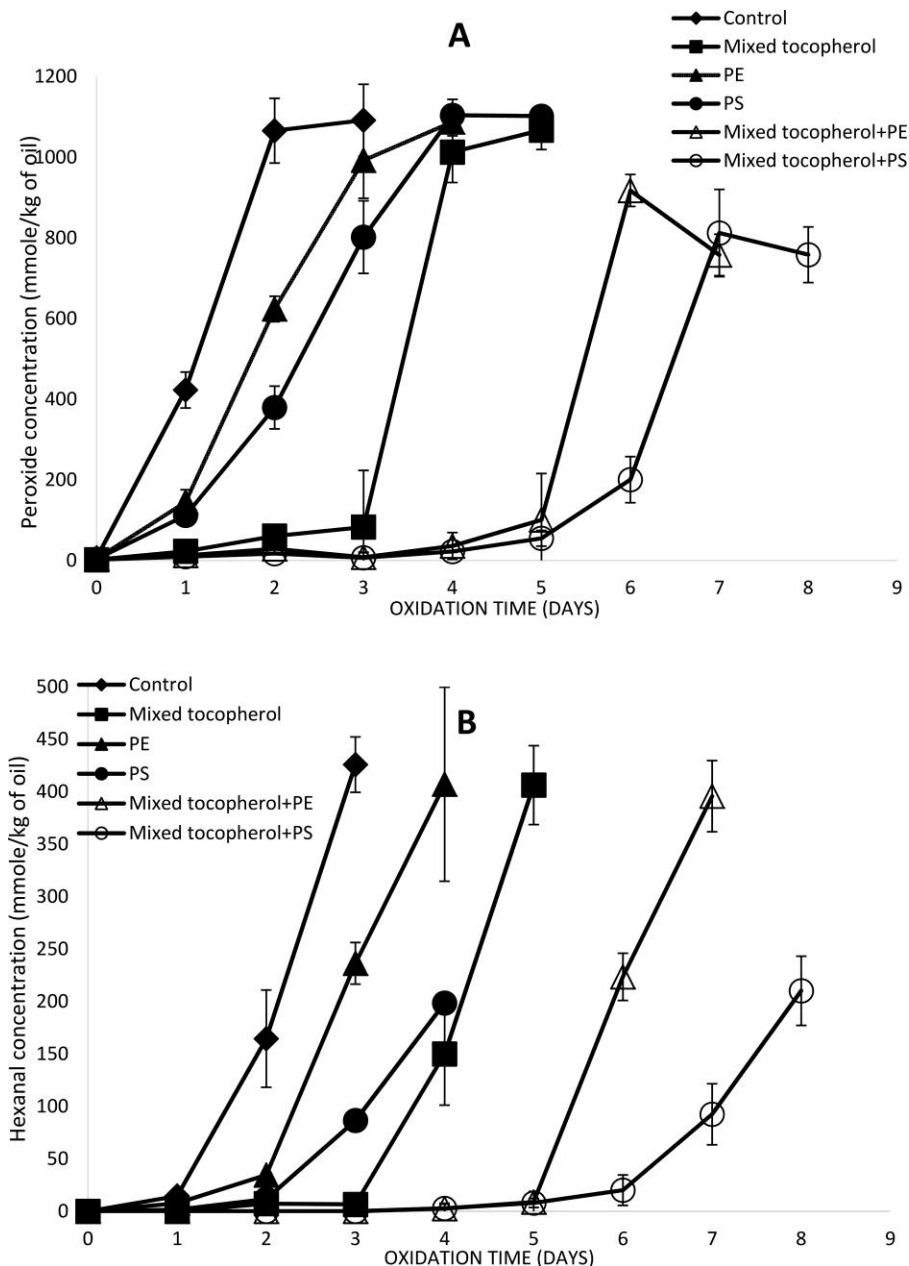


Figure 2.15. Formation of (A) lipid hydroperoxides and (B) hexanal in 1% stripped soybean oil-in-water emulsions stabilized with Tween 20 containing 3.0  $\mu\text{mol}$  of mixed tocopherol/kg of emulsion, 15.0  $\mu\text{mol}$ /kg of emulsion of PE or PS, or both<sup>32</sup>.

In the present study, mixed tocopherols will be used because their low cost makes them a more viable additive to a commercial food product. Since there will still be some residual PC present in the modified lecithin, in addition to the potential to introduce prooxidative contaminants during the modification process, it is possible that the increase in lag phase caused by modified lecithin will be less pronounced.



## Chapter 3

# IMPACT OF POLYUNSATURATED FATTY ACID DILUTION AND ANTIOXIDANT ADDITION ON LIPID OXIDATION KINETICS IN OIL/WATER EMULSIONS

### 1 Introduction

It is important to include unsaturated fatty acids in the diet such as omega-6 fatty acids, which have been associated with lowering levels of low density lipoproteins (LDL) and thus with decreasing the incidence of heart disease<sup>90,91</sup> and with omega-3 fatty acids which improve heart and brain health<sup>92,93</sup>. This was originally done in processed foods with synthetic antioxidants such as butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and ethylenediaminetetraacetic acid (EDTA). Recent consumer demand for clean label products, however has increased pressure on food manufacturers to remove synthetic antioxidants. With this in mind, food manufacturers are constantly examining techniques for delaying lipid oxidation while maintaining a clean label. One potential avenue is diluting an oil containing primarily polyunsaturated fatty acids with a more oxidatively stable oil.

It has been suggested that when diluting oils rich in polyunsaturated fatty acids in oils high in saturated fatty acids, the rate of lipid oxidation becomes slower because it takes longer for lipid radicals to diffuse to and oxidize unsaturated fatty acids. Previous work from Shiota et. al.<sup>94</sup> reported that the oxidative stability of bulk fish oil increased when it was blended with increasing concentrations of unsalted butter oil. Their results showed that with a ratio of 1:40 butter oil to fish oil, the oxidative stability was doubled compared to fish oil alone. At a 50:50 ratio, the lag phase was extended from less than three to 20 days.

The majority of work in this area has focused on lipid oxidation in blends of bulk oils and has attributed much of the oxidative protective effect to the presence of minor components in the

oils. For example, when virgin olive oil (which contains high concentrations of endogenous antioxidants and high amounts of oxidatively stable oleic acid) was blended with soybean oil and sunflower oil, the lag phase of lipid oxidation was extended compared to soybean or olive oil alone<sup>95</sup>. Blending high linoleic sunflower oil with cold pressed black cumin oil, cumin oil, coriander oil, and clove oil resulted in increased oxidative stability compared to the sunflower oil alone, which authors attributed to increased tocopherol concentration in the cold pressed oils as well as modification of the overall fatty acid profile<sup>96</sup>. Similar conclusions were also reached with sunflower kernel oil blended with sclerocarya birrea oil and melon bug oil<sup>97</sup>. Eidhin and O'Beirne found that when bulk camelina oil was blended with tuna and salmon oil, respectively, the peroxide values (PV) were in between the values observed for the oils individually<sup>98</sup>. Authors attributed this observation to the effect of diluting the oils as well as the presence of minor components.

While the majority of research in this area has focused on blending bulk oils, the authors of this study are not aware of published systematic research examining the oxidation of blended oils in oil-in-water emulsions. Let et. al. blended rapeseed oil with cod liver oil (1:1) prior to emulsification in 1% milk to determine the oxidative stability of blended oils in a food emulsion system<sup>99</sup>. Authors found that milk containing the 1:1 ratio of cod liver oil and rapeseed oil had a concentration of volatile lipid oxidation products between 3-19% less than the milk with fish oil alone. Authors attributed this to the increased concentration of minor components such as tocopherol in the rapeseed oil. However, it should be noted that milk is an extremely complex system with a multitude of minor components which could affect lipid oxidation such as metal chelating proteins, free radical scavenging amino acids and low molecular weight antioxidants that would impact these observations<sup>100-102</sup>.

It is unclear from the published literature if the inhibition of oxidation in oil-in-water emulsions was due to dilution or was due to other antioxidant factors originating food systems used. Therefore, the purpose of this study was to determine if fish oil stability could be increased by dilution into a more oxidatively stable oil and if increased stability was due to dilution or components of the diluting oil.

## **2 Materials and Methods**

Medium chain triglycerides (MCT) and fish oil was donated by DSM (Heerlen, Netherlands). Tween 80 was purchased from Sigma-Aldrich. High-oleic sunflower oil (HOSO) was procured from Amazon.com. Barium chloride (Sigma B-0750) was purchased from Sigma-Aldrich. Iron (II) sulfate heptahydrate (Sigma 215422) and activated charcoal (Sigma C3345) were obtained from Sigma-Aldrich (St. Louis, MO).

### **2.1 Emulsion Preparation**

A stock 0.1 M sodium phosphate buffer solution at pH 7 was stored at 4°C. Samples were prepared by combining appropriate amounts of fish oil, MCT oil, Tween 80 and sodium phosphate buffer as shown in Table 3.1 for MCT emulsions and Table 3.2 for high oleic sunflower (HOSO) emulsions. Solutions were homogenized for two minutes using a handheld homogenizer to create a pre-emulsion and were then passed through a microfluidics M-110L homogenizer for three passes at 83 MPa. One milliliter samples from all treatments were aliquoted into 10 mL glass GC vials which were sealed with metal caps and stored in a light-blocking container at 32°C. For emulsions with oils contained in separate droplets, the emulsions were prepared separately with higher concentrations of oil so that they could be mixed and diluted with buffer to achieve the proper oil ratios and total concentrations of the fish oil and diluting oils.

**Table 3.1.** Compositions of each emulsion by weight containing MCT.

Total Oil Content (% wt.)	MCT (% wt.)	Fish Oil (% wt.)	Tween 80 (% wt.)	Phosphate Buffer (% wt.)
1	0	1	0.1	98.9
2	1	1	0.2	97.8
5	4	1	0.5	94.5
10	9	1	1	89
20	19	1	2	78

**Table 3.2.** Compositions of each emulsion by weight containing HOSO.

Total Oil Content (% wt.)	HOSO (% wt.)	Fish Oil (% wt.)	Tween 80 (% wt.)	Phosphate Buffer (% wt.)
1	0	1	0.1	98.9
2	1	1	0.2	97.8
5	4	1	0.5	94.5

Emulsion characteristics were measured using a ZetaSizer Nano-ZS (Malvern Instruments, Worcestershire, UK) to determine the particle size and zeta potential. Samples were diluted to the appropriate level with the same phosphate buffer as was used to prepare the samples.

All measurements were taken as soon as possible after the sample emulsions were prepared to obtain day 0 measurements and subsequently at 24 hour intervals until lipid oxidation products were out of lag phase at which time the experiments were terminated.

## 2.2 Oil Stripping

Endogenous antioxidants/prooxidants were removed from the high oleic sunflower oil according to the method described by Boon et. al <sup>103</sup>. Briefly, 30g of high oleic sunflower oil was diluted 1:1 with hexane and added to a column covered to block light and containing two

outer layers of 22.5g silicic acid (CAS#1343-98-2) and a middle layer of 5.25g activated charcoal. The column was then eluted with 270 mL of hexane to recover the stripped oil. Finally, the mixture was rotary evaporated to remove hexane and the resulting oil was stored at -80°C until emulsion preparation.

### **2.3 Analysis of primary oxidation products**

A modified lipid hydroperoxides assay was performed based on the method described by Shantha and Decker<sup>104</sup>. To summarize briefly, 1.5 mL of iso-octane:isopropanol (3:1) solution was mixed with 0.3 mL of sample emulsion. This mix was vortexed for 30 seconds and then centrifuged at 1785 G for 3 minutes. The upper phase (200uL) was then combined with the supernatant resulting from centrifuging a 1:1 mixture of 0.132 mM BaCl<sub>2</sub> solution and 0.144 M FeSO<sub>4</sub> solution. Thirty uL of a 1:1 mixture of NH<sub>4</sub>SCN and FeCl<sub>2</sub> was then added to each sample and allowed to react for 20 minutes after which the absorbance was measured using a Genesys 20 spectrophotometer (ThermoSpectronic, Waltham, MA, USA) at 510 nm. A standard curve, prepared from cumene hydroperoxide, was used to determine hydroperoxide concentrations.

### **2.4 Analysis of secondary oxidation products**

Gas chromatography (GC) headspace analysis was performed using a Shimadzu GC-2010 (Shimadzu, Kyoto, Japan). Volatile compounds were separated using a 30 m x 0.32 mm Equity DB-1 column with a 1 µm film thickness. Samples were heated for 20 minutes in the autosampler heating block at 55°C. A 50/30 µm DVB/Carboxen/PDMS solid-phase microextraction (SPME) fiber needle was injected into the sample headspace for 3 min to adsorb volatiles. The fiber was placed into the injector port at 250°C for 3 min at a split ratio of 1:5.

Propanal concentrations were determined from standard curves prepared using either MCT or HOSO emulsions with added propanal ranging from 0 – 50  $\mu\text{M}$ .

## **2.5 Statistical Analysis of Data**

Lag phases were calculated using Dunnet's test in Minitab 19 (State College, PA) to compare the day 0 confidence interval to the confidence interval generated from the triplicate samples from each subsequent day. Lag phases were defined as the number of days between the start of the experiment and the last day that the 95% confidence interval for a given time point overlapped with the 95% confidence interval for day 0 ( $p < 0.05$ ).

## **3. Results and Discussion**

### **3.1 Blended Fish Oil and Medium Chain Triacylglycerols (MCT) Emulsion Droplets**

Particle size measurements were taken for all emulsion preparations. The purpose of measuring particle size was to ensure that the particles at the start of the experiment were of similar size in the emulsions made with different oils (data not shown). For all treatments, the average particle size ranged from approximately 150 to 200 nanometers and did not change during the storage studies (data not shown).

In the first experiment, emulsions were made containing fish oil alone or fish oil blended with increasing concentrations of MCT for total oil concentrations between 1 and 20% of the total weight of the emulsion, with fish oil maintained at 1% for all treatments. The purpose of this experiment was to determine whether blending fish oil (high in polyunsaturated fatty acids) with a more oxidatively stable oil such as MCT oil would increase the lag phase of lipid oxidation during storage. MCT was chosen for these experiments because it only contains saturated fatty acids and therefore is not prone to oxidation and because it does not contain endogenous antioxidants such as tocopherols as would be found in refined oils. The lag phase

for the control emulsion (containing just fish oil without MCT) lasted 1 day ( $p < 0.05$ ) for both hydroperoxides and propanal, (Figure 3.1A and B). As the concentration of MCT was increased across the treatments to 19% of the total emulsion, the lag phase was extended up to 5 days ( $p < 0.05$ ) for hydroperoxides and 6 days ( $p < 0.05$ ) for propanal formation. Dilution as low as 1:1 fish oil to MCT doubled the hydroperoxide and propanal lag phases from 1 to 2 days ( $p < 0.05$ ). Presumably, the extension in lag phase occurred because the transfer of lipid radicals between or within triacylglycerols was slower in the emulsion droplets with higher amounts of saturated MCT.

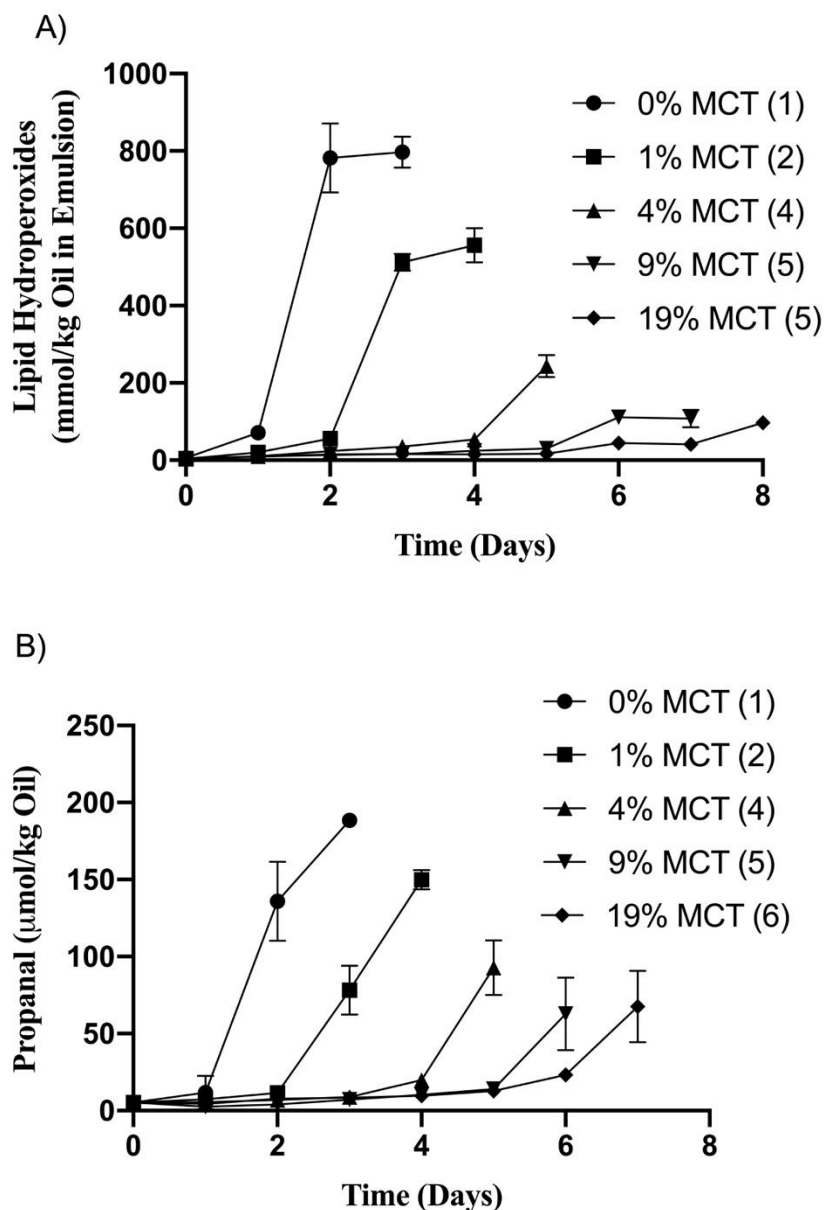


Figure 3.1. Lipid hydroperoxide (A) and propanal (B) formation in emulsions where medium chain triacylglycerol (MCT) oil and fish oil were in the same emulsion droplets. Data points are the average of 3 measurements  $\pm$  standard deviations. The calculated lag phases for each treatment are shown in parentheses.

### 3.2 Fish Oil and MCT in Separate Emulsion Droplets

In this experiment, fish and MCT oil-in-water emulsions were made separately and then were mixed together to give the same total lipid concentrations as the previous experiments. The purpose of these experiments was to determine if the extension in lag phase observed in the



blended oil droplet emulsions (Figure 1) was due to the dilution that occurred when the oils were blended. The lag phase of hydroperoxide and propanal formation was 2 days ( $p < 0.05$ ) for all MCT and fish oil concentrations (Figure 3.2A and B), thus when fish oil and MCT were in separate emulsion droplets, the large increase in lag phase in emulsions where fish oil and MCT were in the same droplets (Figure 3.1) was not observed. Coupland and coworkers<sup>16</sup> found that triacylglycerols in Tween-stabilized corn oil-in-water emulsions did not transfer between emulsion droplets and since our experiments used Tween 80 as the emulsifier and were conducted at similar temperatures (30 vs 32°C) and the same pH (7.0), this provides strong evidence that triacylglycerol transfer also did not occur in the experiments conducted in this study. Additional support for lack of triacylglycerol transfer between droplets is the lack of changes in lipid oxidation kinetics in these experiments. If triacylglycerol transfer did occur, then lipid oxidation rates should have decreased and become more like the emulsions where MCT and fish oil were in the same droplets (Figure 3.1). Since the oils are maintained in separate emulsion droplets, the rate of diffusion of lipid radicals formed from the oxidation of the FO is not altered by the presence of MCT in separate emulsion droplets. Thus, oxidation is just occurring in the fish oil droplets and the presence of MCT droplets had minimal impact on oxidation kinetics.

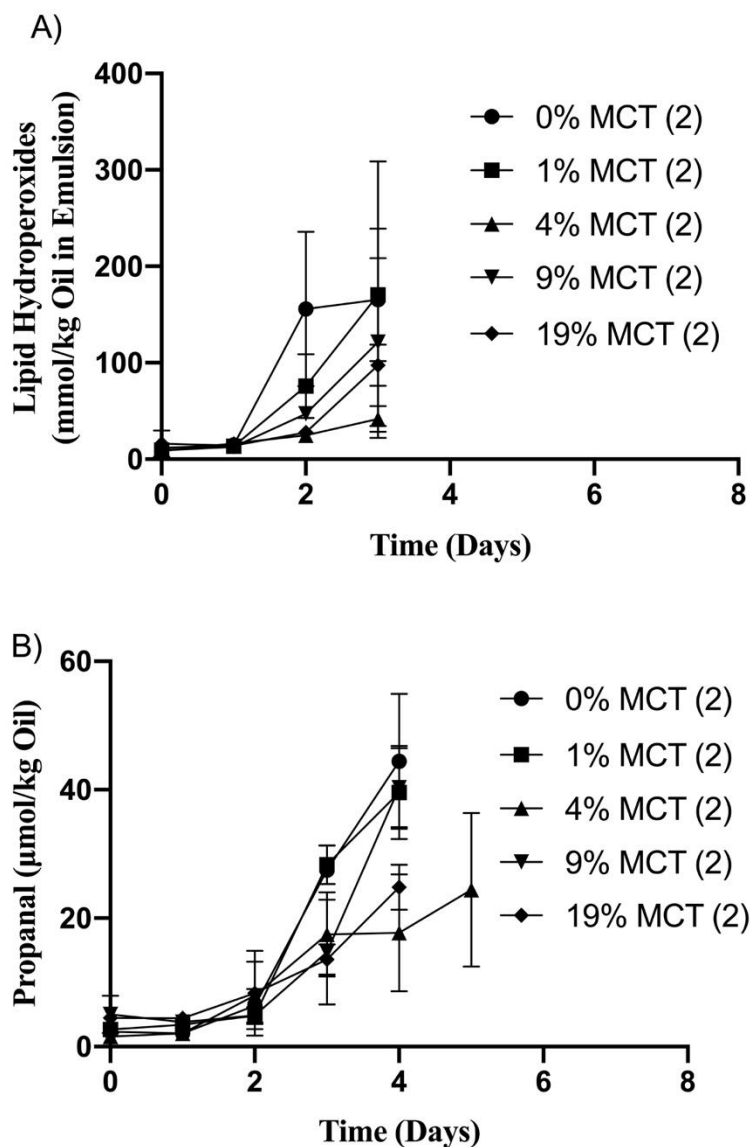


Figure 3.2. Lipid hydroperoxide (A) and propanal (B) formation where medium chain triacylglycerol (MCT) oil and fish oil were in separate emulsion droplets. Data points are the average of 3 measurements  $\pm$  standard deviations. The calculated lag phases for each treatment are shown in parentheses.

### 3.3 Blended and Separate Fish and High-Oleic Sunflower Oil (HOSO) Emulsions Droplets

The purpose of this experiment was to determine if the extension in lag phase due to the dilution of FO with MCT shown in the previous section (Figure 3.1) could be replicated with a less expensive oil more frequently used in food products. HOSO was chosen because oleic acid

is the most oxidatively stable unsaturated fatty acid common in oils and also contains tocopherols which could further inhibit oxidation of the fish oil.

For the emulsions where both oils were in the same droplets, the hydroperoxide lag phase increased from 2 to 3 to 4 days ( $p < 0.05$ ) and the propanal lag phases were 1, 2 and 4 days ( $p < 0.05$ ) as the concentration of HOSO was increased from 0% to 1% and 4% as shown in Figure 3.3A and B. This shows a dose-dependent increase in lag phase similar to what was observed in the MCT dilution experiment (Figure 3.1).

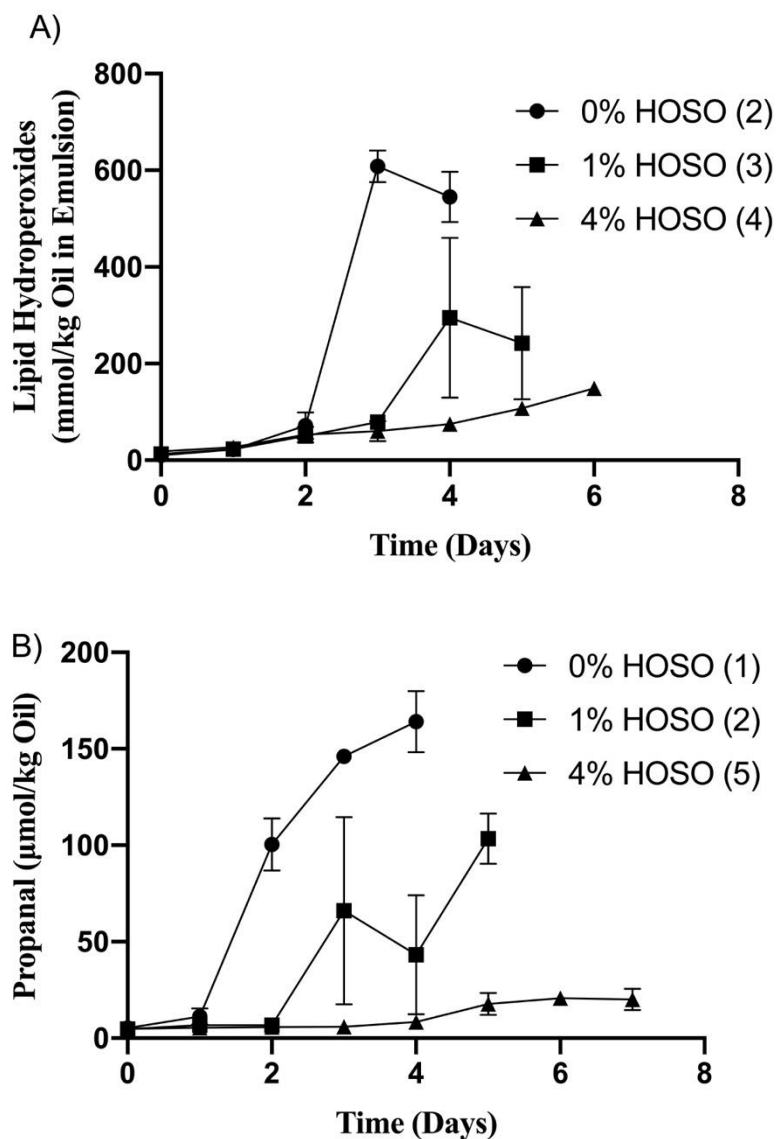


Figure 3.3. Lipid hydroperoxide (A) and propanal (B) formation in emulsions where high oleic sunflower oil (HOSO) and fish oil were in the same emulsion droplets. Data points are the average of 3 measurements  $\pm$  standard deviations. The calculated lag phases for each treatment are shown in parentheses.

For the emulsions where the 2 oils were in separate oil droplet, the hydroperoxide lag phase was 2, 2 and 4 days ( $p < 0.05$ ) and the propanal lag phases were 3, 3, and 5 days ( $p < 0.05$ ) for the respective 0%, 1% and 4% HOSO concentrations, as shown in Figure 3.4A and B.

In the previous experiment with MCT, the lag phases remained constant when the fish and MCT oils were in separate emulsion droplets (Figure 3.2). However, when the HOSO and

fish oil were in separate emulsion, the lag phase of hydroperoxide and propanal formation increased at 4% HOSO. Previous work <sup>105</sup> found that Tween 20 in soybean O/W emulsions was able to facilitate the transfer of tocopherols between emulsion droplets. Therefore, it is possible that the tocopherol from the HOSO [16] was able to transfer to the fish oil droplet and increase the hydroperoxide and propanal lag phases when the two oils were in separate emulsion droplets.

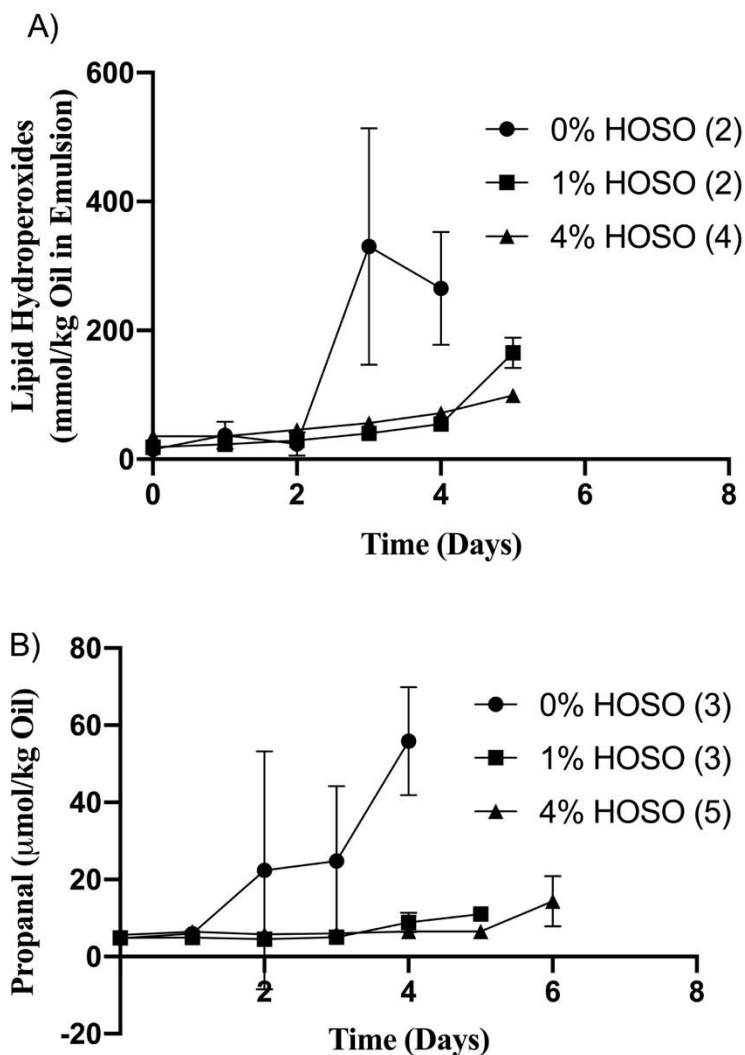


Figure 3.4. Lipid hydroperoxide (A) and propanal (B) formation in emulsions where high oleic sunflower oil (HOSO) and fish oil were in separate emulsion droplets. Data points are the average of 3 measurements  $\pm$  standard deviations. The calculated lag phases for each treatment are shown in parentheses.

### **3.4 Blended and Separate Fish and Stripped High-Oleic Sunflower Oil (SHOSO)**

#### **Emulsions Droplets**

In order to determine if the minor components of the HOSO influenced the oxidative stability of the fish oil, the HOSO was stripped of polar components including antioxidants such as tocopherols and then blended with the fish oil as was done in the previous experiment. In the emulsions where fish oil and stripped high oleic sunflower oil (SHOSO) were in the same droplets, lipid hydroperoxides had a 3 day ( $p < 0.05$ ) observed lag phase while propanal had a 2 day ( $p < 0.05$ ) lag phase (Figure 3.5A and B). Addition of the SHOSO increased the hydroperoxide lag phase to 4 days for both the 1:1 and 1:4 blends. Propanal lag phase was increased to 4 days and 4 days ( $p < 0.05$ ) for the 1:1 and 1:4 blends, respectively. These trends were similar to what was observed in the MCT and non-stripped HOSO blends, however the increase was less pronounced than emulsions with MCT. Since SHOSO contains unsaturated fatty acids while MCT is completely saturated, this suggests that the presence of oxidizable fatty acids in the SHOSO could cause protection by dilution to be less effective.

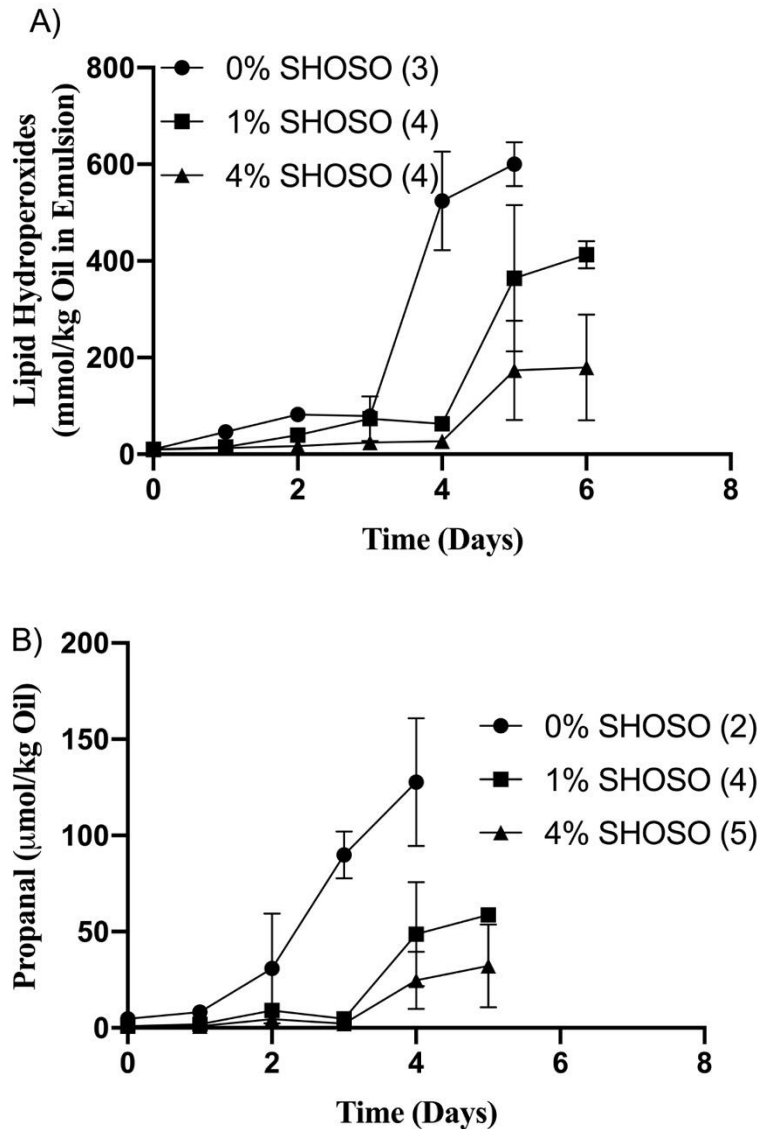


Figure 3.5. Lipid hydroperoxide (A) and propanal (B) formation in emulsions containing where stripped high oleic sunflower oil (SHOSO) and fish oil were in the same emulsion droplets. Data points are the average of 3 measurements  $\pm$  standard deviations. The calculated lag phases for each treatment are shown in parentheses.

Finally, Figure 3.6A and Figure 3.6B respectively show the formation of lipid hydroperoxides and propanal in oil-in-water emulsions with SHOSO and FO in separate emulsion droplets. The lag phase for hydroperoxides at all concentrations of SHOSO was 2 days ( $p < 0.05$ ) and 3 days ( $p < 0.05$ ) for propanal formation. There was not an observed increase in hydroperoxide and propanal lag phases in the presence of SHOSO in separate emulsion droplets.

As mentioned in Section 3.3, this suggests that the increase in oxidative stability in the experiments with non-stripped HOSO and fish oil in separate droplets was due to transfer of antioxidants from the HOSO droplet to the fish oil droplets.

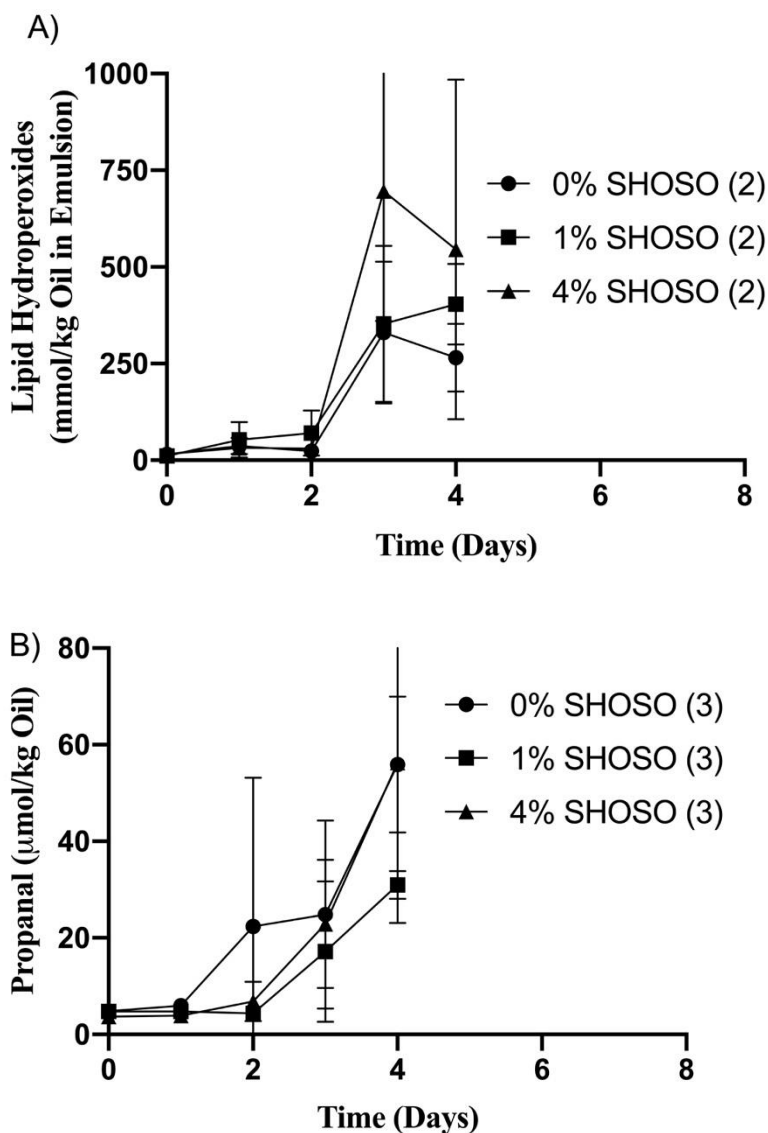


Figure 3.6. Lipid hydroperoxide (A) and propanal (B) formation in emulsions where stripped high oleic sunflower oil (SHOSO) and fish oil were in the separate emulsion droplets. Data points are the average of 3 measurements  $\pm$  standard deviations. The calculated lag phases for each treatment are shown in parentheses.

Since dilution of fish oil with MCT, HOSO and SHOSO (Figure 3.1, Figure 3.3, and Figure 3.5) all inhibited lipid oxidation, inhibition of the propagation step of lipid oxidation by



the slower diffusion of lipid radicals seems to be a major mechanism by which the oils were protected. The lack of increases in hydroperoxide and propanal lag phases when unstripped HOSO was blended with fish oils compared to MCT was somewhat surprising since the HOSO would be increasing the concentration of antioxidants such as tocopherols in the emulsion droplets. However, it is possible that the HOSO also brought prooxidative factors such as unsaturated fatty acids and possibly transition metals into the emulsions and these overcame the protective effects of the antioxidants.

Finally, the adequate intake for adults of DHA and EPA are 0.65g per day, with a minimum of 0.22g of either <sup>106</sup>. Meeting this requirement with menhaden oil, which contains approximately 11 and 12% of EPA and DHA respectively <sup>107</sup>, would require approximately 3 grams of fish oil per day. Table 3.3 shows the dilution that would occur if 3 grams of the total fat content in one serving of each product were replaced by menhaden oil. For example, a single serving of thousand island dressing contains 16g of fat. If 3g of the total fat content were FO, the dilution would be 1:4.3, a dilution ratio shown in this study to increase the oxidative stability of the FO. All of the dilution levels in the food products shown in Table 3.3 were found to be effective at delaying lipid oxidation in the present study. Therefore, dilution of fish oils with more oxidatively stable oils has potential for increasing the shelf-life of omega-3 fortified food products without the labeling of synthetic or natural antioxidants.

**Table 3.3.** The levels of dilution when a nutritionally significant amount of menhaden oil (3g) was used to replace the oil naturally occurring in a variety of food products.

Food Matrix	Serving Size	Total Fat Content (g)	Potential Dilution of 3 g of Fish Oil
Thousand Island Dressing	2 Tbsp (28g)	16	1:4.3
Mayonnaise	2 Tbsp (28g)	9	1:2
Hot Dogs	1 Link (57g)	14	1:3.6
Plain Wole Yogurt	1 Cup (245g)	8	1:1.7

## Chapter 4

# ENZYMATIC MODIFICATION OF LECITHIN FOR IMPROVED ANTIOXIDANT ACTIVITY IN COMBINATION WITH TOCOPHEROL IN EMULSIONS AND BULK OIL

### Abstract:

Oxidized  $\alpha$ -tocopherol can be regenerated by phosphatidylethanolamine (PE) but current commercial sources of PE are too expensive for use as a food additive. The present study aims to determine the optimal reaction conditions for generating high PE lecithin (MHPEL) enzymatically and to validate the MHPEL's synergism with tocopherol in delaying lipid oxidation in an oil-in-water emulsion systems at pH 7, and 4, and in bulk oil. Under optimal conditions of pH 9.0, 37°C and 4h, a MHPEL with ~71.6% PE was obtained from 96% phosphatidylcholine lecithin using phospholipase D from *Streptomyces chromofuscus*. Mixed tocopherols (300  $\mu\text{mol/kg}$  oil) and MHPEL (1500  $\mu\text{mol/kg}$  oil) synergistically increased both the hydroperoxide and hexanal lag phase of lipid oxidation in stripped soybean oil-in-water emulsions at pH 7 by 3 days. At pH 4, this combination increased the lag phases by 3 and 2 days respectively. The combination of 50  $\mu\text{mol/kg}$  oil of  $\alpha$ -tocopherol and 1000  $\mu\text{mol/kg}$  oil MHPEL also synergistically increased the hydroperoxide (5 days) and hexanal (4 days) lag phases in stripped bulk soybean oil. This approach represents a potential clean-label antioxidant system that could have commercial applications to decrease food waste.

Keywords: tocopherol, oxidation, regeneration, phosphatidylethanolamine, *Streptomyces chromofuscus*, transphosphatidylation,

## 1. Introduction

Consumers are currently looking to increase their consumption of unsaturated fats while simultaneously decreasing their consumption of ingredients perceived to be “synthetic” or “unnatural.” This creates a unique challenge for food manufacturers because polyunsaturated fats are prone to oxidative deterioration and thus are often stabilized using synthetic antioxidants such as butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) or ethylenediaminetetraacetic acid (EDTA). In some cases, evidence has shown that BHT and TBHQ may pose health risks to consumers<sup>1</sup>. The challenge of developing foods high in unsaturated fatty acid and low in synthetic antioxidants is also complicated by the fact that the physical properties of foods impact antioxidant partitioning and therefore antioxidant efficacy, meaning that most antioxidants are not equally effective in all foods.

Phosphatidylcholine (PC) from soybean has been shown to have pro-oxidant activity in bulk oil<sup>2</sup> whereas phosphatidylethanolamine (PE) and phosphatidylserine (PS) have been reported to be antioxidative under certain conditions<sup>108</sup>. This difference is thought to be due to the ability of PE and PS to convert  $\alpha$ -tocopherol quinone back to  $\alpha$ -tocopherol, thus extending tocopherol's ability to delay lipid oxidation<sup>2,32,33</sup>. Unfortunately, commercial sources of lecithin are typically low in PE and PS and thus are not strong antioxidants alone or in combination with tocopherols. Purified phospholipids are typically too expensive for usage in food and might not be “label friendly” to consumers due to their complicated scientific names.

PC's prooxidant activity is thought to be due to its negative charge which can bind transition metals and solubilize them into lipids or concentrate them at emulsion droplet surfaces. Therefore, an antioxidative lecithin would not only need to be high in PE or PS but would also

have to be low in PC. In addition, methods to modify phospholipid headgroups can also result in the formation of the negatively charged phosphatidic acids (PA).

An economically and regulatory-friendly modified high PE lecithin (MHPEL) could potentially be produced using phospholipase D (PLD) which can convert PC to PE in the presence of ethanolamine. For this reaction, the starting lecithin would ideally be high in PC, the preferred substrate for PLD. Previous work was done to convert egg lecithin to a MHPEL<sup>32</sup> but this product would not be suitable for vegan diets so it might not have widespread acceptance by the food industry. Finally, the ability of MHPEL to inhibit lipid oxidation needs to be evaluated in food systems such as oil-in-water emulsions as it has only been tested in bulk oil to date<sup>33</sup>.

PLD is a wide family of enzymes found in plants, animals and bacteria that cleaves off the polar head group on a phospholipid and then catalyzes the transphosphatidylation of a different headgroup. Therefore, PLD is capable of converting PC to PE in the presence of ethanolamine or PC to PS in the presence of serine with PA as a byproduct under certain conditions. Therefore, conversion of PC to PE or PS in lecithin could provide a novel food antioxidant when used in combination with tocopherols. However, much of the research published on the characterization of the activity of PLD only measures the release of choline or formation of PA and predominately uses pure phospholipids that are not practical for use as a food additive. Therefore, further fundamental research on the characterization of PLD to produce a high PE or PS lecithin for the purpose of producing a novel antioxidant is required.

The first objective of the present research is to characterize the modification of lecithin using phospholipase D from *Streptomyces chromofuscus* (PLD<sub>SC</sub>). Conversion to PE was determined instead of PS as high PE lecithin has already been shown to inhibit lipid oxidation in bulk oil<sup>33</sup> and PE and tocopherol was found to be an effective antioxidant combination in oil-in-

water emulsions<sup>32</sup>. Characterization was evaluated using a commercially available lecithin (Phospholipon 90G) with a very high (>96%) PC content to show the potential for conversion to PE. Since the PA content has a significant chance of impacting the MHPEL's effectiveness as part of an antioxidant system, and since the conditions favoring hydrolysis may differ from those that favor transphosphatidylolation, the modification of lecithin at varying temperatures and pH levels was investigated by measuring PC, PE, and PA content by HPLC.

After the optimization in the first objective is completed, it will be important to determine the antioxidant efficacy of the MHPEL. This will be tested in stripped soybean oil-in-water emulsions at pH 7 as well as pH 4 to represent a range of different food emulsion systems such as milk (pH 6.7-6.9) and salad dressings (pH 2.9 - 4.4)<sup>109</sup>. Additionally, the antioxidant effect of high PE lecithin was also tested in a bulk oil system. These findings have significant relevance for food manufacturers attempting to increase the shelf-life of their products.

## **2. Materials and Methods**

### **2.1 Materials**

Phospholipase D from *Streptomyces chromofuscus* (>50,000 units/mL), silicic acid, iron (II) sulfate heptahydrate, barium chloride, ethyl acetate, Tween 80, activated charcoal, and  $\alpha$ -tocopherols were all purchased from Sigma Aldrich (St. Louis, MO). Phospholipon 90g was donated by American Lecithin Company, Oxford, CT, and was selected as a commercially available lecithin with a high PC content (>96%) so that there would be an abundance of PC to convert to PE. Mixed tocopherols (Decanox MTS-90G) containing  $\alpha$ - (117 mg/g),  $\beta$ - (19 mg/g),  $\gamma$ - (634 mg/g),  $\delta$ - (141 mg/g) were donated by Archer Daniels Midland (Decatur, Illinois). Purified 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) standard was purchased from Avanti Polar Lipids (Birmingham, AL).

## 2.2 Enzymatic Modification of Lecithin

Enzymatic modification was carried out using a modified version of the method described by Xu et al. (2019). Ethanolamine (1 mL) was combined with a sufficient quantity of HCl to reach the desired pH (7, 8, 9, or 10) for each experiment. The volume was then made up to 2.5 mL using double distilled H<sub>2</sub>O, and the pH was reconfirmed after 1 hour. The pH adjusted ethanolamine was added to a solution of 0.3 g lecithin in 30 mL ethyl acetate.

An enzyme solution was prepared with 1000 units of PLD from *Streptomyces chromofuscus* (PLD<sub>SC</sub>) in addition to calcium chloride (50 mM) as an enzyme cofactor. Enzyme solution (250 µL) was added to the ethanolamine/lecithin mixture. The reaction mixture was placed into an incubator at the appropriate temperature (32, 37, 55, or 65°C). The reaction mixture was removed from the incubator after a designated periods of time (0, 5, 20, 40, 60, 120, or 240 min) and placed into a boiling water bath for 10 minutes to stop the enzyme activity. The ethyl acetate was removed with a rotary evaporator. Phospholipids were extracted by combining the remaining solution with 80 mL chloroform, 30 mL methanol, and 20 mL water. The organic phase was isolated with a separatory funnel and the organic solvent was removed by rotary evaporator until only the extracted lipids remained. Experiments were completed in triplicate (see statistical analysis section).

## 2.3 Phospholipid Determination

Phospholipid quantitation was conducted using a modified version of the method described by Zhang et. al. for determining phospholipid content<sup>110</sup>. A Shimadzu 2030C PLUS HPLC with an evaporative light scattering detector (ELSD) was used with a gradient mobile phase consisting of isopropanol, hexane and water as shown in Table 4.1. Using a 4.6×150 mm, 5 µm Agilent Zorbax SIL (Part No. 883952-701), it was possible to get clear peak definitions for

PC, PA, and PE on a single HPLC run. Column temperature was set for 35°C while the ELSD temperature was set for 63°C with a gain of 5 and a 0.5 second filter.

Table 4.1. Solvent gradients used during the HPLC method to separate phospholipids.

Time (min)	Flow Rate (mL/min)	Isopropanol Content (vol. %)	Water Content (vol. %)	Hexane Content (vol. %)
0	0.8	42	5	53
8	0.8	42	5	53
8.1	1.2	42	5	53
25	1	54	10	36
25.1	0.8	66	17	17
35	0.8	66	17	17

## 2.4 Oil Stripping

Commercial soybean oil was purchased from a local food store. Endogenous antioxidants/prooxidants were removed from the soybean oil according to the method described by Boon et. al.<sup>103</sup>. Briefly, 30 g of soybean oil was diluted 1:1 with hexane and added to a column covered to block light and containing two layers of 22.5 g silicic acid and a middle layer of 5.25 g activated charcoal. The column was then eluted with 270 mL of hexane to recover the stripped oil. Finally, the mixture was rotary evaporated to remove hexane and the resulting oil stored at -80°C until use.

## 2.5 Oxidation Studies

The MHPCL used for all shelf-life experimentation was reacted according to the schedule described in Section 2.2 at pH 9, 37°C, and 4 hours. The resultant lecithin's composition was 71.6% PE, 14.8% PA, and 13.6% PC which all fell within the standard deviations shown in Figure 4.1 for that set of reaction conditions. A 0.1 M sodium phosphate buffer solution was prepared for experiments at pH 7 and a 0.1 M sodium acetate buffer solution was prepared for experiments at pH 4.0. Both were stored at 4°C. Samples were prepared by first dissolving

phospholipids and tocopherol in chloroform or hexane and pipetting them into acid-washed beakers. The solvents were evaporated with nitrogen, and then appropriate amounts of stripped soybean oil, Tween 80 and buffer were added as shown in Table 4.2. The concentration of MHP EL was calculated on the basis of PE content in the sample. Samples were stirred for 3 hours in the dark at 4°C to allow incorporation. Emulsion samples were homogenized for two minutes using a handheld homogenizer to create a pre-emulsion and then passed through a microfluidics M-110L homogenizer for three passes at 83 MPa. One milliliter samples from all treatments were aliquoted into 10 mL glass GC vials which were then sealed with PTFE/silicone septum metal caps and stored in a light-blocking container at 32°C.

Stripped bulk oil samples were prepared by adding appropriate amounts of tocopherol in chloroform and/or phospholipids in hexane to acid washed beakers. Solvents were evaporated and then stripped soybean oil was added. Samples were stored at 4°C for 3 hours with light stirring to allow the antioxidants to be completely dissolved in the stripped oil. Subsequently, 1 mL samples were aliquoted into 10 mL GC vials, capped with PTFE/silicone septum metal caps and stored in the dark at 55°C.

Table 4.2. Summary of the experimental treatments used for the shelf-life experiments. Emulsions were prepared used mixed tocopherols (mixed toc.) while experiments with bulk oil were conducted with  $\alpha$ -tocopherol ( $\alpha$ -toc.).

<b>Emulsions (pH 7 + pH 4)</b>	<b>Buffer (% w/w)</b>	<b>SSO (% w/w)</b>	<b>Tween 80 (% w/w)</b>	<b>Tocopherol (<math>\mu</math>mol/kg oil)</b>	<b>DOPE (<math>\mu</math>mol/kg oil)</b>	<b>MHP EL (<math>\mu</math>mol/kg oil)</b>
Control	98.9	1	0.1	-	-	-
Mixed Toc.	98.9	1	0.1	300	-	-
MHP EL	98.9	1	0.1	-	-	1500
DOPE	98.9	1	0.1	-	1500	-
MHP EL + Mixed Toc.	98.9	1	0.1	300	-	1500
DOPE + Mixed Toc.	98.9	1	0.1	300	1500	-
<b>Bulk Oil</b>						
Control	-	100	-	-	-	-



50 $\alpha$ -Toc.	-	100	-	50	-	-
500 DOPE	-	100	-	-	500	-
500 MHPPEL	-	100	-	-	-	500
1000 MHPPEL	-	100	-	-	-	1000
500 DOPE + $\alpha$ -Toc.	-	100	-	50	500	-
500 MHPPEL + $\alpha$ -Toc.	-	100	-	50	-	500
1000 MHPPEL + $\alpha$ -Toc.	-	100	-	50	-	1000

## 2.6 Analysis of primary oxidation products

A modified lipid hydroperoxides assay was performed based on the method described by Shantha and Decker<sup>104</sup>. To summarize briefly, 1.5 mL of iso-octane:isopropanol (3:1) solution was mixed with 0.3 mL of sample emulsion. This mixture was vortexed for 30 seconds and then centrifuged at 1785 G for 3 minutes. The upper phase (200  $\mu$ L) was combined with the supernatant resulting from centrifuging a 1:1 mixture of 0.132 mM BaCl<sub>2</sub> solution and 0.144 M FeSO<sub>4</sub> solution. Thirty  $\mu$ L of a 1:1 mixture of NH<sub>4</sub>SCN and FeCl<sub>2</sub> were then added to each sample and allowed to react for 20 minutes after which the absorbance was measured using a Genesys 20 spectrophotometer (ThermoSpectronic, Waltham, MA, USA) at 510 nm. A standard curve, prepared from cumene hydroperoxide, was used to determine hydroperoxide concentrations.

## 2.7 Analysis of secondary oxidation products

Gas chromatography (GC) headspace analysis was performed using a Shimadzu GC-2010 with a Shimadzu AOC-6000 autosampler (Shimadzu, Kyoto, Japan). Volatile compounds were separated using a 30 m x 0.32 mm Equity DB-1 column with a 1  $\mu$ m film thickness. Samples were heated for 20 minutes in the autosampler heating block at 55°C. A 50/30  $\mu$ m DVB/Carboxen/PDMS solid-phase microextraction (SPME) fiber needle was injected into the sample headspace for 3 min to adsorb volatiles. The fiber was placed into the injector port at

250°C for 3 min at a split ratio of 1:5. The column oven temperature was held at 65°C, and the FID was set for 250°C with a 40 msec sampling rate with helium at 30mL/min, H<sub>2</sub> at 40 mL/min and air flow at 400 mL/min.

## 2.8 Statistical Analysis of Data

Results are presented as the mean ± standard deviation of analyses conducted in triplicate. Lag phases were calculated using Dunnet's test in Minitab 19 (State College, PA) to compare the day 0 confidence interval to the confidence interval generated from the triplicate samples from each subsequent day. Lag phases are defined as the number of days between the start of the experiment and the last day that a 95% confidence interval was not significantly different from the 95% confidence interval for day 0 (p<0.05).

## 2.9 Calculation of Interaction Indexes

In order to determine whether the combination of tocopherol and either DOPE or MHPEL was synergistic (as opposed to additive or antagonistic), Equation 2 was used based on the statistically calculated lag phases. An interaction index >1 indicates a synergistic interaction between tocopherol and the phospholipid (either DOPE or MHPEL), a value =1 indicates an additive effect, and a value <1 indicates an antagonistic interaction between the two additives.

$$\text{interaction index} = \frac{\text{lag phase}_{(\text{toc \& PL})}}{(\text{lag phase}_{\text{toc}}) + (\text{lag phase}_{\text{PL}})} - \text{lag phase}_{\text{control}}$$

Equation 2. The equation for calculating the interaction index of tocopherol (toc) with 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) or the modified high phosphatidylethanolamine (MHPEL).

### 3. Results and Discussion

#### 3.1 Effect of pH on Phospholipase D Conversion of Phosphatidylcholine to Phosphatidylethanolamine and Phosphatidic Acid

PLD's ability to convert PC to PE or PA was determined at pH 7, 8, 9, and 10 after incubation for 1 and 4 hours (Figure 4.1). As expected, the increase in incubation time allowed for more enzymatic activity at every pH level. The highest conversion of PC to PE in the lecithin was 73.3% at pH 9 after 4 hours with 9.8% PA also being produced. The second highest was at pH 10 which resulted in a modified lecithin containing 46.7% PE. Virtually no PE was formed at pH 7.0. After incubation at pH 8, the resulting lecithin contained only 14% PE, with 61.4% PA.

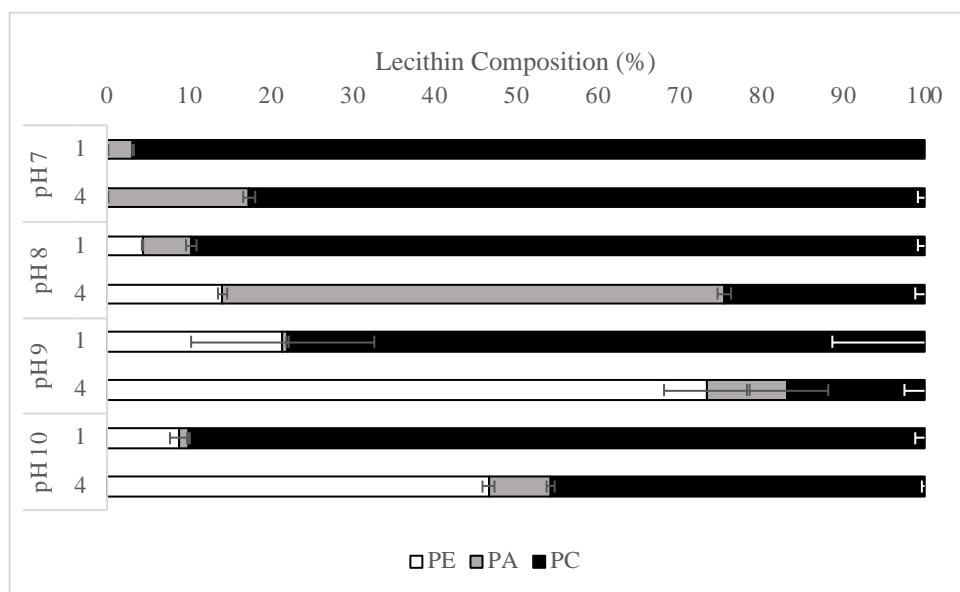


Figure 4.1. Phospholipid compositions after incubating high PC soybean lecithin with phospholipase D from *Streptomyces chromofuscus* at pH 7, 8, 9, and 10 for 1 and 4 hours.. Experiments were completed in triplicate and error bars represent the standard deviation of the 3 replicates.

Many previous studies on PLD pH optimums have focused on hydrolysis rather than transphosphatidylation. For example, Qin et. al. (2018) used a colorimetric assay to measure the amount of choline liberated by *Streptomyces chromofuscus* to determine an optimum pH for

PLD<sub>sc</sub> activity of 8.0<sup>69</sup>. Geng et. al. (1999) used two methods to measure the hydrolysis of L-alpha-dibutyryl-glycero-3-phosphatidylcholine (diC<sub>4</sub>PC) catalyzed by two fractions of PLD<sub>sc</sub>, and found activity maximums of pH 7.5 and pH 8.0, respectively<sup>78</sup>. These authors employed NMR spectroscopy to measure the increase in choline N-methyl resonance intensity as PC is hydrolyzed and a Radiometer pH-stat to measure the generation of PA. Our study showed very little loss of PC at pH 7.0 while at pH 8.0, PC was primarily converted to PA. Significant PE formation was only seen at pH values greater than 8.0.

The finding that PA formation is favored at pH 8.0 while PE formation is favored at pH 9 suggests that at pH 8.0, PC hydrolysis to PA predominates the reaction and/or that if PE is formed it is also hydrolyzed to PA. An early study concluded that PLD from *Streptomyces chromofuscus* showed “poor transphosphorylation” activity in comparison to PLD from other *Streptomyces* origins<sup>73</sup>. Their conclusion was based on hydrolysis and transphosphorylation activity assays that were only performed at pH 8 where we also found little formation of PE. Notably, when authors calculated substrate specificity ratios for all enzymes tested, PLD<sub>sc</sub> had the highest, suggesting that it had the highest affinity to hydrolyze its byproduct (in this case PE) back to PA<sup>73</sup>. With increasing pH, the amine group on PE (pK<sub>a</sub> of 9.5) would become less charged<sup>111,112</sup>. Müller et. al. reported low transphosphorylation activity by PLD from *Streptomyces* sp. when the acceptor molecule was uncharged<sup>113</sup>. This could help explain why transphosphorylation of PC to PE was higher at pH 9.0 than 8.0.

### **3.2 Effect of Temperature and Time on Lecithin Composition**

Figure 4.2 shows the results of lecithin modification with PLD<sub>sc</sub> at pH 9 over time at 20, 37, 55 and 65°C. Although some transphosphatidyl activity was observed at all

temperatures, the most complete conversion of PC to PE occurred at 37°C. After 240 minutes, 73.3% of the starting PC had been converted to PE with only 12.6% converted to PA. With a lower temperature of 20°C, and thus a slower reaction rate, only 32.9% of the PC was converted to PE in the same time. At 55°C, the reaction initially progressed at a faster rate than at 37°C, however the rate slowed and eventually plateaued at ~42% PE. PA formation was observed at all temperatures reaching a maximum of 8.8% after 240 min at 55°C. Minimal transphosphatidylation and hydrolysis was observed at 65°C, presumably due to enzyme denaturation.

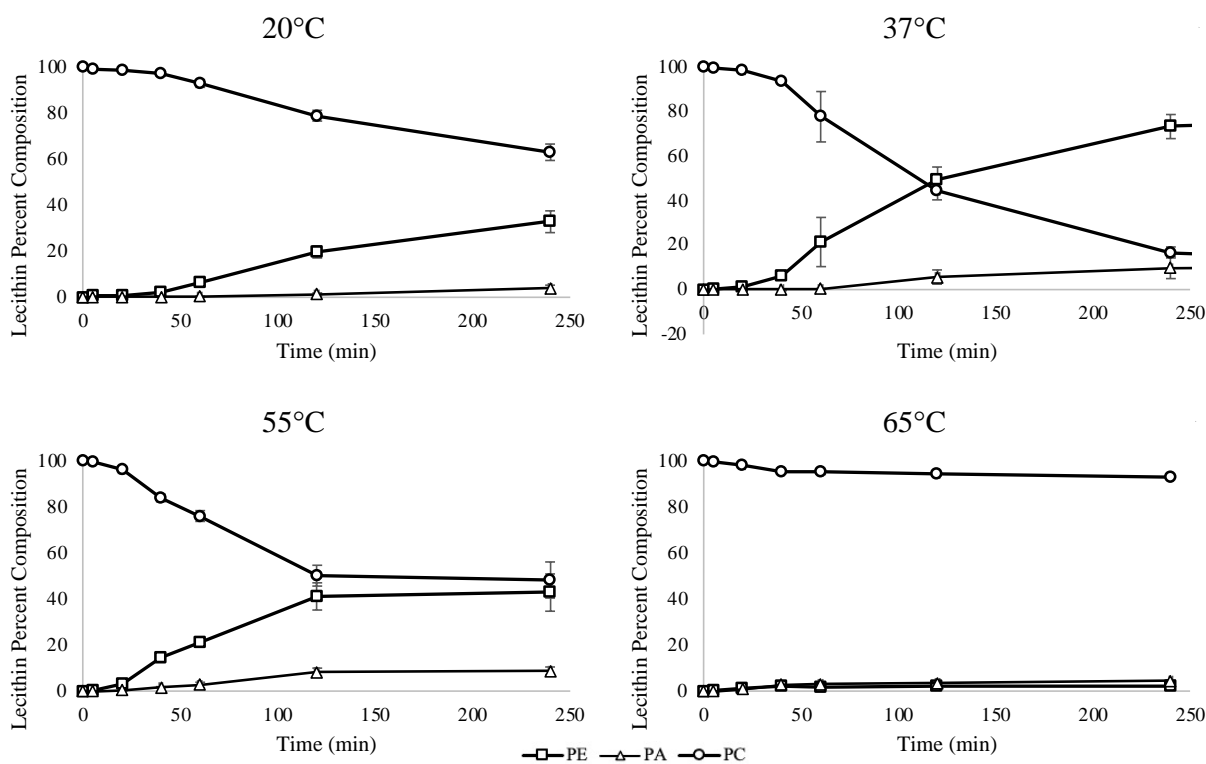


Figure 4.2. The effect of temperature on the conversion of high phosphatidylcholine (PC) lecithin to phosphatidylethanolamine (PE) and phosphatidic acid (PA) by phospholipase D from *Streptomyces chromofuscus* (PLD<sub>SC</sub>) as a function of time. Data points represent the average of three different lecithin conversions with error bars  $\pm$  the standard deviation.

Studies using hydrolysis assays to measure the liberation of choline from standard PC found that PLD<sub>SC</sub> activity increased with temperature until approximately 60-65°C when activity was diminished, presumably due to enzyme denaturation. For example, Liu et. al.<sup>70</sup> incubated PLD<sub>SC</sub> with PC standard at temperatures ranging from 30 to 65°C for one hour before measuring enzyme activity using a hydrolysis assay to measure the amount of choline released from PC and found an activity maximum of 50°C. After being incubated at 65°C, the PLD<sub>SC</sub> had almost no activity. Zambonelli et. al. incubation PLD<sub>SC</sub>'s 20-60°C for 3 h before measuring PLD<sub>SC</sub>'s activity with PC at pH 8 using a colorimetric assay based on phosphatidyl p-nitrophenol hydrolysis<sup>80</sup>. While subjecting the enzyme to this 3 hour incubation at 50°C prior to analysis had no effect on the enzyme's activity when measured under their assay conditions, the enzyme that was pre-incubated at 60°C for 3 hours before being assayed had no hydrolysis activity.

### **3.3 Determining the antioxidant activity of tocopherols and modified high phosphatidylethanolamine lecithin in oil-in-water emulsions and bulk oil.**

Figure 4.3 shows the results of the shelf-life study conducted at pH 7 in 1% soybean oil-in-water emulsions. The control emulsion had a 0 day lag phase for lipid hydroperoxides and a 2 day lag phase for hexanal formation. Lag phases were similar to the control when MHPPEL or purified DOPE (1500 µmol/kg oil) were added alone. When 300 µmol/kg oil of mixed tocopherols were added, the lag phases were extended to 2 and 4 days for hydroperoxide and hexanal formation, respectively. The combination of mixed tocopherols and MHPPEL increased the lipid hydroperoxide lag phase to 5 days and the hexanal lag phase to 7 days. The interaction indexes demonstrate a synergistic interaction of 2.5 for hydroperoxide formation and 1.66 for hexanal formation when mixed tocopherols and MHPPEL are used in combination. Purified DOPE in combination with mixed tocopherols had a lag phase for both lipid hydroperoxides

(IE=3.5) and hexanal (IE=1.66) formation of 7 days. These interaction indexes also indicate a synergistic reaction between the mixed tocopherols and DOPE.

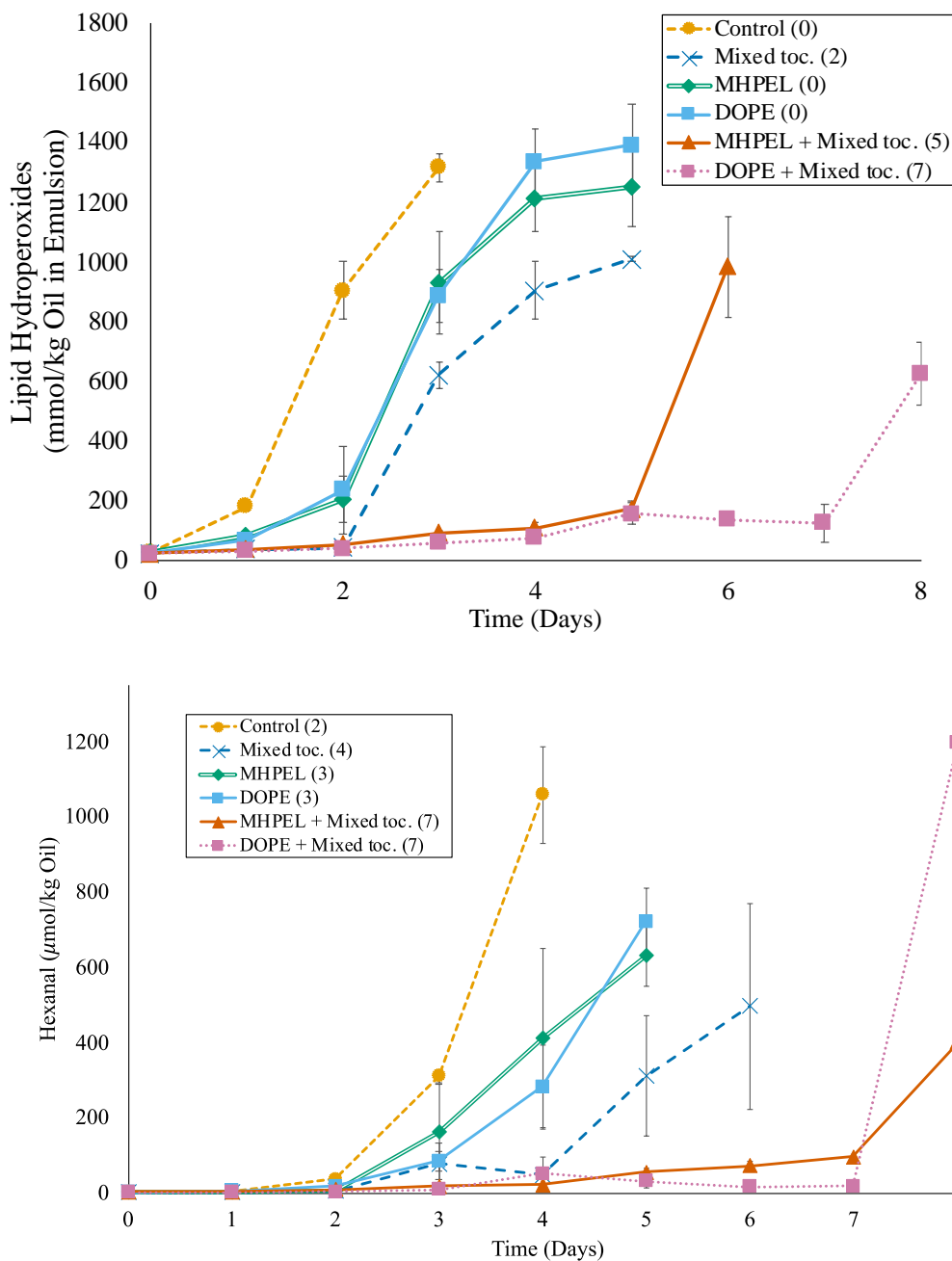


Figure 4.3. Formation of lipid hydroperoxides and hexanal during storage of 1% soybean oil-in-water emulsions at pH 7. Treatments include mixed tocopherols (300 µmol/kg oil), modified high phosphatidylethanolamine lecithin (MHPREL; 1500 µmol/kg oil), purified 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE; 1500 µmol/kg oil), mixed tocopherols (300 µmol/kg oil) + MHPREL (1500 µmol/kg oil) and mixed tocopherols (300 µmol/kg oil) + DOPE

(1500  $\mu\text{mol/kg}$  oil). Data points represent the average of triplicate samples  $\pm$  standard deviations. Statistically calculated lag phases are shown in parentheses in figure legend.

After validating the synergistic effect of tocopherol and MHPEL at neutral pH, a study was performed at pH 4 to more closely mirror the pH found in many emulsion-based foods. Mayonnaise, for example, is a high fat-content oil-in-water emulsion with a pH of around 4 due to the addition of vinegar<sup>114</sup>. As shown in Figure 4.4, the samples containing no added mixed tocopherols or lecithin (control), MHPEL alone and DOPE alone were all out of lag phase after day 0 for both hydroperoxides and hexanal formation. When 300  $\mu\text{mol/kg}$  oil mixed tocopherols was added, the lag phase was extended to 3 and 4 days for hydroperoxides and hexanal, respectively. The addition of 300  $\mu\text{mol/kg}$  oil mixed tocopherols + MHPEL increased the lag phase to 6 days for both hydroperoxides and hexanal, resulting in an interaction index of 2 for hydroperoxides and 1.5 for hexanal formation. The combination of 300  $\mu\text{mol/kg}$  oil mixed tocopherols + DOPE resulted in 9 day lag phases and interaction indexes of 3 and 2.25 for peroxides and hexanal. This indicates a synergistic interaction between mixed tocopherols and DOPE or MHPEL at pH 4, with the antioxidant activity being stronger for pure DOPE and mixed tocopherols.



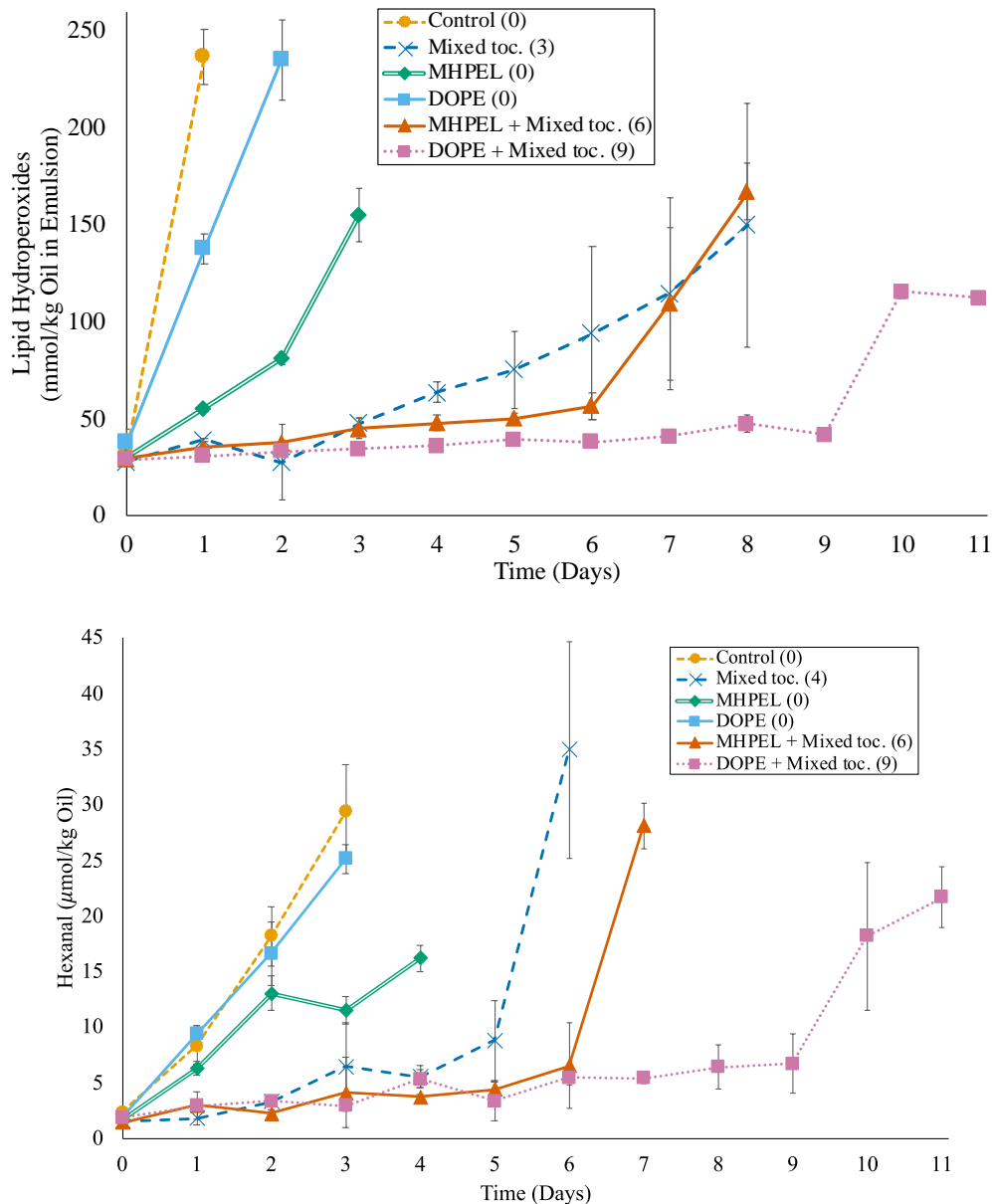


Figure 4.4. Formation of lipid hydroperoxides and hexanal during storage of a 1% soybean oil-in-water emulsions at pH 4. Treatments include mixed tocopherols (Mixed toc.; 300  $\mu\text{mol/kg}$  oil), modified high phosphatidylethanolamine lecithin (MHPPEL) (1500  $\mu\text{mol/kg}$  oil), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (1500  $\mu\text{mol/kg}$  oil), mixed toc. (300  $\mu\text{mol/kg}$  oil) + MHPPEL (1500  $\mu\text{mol/kg}$  oil) and mixed toc. (300  $\mu\text{mol/kg}$  oil) + DOPE (1500  $\mu\text{mol/kg}$  oil). Data points represent the average of triplicate samples  $\pm$  standard deviations. Statistically calculated lag phases are shown in parentheses in figure legend.

Purified PE was more effective than MHPPEL in the oil-in-water emulsions especially at pH 4.0. Aleksandrovna et al.<sup>115</sup> reported that egg, soybean and sunflower lecithin all have iron and copper and that soybean lecithin had 0.4 mg iron/kg lecithin and 0.3 mg copper/kg lecithin.

Addition of the unmodified or modified soybean lecithin by itself did not increase oxidation rates even though the emulsions could contain different levels of iron and copper. In all phospholipid alone samples the hydroperoxide and hexanal lag phases were 0 days so it would not be possible to observe prooxidant activity. However, since transition metals are such strong prooxidant in oil-in-water emulsions, this could explain why MHPEL was less effective than purified PE. This could be especially true at pH 4.0 where transition metals are more soluble and could have a stronger prooxidant effect.

While the demonstrated synergy of the combination of MHPEL and tocopherol presents an opportunity to increase the shelf-life of emulsion-based foods, the aim of this next experiment was to validate the combination's effectiveness in stripped bulk soybean oil. From the results shown in Figure 4.5, the bulk oil control, DOPE, 500  $\mu\text{mol/kg}$  oil MHPEL and the 1000  $\mu\text{mol/kg}$  oil MHPEL all had 0 or 1 day lag phases for lipid hydroperoxides and hexanal formation.  $\alpha$ -Tocopherol (50  $\mu\text{mol/kg}$  oil) by itself extended the lag phase to 3 and 4 days for PV and hexanal formation, respectively. The combination of  $\alpha$ -tocopherol with 500  $\mu\text{mol/kg}$  oil DOPE had a lag phase of 5 (hydroperoxides) and 4 days (hexanal), with interaction indexes of 1.66 and 1. The combination of 50  $\mu\text{mol/kg}$  oil  $\alpha$ -tocopherol and 500  $\mu\text{mol/kg}$  oil MHPEL had lag phases of 7 days for both peroxide and hexanal formation with calculated interaction indexes of 1.75 and 1.4, respectively. Both lag phases were increased to 8 days with the addition of 1000  $\mu\text{mol/kg}$  oil MHPEL in combination with 50  $\mu\text{mol/kg}$  oil  $\alpha$ -tocopherol, giving interaction indexes of 2 (peroxides) and 1.6 (hexanal). The interaction indexes support the conclusion that the suppression of lipid oxidation by the combination of  $\alpha$ -tocopherol and MHPEL is synergistic.

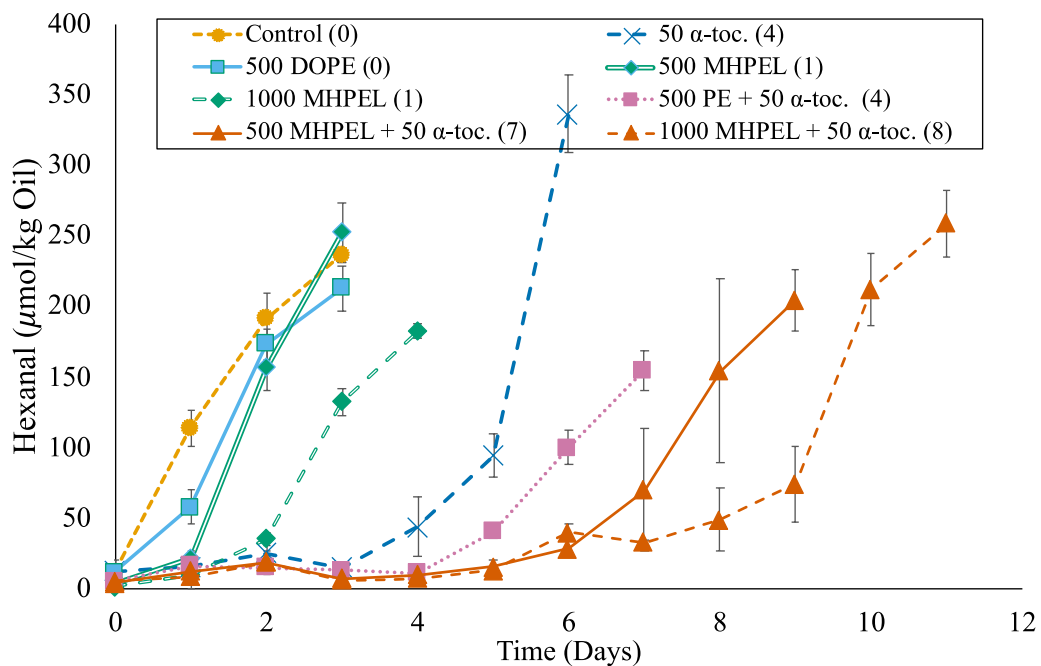
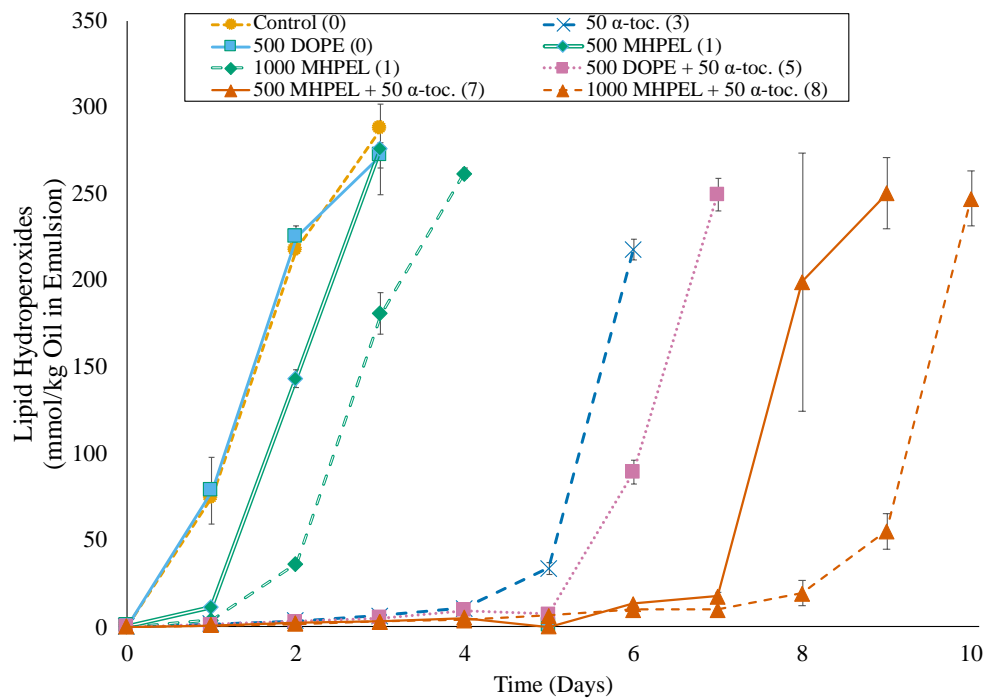


Figure 4.5. Formation of lipid hydroperoxides and hexanal during storage of stripped high oleic soybean oil. Treatments include  $\alpha$ -tocopherol ( $\alpha$ -toc), modified high phosphatidylethanolamine lecithin (MHPPEL), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE),  $\alpha$ -tocopherol + MHPPEL and  $\alpha$ -tocopherol + DOPE. Data points represent the average of triplicate samples  $\pm$  standard deviations. Statistically calculated lag phases are shown in parentheses in the figure legend. All concentrations reported in the legend are in  $\mu\text{mol/kg}$  oil.

In bulk oil, the combination of MHPEL and  $\alpha$ -tocopherol was found to be slightly more effective at extending the oxidative lag phase than the combination of purified DOPE and  $\alpha$ -tocopherol at the same PE concentration. The composition of the MHPEL (which also contained PC and PA) could be responsible for this.

PC, which was present in the MHPEL at approximately 16.8%, has been found to increase the antioxidant activity of low concentrations of  $\alpha$ -tocopherol (10  $\mu$ M), while decreasing the antioxidant activity of 100  $\mu$ M  $\alpha$ -tocopherol<sup>116</sup>. Additionally, PA has been suggested to inhibit iron-induced oxidation in bulk oils<sup>86,87</sup> and was present in the MHPEL at 9.8%. Therefore, the presence of PC and PA could improve the antioxidant activity of MHPEL and increases its efficacy compared to purified PE. This could mean that incomplete conversion of PC to PE could actually be beneficial to inhibiting lipid oxidation in bulk oil.

While the current work establishes the efficacy of MHPEL for usage in food systems to increase the antioxidant efficacy of tocopherol, additional work is needed to commercialize this process. The solvents used in the current work (e.g. chloroform, hexane) are not food-safe, are highly flammable, and are not environmentally friendly. To this end, Duan and Hu used  $\gamma$ -valerolactone as a solvent for the transphosphatidylation of  $\geq 99\%$  soybean PC to PS and achieved a 95% conversion without detection of PA<sup>60</sup>. Iwasaki et. al. (2003) achieved an 80% conversion of lecithin to PS by adding calcium sulfate to an aqueous mixture of lecithin, L-serine, and PLD, however recovery of the products still required n-hexane, ethanol and diluted HCl<sup>117</sup>. Cyclopentyl methyl ether, which has a low acute toxicity and potential for use as a green and sustainable solvent<sup>118</sup>, was used to achieve a 93.4% conversion of PC to PS which could then be easily separated by evaporation<sup>69</sup>. A recent work achieved complete conversion of PC to PE using the reactive deep eutectic solvents choline chloride/trimethyl glycine as hydrogen-bond

acceptors and glycerol/ethylene glycol as hydrogen-bond donors in addition to nucleophiles for choline substitution<sup>119</sup>.

Additional work has focused on parameters needed to scale-up the reaction for commercial production. PLD<sub>SC</sub> was found to retain 81% of its initial enzyme activity after being used to transphosphatidylate PC to PS for 10 batches<sup>68</sup>. A separate study also examining the conversion of PC to PS using PLD<sub>SC</sub> found the enzyme retained a conversion rate above 50% after 7 repeated batch cycles<sup>70</sup>. This researcher suggests that the reaction could be designed to reuse PLD<sub>SC</sub> which would result in significant savings. Increased temperature resistance of PLD<sub>SC</sub> was found when the enzyme was manufactured and displayed on a bacterial surface during a reaction which could aid in speeding up commercial-scale processing<sup>70</sup>.

An additional consideration would be how this system would be labelled if used in a food product. Several applications for GRAS (generally recognized as safe) status for PS formed by transphosphorylating lecithin of various plant sources with PLD and L-serine have been filed and have reached the “FDA has no questions” stage of evaluation (GRN No.: 637, 636, 545, 186). So far, none have been filed for PE. An additional consideration would be how this system would be labelled if used in a food product. The GRAS petitions list “phosphatidylserine” or “phosphatidylserine derived from sunflower” as the proposed common name for the product.

The present work explores a viable way to modify lecithin to increase PE to work synergistically with tocopherols as a food antioxidant. It was found that the most efficient transphosphatidylation by PLD<sub>SC</sub> of commercial high-PC lecithin to MHP<sub>EL</sub> occurs at pH 9 and 37°C. The combination of the MHP<sub>EL</sub> with tocopherol was found to be synergistic in model oil-in-water emulsion systems at pH 4 and 7 and in bulk oil. These findings validate that the

combination of MHPEL and tocopherol could provide a novel antioxidant technology that could be effective in multiple commercial food and cosmetic applications.

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