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Pro- and Antioxidant Effects of Phospholipids on Lipid Oxidation in Bulk Oil

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PRO- AND ANTIOXIDANT EFFECTS OF PHOSPHOLIPIDS ON LIPID OXIDATION IN BULK OIL

A Dissertation Presented

by

LEQI CUI

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DEDICATION

To everyone that loves me and everyone that I love, including science

ACKNOWLEDGMENTS

Two decades ago, when I just started to go to school I couldn't realize how much science was going on and will be going on, and would have never thought to become a scientist to get involved in all of this. With two decades of education, I just realized that this time-point now is only a start to explore the unknown, to answer questions raised by our own curiosities.

At this point, I would like first to thank Dr. Eric Decker for opening the door of science for me and for his patiently and intelligently guidance of inspiring me on the beginning of my journey to science. His wisdom in both academics and life shall always be my best role model.

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Lastly, special thanks to my family, including my girlfriend Jing, for their unconditional support.

ABSTRACT

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The aim of this thesis is to explore how phospholipids at concentrations similar as in refined vegetable oils impact bulk oil lipid oxidation. The possible formation of association colloids and synergism with primary antioxidants are considered. The results provided a better understanding of the pro- and antioxidant activities of phospholipids.

Lipid oxidation leads to quality deterioration by generating off-flavor, nutrient loss, color alteration, texture changes, and even generation of potential toxic products. Phospholipids are important minor components in edible oil that play a role in lipid oxidation. Surface active phospholipids have an intermediate hydrophilic–lipophilic balance value, which allows them to form association colloids such as reverse micelles in bulk oil. These association colloids can influence lipid oxidation since they create lipid–water interfaces where prooxidants and antioxidants can interact with triacylglycerols. In this study, we examined the formation of reverse micelles in a stripped oil system by dioleoyl phosphoethanolamine (DOPE) and the effect of these physical structures on lipid

oxidation kinetics. The critical micelle concentration (CMC) of DOPE was approximately 200 μmol/kg oil at 45 °C. Oxidation kinetics studies showed that DOPE was prooxidative when it was above its CMC (400 and 1,000 μM), reducing the lag phase from 14 days (control) to 8 days. The addition of combinations of DOPE and dioleoyl phosphocholine (DOPC) resulted in formation of mixed micelles with a CMC of 80 μmol/kg oil at 45 °C. These mixed micelles were also prooxidative when concentrations (100 and 500 μM) were above the CMC, decreasing the lag phase from 14 to 8 days.

DOPC and DOPE reverse micelles were examined on their impacts on the activity of primary antioxidants such as the nonpolar α-tocopherol and the polar trolox in stripped and commercial soybean oils. The results showed that DOPC reverse micelles decreased the activity of 100 μM α-tocopherol or trolox. On the other hand, DOPE increased the antioxidant activity of both α-tocopherol and trolox. The polar trolox exhibited better antioxidant activity than the nonpolar α-tocopherol in the presence of both DOPC and DOPE reverse micelles because trolox partitioned more at the water-lipid interface, which was confirmed by a fluorescence steady state spectroscopy. Different ratios of DOPE to DOPC were added to oil containing 100 μM α-tocopherol, and antioxidant activity increased with increasing DOPE/DOPC ratio. Addition of DOPE to commercial oil inhibited lipid oxidation, where as DOPC was ineffective. HPLC showed that DOPE regenerated α-tocopherol.

Overall, these findings provide a better understanding of the role of phospholipids reverse micelles in lipid oxidation in edible oil and indicating that the

antioxidant activity of tocopherols could be improved by utilizing phosphatidylethanolamine (PE) to engineer the properties of reverse micelles in bulk oil.

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CHAPTER 1

INTRODUCTION

Lipid oxidation leads to quality deterioration by generating off-flavor, nutrient loss, color alteration and texture changes and even generation of potential toxic products [1]. Due to these quality and safety concerns, lipid oxidation shortens the shelf life of food products, which results in a loss of food.

Strategies to inhibit lipid oxidation in bulk oils can extend the shelf life of oil-containing products and thus benefit both the food industry and consumers. One effective traditional method to inhibit lipid oxidation is the addition of antioxidants such as t-butyl-4-hydroxyanisole (BHA), 2,6-di-t-butyl-p-hydroxytoluene (BHT) and t-butyl hydroquinone (TBHQ). However, though these antioxidants are economic for food manufacturers, there have been some debates on their potential safety risks [2-4]. In addition, as consumers desire simpler and cleaner labels, there is a growing demand for replacing these synthetic antioxidants with natural antioxidants.

Phospholipids are naturally presented in bulk oils as minor components [5]. However, the role of phospholipids in lipid oxidation is often conflicting. For example, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) have been postulated to act as prooxidants, antioxidants, or have no effect on oxidation reactions [6-10]. Kashima et al. [6] found that in tocopherol-free perilla oil, 500 ppm PE had no effect. Weng and Gordon [7] also reported no effect of 2,600 ppm l-α-dilauryl PE in lard. Koga and Terao [11] found that 5 mM dimyristoyl phosphoethanolamine (DMPE) had little impact on hydroperoxides accumulation in a mixture of methyl linoleate and methyl laurate. In contrast, prooxidative activity of

PE has also been reported. Takenaka et al. [12] found that in the absence of α-tocopherol, 10,000 ppm (1%) 1-palmitoyl- 2-oleoyl-PE and 1,2-dioleoyl-PE decreased bonito oil stability. Lee and Choe [13] also reported that in tocopherol-stripped canola oil containing 4 ppm chlorophyll, 50 ppm PE from soybeans increased oil oxidation.

In contrast, most of the claimed antioxidant properties of PE were in the presence of other primary antioxidants, especially tocopherols [8, 14-16]. Tocopherols are important natural antioxidants as they exist in many vegetable oils such as rapeseed, sunflower, cottonseed, soybean, and corn oils. Several studies have shown that phospholipids help increase their effectiveness. Judde et al. [14] reported the ability of lecithin containing high proportions of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) to increase the activity of γ- and δ-tocopherols. Bandarra et al. [16] reported that PC, PE, and cardiolipin combinations improved the effectiveness of α-tocopherol. However, PC has not always been found to increase the activity of tocopherols in all studies. For example, Takenaka et al. [12] found that only PE, but not PC, increased the effectiveness of α-tocopherol. Kashima et al. [6] reported that PE and phosphatidylserine (PS) suppressed lipid oxidation of perilla oil containing 866 ppm of mixed tocopherols, whereas PC showed no effect.

One possible mechanism accounting for the cooperative activity of phospholipids and tocopherols could be due to the headgroup of PC and PE regenerating tocopheroxyl radical to tocopherol. Doert et al. [17] recently reported that primary amines can regenerate oxidized tocopherols through an ionic mechanism rather than a radical mechanism. A second proposed mechanism for

phospholipids enhancing the activity of tocopherols is the nonenzymatic browning reaction products from amine-containing phospholipids, and oxidation products such as aldehydes are antioxidative and thus provide an additional source of antioxidants. Hidalgo et al. [18] reported that the antioxidative activity of carbonyl–amine products was greatly increased with the addition of tocopherols. Shimajiri et al. [19] further suggested that the formation of antioxidant nonenzymatic browning compounds from amine-containing polar lipids and oxidation products might require the presence of α-tocopherol. Nevertheless, the exact mechanism of how the combination of phospholipids and tocopherols influences bulk oil oxidation is still not fully understood.

Recently, Chen et al. [10, 20] reported that dioleoyl phosphocholine (DOPC) formed reverse micelles in stripped soybean oil. These reverse micelles increased oxidation rates by increasing the activity of iron but also enhanced the activity of antioxidant such as α-tocopherol and Trolox. To better understand how phospholipids and their reverse micelle structures affect oil oxidation, this research focused on 1) the role of PE and its combination with PC in the formation of association colloids and how these structures impacted stripped soybean oil oxidation and 2) the impact of DOPC and DOPE both individually and in combination on the antioxidant activity of α- tocopherol or trolox in stripped soybean oil. In addition, α-tocopherol regeneration by different types of phospholipids was also examined. Finally, the potential of electron paramagnetic resonance to predict the lag phase of bulk oil oxidation was also investigated.

CHPTER 2

LITERATURE REVIEW

2.1 Introduction

Lipids, as one of the major macronutrients required for our growth and maintenance, are therefore important constituents in foods. They provide unique properties of texture, appearance, flavor and caloric density to foods.[21] However, lipids at the same time are prone to oxidation, which negatively impacts not only quality and nutritive values of foods, but also consumer health. Thus, lipid oxidation is a great concern to both food manufacturers and the general public.

There are a variety of factors influencing lipid oxidation susceptibility, such as water activity, transition metal type and concentration, singlet oxygen, fatty acids composition, presence of antioxidants and environmental conditions like light, temperature and oxygen concentration.[22] Some of these factors are considered to be prooxidant, which is defined to cause or accelerate lipid oxidation. Prooxidants act by promoting lipid hydroperoixdes formation (e.g. singlet oxygen), free radicals formation (e.g. irradiation) or hydroperoixdes decomposition (e.g. transition metals). In contrast, compounds that can slow down lipid oxidation are known as antioxidants. Antioxidants are classified into primary and secondary antioxidants, according to their chemical mechanisms. Broadly speaking, primary antioxidants scavenge the free radicals that promote oxidation while secondary antioxidants retard lipid oxidation by decreasing other prooxidative factors (e.g. metal chelation) or regenerating primary antioxidants.[23]

The use of antioxidants in foods has been an effective way to inhibit lipid oxidation because a variety of other methods have shown their limitations. For example, reducing polyunsaturated fatty acid (PUFA) concentrations, partial hydrogenation or exclusion of oxygen from products can be utilized to increase oxidative stability of food products. However, nutritionists do not recommend replacing PUFAs with saturated fatty acids because dietary PUFAs are linked to many health benefits.[24] Partial hydrogenation would also not be an ideal method to decrease lipid oxidation because it converts PUFAs to *trans* fatty acids, which are more atherogenic than saturated fats because they both increase low density lipoprotein (the bad lipoprotein) and decrease high density lipoprotein (the good lipoprotein). Excluding oxygen can be effective, but these techniques must produce very low oxygen concentrations and are not practical for many types of foods.[25] For all these reasons, the use of antioxidants is widely accepted as a reliable technique to control lipid oxidation in a wide variety of food products. But, primary and secondary antioxidants are not the perfect solution either. One problem is that the most powerful and economical antioxidants are synthetic (e.g. butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ) and ethylenediaminetetraacetic acid (EDTA)), whose use is contrary to current consumers' preference for cleaner and simpler labels. Thus, searching for novel natural antioxidants or increasing the antioxidant activity of current available natural antioxidants is of great importance.

Phospholipids are an essential part of biological membranes and thus are presented in all living species from which foods are derived. The concentration and

composition of phospholipids endogenous to foods can vary greatly in foods from animal or plant sources and are dependent on the origin of the food and how it's processed. For example, phospholipids from cold water marine animals will be highly unsaturated and high in omega-3 fatty acids compared to warm, fresh water species.[26] Phospholipid concentrations can also be increased during processing operations such as the drying of milk or whey.[27] In addition, phospholipid, or as commonly referred to as lecithin, possess many desirable functional properties such as emulsification, crystallization inhibition, acting as a non-stick releasing agent, improving wetting properties and acting as a anti-spattering agent in margarines meaning that they are commonly added to foods.[28]

Phospholipids are sometimes used as antioxidants in foods. Several mechanisms of how phospholipids could influence lipid oxidation have been proposed. In general, phospholipids could bind prooxidative metals,[29] produce antioxidative compounds through Maillard reactions during lipid oxidation,[30] alter the location of other antioxidants[11] and regenerate primary antioxidants like tocopherols.[31] However, phospholipids could also serve as oxidation substrates themselves. Due to their high degree of unsaturation, negative charge that attracts prooxidant metals and large surface area when they exist as dispersions, they can be an important substrate for oxidation in foods containing considerable amounts of biological membranes like meats[32]. In addition, there were also times when phospholipids showed no antioxidant activity or even act as prooxidants.[33, 34] One possible prooxidant mechanism of phospholipids in bulk oil could be their

formation of association colloids like reverse micelles which can increase metal-lipid interactions.[9]

To best understand the many facets of how phospholipids influence lipid oxidation in food products, a comprehensive review is needed. In this review, the source and compositions of phospholipids in foods will be summarized. The impact of phospholipids in different food systems on lipid oxidation and the different anti- and prooxidant mechanisms will be discussed.

2.2 Phospholipids properties and sources

2.2.1 Properties

2.2.1.1 Structures

Phospholipids consist of a glycerol backbone and a phosphate head group, which is typically found at the sn-3 position (Figure 2.1). The simplest phospholipid is phosphatidic acid (PA) and others are named after the group attached to the phosphate group. For example, if the group attached to the phosphate group is choline, this phospholipid is called phosphatidylcholine (PC). Other substitution groups on the phosphate group include ethanolamine, serine or inositol, thus the phospholipid are named phosphatidylethanolamine (PE), phosphatidylserine (PS) or phosphatidylinositol (PI). Lysophospholipids refer to phospholipids whose fatty acid chain has been removed from sn-2 position. In addition, there is another type of lipids, sphingolipids, which are sometimes considered to be phospholipids because they could contain a phosphatidylcholine or phosphatidylethanolamine group in the

molecules. For example, sphingomylein contains a phosphotidylehtanolamine group.[35]

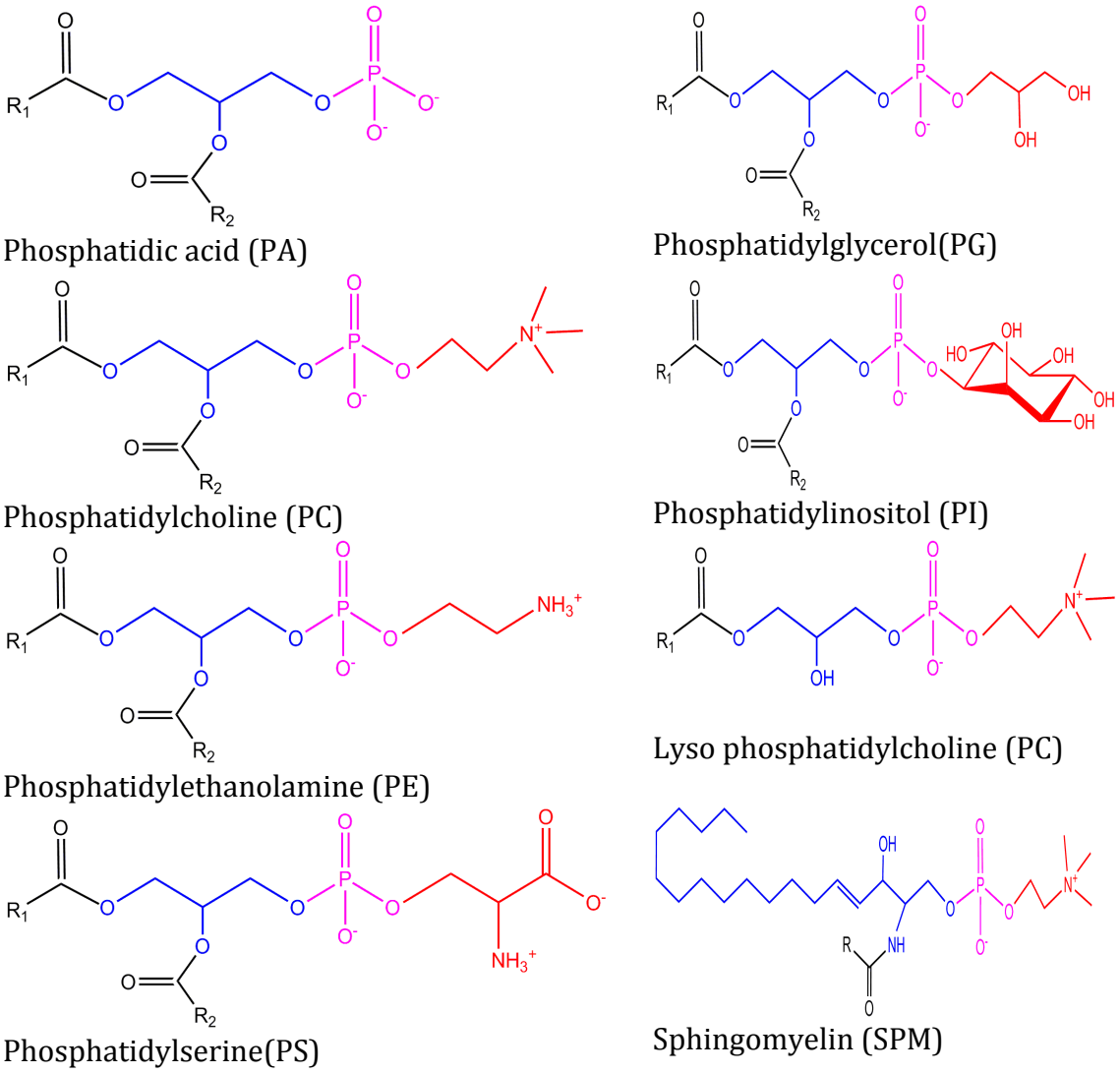


Figure 2.1 Phospholipid structures

The fatty acid composition of phospholipids varies depending on their origin. Moreover, it is worth noting that dietary lipids can influence phospholipid fatty acid composition.[36-38] In general, saturated fatty acids are more often found at sn-1

position while unsaturated fatty acids tend to be esterified at sn-2 position. A proper ratio of saturated to unsaturated fatty acids of phospholipids is important to living cells since the saturation degree would determines the physical state (e.g. fluidity) of the cell membrane. Table 2.1 listed the phospholipids fatty acid compositions from different sources.

Table 2.1 Fatty acid compositions (wt%) of phospholipids from different sources

	Chicken egg yolk	Bovin whole milk[a]	Chicken breast muscle[b]	Pig	Cattle	Tuna[a, c]	Salmon[d]	Soybean lecithin[a]	Egg lecithin[a]
10:0		0.4							
12:0		1.3							
14:0	28.7	5.7	0.8	0.3	0.2	1.43	5.78		
14:1			0.2						
14:2			1.4						
15:0		1.5				0.68			
16:0	1.65	34.7	23.7	16.6	14.6	19.26	13.0	11.2	35.0
16:1		1.0	1.8	0.8	0.8	2.09	7.33		
16:2			0.8						
17:0		2.0				1.23			
18:0	14.1	9.5	11.9	12.1	11.0	3.6	3.15	11.9	13.4
18:1	31.3	26.7	21.1	9.4	15.8	12.9	14.13	8.6	30.4
18:2	16.3	15.0	20.7	31.4	22.0	0.7	3.42	58.6	18.0
18:3			0.8	0.6	0.7	0.49	1.12	9.9	
20:0						0.47			
20:1						0.64	7.09		
20:2							0.35		
20:3		0.9	1.8				0.29		
20:4	5.38		9.4	10.5	10.0	4.93	1.15		3.2
20:5		0.8	0.4	1.0	0.8	5.75	3.22		

21:5							0.44		
22:1							5.72		
22:4							0.06		
22:5			1.0				2.01		
22:6	2.68		1.2			41.63	19.38		
UnS/S[e]	1.25	0.8	1.66	1.85	1.94	3.09	2.99	3.29	1.07
Ref	[39]	[40]	[41]	[42]	[42]	[43]	[44]	[45]	[45]

a. The values are of phosphatidylcholine (PC)

b. Chickens are 21 days old and fed a diet with no added fat

c. The species is Yellowfin. The values are of mol%

d. The species is Anadromous Atlantic salmon and the age is 77 days after hatching

e. The ratio of unsaturated fatty acids to saturated fatty acids

2.2.1.2 Molecular charge

pK_a is the negative logarithm of acid dissociation constant K_a. The value of pK_a is the pH at which the molecule is exactly half dissociated. This indicates how acidic (or not) a given hydrogen atom in a molecule is at a given pH. For example, if the pH of the environment is above the pK_a, the molecule exists more as dissociated form, which is the case where a chelating molecule is charged and thus is able to bind metals. Table 2.2 summarizes reported pK_a values of the major phospholipids. However, it is worth noting that because of the different measurement methods (e.g. indirect calorimetric and turbidity measurements, surface potential measurement by radioactive electrode, transmembrane potential by potential dynamic and proton

binding by acid acid-base titration) and physical systems (e.g. dispersions, monolayers, bilayer membrane and vesicles), different pK_a values are often reported. For example, pK_a values ranging from 2.1 to 4 for carboxyl group of PS were reported.[46-48] Similarly for PC, the pK_a values from 0.8 to 4.5 were seen in different studies.[49-51]

Table 2.2 pK_a of different functional groups of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS)

	pK_a (phosphoate)	pK_a (amino)	pK_a (carboxyl)
PC	0.8[a]		
PE	0.5[a]	9.6[c]	
PS	2.6[b]	9.8[c]	3.6[c]

a. Data from Moncelli et al.[49] A phospholipid monolayer model was used. The value was measured by differential capacity of an electrode coated with phospholipid monolayers.

b. Data from Petelska et al.[50] A phospholipid bilayer membrane model was used. The value was measured by acid acid-base titration.

c. Data from Tsui et al.[52] The value of PE was determined by surface potential measurements of PE-PC mixed vesicle. The values of PS were determined by potentiometric titrations and surface potential measurements of PS-PC mixed vesicles.

2.2.2 Sources

All foods that originate from living plants/animals contain phospholipids. This is because all living plants/animals have cells and phospholipids are integral component of cell membranes. The major animal-based sources of phospholipids include eggs, milks, meats and marine phospholipids. Eggs of chicken, duck and turkey all contain considerable amount of phospholipids. Egg yolks are especially rich sources of phospholipids with a weight percent up to 10%, with the majority of the phospholipids being PC (66 %) and PE (19 %).[53] Egg yolks are a common source of non-vegan food grade lecithin. Raw meats contain large amounts of biological membranes and thus generally contain 0.5 - 1% phospholipids. In addition to muscle foods, animal organs contain even higher phospholipids. For example, pig and chicken kidney contain 2.9 % and 2.5 % phospholipids respectively.[53] Seafood has similar phospholipids concentrations as warm-blooded animals but marine phospholipids are much higher in omega-3 fatty acids (Table 1) which makes them a promising functional food ingredient in foods.[26] Krill oil is a unique source of marine phospholipids that originate from small marine crustaceans. Krill oil is high in phospholipids and thus are used in highly bioavailable omega-3 supplements.[54] Vegetable seeds and cereal grains are also rich sources of phospholipids. These include soybean, corn, cottonseed, rapeseed, sunflower, peanut and oats with commercial lecithin being obtained from some of these sources during oil refining (see below). In addition, it is worth noting that even in some vegetable, fruit and carbohydrate related food products such as

spinach, orange juice, lemon juice and wheat starch, phospholipids are prevalent. The phospholipid types and content of common foods are summarized in Table 2.3.

Table 2.3 Phospholipids content of common foods.[a] (abbreviations: PL = phospholipid, PC = phosphatidylcholine, PE = phosphatidylethanolamine, PS = phosphatidylserine, PI = phosphatidylinositol, SPM = sphigomyelin, LPC = lysophosphatidylcholine, LPE = lysophosphatidylethanolamine, CL = cardiolipin, PG = phosphatidylglycerol)

	Total PL	PC	PE	PS	PI	SPM	LPC	LPE	CL	PG	Ref
Chicken whole egg	3.49	77	16.6			2.4	1.6				[53]
Bovine whole milk	0.02	32.7	28.5	14.1[b]		23.0	1.8				[40]
Beef	0.7	49.3[c]	18.0	13.9[d]	4.6	6.4					[53]
Pork	0.6	42.9	26.7	4.9	6.8	7.5	2.9		8.3		[55, 56]
Chicken breast	0.4	61.0	19.4	4.0	6.7	5.5					[57]
Chicken thigh	0.6	50.0	22.8	5.0	7.3	7.7					[57]
Salmon (head)	0.54	54.7	14.0	10.4	2.5	8.3	1.4				[58]
Tuna	0.6	37.9	21.0	5.4	8.5	4.0	21.5				[43, 53]
Soybean	2.0	45.0	26.3	5.0[e]	14.1				3.5[f]		[59]
Corn germ	1.1	30.7	14.2	27.1[e]	18.7		0.7				[60]
Rapeseed	1.5	48.7	8.3		18.4						[61]

Peanut	0.6	43.5	8.1	4.0[e]	24.2					[53]
Lemon juice	0.03	38.7	35.5	5.5[e]	16.1					[62]
Orange juice	0.03	32.3	38.7	13[e]	6.5					[62]
Wheat starch	0.7						80.9	10.6		[63]
Spinach	0.2	23.6	22.9	8.9[e]	7.0				31.2	[53]
Soybean lecithin		38.6	16.4	0.6	19.2				1.2	[64]
Egg lecithin		75.4	18.3			1.9	2.5	1.2		[65]

a. The values of total phospholipids are wt % of total food. The values of individual phospholipids are wt% of total phospholipids

b. The value includes PI

c. The value includes LPC

d. The value includes PA and CL

e. The value includes PA

f. The value includes PG

2.2.3 Lecithin

Lecithin refers to a mixture of phospholipids extracted from animal (e.g. eggs) and vegetable (e.g. soybean, sunflower, rapeseed and cottonseed) sources. Soybean has been the primary commercial source of food grade lecithin because it economical to produce and can be used in vegan applications. Lecithin is not only an important additive in food but also in cosmetics and lubricants industries. In the food industry, lecithin is the most important natural emulsifier with an estimated

world market at 150,000 – 170,000 metric tons. In addition to the use of lecithin as a food emulsifier, manufacturers often also expect to receive the benefit of its antioxidant activity.

2.2.3.1 Production

Soybean lecithin is a co-product of soybean oil processing. Figure 2.2 is a schematic description of the operations for lecithin production. Crude soybean oil contains 1.5 - 3.0% phospholipids, which can decrease oil quality due to its susceptibility to nonezymatic browning and its ability to trap water in the oil. Phospholipids are removed by the degumming process which utilizes a weak acid solution to partition out of the oil and into the water fraction. The lecithin fraction would be dried and in some cases, bleached to remove the natural brown color of lecithin.

The composition of lecithin can be modified by means of solvent extraction as well as chemical and enzyme modification to produce specialty, value added products.[28] For example, crude dried lecithin contains 30 - 40% neutral oil, which can be modified to 2 - 3% with solvent extraction. The product is called de-oiled lecithin. This is often done with acetone which can selectively extract neutral oil (triacylglycerides) out of the lecithin. Individual phospholipids have different solubility properties. For example, PC dissolves well in ethanol while PI and PA do not. Taking advantage of this difference, PC enriched lecithin can be produced. Increasing the PC content of lecithin results in increased hydrophilicity, which is

preferred in oil-in-water emulsions like salad dressings. Besides modifications with solvent extraction, chemical modification (e.g. hydrogenation) is used to improve the oxidative stability, color and odor of lecithin. Hydrogenated lecithin is currently used in cosmetics, dye and lubricants industry. One example of lecithin that is enzyme modified is lyso-lecithin. This product requires phospholipase A_2 that hydrolyzes the fatty acid of phospholipids at sn-2 position. With the removal of fatty acid chain from phospholipids, lyso-lecithin exhibits increased hydrophilic and emulsifying properties under lower pH values and a broader range of temperatures and salt concentrations.[66]

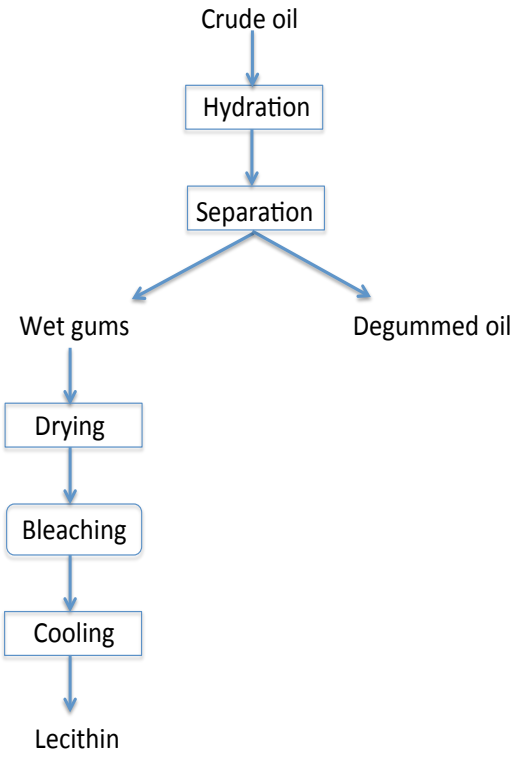


Figure 2.2 Schematic description of lecithin production

2.2.3.2 Composition

Lecithins from different sources have different compositions. An example of fatty acid composition and phospholipids composition differences between lecithin from eggs and soybeans were included in Table 1 and 2. However, it is worth noting that lecithins with a variety of phospholipid composition are commercially available (as different types of lecithin mentioned in production section) and lecithin mixtures still contain some portion of compounds other than phospholipids (Table 2.4).

Table 2.4. Composition of different types of soybean lecithin[a]

	Crude lecithin	De-oiled lecithin	PC-enriched fraction
Phospholipids (total)	47	74	51
PC[b]	31.9	32.4	74.5
PE[b]	23.4	23.0	15.7
PI[b]	21.3	21.6	5.9
PA[b]	8.5	8.1	2.0
Others[b]	14.9	14.9	2.0
Triacylglycerides	37	3	37
Glycolipids	11	17	9
Carbohydrates	3.5	5.5	2.5
Water	< 1	< 1	< 1

a. Adapted from Nieuwenhuyzen and Tomas [28]

b. The values are percentage of total phospholipids

Abbreviations: PC = phosphatidylcholine, PE = phosphatidylethanolamine, PI = phosphatidylinositol, PA = phosphatidic acid

2.2.3.3 Uses

The applications of lecithin mainly associate with the molecular characteristics of its phospholipids. The presence of both hydrophilic and lipophilic groups within a phospholipid molecule makes it surface active. This surfactace active property plus the natural nature of lecithin gives it prominent applications in foods such as emulsification, antispattering, wetting, antistaling, dough conditioning and antioxidant. Emulsifiers are used in many food products due to their ability to stabilize oil and water dispersions. The emulsification property of lecithin depends on its affinity to oil or water molecules, which can be identified as hydrophilic lipophilic balance (HLB). The HLB ranges from 0-20 with a higher value representing higher hydrophilic affinity. By modifying lecithin composition (e.g. de-oiled, PC-enriched and enzyme-hydrolyzed lysolecithin), different HLB can be created for different applications. For example, standard crude lecithin and de-oiled lecithin with 45% PC have HLB values of 3.5 and 6.5 respectively.[67] Another use of lecithin that directly relates to its emulsification property is antispattering. In margarine, which is a water-in-oil emulsion, spattering happens when water droplets coalesce during heatin. Lecithin surrounds water particles to slow down this coalescence and thus deceasing spattering. Lecithin also serves as wetting agent in powdered or granular products. For example, the addition of lecithin helps cocoa

powder disperse easily in water. Another advantage of lecithin is its ability to form complexes with starch and protein. One example is the ability of lyso-lecithin to form lipid-amylose complex that decreases wheat starch retrogradation.[68] Also, linking to wheat gluten through hydrogen bonds makes lecithin a good dough conditioner in that it can improve bread elasticity, baking volume and fermentation tolerance.[69] Another attractive trait of lecithin is its antioxidant properties.[17, 70, 71] However, application of lecithin for the purpose of inhibiting lipid oxidation is not always successful. This will be discussed in more details in later sections.

2.3 Lipid oxidation mechanisms

Unsaturated lipids undergo oxidation in the presence of oxygen in a pathway which involves free radical chain reactions. These reactions can be divided into three stages including initiation, propagation and termination.[1] A brief view of this pathway is shown in Figure 2.3. An initiator such as light, heat, transition metals or reactive oxygen species is required to initiate the reaction to convert a fatty acid substrate (L_1H) to a free radical. After the initiation happens, an alkyl radical ($L^{\cdot}$) is formed and the reaction enters the propagation phase. In the propagation step, the alkyl radical reacts with bi-radical triplet oxygen (O_2) to form peroxyl radical ($LOO^{\cdot}$). This reaction is diffusion limited because it is a radical-radical reaction with minimal activation energy. Peroxyl radical ($LOO^{\cdot}$) abstracts hydrogen from a new unsaturated fatty acid substrate (L_2H) and thus causing the formation of an additional alkyl radical which can enter a new round of propagation reactions. The

peroxyl radical itself forms lipid hydroperoxide (LOOH), which is a primary oxidation product. The susceptibility of the fatty acid substrate to lose hydrogen (LH → L•) increases with increasing unsaturation, which is why more unsaturated lipids are more easily oxidized. Finally, the reaction won't terminate until two radicals combine to form non-radical species. However, in food systems the termination step is not important because most foods are rancid before there is significant termination.

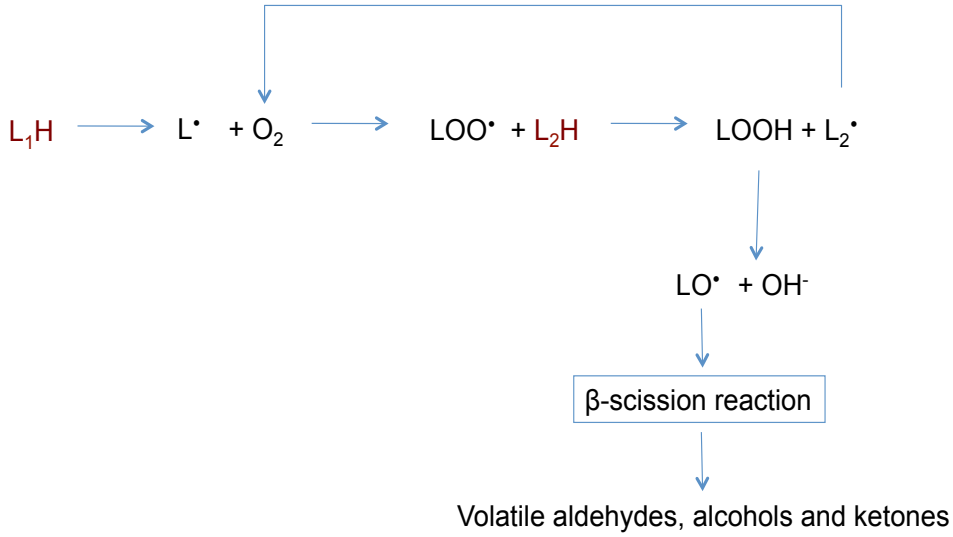


Figure 2.3 Schematic description of lipid oxidation chain reaction

The development of oxidative rancidity originates from formation of volatile secondary oxidation compounds coming from the decomposition of lipid

hydroperoxides (LOOH), which is known as β-scission reactions. Lipid hydroperoxides (LOOH) decompose in the presence of heat, ultraviolet radiation and transition metals to form alkoxyl radicals (LO•). Alkoxyl radicals are so energetic that they can abstract electron from the covalent bonds adjacent to the alkoxyl radical to cleave the aliphatic chain, resulting in the formation of low molecular weight, volatile compounds such as aldehydes and ketones. β-scission reactions are greatly accelerated by transition metals like iron and copper which is why transition metals are so important in lipid oxidation in foods.[72] More details on products from decomposition of lipid hydroperoxides can be found in Frankel.[1]

2.4 Phospholipids in lipid oxidation

2.4.1 Phospholipids as oxidation substrates

Due to the presence of unsaturated fatty acids in phospholipids, phospholipids themselves are susceptible to lipid oxidation. In fact, in many foods the phospholipids are more unsaturated than the triacylglycerides since they must provide fluidity in cell membranes. Examples of food system that involve phospholipid as an oxidation substrate are meats and dried milk products.
Meats are susceptible to lipid oxidation not only because they are exposed to oxygen and contain unsaturated fatty acids, both of which are major substrates of lipid oxidation, but also because they contain prooxidative metals, enzymes and reactive oxygen species. Total lipid content of meats varies depending on species, animal diet, muscle groups and types of processing, which was summarized elsewhere.[53, 73]

Phospholipids in muscle are very susceptible to lipid oxidation because they are highly unsaturated and are more exposed to prooxidants than triacylglycerides because they are within membranes which have a very high surface area.[74, 75] Generally, the phospholipid content of muscle is about 0.5%.[76] Red meat have greater proportions of phospholipids than white meat which in part is due to higher levels of mitochondria.[77]

Oxidation in meats leads to negative impacts on flavor [75, 78-80] and protein integrity, [81-83] including discoloration.[84, 85] Keller et al. [74] observed the loss of unsaturated PC and PE with increasing TBA values in raw and cooked beef patties during frozen storage. This was especially true during frozen storage where they found that the total lipids of hamburgers remained constant while the amount of PC and PE decreased and this loss of PC and PE was accompanied by an increase of TBA values of ground beef during first two weeks. Igene et al. [79] found that phospholipids oxidized before triaclyglycerides during frozen storage of lipid-free muscle fibers where phospholipids and triacylglycerides were added. Igene et al. also reported [86] that PE was associated with high susceptibility to oxidation and was important in the autooxidation of cooked meat. Pikul et al. [87] found that the phospholipids fraction of chicken meat contributed approximately 90 % of malonaldehyde during lipid oxidation.

Individual phospholipids have varying degrees of susceptibility to lipid oxidation differently due to differences in polar head groups and level of unsaturation. Pikul et al. [88] found that in chicken meat, PC and PE produced 70-77% malondialdehyde, followed by 16-25% from PI and PS. Yin et al. [89] found that in a

liposome model system where PC and PE had the same fatty acid composition, PE liposomes oxidized faster than PC liposomes. The rapid loss of PE during oxidation of muscle cell membranes could be due to its higher level of unsaturation than PC.[89] In addition, PE loss could also be due to their ability to regenerate α-tocopherol. In general, the fat content of meat does not greatly influence lipid oxidation rates.[90] This further supports that phospholipids are the major lipid substrates since fat contents in meats are changed by varying triacylglyceride levels.

In addition, food products that have low lipid concentrations like nonfat dry milk can also have rancidity problems caused by phospholipid.[91, 92] Polar phospholipids are essential components in milk as they, together with proteins, form milk fat globule membrane (MFGM) which surrounds the lipid droplets secreted by the mammary gland cells. During processing, the milk-fat globule membranes can partition into the aqueous phase of milk and are thus present in nonfat dry milk and whey.[93] Two major milk phospholipids, PC and PE, are about one third polyunsaturated and thus have poor oxidative stability.[94] PC in milk can be hydrolyzed by milk lipase to generate lyso PC and unsaturated free fatty acids, which can undergo further oxidative deterioration and yield off flavors.[95] Volatile lipid oxidation compounds including C_6 - C_{14} aldehydes have been identified in nonfat dry milk,[91, 92] whey protein isolate and whey protein concentrate.[96] In the latter study, hexanal was found being responsible for over 90% of total aldehydes in both products. This high concentration of hexanal is believed coming from linoleic acid of phospholipids. This is because milk fat only contains 2% linoleic acid while the phospholipid from MFGM contains up to 6%.[96] Again, due

to its high unsaturation and surface area, phospholipids in dried dairy products present a major problem by causing oxidative rancidity.

2.4.2 Prooxidant Properties of Phospholipids

A typical example of food systems where phospholipid acts as antioxidants in some situations while being prooxidantive in other situations is bulk oil. Bulk oil is a heterogeneous system that contains more than just triacylglycerides including 200 - 800 ppm water, and a variety of amphiphilic minor compounds like monoacylglycerides, diacylglycerides, free fatty acids, phospholipids, phytosterols and oxidation products (details can be found in Chaiyasit et al.[23]). The combination of amphiphilic molecules and water in the triacylglycerides will lead to the spontaneous formation of nanostructures. For example, phospholipids in bulk oils form association colloids like reverse micelles. For example, Gupta et al.[97] found that native soybean phospholipids could form reverse micelles in the mixture of hexane and soybean oil containing less than 3% water. Danino et al.[98] later applied cryo-TEM in a similar system (soybean phospholipids/soybean oil/hexane/water) for direct visualization of phospholipids reverse micelles and reported the size of the aggregates to be 5-9 nm. Shtykova et al.[99] reported reverse micelles formed by dilinoleoyl PC (DLPC) and dilinoleoyl PE (DLPE) in hexane. By synchrotron small angle X-ray scattering, they found spherical aggregates with an outer radius of 1.5 nm. They attributed the smaller size of reverse micelles to the low water content in their system (less than 0.1%). The

authors also found that the amount of reverse micelles increased during the oxidation of phospholipids oxidation. Subramanian et al.[100] reported the presence of reverse micelles structures in crude soybean oil and high oleic sunflower oil which contained 245 and 400 ppm water, respectively. Recently, studies in our lab also found evidence of reverse micelles formed by phospholipids in oils stripped of their polar compounds by means of small angle X-ray scattering.[10, 101]

Reverse micelles can act as nano-reactors that can alter chemical reaction rates by bringing hydrophilic and lipophilic compounds into close contact allowing increased interactions.[102] Kasaikina et al. had several reports [103-107] where they used different surfactants in non-aqueous medium including bulk oil as a simple self-assembling model to investigate the impact of physical structures on lipid oxidation. They indicated that surfactants in heterogeneous system could spontaneously group into micro/nano-structures like reverse micelles and lipid hydroperoxides could participate into these structures as co-surfactant. The ability of hydroperoxides to reduce interfacial tension and thus being amphiphilic was also confirmed by Nuchi et al.[108]. Trunova et al.[103] reported that both cationic reverse micelles formed by cetyltrimetyl ammonium bromide (CTAB) and anionic reverse micelles formed by sodium dodecylsulphate (SDS) increased the decomposition of ethylbenzene and limonene hydroperoxides.[104] In another reverse micelle system that used AOT as surfactant (AOT/water/hexadecane), the authors found that lipid oxidation rates of methyl linolenate were altered upon the addition of cumene hydroperoxides, water, oleic acid or PC.[109]

The presence of phospholipid reverse micelles in bulk oils creates oil-water interfaces where hydrophilic prooxidants (e.g. iron) and amphiphilic prooxidants (e.g. lipid hydroperoxides) and triacylglyceride substrate are driven to close contact with each other resulting in increased lipid oxidation rates (Figure 2.4). Chen et al.[10, 20, 110] had several studies on the impact of reverse micelles formed by dioleoyl PC on soybean oil oxidation. To minimize the influence of minor components present in commercially refined oil, they used stripped soybean oil so that it contained ultra-low polar lipids (free fatty acids, phospholipids, MAG and DAG) and antioxidants (tocopherols) concentrations. They found that dioleoyl PC could spontaneously form reverse micelle structures when its concentration was above its critical micelle concentration and the reverse micelles would accelerate lipid oxidation. In contrast, when dibutyryl PC was added at the same concentration as dioleoyl PC, no prooxidant effects were observed. They suggested that the lack of prooxidant effect of dibutyryl PC was due to its short fatty acid chains, which were too short and thus not lipophilic enough to form the reverse micelle structures.[10] A similar prooxidant activity of PE was also reported by Cui et al..[111] In their study, they found dioleoyl PE promoted lipid oxidation of stripped soybean oil by forming reverse micelles while dihexanoyl PE was unable to form the reverse micelle and thus had no impact on lipid oxidation rates. They also reported that the critical micelle concentration of dioleoyl PC and dioleoyl PE decreased with increasing temperatures and that the critical micelle concentration decreased when when dioleoyl PC and dioleoyl PE were combined to form mixed reverse micelles. Dioleoyl PC and dioleoyl PE mixed reverse micelles were also prooxidantive and

they decreased the effectiveness of α-tocopherol and trolox upon their addition into stripped oils.[20, 31] In addition, prooxidative reverse micelles could also be formed in stripped oil by a combination of multiple polar/amphiphilic minor components found in commercial refined oils such as phospholipids, free fatty acids, phytosterols and DAG.[98] The prooxidant activity of phospholipids has also been reported in other systems. For instance, Hudson and Mahgoub et al.[112] found that addition of PC and PE to lard promoted oxidation as measured by oxygen absorption induction periods. Yoon and Min[33] found that in stripped soybean oil, 300 ppm of phospholipids increased lipid oxidation. Lee and Choe[13] found that chlorophyll b increased oil oxidation as well as chlorophyll b degradation by promoting singlet oxygen production. The addition of PC and PE retarded the decomposition of chlorophyll b. In this way, chlorophyll b could promote more photooxidation of canola oil because chlorophyll b was protected by PC and PE.

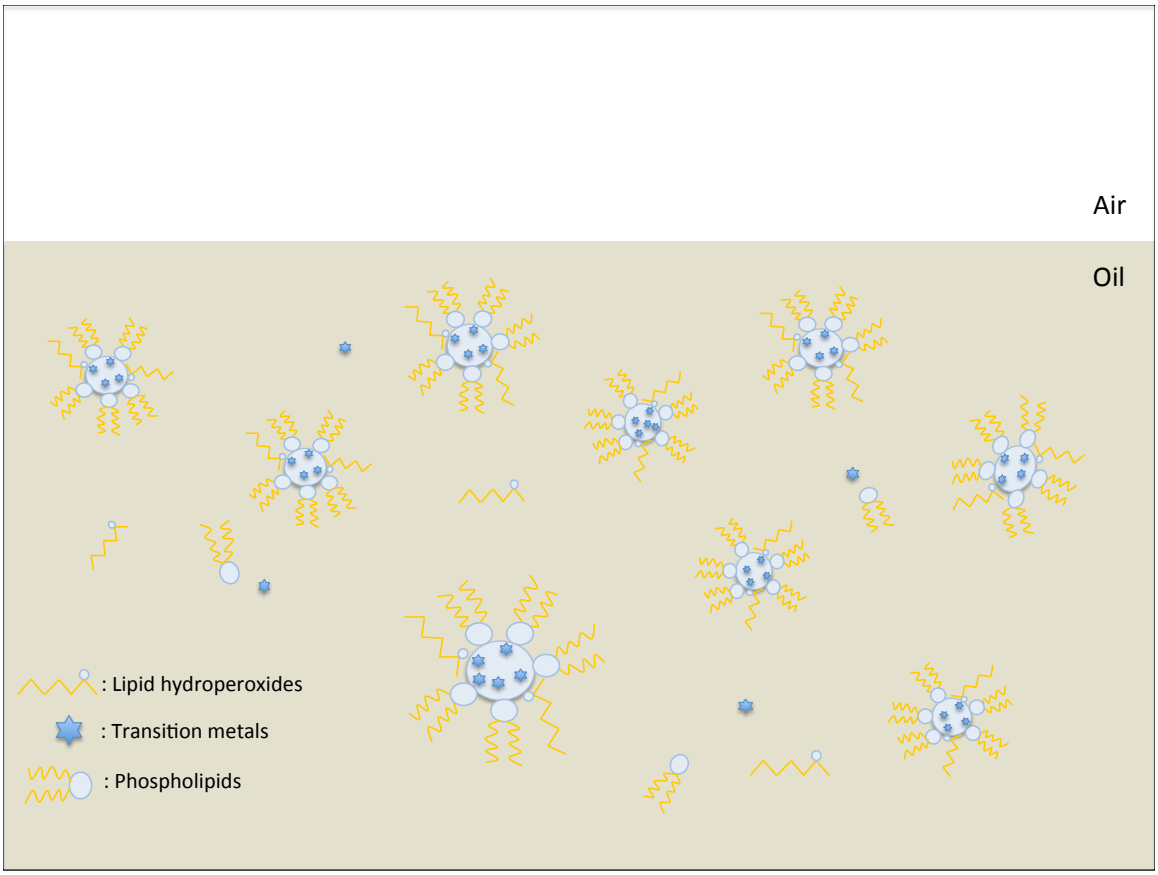


Figure 2.4 Bulk oil containing phospholipid reverse micelles

2.4.3 Antioxidant Properties of Phospholipids

The importance of understanding the food system when investigating the antioxidant properties of phospholipids should be emphasized. For example, bulk oil should not be treated as homogeneous because in different oil systems containing different minor components, phospholipid acts differently. King et al.[15] reported antioxidant properties of individual phospholipids in a salmon oil model system. However, the salmon oil used in their study contained 280 ppm tocopherols,

which made it hard to learn whether the antioxidant activity of phospholipids came from the phospholipids themselves or synergism with tocopherols. If the latter were the reason, one would not expect phospholipids to be antioxidant in another salmon oil where tocopherols were depleted. A similar case is with egg yolk phospholipids which slowed down lipid oxidation of DHA rich oil whereas the oil contained 3000 ppm tocopherols.[113] The importance of understanding the food system to learn what roles phospholipids play is also evidenced by Yoon and Min[33] who also reported different activities of phospholipids in different oil environments. The authors found that phospholipids acted as antioxidant when 1 ppm ferrous iron was added into stripped soybean oil which was depleted of tocopherols and other polar compounds. However, when in the absence of added iron, the phospholipids exhibited prooxidant activity. This again emphasized the importance of understanding the food system when investigating the antioxidant activity of phospholipids. More details of the reasons for the above differences will be discussed in the next sections.

2.4.3.1 Chelation

Theoretically, phospholipid can bind prooxidative metals through the negative charges presented on the phosphate headgroup and thus inhibit lipid oxidation. A simple test can be conducted to determine the binding ability of phospholipids membranes.[29] Briefly, phospholipid liposome or microsomes are incubated with metals followed by centrifugation to precipitate the liposomes. Unbound metals are

then determined in the aqueous phase. Using this method, iron was found to bind phospholipids membranes of microsome and liposome in the absence of chelators.[114] Zago and Oteiza[115] also reported that ferrous iron bound to PC/PS liposomes and that addition of zinc could displace iron from the membrane. They further suggested that zinc preferentially bound to PS over PC because PS has an additional carboxyl group and thus is more negatively charged. Dacaranhe and Terao[116] reported that the iron binding capacity of individual phospholipids in egg yolk PC unilamellar liposomes was in the following order: PA ≥ PS ≥ PG > PE = PC by a method similar as described earlier. They further determined that iron promoted decomposition of PC hydroperoxides in unilamellar liposomes were inhibited by the addition of PS. Likewise, Yoshida et al.[29] claimed that both saturated and unsaturated PS protected egg yolk PC liposomes from oxidation by binding free iron. Viani et al.[117] used a PC liposome inserted with arachidonic acid and found that addition of PA significantly retarded iron-induced oxidation. In an oil-in-water emulsion system where sardine oil was stabilized with Triton X-100 in Tris-HCl buffer, the authors found that addition of PA and PS effectively inhibited iron-induced lipid oxidation.[118] Cardenia et al.[34] also believed chelating property was responsible for the observed antioxidant activity of PC in stripped soybean oil-in-water emulsion at pH 7 where Tween 20 was used as emulsifier. They reported that at pH 3 which was near or even below the pK_a of PC, PC was not charged and thus unable to chelate metals and consequently its antioxidant activity disappeared. In a bulk oil system where 1 ppm ferrous iron was added, it was found

that phospholipids (PC, PE, PI, PA and PG) acted as antioxidants as they chelated iron.[33]

It is worth noting that just because phospholipids can bind iron does not always guarantee that they will inhibit lipid oxidation. One reason is that similar to EDTA and organic acids, the chelation of iron can increase its solubility and thus increase metal-lipid interaction which promote lipid oxidation.[119] Different reactivity's of metals bound to chelators could also influence the ability of chelators to act as antioxidants. For example, in a liposome system, EDTA, citrate and adenosine triphosphate (ATP) all removed iron bound to phospholipid membranes, but only EDTA and citrate inhibited lipid oxidation presumably because the iron bound to ATP was still reactive.[114] It is possible that metals bound to phospholipid are reactive since when the negative charge of the phospholipid attracts metals to the membrane surface lipid oxidation is accelerated. For example, Tampo et al.[120] reported that iron-promoted lipid oxidation was affected by the surface charge of liposomal membrane and PS was most sensitive to iron-promoted oxidation. Gal et al.[121] also found that increasing PS or PA to PC ratio in liposome produced more negative charges which resulted in more copper bound to the membrane surface and more lipid oxidation.

One reason for the contradicting reports of prooxidant and antioxidant activities of phospholipid bound metals could be due to different metal types and concentrations in different studies. For example, in the study by Gal et al.,[121] they found that increasing copper concentrations decreased the prooxidant activity of PS. They suggested that copper at high concentrations might cause lateral phase

separation of PS and PC in mixed liposome, where most of the copper bound to PS rich domains and thus was less available to oxidize PC substrates. Alternatively, copper at low concentrations might bind to both the amine and the carboxyl groups of PS forming a 2:1 PS:copper complex while at higher concentrations could forma a 1:1 complex. In general, chelators like EDTA are more effective when the chelator concentration exceed the metal concentrations because the binding of multiple chelators to a metal can tie up all the metal coordination sites and make the metal unreactive.[122]

2.4.3.2 Antioxidative properties of phospholipid Maillard reaction products

Maillard reactions are very important for the food industry because they can both positively or negatively impact food aroma, taste, color and nutritional attributes. The Maillard reaction occurs in the presence of carbonyls (e.g. reducing sugars, ascorbic acid and lipid oxidation aldehydes) and free amine groups (e.g. lysine).[123] The antioxidant properties of Maillard reaction products from reducing sugars and amino acids have been studied extensively since they can scavenge free radicals[124-126] and act as metal chelators.[127, 128]

At first glance, one might not expect Maillard reactions to be important in bulk oils. However, phospholipids like PE have a primary amine groups that can serve as a Maillard reaction substrate. In addition, carbonyls produced from the β-scission reactions of lipid oxidation (e.g. aldehydes and ketones) can provide the other substrate allowing Maillard reactions to occur. One of the reasons why

phospholipids are removed during the degumming step of oil refining is to decrease browning. More details of these reactions were described by Zamora and Hidalgo.[129]

Alaiz et al.[130] examined the antioxidative property of amine groups in stripped soybean oil with octylamine, methylheptylamine and dimethylhexylamine representing primary, secondary and tertiary amine groups. The authors found that primary and secondary amines inhibited lipid oxidation while tertiary amine had no effect. They further identified several oxidized lipid/amine reaction products like pyrrole derivatives that were formed by the reaction between octylamine (primary amine) and 4,5-epoxy-2-heptenal (a lipid oxidation products) in the oil samples and attributed the inhibitory effects of primary and secondary amines to these Maillard reaction products. Similar to octylamine, PE also contains a primary amine group and was also shown to react with 4,5-epoxy-2-heptenal to generate similar antioxidative Maillard reaction products.[131, 132] PC with a tertiary amine group, on the other hand, did not show inhibitory effect on lipid oxidation in a manner similar to dimethylhexylamine.[30] Since these Maillard products also produced color compounds, the oxidative stability of the oil was correlated to its yellowness index.[30] King et al. [133] also reported a relationship between the oxidative stability of salmon oil and color intensity from Maillard-type reaction products.

Bandarra et al.[16] suggested that the synergism between PC/PE and α-tocopherol in sardine oil could be due to Maillard reaction products measured at 430 nm. In a marine phospholipid liposome system, Maillard reaction in the presence of α-tocopherol was again confirmed through measurement of Strecker

aldehydes, color changes, and pyrrole content and proved to suppress the formation of volatile lipid oxidation[134]. Shimajiri et al.[19] who reported antioxidant activity of amine-containing phospholipids (PC, PE and SPM) further suggested that the presence of α-tocopherol was essential to the reaction that produced antioxidative Maillard reaction products.

2.4.3.3 Synergism with tocopherols

Many of the antioxidant properties of phospholipid reported in the literature are related to their ability to inhibit lipid oxidation synergistically with primary antioxidants, especially the tocopherols. Although many studies reported increased oxidative stability of food products when phospholipid and tocopherols were added together, the evidence of synergism is better demonstrated in studies showing that phospholipid alone do not inhibit lipid oxidation, but when they are in combination with tocopherols a strong antioxidant effect is observed. For instance, in perilla oil that was depleted of mixed tocopherols and stored in dark at 37 °C, neither 500 ppm PC, PE nor PS affected lipid oxidation. However, when 366 and 866 ppm mixed tocopherols were present, PE and PS prolonged the oxidation lag phase of the oil.[6] When canola underwent singlet oxygen-induced lipid oxidation at 10 °C, neither PC nor PE at 50 ppm decreased lipid hydroperoxides formation. Nevertheless, upon the addition of either 50 and 100 ppm α-tocopherol, synergistic activities were observed.[135] Takenaka et al. found that 1% unsaturated PE and PC were prooxidative when added alone in stripped bonito oil which was stored in dark at

40 °C. But when combined with 500 ppm α-tocopherol, PE exhibited synergistic antioxidant activity, while PC still had no effect. This absence of phospholipid antioxidant activity when used alone but enhanced antioxidant activity when present with α-tocopherol was also supported by the same research group in another study.[19] In stripped soybean oil, it has also been reported PE alone promoted lipid oxidation but inhibited lipid oxidation upon the addition of α-tocopherol.[31]

Synergism between phospholipid and tocopherols could be due to ability of phospholipids to: 1) form antioxidative Maillard reaction products in the presence of tocopherols, 2) altering the physical location of tocopherols and/or 3) regenerate tocopherols. As for Maillard reaction mechanism hypothesis, several studies[16, 19, 30, 133, 134] reported increased formation of phospholipid Maillard products in the presence of tocopherols, which has been discussed above.

Phospholipid can alter the physical location and thus the effectiveness of tocopherols. Physical location of antioxidants is known to influence their activity. For example, Huang et al.[136] showed that the distribution of α-tocopherol and trolox was different in different lipid systems (e.g. triacylglycerides, methyl linoleate and linoleic acid in bulk or emulsified form) which resulted in differences in antioxidant activities. Losada-Barreiro et al.[137] examined the impacts of emulsifiers with different HLB and at different concentrations on the distribution of antioxidants in oil-in-water emulsion. They found that increasing emulsifier concentration and decreasing HLB both promoted the incorporation of α-tocopherol and propyl gallate into the interfacial region of the emulsion. In biological

membranes that consist of saturated and unsaturated phospholipids, cholesterol, sphingomyelin and proteins, α-tocopherol is believed to concentrate at a polyunsaturated phospholipid domain.[138]

As both tocopherols and phospholipid have surface activity properties, their combination could influence the physical location of tocopherols as well as other primary antioxidants and the resulting change in location could impact their antioxidant activity. Koga and Terao[139] examined the impact of α-tocopherol and its phosphatidyl derivative (α-tocopherol conjugated to the head group of PC) on lard oxidation. They found that while both chemicals had the same radical scavenging ability, the phosphatidyl derivative of α-tocopherol had a better antioxidant activity than α-tocopherol alone. They suggested this improvement was due to better accessibility of the functional group of α-tocopherol to the site where iron-dependent oxidation reactions took place. In another study the same authors monitored the oxidation of methyl linoleate by measuring methyl linoleate hydroperoxides. They found that in the presence of a water-soluble compound that generates free radicals only in the aqueous phase, PC/PE had no impact alone but increased α-tocopherol antioxidant activity, showing a synergistic activity. In this case, PC/PE increased the consumption of α-tocopherol, meaning more α-tocopherol interacted with prooxidant due to a higher association with the aqueous phase. In contrast, when a lipid-soluble compound that generates free radicals only in the lipid phase was used, PC/PE had no impact on the consumption of α-tocopherol, meaning α-tocopherol interaction with prooxidant was minimal. These

results together suggested that phospholipids could alter the physical location of α-tocopherol and bring it to close proximity of the site of greatest oxidative stress.[11]

Since oxidized tocopherols (α-tocopherolquinone, α-tocopherolhydroquinone, and epoxy-α-tocopherolquinone) have been reported to accelerate lipid oxidation,[140, 141] and since oxidized tocopherols cannot scavenge free radicals, regeneration of oxidized α-tocopherol would help inhibit lipid oxidation by: 1) eliminating prooxidative oxidized α-tocopherol and 2) reforming antioxidative α-tocopherol. Regeneration of α-tocopherol by phospholipids is at least partially responsible for the observed synergism between tocopherols and phospholipids. While many studies mention tocopherol regeneration by phospholipids, few actually explain how this occurs. Oxidation-reduction potential is one potential parameter to determine the possibility that tocopherol can be regeneration by phospholipids. . However, this direct electron transfer between phospholipid and tocopherol is unlikely since they have similar reduction potential around 600 mV (data not shown). In contrast, there are several reports of phospholipids containing a primary amine group like PE and PS interacting with α-tocopherolquinone, an oxidation product of α-tocopherol.[7, 17, 31] Doert et al.[17] monitored the reaction between α-tocopherolquinone and different types of phospholipids (PC, PE, PI, PA and PS) in toluene at 100°C. They found that all tested phospholipids except PC were able to convert α-tocopherolquinone to α-tocopherol. The authors identified an intermediate PE-α-tocopherone condensation product by examining reaction products of PE and α-tocopherolquinone with mass spectrometry. They further suggested that PE-α-tocopherone would subsequently go through heterolytic

cleavage to form a carbenium ion which regenerated a-tocopherol. This reaction between PE and α-tocopherolquinone was also recently confirmed in a stripped soybean oil and medium-chain triacylglycerides system at a lower temperature of 55°C.[31] The synergism reported from literature between phospholipid and primary antioxidants (mainly tocopherols) was summarized in Table 2.5.

Table 2.5. Synergism between phospholipid and primary antioxidant

Phospholipid	Antioxidant	System	Ref
PC, PE	ethoxyquin	refined menhaden oil	[142]
PC, PE	α-tocopherol, quercetin	lard	[112]
PC, PE	polyhydroxyl flavonoids	lard	[143]
PC, PE, PI	mixed tocopherols	refined soybean oil	[144]
PE, PS, not PC[a]	mixed tocopherols	refined perilla oil	[6]
SPM, LPC, PC, PE[b, c]	endogenous mixed tocopherols	salmon oil	[133]
PE, ethanolamine, PS, not PC	α-tocopherol	sardine and mackerel lipids	[145]
PS, PE, soybean lecithin, not PC	α-tocopherol	methyl linolenate	[146]
PE, PS, PC[c]	mixed tocopherols	fish oil	[147]

Egg yolk phospholipid	endogenous mixed tocopherols	DHA oil	[113]
PE, PC, CL[b, c]	α-tocopherol	sardine oil	[16]
Soybean lecithin	endogenous mixed tocopherols	rapeseed, soybean, walnut, palm oil and lard	[14]
PE, not PC	endogenous mixed tocopherols	refined olive oil	[30]
Soybean lecithin	endogenous mixed tocopherols	virgin olive oil containing	[8]
PE	gallic acid, propyl gallate, caffeic acid, α-tocopherol, BHA, BHT, TBHQ	lard	[148]
PE	propyl gallate	lard	[149]
PE, not PC[a]	α-tocopherol	bonito oil	[12]
Soybean lecithin	α-tocopherol	fish oil	[150]
PE, PC[a]	α-tocopherol	canola oil	[135]
Soybean lecithin, rapeseed lecithin, sunflower lecithin, not soybean PC	α-tocopherol	ethyl linoleate	[17]

PE, PC, SPM[a]	α-tocopherol	fish oil	[19]
PE, not PC[a]	α-tocopherol, trolox	stripped soybean oil	[31]

a. Studies where phospholipids alone had no antioxidant affect, but showed synergism with α-tocopherol

b. Studies where Maillard reaction products were measured as color index and correlated with oxidative stability

c. Studies where the antioxidant activity was ordered and was as the sequence in the table

abbreviations: PC = phosphatidylcholine, PE = phosphatidylethanolamine, PS = phosphatidylserine, SPM = sphigomyelin, LPC = lysophosphatidylcholine, CL = cardiolipin,

CHAPTER 3

IMPACT OF PHOSPHATIDYLETHANOLAMINE REVERSE MICELLES ON LIPID OXIDATION IN BULK OILS

3.1 Introduction

Lipid oxidation leads to quality deterioration by generating off-flavor, nutrient loss, color alteration and formation of texture changes and even generation of potential toxic products [1]. Due to these quality and safety concerns, lipid oxidation shortens the shelf life of food products, which results in a loss of food.

Numerous researchers have identified lipid oxidation mechanisms in bulk oil [1, 142, 144, 151, 152]. However, the mechanism of lipid oxidation in bulk oil is still not fully understood and the role of minor components on lipid oxidation is often conflicting. For example, phosphocholine (PC) and phosphoethanolamine (PE) role in oxidation is unclear since they have been postulated to act as prooxidants, antioxidants or have no effect on oxidation reactions [6-17]. PE has been reported to have no effect on oxidation reactions when added to oils without other primary antioxidants. Kashima et al. [6] found that in tocopherol-free perilla oil, 500 ppm PE had no effect. Weng and Gordon [7] also reported no effect of 2600 ppm L-α-dilauryl PE in lard. Koga and Terao [11] found that 5 mM dimyristoyl phosphoethanolamine (DMPE) had little impact on hydroperoxides accumulation in a mixture of methyl linoleate and methyl laurate. In contrast, prooxidative activity of PE has also been reported. Takenaka et al. [12] found that in the absence of α-tocopherol, 10000 ppm (1 %) 1-palmitoyl-2-oleoyl-PE and 1,2-dioleoyl-PE decreased bonito oil stability. Lee

and Chloe [135] also reported that in tocopherol-stripped canola oil containing 4 ppm chlorophyll, 50 ppm PE from soybeans increased oil oxidation.

Most of the claimed antioxidant properties of PE were in the presence of other primary antioxidants such as tocopherols or biophenols so it is hard to know the role PE plays in antioxidant activity. These experiments include virgin olive oil (155-178 ppm mixed tocopherols) [8], rapeseed oil (637 ppm mixed tocopherols), soybean oil (1106 ppm mixed tocopherols) [14], perilla oil (836 ppm mixed tocopherols) [6], salmon oil (280 ppm mixed tocopherols) [15], lard (1000 ppm α-tocopherol) [7], sardine mince (400 ppm α-tocopherol) [16], olive oil (120 ppm of α-tocopherol and 4 ppm of γ-tocopherol) [30], DHA oil (3000 ppm mixed tocopherol) [113], methyl linoleate and methyl laurate (1 mM α-tocopherol) [11], ethyl linoleate (500 ppm α-tocopherol) [17] and bonito oil (500 ppm mixed tocopherols) [12].

Recently, Chen et al. [10, 20] reported that dioleoyl phosphocholine (DOPC) formed reverse micelles in stripped oil. These reverse micelles increased oxidation rates by increasing the activity of iron but also enhanced the activity of antioxidant such as α-tocopherol and Trolox. To better understand how phospholipids and their reverse micelles affect oil oxidation, this research focused on the role of PE and its combination with PC on the formation of association colloids and how these structures impacted soybean oil oxidation.

3.2 Materials and methods

3.2.1 Materials

Soybean oil was acquired from a local supermarket and stored at -20°C. Medium-chain triacylglycerol (MCT) were purchased from Sasol North America Inc. (Houston, TX, USA) and stored at -20°C. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dihexanoyl-sn-glycero-3-phosphoethanolamine (DHPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Silicic acid (100-200 mesh), activated charcoal (100-400 mesh), 7,7,8,8-Tetracyanoquinodimethane (TCNQ) and hexane were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). All chemicals and solvents were of analytical grade or purer. Distilled and deionized water was used in all experiments.

3.2.2 Preparation of stripped soybean oil

Stripped soybean oil (SSO) was prepared according to Boon et al. [153]. Basically, a chromatographic column (3.0 cm diameter, 35 cm long) was used to isolate soybean oil triacylglycerols. Three layers were packed into the column sequentially. The bottom layer was packed with 22.5 g silicic acid (washed with distilled and deionized water and activated at 110°C for 20 h). Activated charcoal (5.6 g) was then used for the middle layer and another 22.5 g silicic acid for the top layer. Commercial soybean oil (30 g) was mixed with 30 mL hexane and the mixture was then passed through the column using 270 mL hexane for elution. The solvent

was removed from the stripped oil with a vacuum rotary evaporator (Rotavapor R 110, Buchi, Flawil, Switzerland) at 38°C and the remaining solvent was evaporated by nitrogen flushing. Thin layer chromatography was used to verify the removal of minor components according to AOAC official method 982.27 [154]. The water content of the stripped oil was determined by a 831 KF Coulometer (Metrohm USA, Inc.). Unless noted, SSO mentioned in the following experiments was a mixture of 25% of stripped soybean oil and 75% MCT.

3.2.3 Determination of critical micelle concentration (CMC) of phospholipids in stripped soybean oil

The CMC of DHPE, DOPC, DOPE and the combination of DOPE and DOPC in SSO were determined using a TCNQ solubilized technique according to Kanamoto et al. [155] at both room temperature and 45°C. Briefly, TCNQ (1.5 mg) was added to 1.5 g SSO containing various concentrations of phospholipids and the mixture was stirred for 5 h. After sedimentation of excess TCNQ by centrifugation at 2000 g for 20 min, the absorbance of all oil samples at 480 nm were measured by a UV-VIS spectrophotometer (Shimadzu, Japan). The absorbance was plotted against the logarithm of phospholipids concentrations and the CMC was calculated as the intersection point of straight lines extrapolated from low and high phospholipids concentrations.

3.2.4 Preparation of SSO containing phospholipids

Various concentrations of phospholipids were dissolved in chloroform and then pipetted into an empty beaker. Chloroform was then removed by flushing with nitrogen. SSO was then added and stirred overnight at 800 rpm at room temperature or 45°C. Three groups of samples were prepared. First, SSO containing DOPE at concentrations below (50 and 100 μM) and above (400 and 1000 μM) CMC were prepared. Second, SSO containing DOPE and DHPE at same concentration (1000 μM) were prepared. Last, SSO containing the mixture of DOPC and DOPE at ratio 1:1 at concentrations below (5 and10 μM) and above (100 and 500 μM) CMC were prepared. For the mixture of DOPC and DOPE, each concentration had half DOPE and half DOPC. For example, 10 μM total phospholipids consisted of 5 μM DOPE and 5 μM DOPC. SSO without addition of any phospholipids was used as control.

3.2.5 Measurement of lipid oxidation

Samples (1 mL) were place in 10 mL headspace vials sealed with PTFE/silicone septa lined aluminum caps and stored at 45°C in the dark. At various sampling times, lipid oxidation was determined by monitoring lipid hydroperoxides and headspace hexanal formation. Lipid hydroperoxides were measured as primary oxidation products according to Shantha and Decker [156]. In short, 2.8 mL of a mixture of methanol/butanol (2:1, v/v) were added to oil samples of known weight. Then 15 μL of 3.94 M ammonium thiocyanate and 15μL of 0.072 M Fe^{2+} (ferrous sulfate)

were added and the solution was vortexed. After a 20 min reaction time, the absorbance was measured at 510 nm using a Genesys 20 spectrophotometer (ThermoSpectronic, Waltham, MA). The concentration of lipid hydroperoxides was calculated from a cumene hydroperoxide standard curve ranging from 0.7 mM to 17 mM. Samples with peroxides value higher than 17 mM were diluted with methanol/butanol (2:1, v/v) before measurements.

Headspace hexanal was measured as a secondary lipid oxidation products using a GC-17A Shimadzu gas chromatograph equipped with an AOC-5000 autosampler (Shimadzu, Kyoto, Japan) as described by Chen et al. [10]. In short, a 30 m × 0.32 mm Equity DB-1 column with a 1 μm film thickness was used for separations. Oil samples (1 mL) in 10 mL headspace vials were heated at 55°C for 15 min in the autosampler heating block. A 50/30 μm DVB/Carboxen/PDMS solid-phase microextraction (SPME) fiber needle was injected into the sample vial for 2 min to adsorb volatiles and then the fiber was placed into the 250°C injector port for 3 min at a split ratio of 1:5. The GC ran for 10 min at 65°C for each sample. Helium was used as a carrier gas, with a total flow rate of 15.0 mL/min. A flame ionization detector at 250°C was used to detect hexanal and concentrations were determined from peak areas using a standard curve made from SSO containing known hexanal concentrations from 2 μM to 800 μM.

3.2.6 Statistical analysis

All experiments were conducted on triplicate samples. Data were presented as mean values ± standard deviation. Data results were analyzed by analysis of variance (ANOVA) using SPSS (SPSS Inc., Chicago, IL). The differences between mean values were compared using Duncan's multiple-range test with a significance level of $p < 0.05$. Lag phases were defined as the first data point statistically greater than time 0.

3.3 Results and Discussion

3.3.1 Determination of CMC of DOPE in stripped soybean oil

Phospholipids (Figure 3.1) like DOPE and DOPC are amphiphilic molecules with medium hydrophilic-lipophilic balance (HLB) values of around 8.0, thus they can form aggregates in both polar and nonpolar solvents at concentrations higher than their CMCs. In nonpolar systems, such as SSO containing about 200-300 ppm water (the concentrations in these experiments), DOPE and DOPC could form reverse micelles with its polar head pointing to the water core while its nonpolar tail pointing to bulk oil. The lipid-water interfaces created during the aggregation could influence lipid oxidation. For example, lipid hydroperoxides could interact with iron at the oil-water interface, promoting the oxidation process [110].

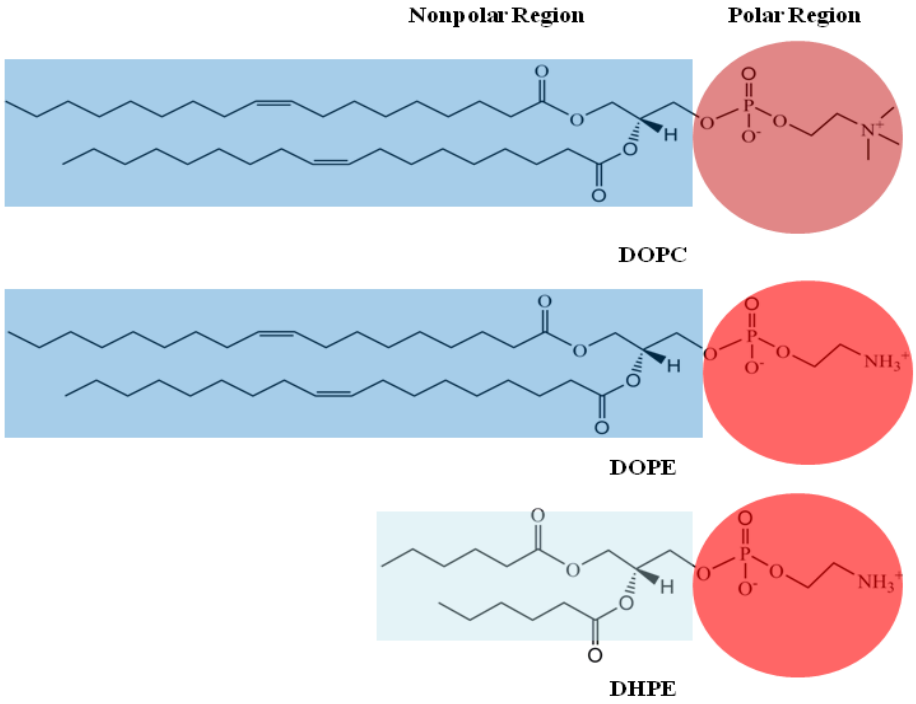


Figure 3.1 Structures of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dihexanoyl-sn-glycero-3-phosphoethanolamine (DHPE).

The absorbance of TCNQ solubilized in SSO containing phospholipids at 480 nm was plotted against the logarithm of phospholipids concentrations. The CMC was taken as the intersection point of straight lines extrapolated from low and high concentrations of DOPE, DHPE, DOPC and DOPC and DOPE combinations. No intersection point was found for up to 10,000 μM DHPE, suggesting that DHPE does not form association colloids. Chen et al. [10] observed a similar behavior for 1,2-dibutyl-sn-glycero-3-phosphocholine (DBPC) and postulated that polarity

differences between these molecules influenced their ability to form reverse micelles. DOPC had a lower CMC than DOPE (Table 3.1), which is probably due to a greater interaction with water, i.e. hydration, than DOPE [155]. The reason that PE is less hydratable than PC is that the amine group in the headgroup of PE tends to form hydrogen bonds directly with adjacent phosphate molecules. Thus, stronger lipid-lipid rather than lipid-water interactions are present for PE [157]. The mixture of DOPE and DOPC at ratio of 1:1 also had a lower CMC than DOPE. Phospholipids content in crude oil can be as high as 3300 μM which are then reduced to 38 μM to 600 μM after refining, bleaching and deodorizing [158]. These findings indicate that the concentrations of PC and PE in refined oils could exist as reverse micelles at levels similar to the phospholipids concentrations in refined oils.

Table 3.1 Critical micelle concentration of DOPE, DOPC and the mixture of DOPE and DOPC at a 1:1 ratio at room temperature and 45°C.

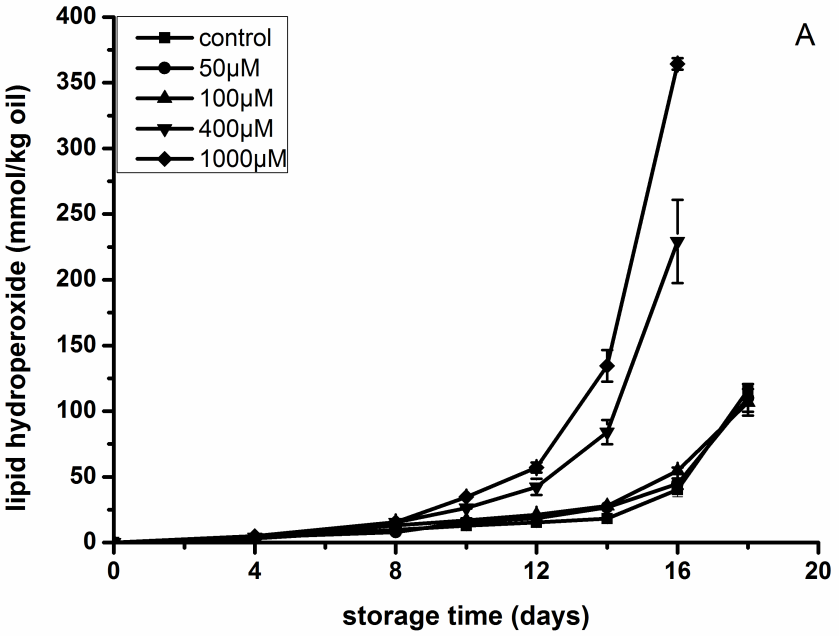
	CMC	
Phospholipids	Room temperature	45°C
DOPE	800 μM	200 μM
DOPC	50 μM	20 μM
DOPE and DOPC at 1:1 ratio	150 μM	50 μM

In SSO, the CMC of DOPE and DOPC and their combinations were lower at 45°C than at room temperature (Table 3.1). This trend was more obvious for DOPE as the CMC drop from 800 μM to 200 μM with increasing temperature. Both an increase and decrease of CMC with increasing temperature have been reported for different compounds and solvents [159, 160]. Kang et al. [161] reported a U-shaped behavior of the CMC of anionic ammonium dodecyl sulfate (ADS) and cationic octadecyltrimethylammonium chloride (OTAC) with increasing temperature. Gupta et al. [97] reported no significant effect of temperature on reverse micellar size with a phospholipid/hexane/soybean oil system in which the surfactants are native soybean phospholipids containing PC, PE and small amounts of phosphatidic acid, phosphatidylinositol, and lysophosphatides.

3.3.2 Oxidation of SSO containing DOPE and DHPE

Primary oxidation products (Figure 3.2A) and secondary oxidation products (Figure 3.2B) were monitored during storage. The lag phase of oxidation was defined as the time when lipid hydroperoxides and hexanal values increased dramatically. Lag phase was used as the key parameter to assess oxidative quality of the samples since the oil would be rancid once oxidation rates increased rapidly. At concentrations above its CMC (400 and 1000 μM), it is expected that the DOPE reverse micelles would increase oxidation rates since they would increase interactions between surface active lipid hydroperoxides and metals resulting in formation of free radicals that would further promote oxidation. DOPE reverse

micelles promoted the formation of both lipid hydroperoxides and hexanal in SSO. The lag phase of both lipid hydroperoxides and hexanal formation in the control was 14 days, compared to 8 to 9 days for both 400 μM and 1000 μM DOPE. DOPE concentrations below the CMC (50 and 100 uM) had no impact on lipid oxidation as indicated by the same lag phase as the control. Under this situation, no association colloids are formed, thus no water-lipid interfaces were present to promote oxidation. Kasaikina et al. [106] also reported that surfactants like cetyltrimethylammonium chloride and bromide could accelerate sunflower-seed oil oxidation kinetics when they formed reverse micelles. The ability of DOPE to have no effect on oxidation rates or act as a prooxidant in bulk oil depending on its concentration and thus ability to form physical structures could be one explanation for some of the contrary results reported for the impact of PE on oxidative rancidity.


A
control
50μM
100μM
400μM
1000μM
lipid hydroperoxide (mmol/kg oil)
storage time (days)
0
50
100
150
200
250
300
350
400
0
4
8
12
16
20

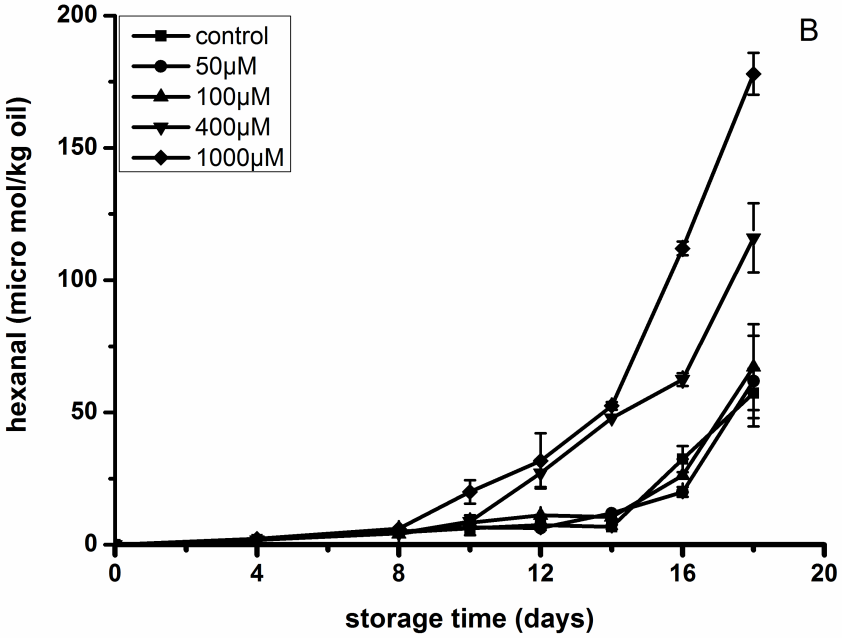

B
control
50μM
100μM
400μM
1000μM
hexanal (micro mol/kg oil)
storage time (days)
0
50
100
150
200
0
4
8
12
16
20

Figure 3.2 Formation of lipid hydroperoxides (A) and headspace hexanal (B) in stripped soybean oil with various DOPE concentrations both below (≤ 100 μM) and above (≥ 400 μM) its critical micelle concentration at 45°C. Data points represent means (n=3) ± standard deviations.

A comparison between DOPE and DHPE at 1000 uM and 45°C were made to better understand the role of PE in oil oxidation. The lag phase of both lipid hydroperoxide and headspace hexanal formation in the presence of DOPE reverse micelles was 8 days, while the lag phases of DHPE and control were both 14 days (Figure 3.3). The two PE used here were at the same concentration and had the same molar concentration of their hydrophilic polar head groups. The only difference between these two molecules is that DOPE has a long hydrophobic tail, which increases its ability to form reverse micelles, while DHPE has a much shorter hydrophobic tail, which decreases its ability to form reverse micelles as seen from the CMC data. This suggests that the higher oxidation found in the DOPE-containing systems was likely related to the reverse micelles formed by DOPE and not the PE head group since both DOPE and DHPE contain the same ethanolamine head group.

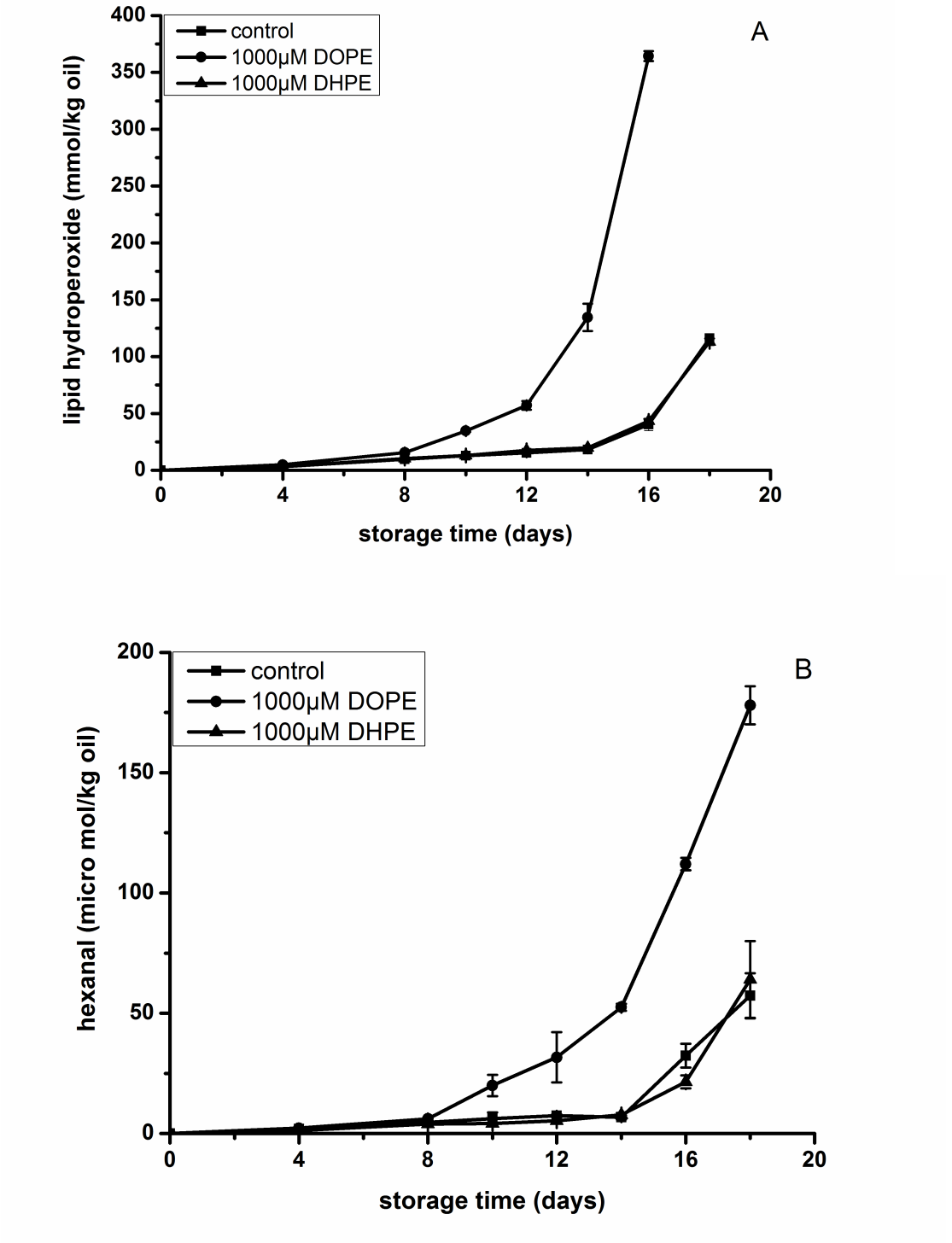

A
control
1000μM DOPE
1000μM DHPE
lipid hydroperoxide (mmol/kg oil)
storage time (days)
0
50
100
150
200
250
300
350
400
0
4
8
12
16
20
B
control
1000μM DOPE
1000μM DHPE
hexanal (micro mol/kg oil)
storage time (days)
0
50
100
150
200
0
4
8
12
16
20

Figure 3.3. Formation of lipid hydroperoxides (A) and headspace hexanal (B) in stripped soybean oil with 1000 μM DOPE and 1000 μM DHPE stored at 45°C. Data points represent means (n=3) ± standard deviations.

3.3.3 Impact of the combination of DOPE and DOPC on oil oxidation

For SSO containing both DOPE and DOPC, a DOPE to DOPC ratio of 1:1 was chosen according to Racicot's et al. study on DOPE and DOPC concentrations in degummed soybean oil [162]. From Table 3.1, the CMC of DOPC was approximately 20 μM and 50 μM at 45°C and room temperature, respectively. Combinations of DOPE and DOPC had CMCs between DOPE and DOPC alone at 50 and 150 μM at 45°C and room temperature, respectively. These intermediate CMCs suggest that the two phospholipids were forming mixed reverse micelles. The impact of the combination of DOPE and DOPC on oil oxidation was then investigated at phospholipid concentrations below (5 μM and 10 μM) and above (100 μM and 500 μM) the CMC (Figure 3.4). These concentrations are again similar to phospholipids concentration in degummed and refined vegetable oil [158].

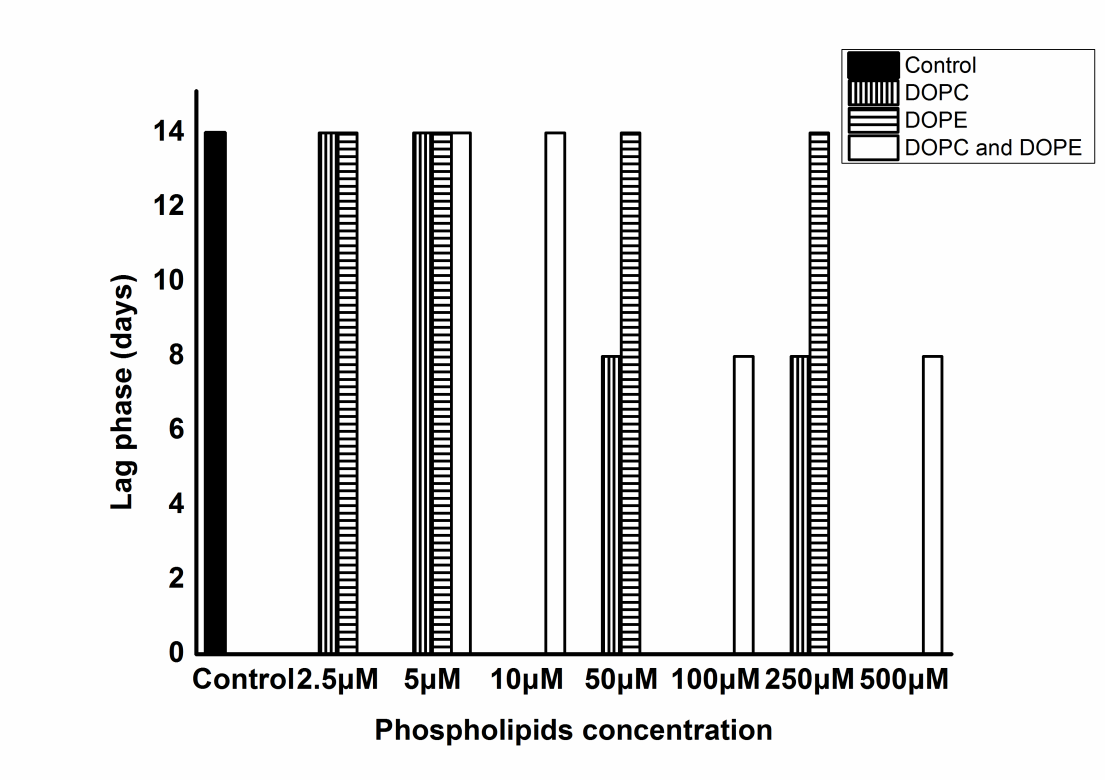


Figure 3.4. Headspace hexanal lag phases in stripped soybean oil with various concentation of DOPE and DOPC and their combination (1:1 ratio) at 45°C.

At 5 μM and 10 μM phospholipids concentrations, DOPC, DOPE and their combinations were all below their CMCs, and the lag phases for lipid hydroperoxides and headspace hexanal formation were the same as control. DOPE alone at 100 μM was also below its CMC and did not increase oxidation rates. This again suggested that when phospholipids do not form association colloids in stripped oil, they have no impact on lipid oxidation. When the phospholipids concentrations were above the CMC (100 μM and 500 μM for DOPC and the DOPE and DOPC combination and 500 μM for DOPE), prooxidant activity was observed.

This agrees with the hypothesis that association colloids serve as reaction sites that accelerate lipid oxidation in stripped bulk oil.

CHAPTER 4

IMPACT OF PHOSPHATIDYLETHANOLAMINE ON THE ANTIOXIDANT ACTIVITY OF A-TOCOPHEROL AND TROLOX IN BULK OIL

4.1 Introduction

Lipid oxidation is a great concern for food manufacturers and consumers as it negatively impacts on not only food quality and nutritive value of bioactive lipids, but also consumers' health [1]. Strategies to inhibit lipid oxidation in bulk oils can extend the shelf life of oil-containing products and thus benefit both the food industry and consumers. One effective traditional method to inhibit lipid oxidation is the addition of antioxidants such as t-butyl-4-hydroxyanisole (BHA), 2,6-di-t-butyl-p-hydroxytoluene (BHT) and t-butyl hydroquinone (TBHQ). However, though these antioxidants are economic for food manufacturers, there have been some debates on their potential safety risks [2-4]. In addition, as consumers desire simpler and cleaner labels, there is a growing demand for replacing these synthetic antioxidants with natural antioxidants.

Among the natural antioxidants, tocopherols are important as they exist in many vegetable oils such as rapeseed, sunflower, cottonseed, soybean and corn oil [163]. Scientists have been investigating on how to maximize the activity of endogenous tocopherols and several studies showed that phospholipids help increase their effectiveness. Judd et al. [14] reported the ability of lecithin containing high proportions of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) to increase the activity of γ- and δ- tocopherols.

Bandarra et al. [16] reported that PC, PE and cardiolipin combinations improved with the effectiveness of α-tocopherol. PC has not always been found to increase the activity of tocopherols in all studies. For example, Takenaka [12] found that only PE, not PC, increased the effectiveness of α-tocopherol. Kashima et al. [6] reported that PE and phosphatidylserine (PS) suppressed lipid oxidation of perilla oil containing 866 ppm mix tocopherols, while PC showed no effect.

One possible mechanism accounting for the cooperative activity of phospholipids and tocopherols could be due to the head group of PC and PE regenerating tocopheroxyl radical to tocopherol. Doert et al. [17] recently reported that primary amines can regenerate oxidized tocopherols through an ionic mechanism rather than a radical mechanism. A second proposed mechanism for phospholipids enhancing the activity of tocopherols is the non-enzymatic browning reaction products from amine-containing phospholipids and oxidation products such as aldehydes are antioxidative and thus provide an additional source of antioxidant [30, 129, 132, 164]. Hidalgo et al. [18] reported that the antioxidative activity of carbonyl-amine products was greatly increased with the addition of tocopherols. Shimajiri et al. [19] further suggested that the formation of antioxidant compounds from amine-containing polar lipids and oxidation products might require the presence of α-tocopherol. Nevertheless, the exact mechanism of how the combination of phospholipids and tocopherols influences bulk oil oxidation is still not fully understood.

Recently, studies from our lab found that surface-active phospholipids such as dioleoyl-PC (DOPC) and dioleoyl-PE (DOPE) can form association colloids such as

reverse micelles in bulk oils, which can influence the oxidative stability of the oil [10, 165]. Chen et al. [20] further reported that DOPC reverse micelles improved the activity of α-tocopherol or trolox, a water-soluble analog of α-tocopherol, at low concentrations (10 μM) but decreased their activity at higher concentrations (100 μM). Since lecithin is a mixture of different types of individual phospholipids, the objective of this study was to examine the impact of DOPC, DOPE both individually and in combinations on the antioxidant activity of α-tocopherol or trolox. The experiments were conducted at PC and PE concentrations above and below their critical micelle concentrations to determine the impact of reverse micelles on the antioxidant activity of α-tocopherol and trolox. In addition to the investigation of the influence of association colloids formed by phospholipid, α-tocopherol regeneration by different types of phospholipids was also examined.

4.2 Materials and methods

4.2.1 Materials

Soybean oil was purchased from a local store and stored at -20 °C. Medium-chain triacylglycerols (MCT) were purchased from Sasol North America Inc. (Houston, TX, USA). 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-PE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Silicic acid, activated charcoal, alpha-tocopherol, alpha-tocopherol quinone and trolox

were purchased from Sigma-Aldrich Co. (St. Louis, MO). All other reagents were of HPLC grade or purer. Distilled and deionized water was used in all experiments.

4.2.2 Preparation of stripped soybean oil

To avoid the influence of the minor compounds such as tocopherols, phospholipids, free fatty acids and mono- and diacylglycerols which are originally in the commercial oil, stripped soybean oil (SSO) was prepared as described by Cui et al. [165]. Briefly, a chromatographic column (3.0 cm diameter, 35 cm long) was used to isolate soybean oil triacylglycerols. Three layers were packed into the column sequentially. The bottom layer was packed with 22.5 g silicic acid (washed with distilled and deionized water and activated at 110 °C for 24 h). Activated charcoal (5.6 g) was then used for the middle layer and another 22.5 g silicic acid for the top layer. Commercial soybean oil (30 g) was mixed with 30 mL hexane and the mixture was passed through the column using 270 mL hexane for elution. The solvent was then removed by a vacuum rotary evaporator (Rotavapor R 110, Buchi, Flawil, Switzerland) at 38 °C and the remaining solvent was evaporated by nitrogen flushing. The removal of polar lipids was verified by a thin layer chromatography method according to Association of Official Analytical Chemists (AOAC) [154]. HPLC analysis also detected no tocopherols in SSO (method described below). The water content decreased from about 250 ppm to about 20 ppm as determined by Karl Fischer analysis using a 831 KF Coulometer (Metrohm, FL, USA) [166]. Unless noted,

SSO mentioned in the following experiments was a mixture of 25 % of stripped soybean oil and 75 % MCT.

4.2.3 Sample preparation

The experiment was separated to 5 parts. Part 1 included the addition of 20 or 1000 μM of DOPC or DOPE into SSO. Part 2 included the addition of 20 or 1000 μM of DOPC or DOPE to SSO containing100 μM α-tocopherol or trolox. Part 3 included addition of 1000 μM DOPC or DOPE to the commercial soybean oil. Part 4 included addition of 1000 μM DOPC or DOPE to SSO containing 0.95 μM NBD-PE and 0-100 μM α-tocopherol or trolox. Part 5 included addition of 800 μM DOPC, DOPE or ethanolamine to MCT or SSO containing 800 μM α-tocopherol quinone. According to different parts, appropriate amount of antioxidants (α-tocopherol, α-tocopherol quinone or trolox), were dissolved in ethanol and then pipetted into empty beakers followed by removal of solvent by nitrogen flushing. SSO was then added and the sample was magnetically stirred overnight. According to different experimental parts, the appropriate amounts of NBD-PE, DOPC and/or DOPE in chloroform, individually or at different ratios, were pipetted into empty beakers and chloroform was removed by nitrogen flushing. SSO containing antioxidants were added and magnetically stirred at room temperature for 5 hours for NBD-PE fluorescence study or overnight for lipid oxidation studies. For the lipid oxidation studies, 1 mL samples were aliquoted into 10-mL headspace vials, sealed with aluminum caps with PTFE/silicone septa, and stored in dark at 55 °C.

4.2.4 Measurement of lipid oxidation

At various sampling times, lipid oxidation was determined by monitoring lipid hydroperoxides and headspace hexanal formation. Lipid hydroperoxides were measured as primary oxidation products according to Shantha and Decker [156]. In short, 2.8 mL of a mixture of methanol/ butanol (2:1, v/v) was added to oil samples of known weight. Then 15 μL of 3.94 M ammonium thiocyanate and 15 μL of 0.072 M Fe^{2+} (ferrous sulfate) were added and the solution was vortexed. After a 20 min reaction time, the absorbance was measured at 510 nm using a Genesys 20 spectrophotometer (ThermoSpectronic, Waltham, MA). The concentration of lipid hydroperoxides was calculated from a cumene hydroperoxide standard curve ranging from 0.7 to 17 mM. Samples with hydroperoxides concentrations higher than 17 mM were diluted with methanol/butanol (2:1, v/v) before measurements.

Headspace hexanal was measured as a secondary lipid oxidation products using a GC-17A Shimadzu gas chromatograph equipped with an AOC-5000 autosampler (Shi- madzu, Kyoto, Japan) as described by Chen et al. [10]. In short, a 30 m × 0.32 mm Equity DB-1 column with a 1 μm film thickness was used for separations. Oil samples (1 mL) in 10 mL headspace vials were heated at 55 °C for 8 min in the autosampler heating block. A 50/30 μm DVB/Carboxen/PDMS solid-phase microextraction (SPME) fiber needle was injected into the sample vial for 2 min to adsorb volatiles and then the fiber was placed into the 250 °C injector port for 3 min at a split ratio of 1:5. The GC separations were 10 min at 65 °C for each sample. Helium was used as a carrier gas, with a total flow rate of 15.0 mL/min. A flame ionization detector at 250 °C was used to detect hexanal and concentrations

were determined from peak areas using a standard curve made from SSO containing known hexanal concentrations from 2 to 800 μM.

4.2.5 Fluorescence measurement of SSO containing reverse micelles and antioxidants

The concentration of NBD-PE in SSO was varied to get appropriate fluorescence intensity with minimal self-quenching, the optimal concentration of the probe was found to be 0.95 μM. The measurements were carried out as described by Kittipongpittaya et al. [167]. Basically, the sample was transferred to a triangular Suprasil quartz cuvette (Sigma-Aldrich Co., St. Louis, MO). The steady-state emission intensity of spectra, determined as the emission signal intensity (counts per second) measured by a photomultiplier, was recorded with a PTI spectrofluorometer (PTI, Ontario, Canada). The spectral band width for both excitation and emission slits were set to 2 nm for excitation at 468 nm. The integration time was set to 1 s.

4.2.6 Determination of α-tocopherol concentration by HPLC

Oil samples (20-30 mg) were dissolved in 1 mL isoproponal and 1 mL methanol and then passed through a 0.2 μm filter (Fisher scientific, PA, USA). Sample (20 μL) was then injected to a Shimadzu HPLC system equipped with a Beckman Ultrasphere C18 reversed phase column (150 mm × 4.6 mm, 5μm). The mobile phase was isocratic methanol at a flow rate of 1 mL/min. A Waters 474 scanning fluorescent detector (Waters, MA, USA) was used to detect α-tocopherol at an

excitation wavelength of 290 nm and an emission wavelength of 330 nm. Peak integration was performed using Shimadzu EZstart software (version 7.2). α-tocopherol in the samples was identified and quantified by comparing their relative retention times and peak areas with authentic compounds.

4.2.7 Cyclic voltammetry measurements

Cyclic voltammetry measurements were performed according to the method described by Wilson and coworkers [168]. Briefly, a BASi model C-3 cell stand with a planar 1 mm diameter glassy carbon working electrode, a Ag/AgCl reference electrode, and a Pt wire auxiliary electrode was used. Tetrabutylammonium hexafluorophosphate (Bu_4NPF_6; 0.5 M) in dichloromethane was used as a salt bridge. DOPC, DOPE and α-tocopherol (1 mM) were freshly dissolved in dichloromethane before measurements.

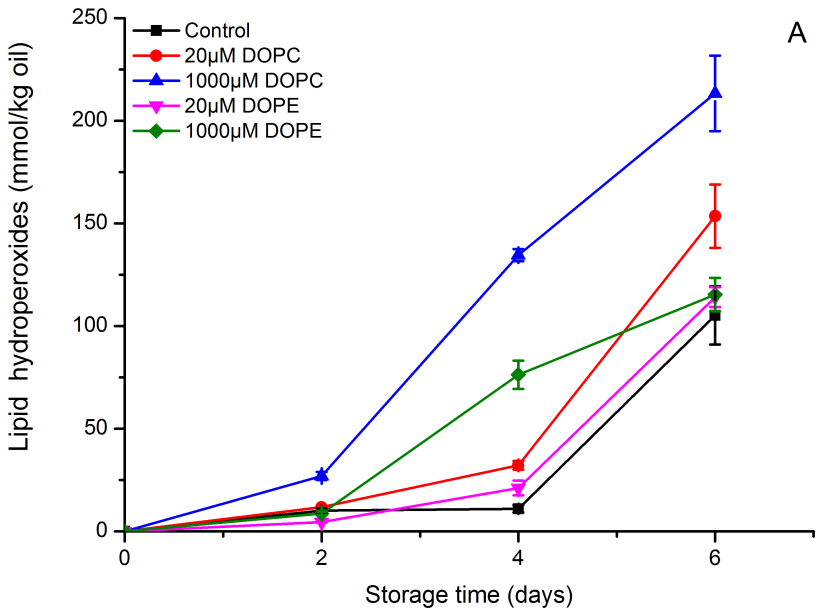
4.2.8 Statistical analysis

All experiments used triplicate samples (three samples taken at each time point) and each experiment was repeated at least 2 times. Data were presented as mean values ± standard deviation. Data results were analyzed by analysis of variance (ANOVA) using SPSS 17.0 (SPSS Inc., Chicago, IL). The differences between mean values were compared using Duncan's multiple-range test with a significance level of $p < 0.05$.

4.3 Results and discussion

4.3.1 Impact of DOPC and DOPE on SSO oxidation

There are two reasons for choosing 20 µM and 1000 µM DOPE in the first experiment. First, 20µM and 1000µM represent concentrations below and above the critical micelle concentration (CMC) for DOPE since the CMC for DOPE is 200µM [165]. Second, these concentrations are within the range of what is found in refined commercial oils. In this experiment, we found that at 55°C, DOPE had the same lag phase (4 days) as the stripped soybean oil control when its concentration (20 µM) was below its CMC (200 µM). On the other hand, when DOPE concentration (1000 µM) was above its CMC (200 µM), it had prooxidant activity with a lag phase of 2 days.



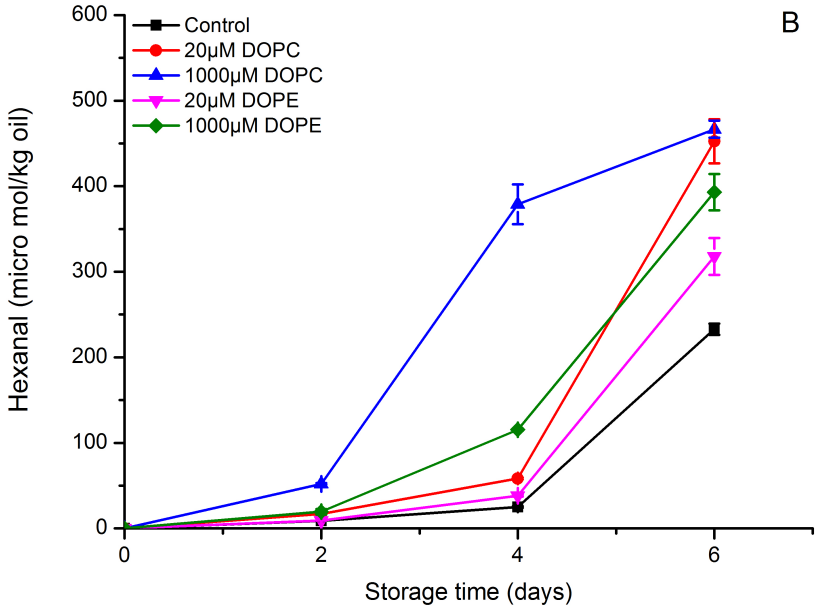


Figure 4.1. Formation of lipid hydroperoxides (A) and headspace hexanal (B) of SSO with 20 or 1000 μM of DOPC or DOPE concentrations at 55°C. Data points represent means (n=3) ± standard deviations.

When 20 μM DOPC was added to the SSO at the same concentration as DOPE, the lag phase was also similar to the control. DOPC above its CMC (1000 μM) was prooxidative, which agrees with previous reports (*17*). Samples containing 1000 μM DOPE showed slower rates of lipid hydroperoxides and hexanal formation than DOPC but both had the same lag phase. Lag phase is the most important indicator of oil quality since after the lag phase the oil is rancid. This indicates that both DOPE and DOPC are prooxidative at concentrations above their CMCs where they form association colloids. This prooxidative activity of phospholipids caused by the

formation of reverse micelles during storage at 55°C agrees with our previous results using SSO stored at 37 and 45°C [10, 165].

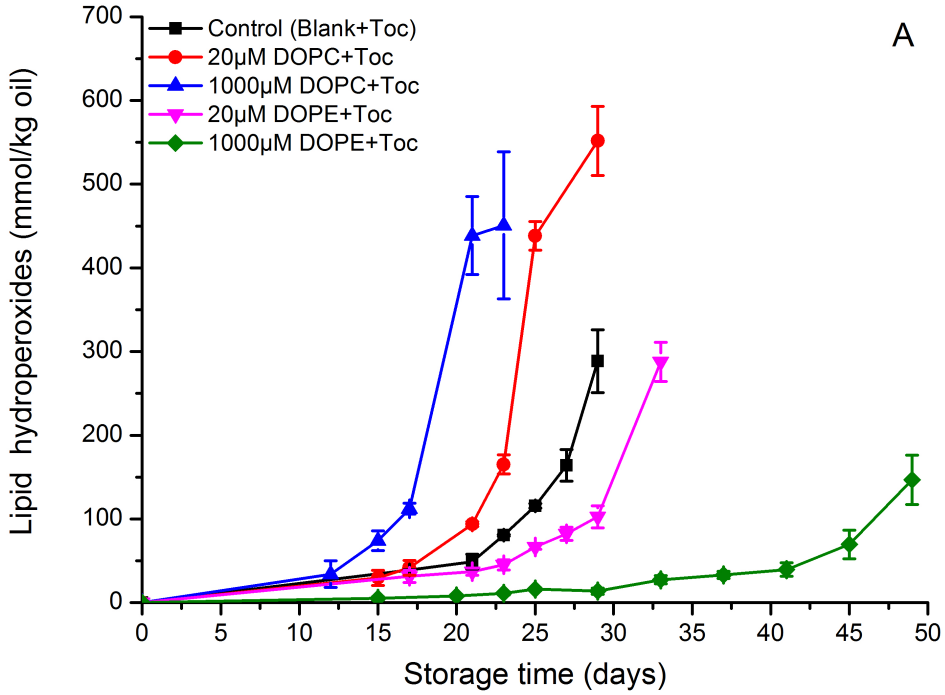
4.3.2 Impact of DOPC and DOPE on the oxidation of SSO containing α-tocopherol or trolox

There has been a lot of research done on the impact of phospholipids on bulk oil oxidation. However, no agreement has been made on their roles as studies report both antioxidative and prooxidative activity [6, 8, 12, 14]. Our previous results showed that when DOPE is above its CMC, it is prooxidative while DOPE below its CMC does not impact oxidation rates. However, those results were based on a SSO system, which was almost pure triacylglycerols. Here, we used a system consisting of SSO and 100 μM α-tocopherol or trolox, which enabled us to investigate on how DOPC and DOPE reverse micelles impact the ability of antioxidants to alter lipid oxidation in bulk oil.

In the case of α-tocopherol (Figure 4.2), the lag phase of hydroperoxides and hexanal formation in the presence and absence of 20 μM DOPC were 17 days and 21 days, respectively. It was somewhat unexpected that 20 μM DOPC decreased the activity of α-tocopherol since 20 μM DOPC had no impact on lipid oxidation rates by itself (Figure 4.1A, 1B). A potential reason that 20 μM DOPC could be prooxidative is that this concentration was very close to the CMC of DOPC and addition of α-tocopherol could cause formation of some prooxidative reverse micelles. In the presence of 1000 μM DOPC, where large amounts of reverse micelles were present, the hydroperoxides and hexanal formation lag phase reduced from 21 days (α-

tocopherol only) to 12 days. DOPC reverse micelles themselves had prooxidant activity (Figure 4.1), which would be expected to diminish the antioxidant activity of α-tocopherol. In addition, it was worth noting that comparing to SSO alone, which had a lag phase of 4 days (Figure 4.1), the combination of 1000 μM DOPC and α-tocopherol still had a longer lag phase (12 days) and thus a net antioxidant effect.

As for DOPE, the activity of α-tocopherol was increased at DOPE concentrations both below (20 μM) and above (1000 μM) its CMC with antioxidant activity increasing with increasing DOPE concentrations as determined by both lipid hydroperoxides and headspace hexanal. This suggested that the ability of DOPE to increase the activity of α-tocopherol was independent from the presence of reverse micelles. The synergy seen between PE and α-tocopherol has been reported earlier [6, 12] but no clear mechanism was presented.



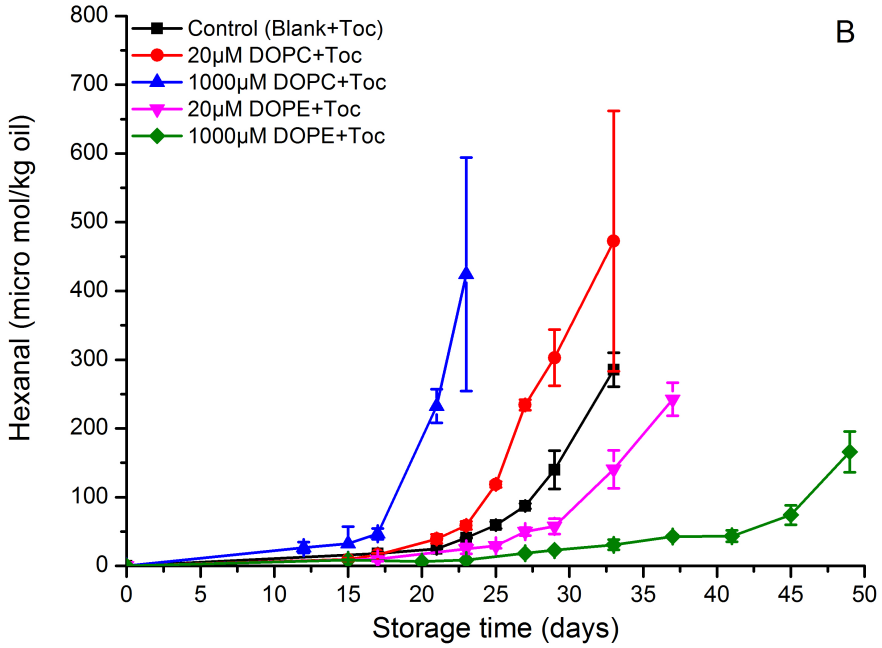
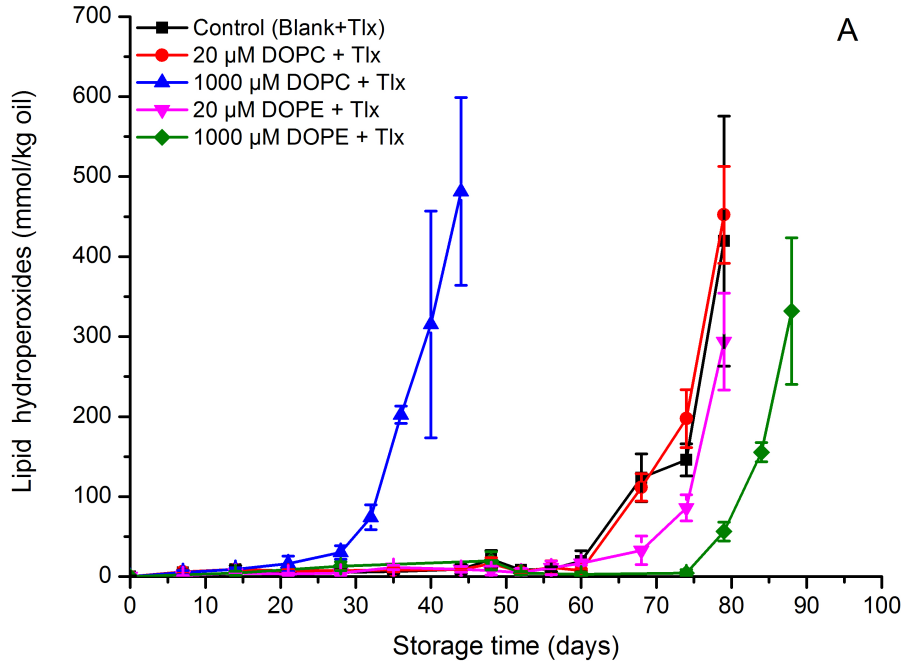


Figure 4.2. Formation of lipid hydroperoxides (A) and headspace hexanal (B) of SSO containing 100 μM α-tocopherol with addition of DOPC and DOPE concentrations both below and above their critical micelle concentrations at 55°C. Data points represent means (n=3) ± standard deviations.


A
Control (Blank+Tlx)
20 µM DOPC + Tlx
1000 µM DOPC + Tlx
20 µM DOPE + Tlx
1000 µM DOPE + Tlx
Lipid hydroperoxides (mmol/kg oil)
Storage time (days)
0
100
200
300
400
500
600
700
0
10
20
30
40
50
60
70
80
90
100

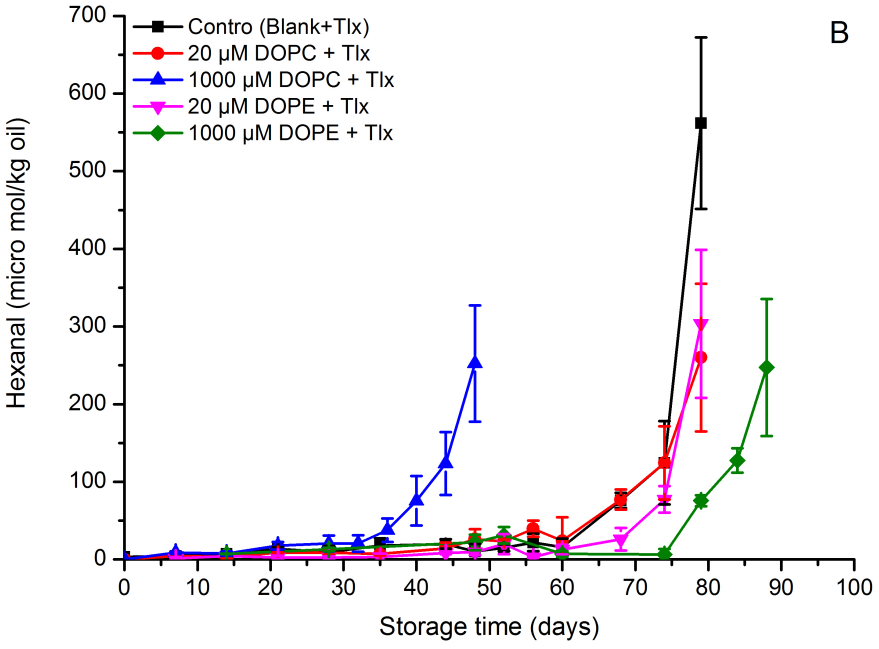

B
Contro (Blank+Tlx)
20 µM DOPC + Tlx
1000 µM DOPC + Tlx
20 µM DOPE + Tlx
1000 µM DOPE + Tlx
Hexanal (micro mol/kg oil)
Storage time (days)
0
100
200
300
400
500
600
700
0
10
20
30
40
50
60
70
80
90
100

Figure 4.3. Formation of lipid hydroperoxides (A) and headspace hexanal (B) of SSO containing 100 μM trolox with addition of DOPC and DOPE concentrations both below and above their critical micelle concentrations at 55°C. Data points represent means (n=3) ± standard deviations.

In the case of trolox (Figure 4.3), the lag phases of both hydroperoxides and hexanal formation in the presence and absence of 20 μM DOPC were the same, being 60 days. In the presence of reverse micelles formed by 1000 μM DOPC, the lag phase reduced from 60 days (trolox only) to 28 days for hydroperoxide formation, and to 31 days for hexanal formation. This was as expected because of the prooxidative activity of DOPC reverse micelles. As for DOPE, the activity of trolox was increased at DOPE concentrations both below (20 μM) and above (1000 μM) the CMC with antioxidant activity increasing with increasing DOPE concentrations as determined by both lipid hydroperoxides and headspace hexanal. This again suggested that DOPE was increasing the activity of trolox regardless of the presence of reverse micelles. In addition, the lag phases of all treatments with trolox were longer than those with α-tocopherol, which agreed with the antioxidant polar paradox theory [169].

4.3.3 Impact of DOPC and DOPE combinations on the oxidation of SSO containing α-tocopherol

Previously, we reported that the combination of DOPC and DOPE at concentrations above their CMC could form mixed reverse micelles and was

prooxidative in SSO [165]. Since PE was found to increase the activity of α-tocopherol, a further investigation on the combinations of these two phospholipids was carried out to see their overall impact on α-tocopherol. The lag phases of hexanal formation of different compositions of DOPC and DOPE above the CMC (1000 μM in total) with 100 μM α-tocopherol were shown in Figure 4.4. As the proportion of DOPE increased from 0 to 10, 30, 50, 60 and 100 %, the corresponding lag phases increased from 17 to 21, 28, 28, 36 and 42 days, respectively. These different overall effects in hexanal lag phases with different DOPC: DOPE ratios were due to a combination of the prooxidative effect of the phospholipids reverse micelles and of the ability of PE to increase the activity of α-tocopherol. This suggested that addition of PE could improve the oxidative stability of refined soybean oil which would naturally contain tocopherols. We tested this hypothesis with commercial soybean oil naturally containing 2000 μM (800 ppm) mixed tocopherols in which we added 1000 μM DOPC or DOPE (Figure 4.5). The control and the oil with addition of DOPC had same lipid hydroperoxides lag phase of 4 days and hexanal lag phase of 9 days, while the oil with addition of DOPE increased the lipid hydroperoxide lag phase to 6 days and hexanal lag phase to 15 days.

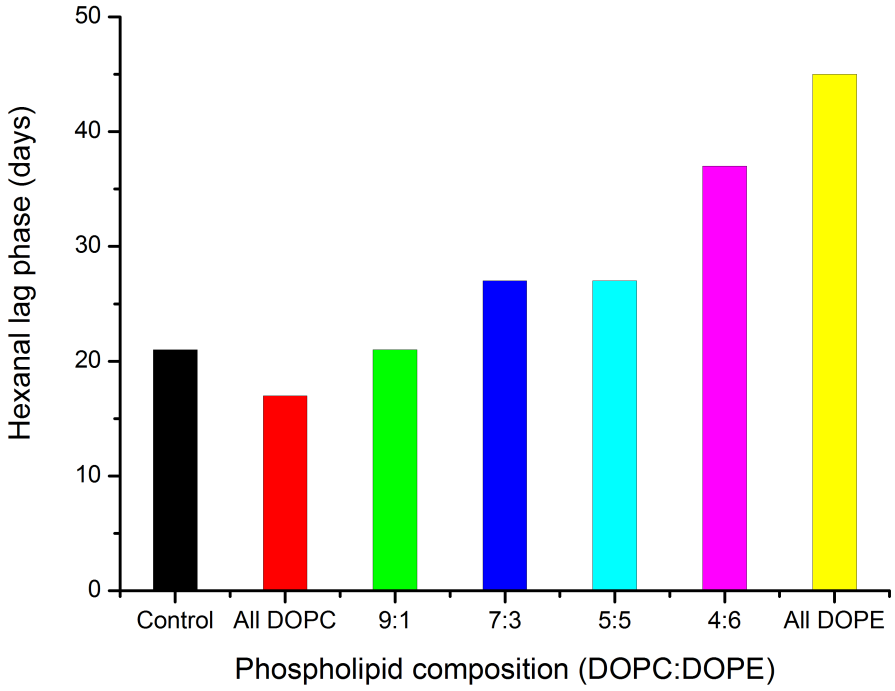


Figure 4.4. Headspace hexanal lag phases of SSO containing 100 μM α-tocopherol with addition of DOPC and DOPE at different ratios (1000 μM in total) at 55°C.

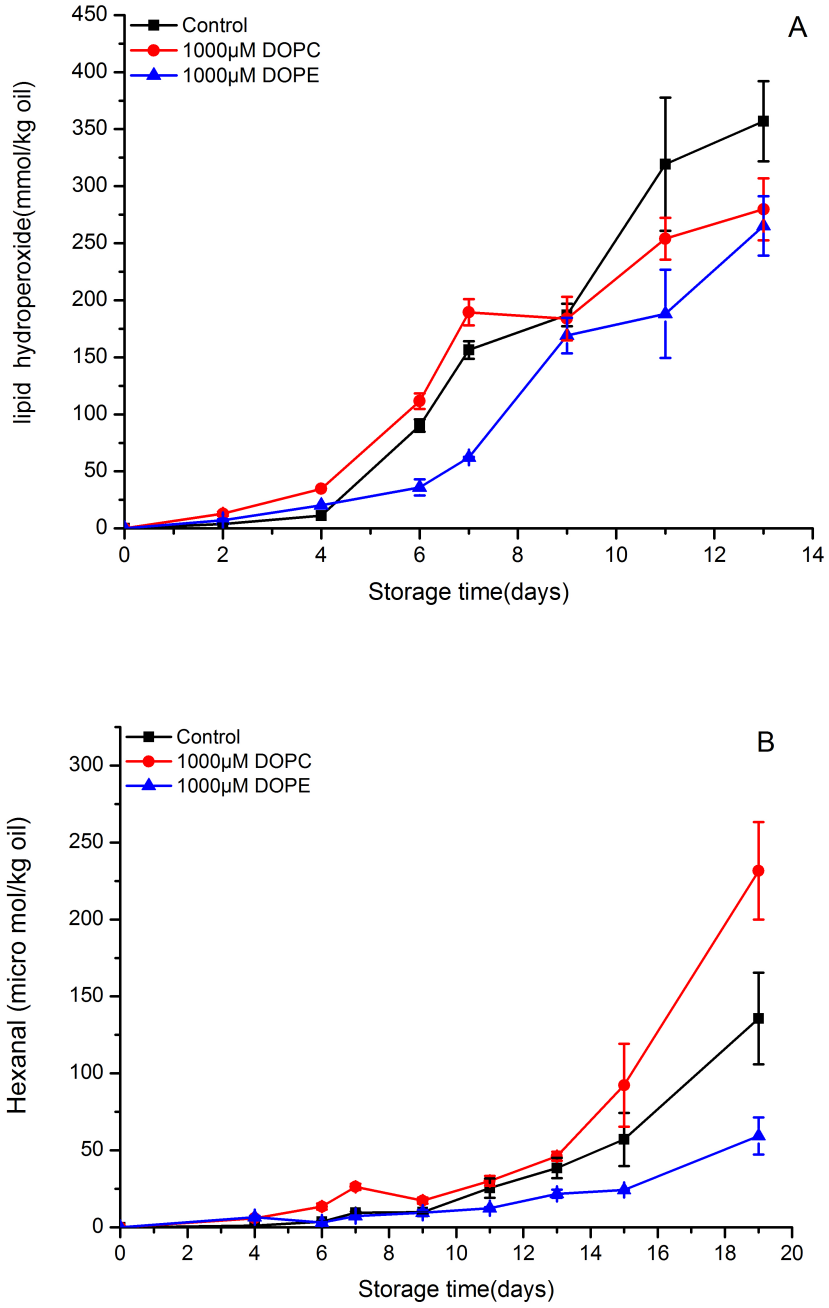


Figure 4.5. Formation of lipid hydroperoxides (A) and headspace hexanal (B) of commercial soybean oil with addition of 1000 μM DOPC or DOPE at 55°C. Data points represent means (n=3) ± standard deviations.

4.3.4 Location of antioxidants in the presence of DOPE and DOPC reverse micelles

Physical locations of antioxidants in bulk oil can influence their antioxidant activities. NBD-PE was used to investigate whether or not DOPC and DOPE reverse micelles have an impact on antioxidants locations. NBD-PE is a surface-active phospholipid with a fluorophore group covalently attached to the head group of PE. This probe has been widely used in biological and model membranes as fluorescent analogues of native lipids and the location of the probe in those models is believed to be at the interfacial region of the membrane [170]. In our experiments, 0.95 μM NBD-PE and 1000 μM DOPE or DOPC were added into bulk oil. With those concentrations, the ratio of fluorophore to surfactant was about 1:1000 which helped avoid probe aggregation and thus self-quenching effects.

When DOPC and DOPE reverse micelles were not present, the emission intensity of the probe decreased with increasing α-tocopherol concentration (data not shown), showing that α-tocopherol has the ability to quench the probe. On the other hand, when α-tocopherol is not present, the phospholipids alone (DOPC or DOPE) did not cause any changes of probe emission intensity (data not shown). In the presence of both DOPC and DOPE reverse micelles, increasing trolox concentration decreased the emission intensity of the NBD-PE probe (Figure 4.6), indicating that trolox was in close contact with the probe thus quenching its fluorescence. However, the combination of DOPE reverse micelles and α-tocopherol did not cause a significant difference in NBD-PE emission intensity with increasing α-tocopherol concentrations. When the combination DOPC reverse micelles and α-tocopherol was present, the emission intensity of the probe decreased with the

addition of α-tocopherol at first, but did not further decrease as α-tocopherol concentration increased from 10 to 100 μM. These results suggested that α-tocopherol did not accumulate in the DOPE reverse micelle but did accumulate in the DOPC reverse micelles. These data indicated that water-soluble trolox partitioned more readily into DOPE reverse micelles. Trolox also partitioned into DOPC reverse micelles and at the concentration used in the antioxidant studies (100 μM) more trolox was in the reverse micelles than α-tocopherol did. Since the micelle interface could be a major site of lipid oxidation reactions due to the presence of surface active hydroperoxides and water soluble transition metals, the greater concentration of the antioxidant at the interface could explain why trolox was more effective than α-tocopherol. However, DOPE was found to increase the activity of α-tocopherol even though DOPE reverse micelles where not found to increase α-tocopherol concentrations at the micelle interface. This suggests that DOPE's ability to increase the activity of α-tocopherol was not due to changes in its physical location.

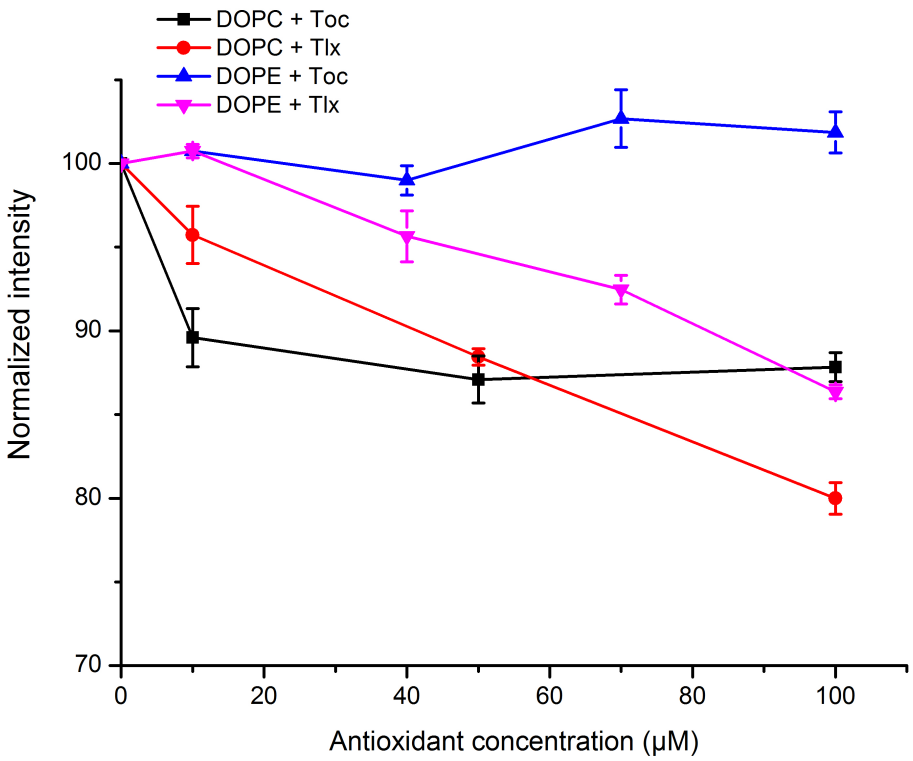
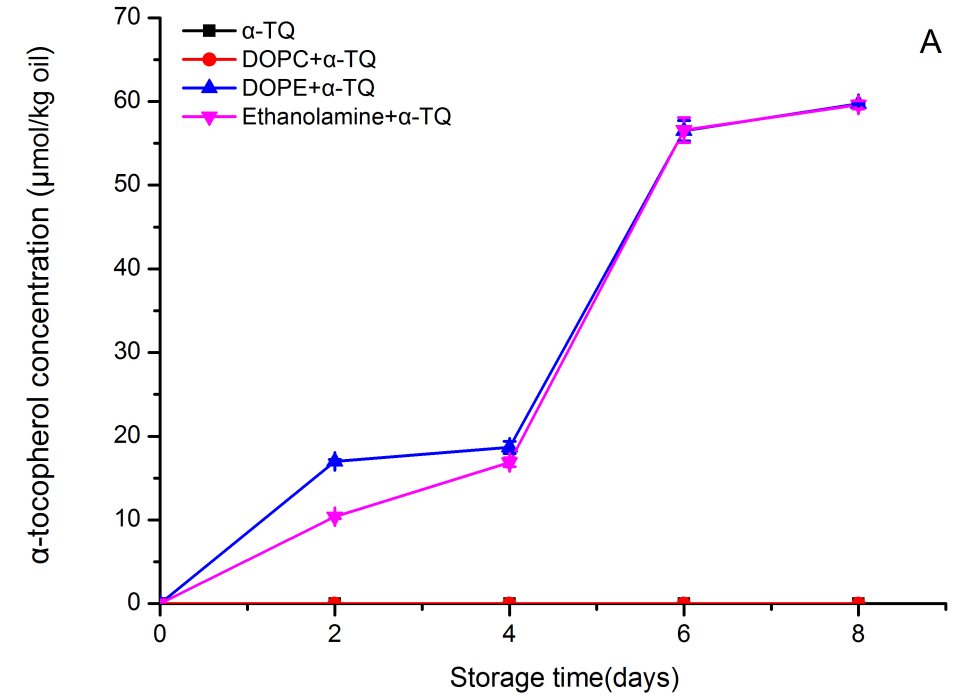


Figure 4.6. Normalized fluorescence intensity of NBD-PE in SSO with addition of 1000 uM DOPC or DOPE and different concentrations of α-tocopherol or trolox.

4.3.5 Effect of DOPE on α-tocopherol regeneration

Another potential mechanism by which DOPE could increase the activity of α-tocopherol would be through its ability to regenerate oxidized α-tocopherol back to its active, reduced form. The hypothesis that phospholipids regenerate tocopherols was tested by monitoring the ability of DOPC, DOPE and ethanolamine, the headgroup of PE, to react with α-tocopherol quinone, an oxidation product of α-tocopherol, in both MCT (Figure 4.7A) and SSO (Figure 4.7B) at 55 °C. In MCT (Figure 4.7A) where no lipid oxidation occurs, the treatments with DOPE and

ethanolamine but not DOPC promoted the regeneration of α-tocopherol quinone to α-tocopherol, indicating that the ethanolamine headgroup of PE can reduce α-tocopherol quinone. In SSO (Figure 4.7B), only the treatment with DOPE and α-tocopherol quinone resulted in the formation of α-tocopherol. Also, the quantity of α-tocopherol regenerated by DOPE in SSO was smaller than that in MCT. This could be because that in SSO, lipid oxidation was occurring, which could consume α-tocopherol once it was produced by DOPE and α-tocopherol quinone. This was confirmed by monitoring the hydroperoxides formation in SSO (Figure 4.7B, right y axis). DOPE with α-tocopherol quinone was still within hydroperoxides lag phase after 8 days of storage, while all other treatments and the α-tocopherol quinone control had a lag phase of 2 or 4 days. This indicated that the newly produced α-tocopherol by DOPE and α-tocopherol quinone was being consumed to retard lipid oxidation in SSO thus decreasing the accumulation of α-tocopherol comparing to the non-oxidizing MCT.


A
α-TQ
DOPC+α-TQ
DOPE+α-TQ
Ethanolamine+α-TQ
α-tocopherol concentration (μmol/kg oil)
Storage time(days)
0
10
20
30
40
50
60
70
0
2
4
6
8

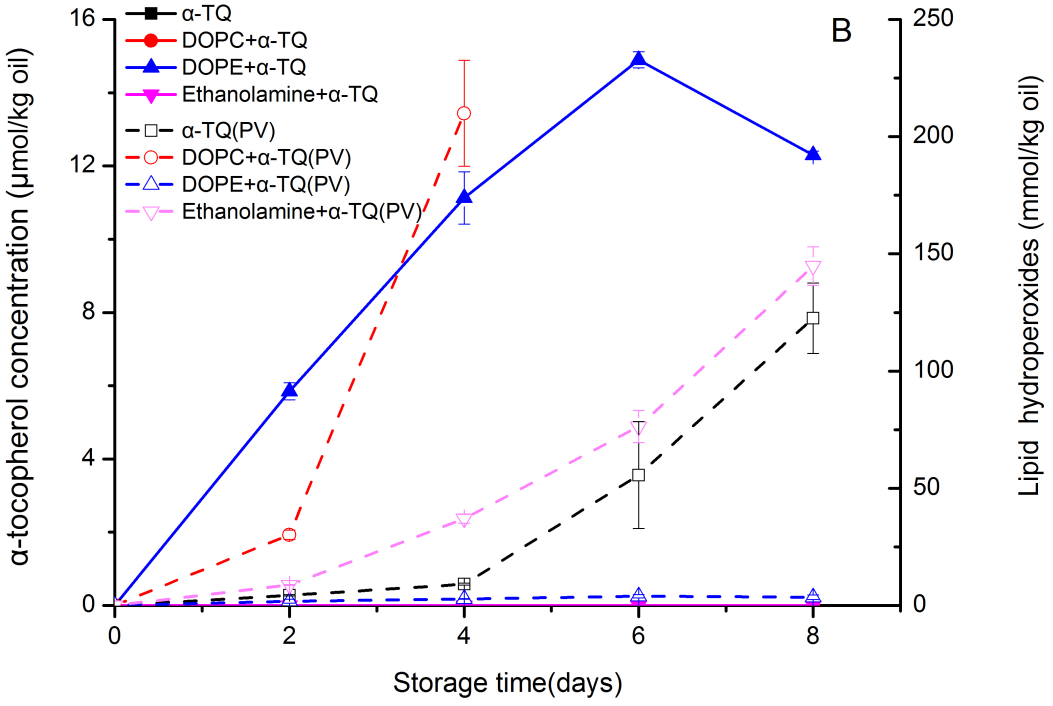

B
α-TQ
DOPC+α-TQ
DOPE+α-TQ
Ethanolamine+α-TQ
α-TQ(PV)
DOPC+α-TQ(PV)
DOPE+α-TQ(PV)
Ethanolamine+α-TQ(PV)
α-tocopherol concentration (μmol/kg oil)
Lipid hydroperoxides (mmol/kg oil)
Storage time(days)
0
4
8
12
16
0
50
100
150
200
250
0
2
4
6
8

Figure 4.7. Formation of α-tocopherol (solid lines) by 800 uM DOPC, DOPE or ethanolamine with 800 uM α-tocopherol quinone at 55°C in MCT (A) and SSO (B). Formation of lipid hydroperoxides (B, dashed lines) of SSO containing 800 uM DOPC, DOPE or ethanolamine with 800 uM α-tocopherol quinone at 55°C.

A potential mechanism by which DOPE could regenerate α-tocopherol quinone is by electron transfer. The potential for such an electron transfer reaction can be evaluated by measuring oxidation-reduction potentials via cyclic voltammetry [171]. Using this method, α-tocopherol exhibited an oxidation peak at about 600 mV, which was close to that reported in the literature [73]. However, there were no oxidation or reduction peaks detected for DOPC or DOPE (data not shown). This suggested that DOPE did not regenerate α-tocopherol through direct electron transfer. Recently, Doert et al. [17] also reported that α-tocopherol regeneration by primary amines can be explained by an ionic mechanism rather than by a radical mechanism. In their study, they found that 1% addition of L-β,γ-dipalmitoyl-α-phosphatidylethanolamine (PE) and 1,2-dipalmitogycero-3-phosphoryl-L-serine (PS), not L-β,γ-dipalmitoyl-α-phosphatidylcholine (PC) or L-β,γ-dipalmitoyl-α-inositol (PI), regenerated α-tocopherol quinone to α-tocopherol in toluene at 100 °C. In our case, this reaction was confirmed with about ten times lower concentration of PE (0.07%) in both bulk oil and MCT at a much lower temperature (55 °C). The concentrations used in this study are much closer to concentrations expected in refined, commercial oils.

CHAPTER 5

MEASURING OXIDATIVE STABILITY OF STRIPPED BULK OIL AND COMMERCIAL EDIBLE OILS WITH ELECTRON PARAMAGNETIC RESONANCE (EPR)

5.1 Introduction

Lipid oxidation is one of the major chemical causes of deterioration of foods. For example, lipid oxidation can induce color changes and generate volatile oxidation products which are responsible for "rancid flavors". These defects negatively impact the shelf life of food products and are a major cause of food waste. Consequently, lipid oxidation continues to be a great concern for manufacturers and consumers.

Lipid oxidation mechanisms have been studied extensively.[1, 23] These reactions involve a series of free radical reactions. For example, unsaturated fatty acids lose a hydrogen to form an alkyl radical in the presence of initiators (e.g. UV light, heat and transition metals) (reaction 1). Alkyl radicals are subsequently converted to peroxyl radicals by the addition of oxygen and to hydroperoxides by hydrogen abstraction from another unsaturated fatty acid (reaction 2 and 3). Lipid hydroperoxides can decompose to peroxyl and alkoxyl radicals (reaction 4 and 5). The alkoxyl radical will undergo β-scission decompositions, resulting in low molecular weight, volatile compounds such as aldehydes and ketones (reaction 6).

$LH \rightarrow L^{\bullet}$ (1)

$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$ (2)

$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$ (3)

LOOH → LOO• (4)

LOOH → LO• + OH^- (5)

LO• → volatile aldehydes and ketones (6)

Based on these reaction products, a variety of methods that measure different parameters have been developed to evaluate the oxidative stability of food products.[172] For example, lipid hydroperoxides (reaction 2) can be measured as a primary oxidation product. On the other hand, hexanal, which can be formed by decomposition of lipid hydroperoxides at the omega-6 position of unsaturated fatty acids (reaction 4), can be a determinate of a secondary oxidation product. Plotting such parameters against time describes the lipid oxidation process. Usually, a lag phase, or induction period, is initially observed and is followed by a rapid increase of oxidation products.[173] The lag phase is an important indicator of the oxidative stability of the food products because after the lag phase off-flavors have developed and the food is rancid.

Electron paramagnetic resonance (EPR), or electron spin resonance (ESR), can be applied to measure oxidative processes in foods .[174] This technique is based on the absorption of electromagnetic radiation in the microwave range produced by unpaired electrons in the presence of a magnetic field. Free radicals produced during lipid oxidation (reaction1, 2, 4 and 5) possess these unpaired electrons but they are short-lived and can rarely be detected directly. These radicals can be captured by spin traps such as N-*tert*-butyl-α-phenylnitrone (PBN) to form more stable radicals that produce an EPR signal that reflects the concentration of free radicals. However, to accurately measure free radical concentrations in foods, the

spin trap should be added to the samples immediately prior to EPR measurements rather than before storing the food lipid. This is because PBN can inhibit lipid oxidation, if it is added to foods in advance, by scavenging free radicals.[175] Velasco and coworkers[175] reported poor linear correlation between radicals concentration and both hydroperoxides and thiobarbituric acid value in rapeseed, sunflower and fish oils as they found radicals concentration increased rapidly before significant changes of these two lipid oxidation parameters. On the other hand, Papadimitriou et al.[176] found a good correlation between the induction period of EPR signal intensity at 70 °C the Rancimat method at 110 °C in extra virgin olive oil, although correlation of the induction period by EPR intensity with tocopherols concentrations was not successful. A major limitation of this study was the use of high incubation temperatures (70 and 110 °C) which would alter lipid oxidation pathways by accelerating hydroperoxides decomposition.[177]

Free radicals in oils could relate to lag phase and thus the shelf-life of foods since the alkoxyl radicals is a major factor in the decomposition of unsaturated fatty acids to the low molecular weight volatile compounds that cause rancidity. However, spin traps are not effective at detecting low energy free radicals such as tocopherol radicals. Therefore, the conditions where EPR could be used to measure lipid oxidation in foods could vary as a factor of the composition of a particular food. Therefore, the objectives of this study were to use EPR to monitor radical concentrations in oil stripped of minor components such as tocopherols as well as stripped oils where tocopherols were added back to the samples. Finally, monitoring the oxidation stability of commercial soybean, corn, sunflower and

canola oil with EPR is reported. In all studies, EPR results were compared to the classic primary and secondary lipid oxidation markers, hydroperoxides and hexanal.

5.2 Materials and methods

5.2.1 Materials

Soybean, canola, corn and sunflower oil were purchased from a local store. N-*tert*-butyl-α-phenylnitrone (PBN) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). All other reagents were of HPLC grade or purer. Distilled and deionized water were used in all experiments.

5.2.2 Preparation of Stripped Soybean Oil (SSO)

SSO was prepared by column chromatography according to Cui et al.[31] The packing materials were silicic acid and activated charcoal. Hexane was used as the eluting solvent. The solvent was separated from the lipid by a vacuum rotary evaporator (Rotavapor R 110, Buchi, Flawil, Switzerland) at 38 °C followed by nitrogen flushing. The removal of polar lipids and tocopherols were verified by a thin layer chromatography method according to the Association of Official Analytical Chemists (AOAC)[154] and HPLC analysis.[31]

5.2.3 Sample Preparation

SSO containing different concentrations of α-tocopherol were prepared as following: α-tocopherol was dissolved in methanol and then pipetted into empty beakers followed by removal of solvent by nitrogen flushing. SSO was then added, and the samples were magnetically stirred overnight. One mL of SSO, SSO containing different concentrations of α-tocopherol and the different commercial oils were pipetted into 10 mL headspace vials sealed with aluminum caps with PTFE/silicone septum, stored at 37, 45 or 55 °C in dark and sampled periodically for EPR signal, lipid oxidation products and α-tocopherol concentrations.

5.2.4 Determination of EPR Signal Intensity

The EPR measurements were carried out with an e-scan EPR from Bruker (Boston, MA, USA). One mL of oil was transferred from 10 mL headspace vials to 15 mL brown vials and then mixed with 2 mL of heptane. PBN in heptanes was subsequently added to the mixture at a final concentration of 30 mM. The vials containing oil and PBN were heated for 75 min at 45 or 70 °C to increase radical production. Samples were transported to the sample cuvette by an autosampler powered by a peristaltic pump. EPR intensity was calculated basing on spectrum peaks, which were recorded and analyzed by Liquids and Beverages Analyzer (ELBA) Software from Bruker (Boston, MA, USA).

5.2.5 Lipid Hydroperoxides Analysis

Lipid hydroperoxides were measured as primary oxidation products according to the method of Cui et al.[31] Briefly, 2.8 mL of a mixture of methanol/ butanol (2:1, v/v) was added to oil samples of known weight. Fifteen µL of 3.94 M ammonium thiocyanate and 15 µL of 0.072 M Fe^{2+} (ferrous sulfate) were added subsequently. After a 20 min reaction time, the absorbance was measured at 510 nm using a Genesys 20 spectrophotometer (ThermoSpectronic, Waltham, MA, USA). The concentration of lipid hydroperoxides was calculated from a cumene hydroperoxide standard curve ranging from 0.7 to 17 mM. Samples with hydroperoxide concentrations >17 mM were diluted with methanol/butanol (2:1, v/v) before measurements.

5.2.6 Hexanal Analysis

Headspace hexanal was measured as a secondary lipid oxidation products using a GC-17A Shimadzu gas chromatograph equipped with an AOC-5000 autosampler (Shimadzu, Kyoto, Japan) as described by Cui et al.[111]. In short, a 30 m × 0.32 mm Equity DB-1 column with a 1 µm film thickness will be used for separations. Oil samples (1 mL) in 10 mL headspace vials were heated at 55 °C for 8 min in the autosampler heating block. A 50/30 µm DVB/Carboxen/PDMS solid-phase microextraction (SPME) fiber needle was injected into the sample vial for 2 min to adsorb volatiles, and then the fiber was placed into the 250 °C injector port for 3 min at a split ratio of 1:5. The GC separations were 10 min at 65 °C for each

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

5.2.7 Determination of α-Tocopherol Concentrations

Oil samples (20-30 mg) were dissolved in 1 mL of isoproponal followed by 1 mL of methanol. Twenty μL of the diluted samples were injected to a Shimadzu HPLC system equipped with a Beckman Ultrasphere C18 reversed phase column (150 mm × 4.6 mm, 5 μm). Isocratic methanol was used as the mobile phase at flow rate of 1 mL/min. A Waters 474 scanning fluorescent detector (Waters, Milford, MA, USA) was used to detect α-tocopherol at an excitation wavelength of 290 nm and an emission wavelength of 330 nm. Peak integration was performed using Shimadzu EZstart software (version 7.2). Tocopherol in the samples were identified and quantified by comparing retention times and peak areas with those of authentic compounds.

5.2.8 Statistical Analysis

All experiments used triplicate samples (three samples taken at each time point), and each experiment was repeated at least two times. Data was presented as the mean value ± standard deviation. Data results were analyzed by analysis of variance (ANOVA) using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Differences between

mean values were compared using Duncan's multiple-range test with a significance level of $p < 0.05$. The lag phase, or induction period, was measured by drawing tangents to the lines.[173]

5.3 Results and discussion

5.3.1 Sample incubation conditions

SSO was stored at 45 for three days and its EPR intensities were subsequently monitored after incubation for 75 min at 45 or 70 °C. The spectra were shown in Figure 1. No signal was observed for SSO at both initial time or after 75 min of incubation at 45 °C. In contrast, after incubating at 70 °C for 75 min, free radicals were detected. Failure to detect radicals of samples heated at 45 °C could be due to very low lipid radical concentrations in SSO[178, 179]. Previous studies using PBN to trap lipid radicals also used thermal treatment to successfully detect free radicals. For example, Ricca et al.[178] failed to detect EPR signal in extra virgin olive oil incubated at room temperature, while radicals were observed after 120 or 360 min heating at 60 °C. Similarly, incubation at 70 °C allowed for detection of free radicals signals in extra virgin olive oil.[176]

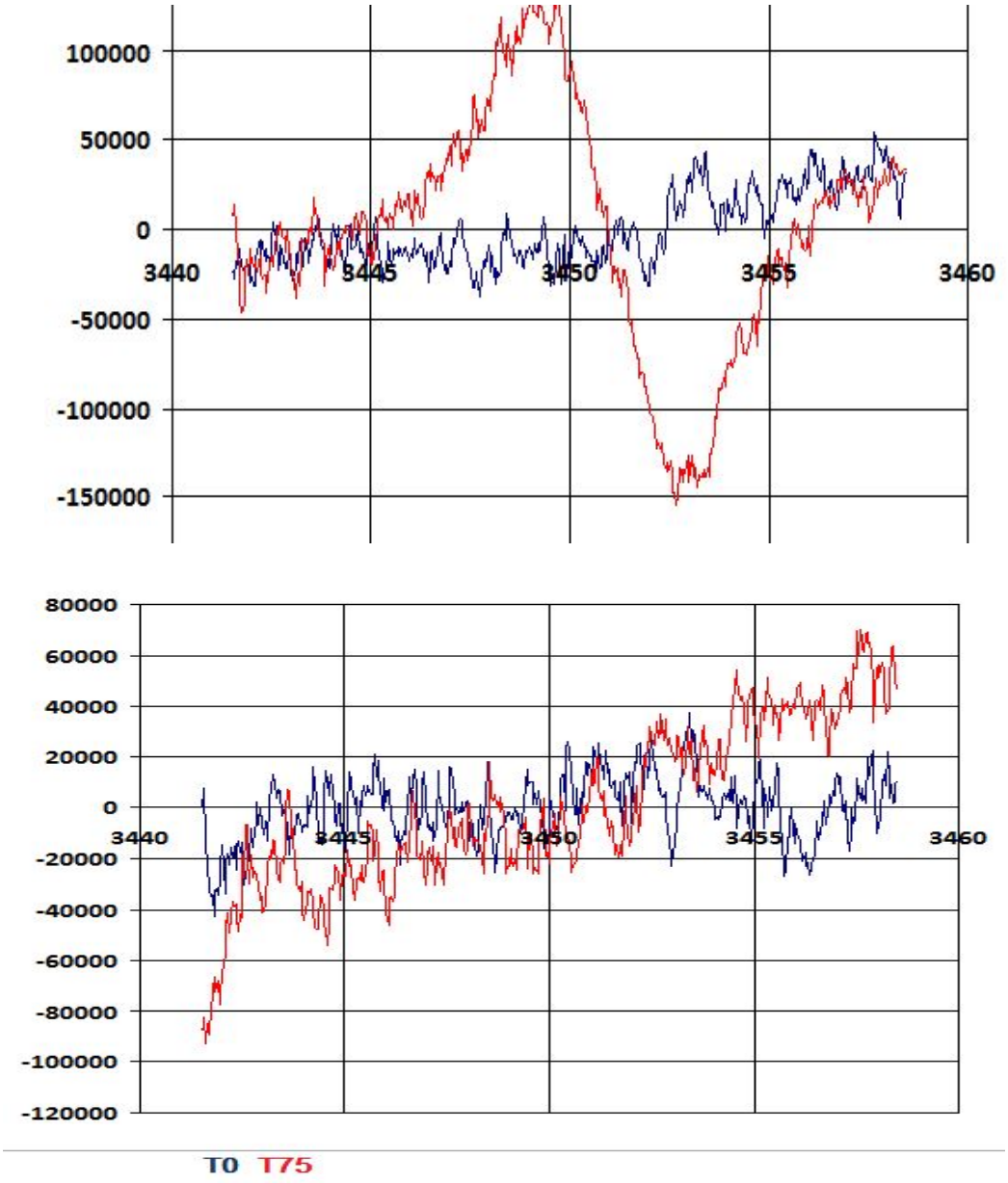


Figure 5.1 Spectra from SSO stored for 3 days at 45 °C and incubated at 70 °C (top) and 45 °C (bottom) at initial time (T0) and 75 min (T75)

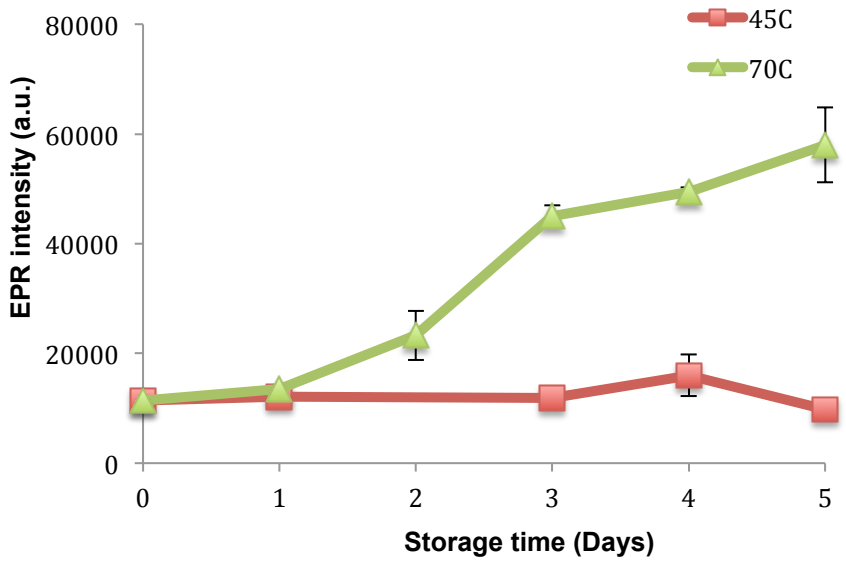
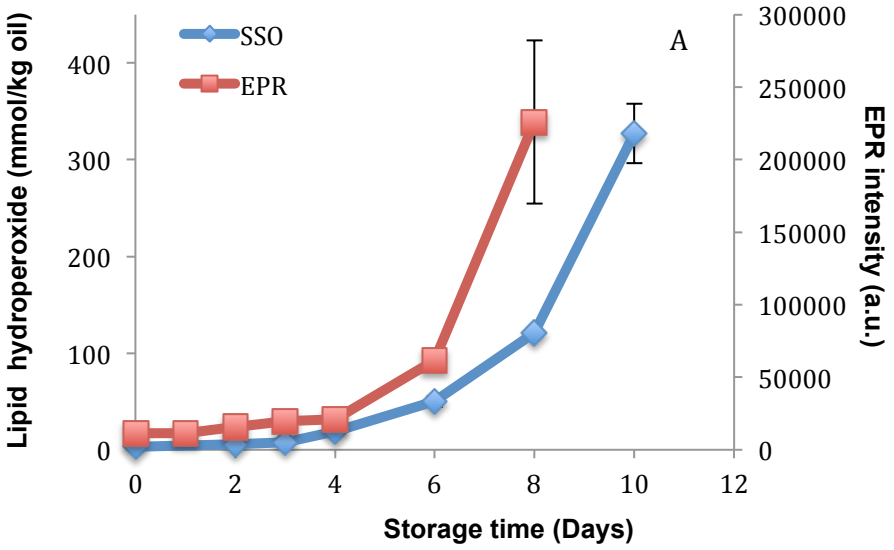


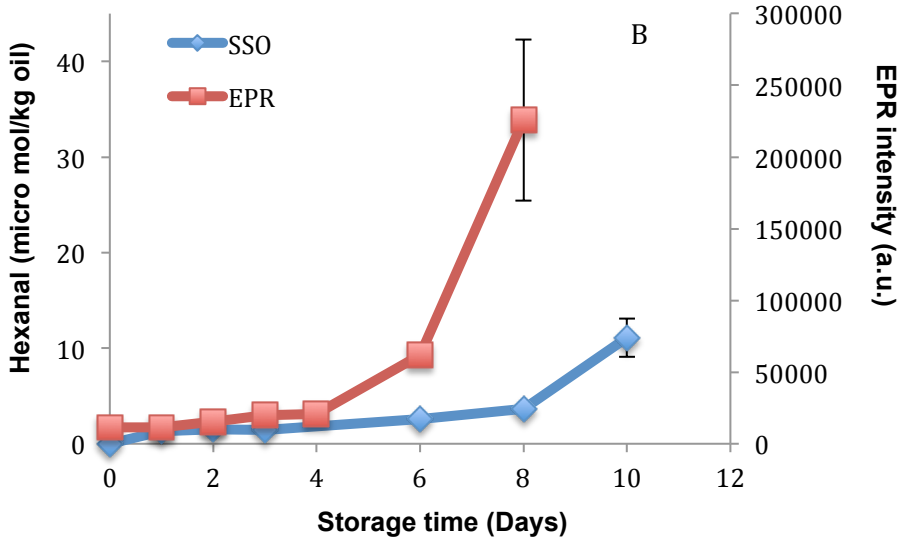
Figure 5.2 EPR intensity of SSO during its storage at 45 °C at EPR incubation temperatures of 45 and 70°C.

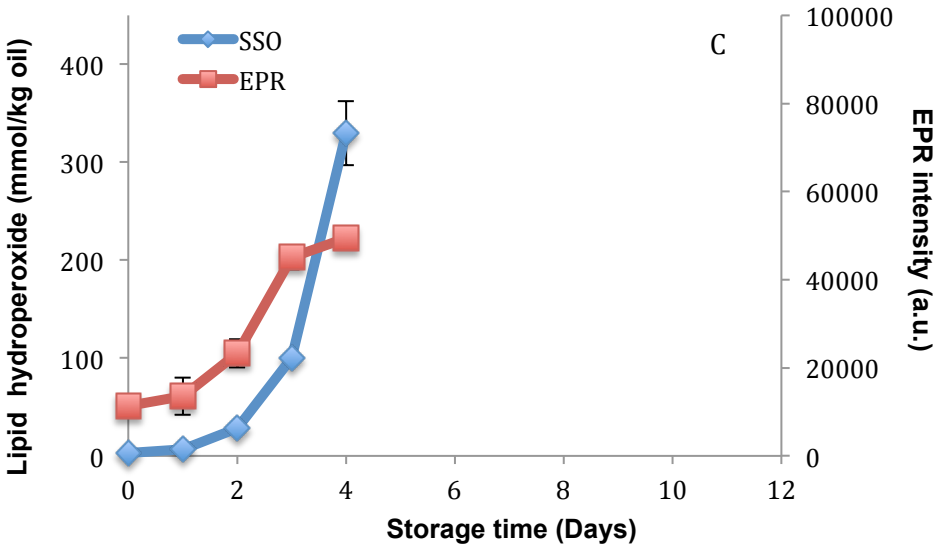
5.3.2 EPR intensity of SSO

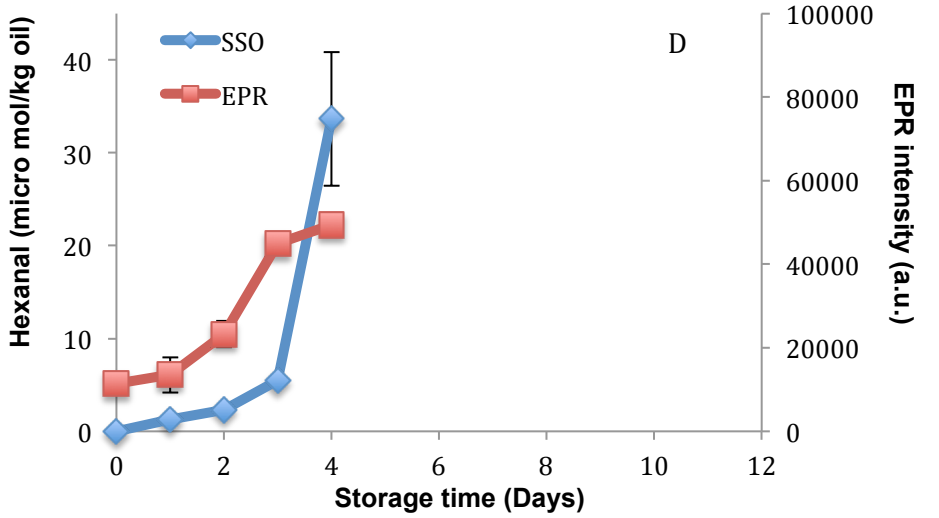
Commercial soybean oil contains minor components such as phospholipids, mono- and diacylglycerides and tocopherols that impact lipid oxidation kinetics.[5, 180] In particular, tocopherols can quench lipid radicals and form lower energy tocopherol radicals that may not be trapped by PBN to form an EPR signal. Thus, soybean oil stripped of tocopherols was first examined for the production of PBN trapped free radicals as well as the formation of the atty acid oxidation products, lipid hydroperoxides or hexanal (Figure 3). When the SSO was incubated at 37 °C, lipid hydroperoxides formation (Figure 3 A, left y axis) and EPR intensity (Figure 3 A, right y axis) both had a lag phase of 4 days. On the other hand, hexanal formation had a longer lag phase of 8 days (Figure 3 B, left y axis), which is not uncommon

since hexanal is formed by the decomposition of lipid hydroperoxides.[22] When the incubation temperature for SSO was increased to 45 and 55 °C (Figure 3 C - F), the lag phase for lipid hydroperoxides and hexanal formation became similar which is likely due to the elevated temperatures increasing lipid hydroperoxides decomposition to produce hexanal. EPR signals at both 45 and 55 °C showed a similar trend as lipid hydroperoxides and hexanal formation.






C
SSO
EPR
Lipid hydroperoxide (mmol/kg oil)
EPR intensity (a.u.)
Storage time (Days)
0
100
200
300
400
0
20000
40000
60000
80000
100000
0
2
4
6
8
10
12


D
SSO
EPR
Hexanal (micro mol/kg oil)
EPR intensity (a.u.)
Storage time (Days)
0
10
20
30
40
0
20000
40000
60000
80000
100000
0
2
4
6
8
10
12

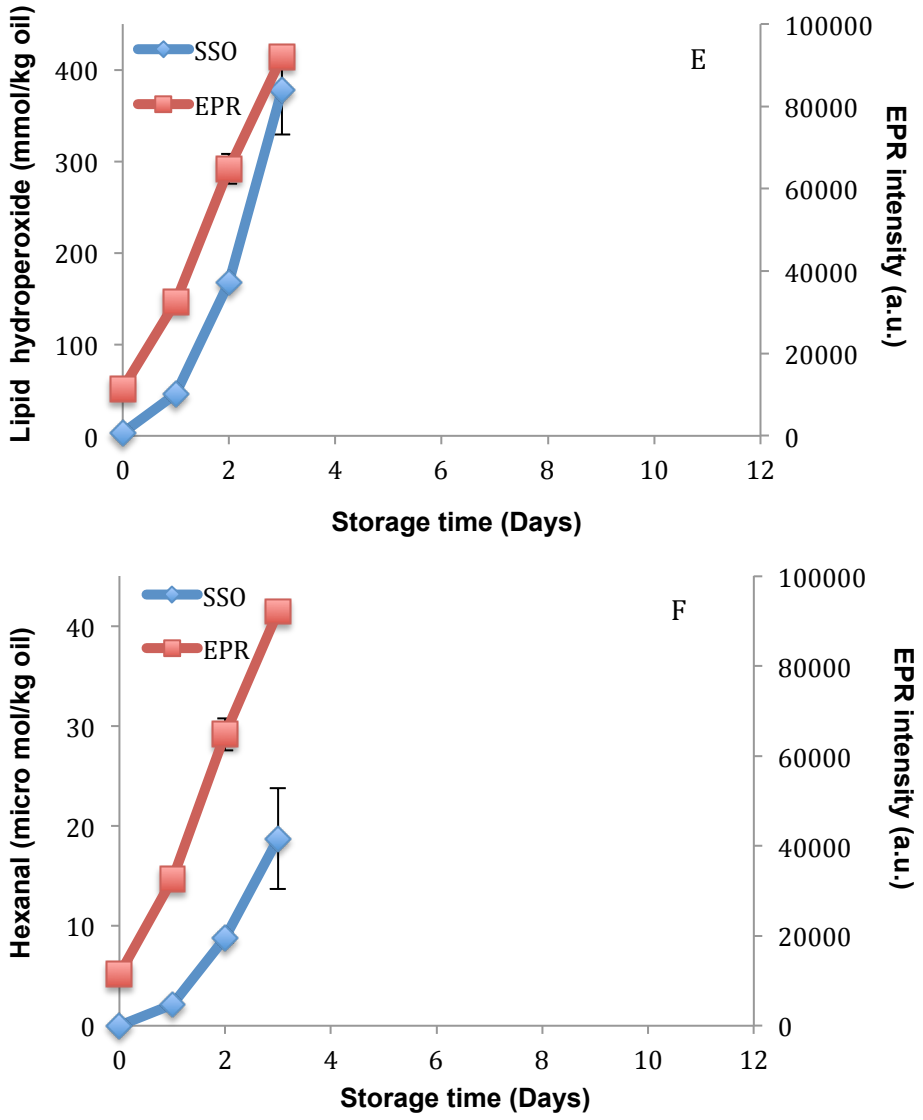


Figure 5.3 EPR intensity (right y axis), lipid hydroperoxides formation (left y axix of figure A, C and E) and hexanal formation (left y axis of figure B, D and F) of SSO stored at 37°C (A and B) , 45 °C (C and D) and 55 °C (E and F).

5.3.4 EPR intensity of SSO containing α-tocopherol

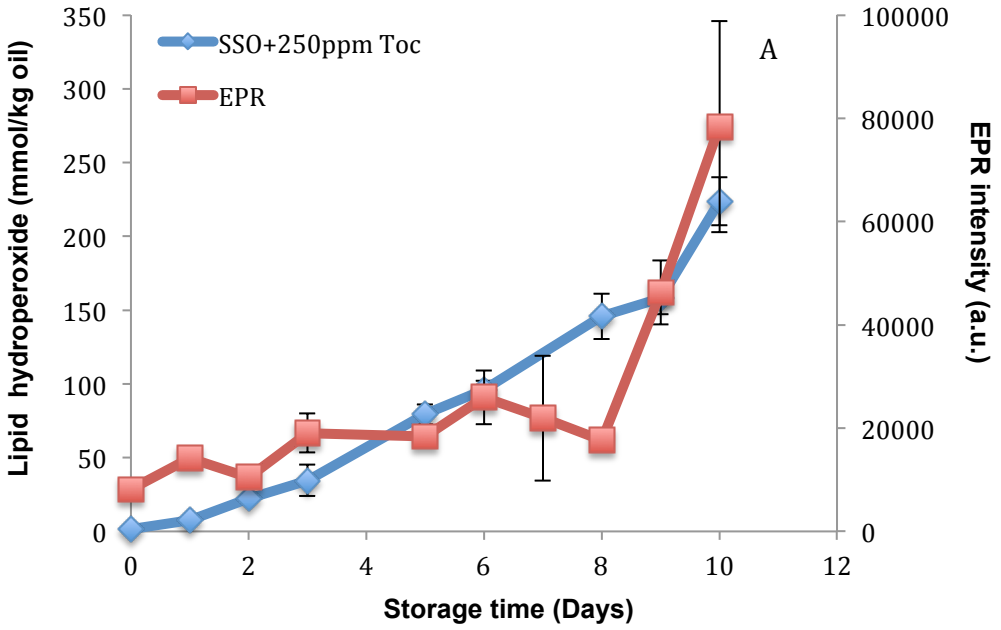
Since tocopherols can trap and decrease the energy of free radicals, this could interfere with the ability of PBN to detect free radicals. Thus, α-tocopherol was

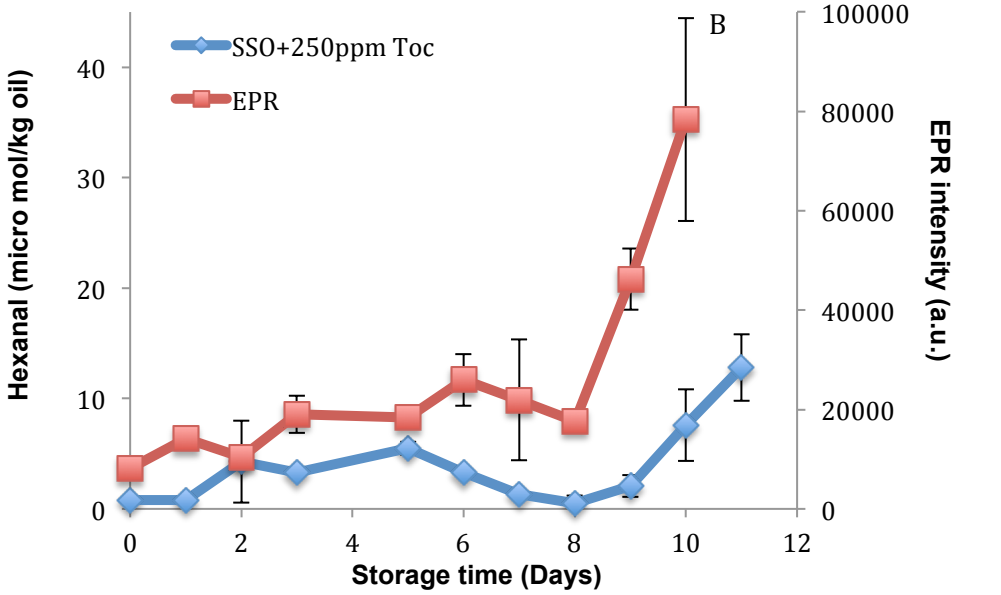
added to SSO at different concentrations and the formation of PBN trapped radicals and conventional oxidation parameters (lipid hydroperoxides and hexanal) were measured (Figure 4 and 5) during storage of the oil at 55 °C.

SSO containing 250 ppm and 500 ppm α-tocopherol had a EPR lag phase of 8 days (Figure 4 and 5). No measurable lag phases were observed for lipid hydroperoxides (Figure 4 A and 5 A). When lipid peroxides are used to monitor lipid oxidation, α-tocopherol can be seen to lack antioxidant activity or in some cases increase lipid hydroperoxides formation.[73, 181-183] This is because carbon based alkyl radicals on fatty acids reacts with oxygen at diffusion limited reaction rates to form peroxyl radicals (reaction 2 in the introduction). Peroxyl radicals have one of the longest lifetimes of common lipid oxidation radicals and thus are able to react with tocopherols to form lipid hydroperoxides. This could explain why addition of tocopherol did not alter lipid hydroperoxides formation during the incubation of the SSO. Although both concentrations of α-tocopherol did not inhibit lipid hydroperoxides formation in our experiments, they showed antioxidant activity in terms of hexanal production (Figure 4 B and 5 B). The lag phase for PBN radical adduct intensities of 8 days was similar to the hexanal formation lag phases of 9 and 8 days for samples containing 250 ppm and 500 ppm α-tocopherol, respectively.

The presence of α-tocopherol in SSO could alter the PBN trapping process and formation of lipid oxidation products. Velasco et al.[175] reported that PBN could act as radical scavenger to inhibit lipid oxidation in bulk oils. During heating at 70 °C in the presence of PBN, lipid hydroperoxides could decompose into free radicals to form PBN radical adducts that produce the EPR signal. In the presence of tocopherol,

PBN and α-tocopherol would compete for these radicals and if tocopherol reacted with the majority of radicals then PBN radical adducts might not be detected[174] and thus the EPR signal would be low despite the existence of high levels of hydroperoxides. However, tocopherols would be consumed by lipid radicals, and as their concentrations decreased it would be expected that the PBN-radical signals would be detected. To get a better understanding of this possibility, the loss of α-tocopherol during the storage of SSO was measured and plotted with EPR intensity as a function of storage time (Figure 4C and 5C). For both tocopherol concentrations tested, the lag phase of PBN radical adducts formation did not occur until 50 - 65 % of the α-tocopherol was consumed (Figure 5), which supported the hypothesis that α-tocopherol was inhibiting PBN radical adducts formation. In addition, decomposition of lipid hydroperoxides leads to the formation of alkoxyl radicals (Reaction 5 from introduction) and tocopherol is able to interact with alkoxyl radical to form lipid alcohols thus preventing fatty acid scission to form products such as hexanal.[8, 16] This could explain why hexanal formation was also related to tocopherol concentrations as the lag phase of hexanal concentrations occurred after 50 - 65 % of the tocopherol was consumed. Thus, when tocopherol levels decreased, free radical formed by hydroperoxide decomposition during storage could lead to the formation of hexanal and PBN radical adducts explaining why the lag phases of PBN radical adduct signals hexanal formation where similar.


SSO+250ppm Toc
EPR
A
Lipid hydroperoxide (mmol/kg oil)
EPR intensity (a.u.)
Storage time (Days)
0
50
100
150
200
250
300
350
0
20000
40000
60000
80000
100000
0
2
4
6
8
10
12


SSO+250ppm Toc
EPR
B
Hexanal (micro mol/kg oil)
EPR intensity (a.u.)
Storage time (Days)
0
10
20
30
40
0
20000
40000
60000
80000
100000
0
2
4
6
8
10
12

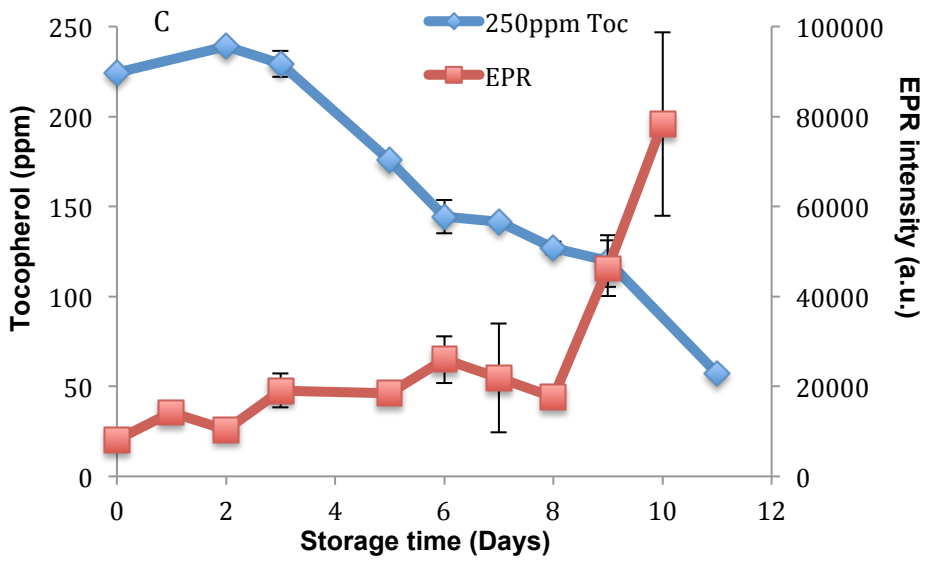
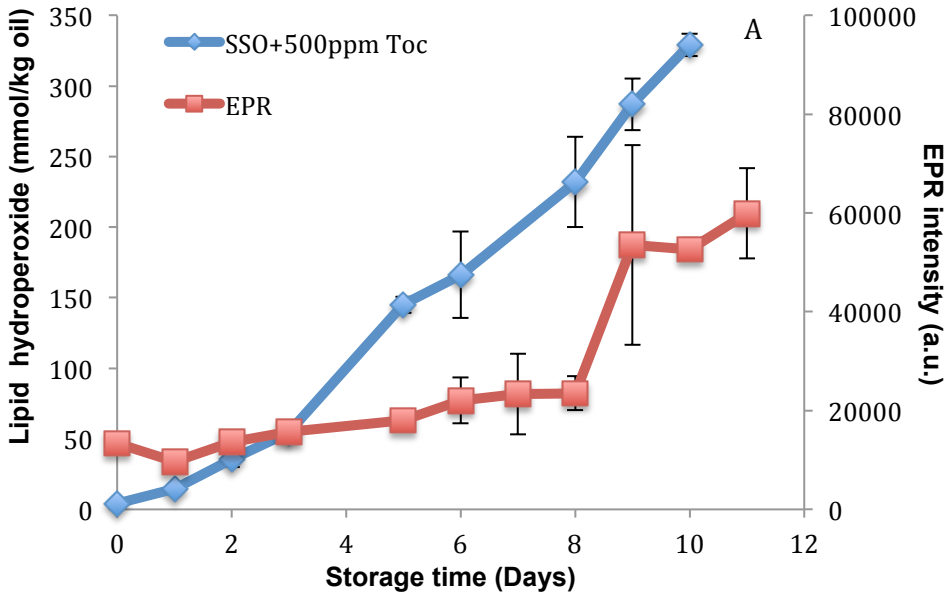
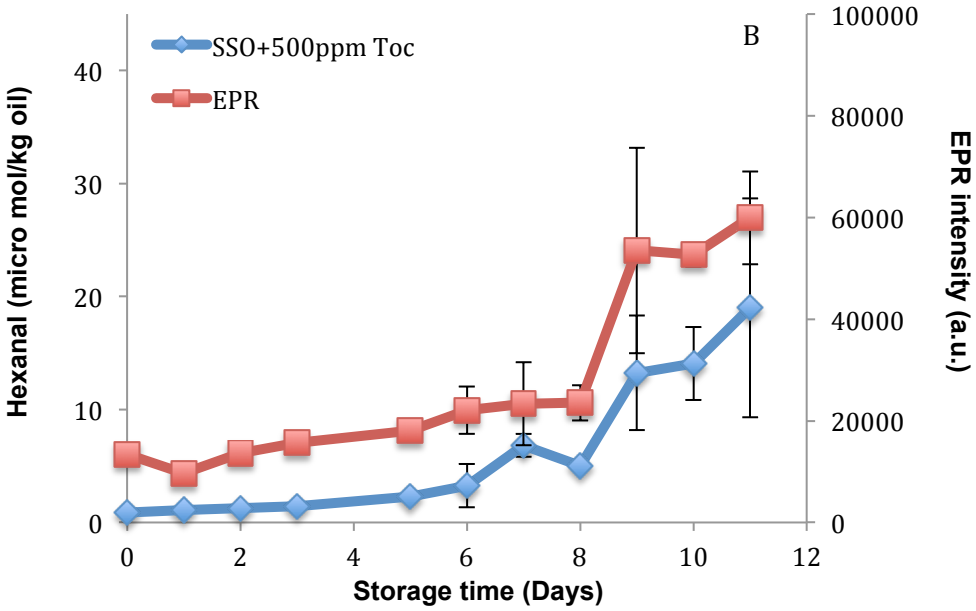


Figure 5.4 SSO containing 250 ppm α-tocopherol was stored at 55 °C. A: lipid hydroperoxides formation (left y axix) and EPR intensity (right y axis). B: hexanal formation (left y axis) and EPR intensity (right y axis). C: α-tocopherol concentration (left y axis) and EPR intensity (right y axis).




B
SSO+500ppm Toc
EPR
Hexanal (micro mol/kg oil)
EPR intensity (a.u.)
Storage time (Days)
0
10
20
30
40
0
20000
40000
60000
80000
100000
0
2
4
6
8
10
12

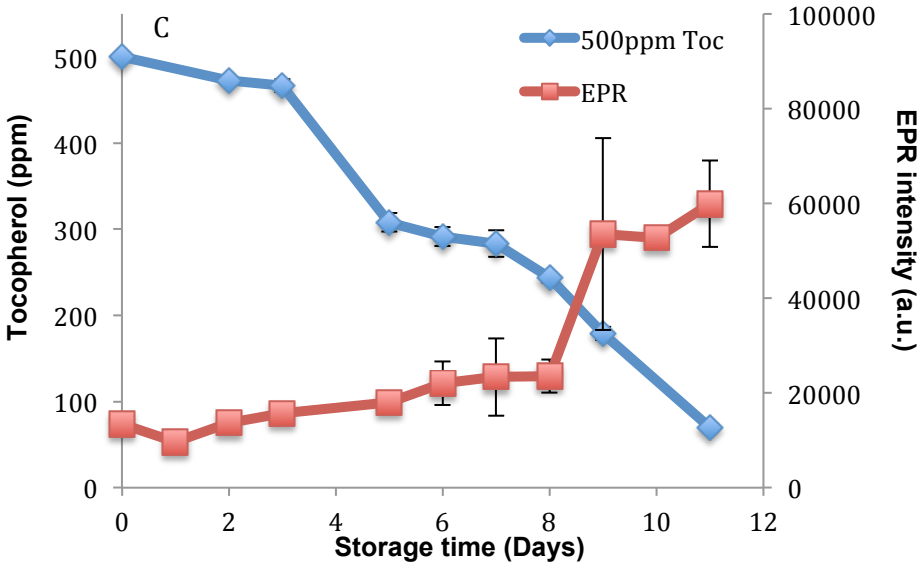

C
500ppm Toc
EPR
Tocopherol (ppm)
EPR intensity (a.u.)
Storage time (Days)
0
100
200
300
400
500
0
20000
40000
60000
80000
100000
0
2
4
6
8
10
12

Figure 5.5 SSO containing 500 ppm α-tocopherol was stored at 55 °C. A: lipid hydroperoxides formation (left y axix) and EPR intensity (right y axis). B: hexanal formation (left y axis) and EPR intensity (right y axis). C: α-tocopherol concentration (left y axis) and EPR intensity (right y axis).

5.3.5 PBN radical adduct formation in commercial soybean, sunflower, canola and corn oil

Commercial soybean, sunflower, corn and canola oil were stored at 55 °C and the PBN radical adduct sigmal, lipid hydroperoxides and hexanal formation were measured during storage (Figure 6 - 9) to examine the feasibility of applying EPR to monitoring lipid oxidation of commercial food oils.

Commercial soybean, canola, sunflower and corn oil had lipid hydroperoxides lag phases of 1, 10, 2 and 12 days (Figure 6A, 7A, 8A and 9A), and a hexanal formation lag phases of 5, 13, 24 and 25 days (Figure 6B, 7B, 8B and 9B) respectively. The EPR intensity for commercial soybean, canola, sunflower and corn oil showed a lag phase of 5, 13, 32 and 27 days (Figure 6 - 9, right y axis).

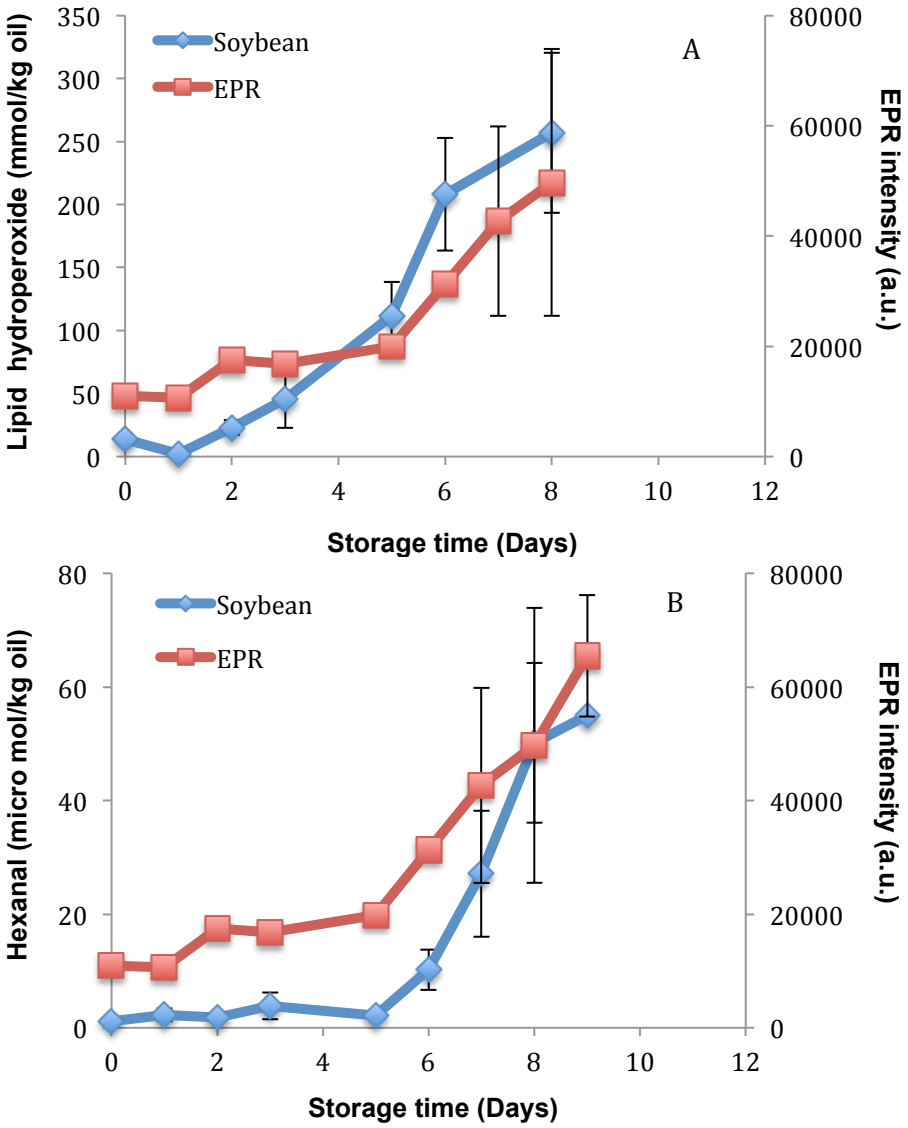


Figure 5.6 Commercial soybean oil was stored at 55 °C. A: lipid hydroperoxides formation (left y axix) and EPR intensity (right y axis). B: hexanal formation (left y axis) and EPR intensity (right y axis).

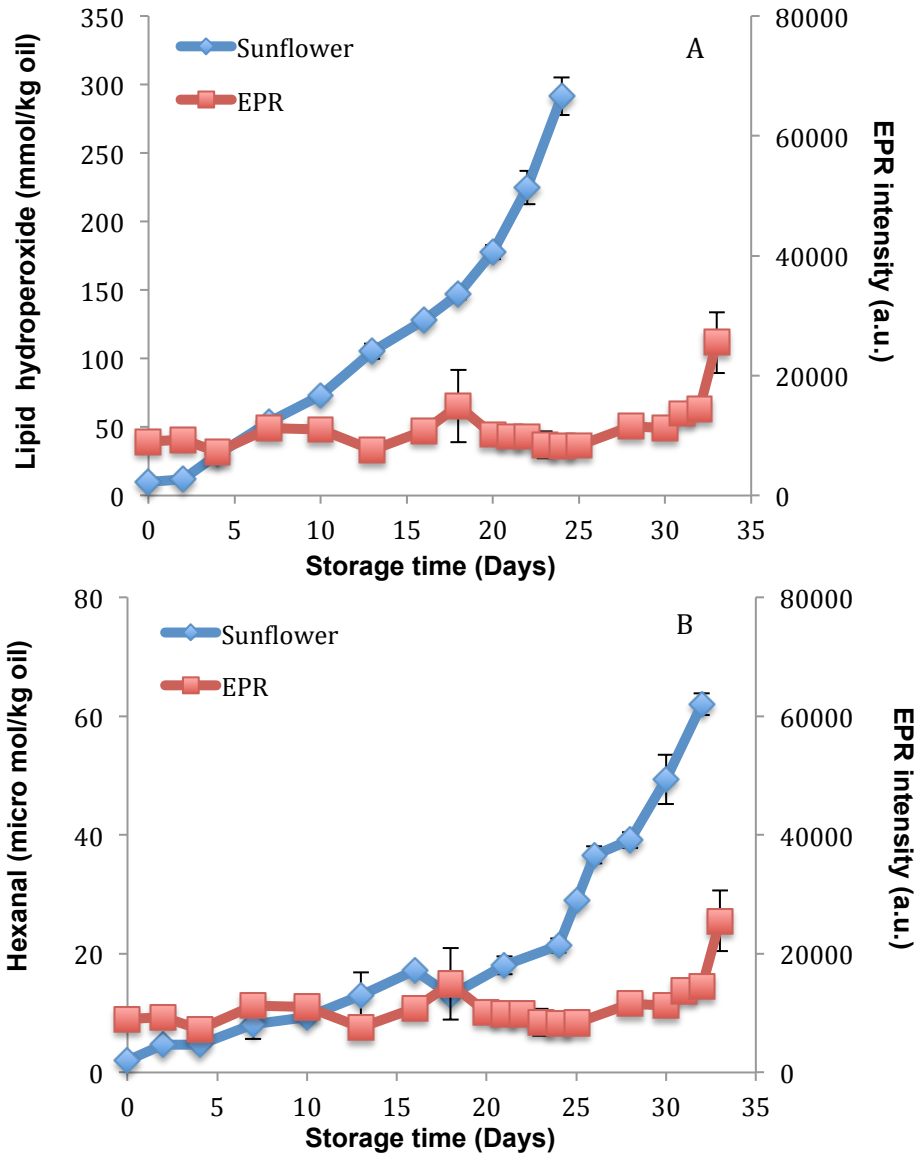


Figure 5.7 Commercial sunflower oil was stored at 55 °C. A: lipid hydroperoxides formation (left y axix) and EPR intensity (right y axis). B: hexanal formation (left y axis) and EPR intensity (right y axis).

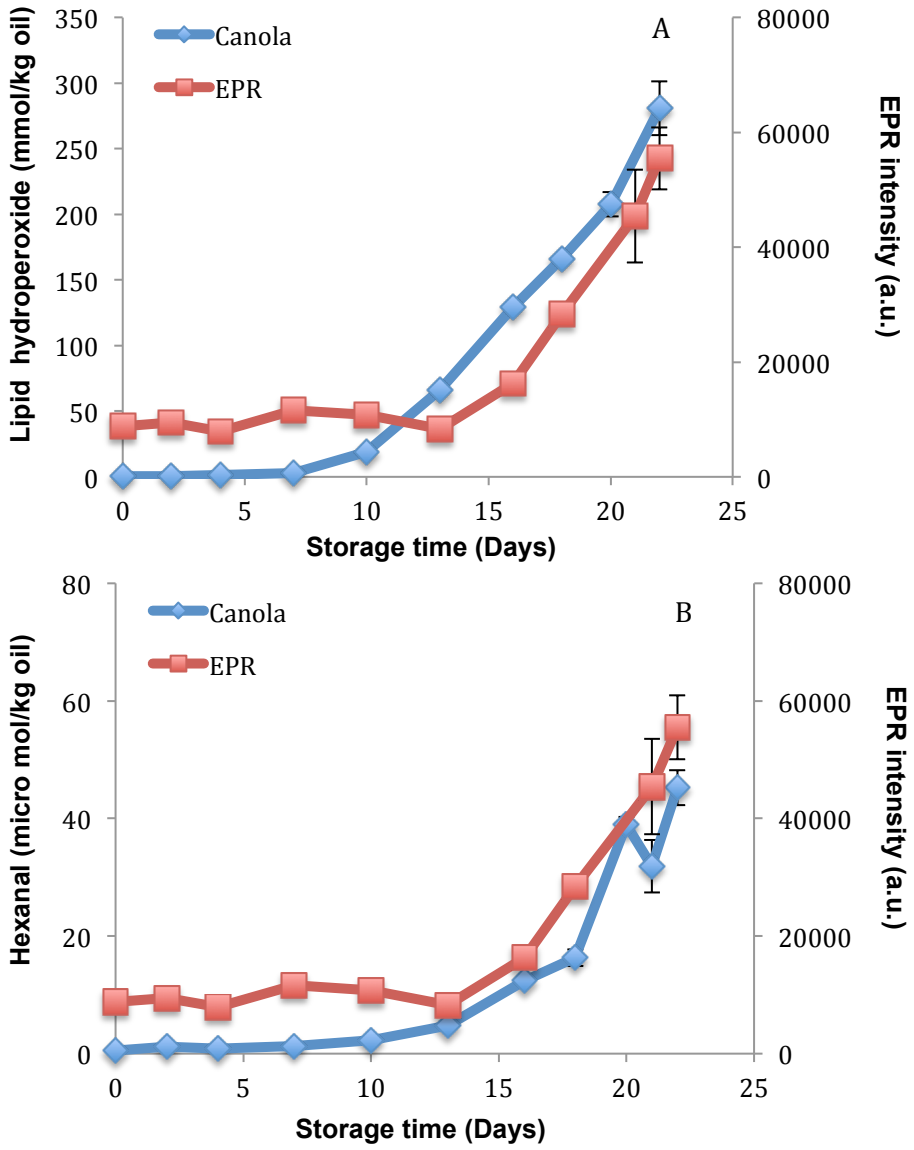


Figure 5.8 Commercial Canola oil was stored at 55 °C. A: lipid hydroperoxides formation (left y axix) and EPR intensity (right y axis). B: hexanal formation (left y axis) and EPR intensity (right y axis).

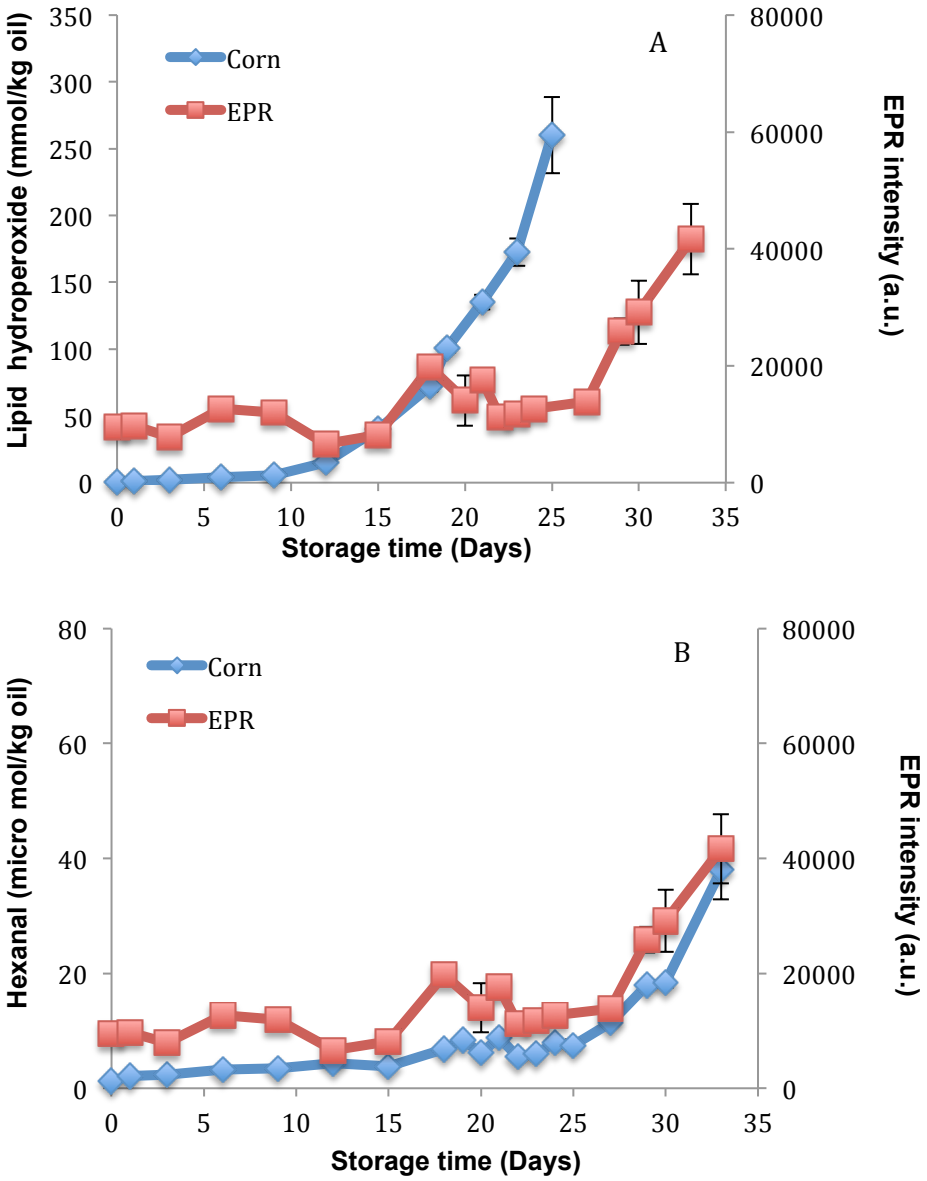


Figure 5.9 Commercial corn oil was stored at 55 °C. A: lipid hydroperoxides formation (left y axix) and EPR intensity (right y axis). B: hexanal formation (left y axis) and EPR intensity (right y axis)

For all four tested commercial oils, the EPR intensity lag phase differed from lipid hydroperoxides values, exhibiting no correlation, which was already seen for SSO containing α-tocopherol. However, the lag phases for hexanal and PBN radical adduct formation was similar in all the oils except for sunflower suggesting that EPR could be a good predictor of quality for some commercial oils. It was unclear why hexanal and PBN radical adduct formation was not similar in sunflower oil. One possibility is that the sunflower oil contained unidentified antioxidant that delayed the formation of PBN radical adducts. Monitoring PBN radical adducts with the Bruker e-scan with autosampler could have some advantages over measuring lipid oxidation volatiles by solid phase microextraction /gas chromatography (SPME/GC). For example, it takes 10 - 15 min per sample to measure hexanal by SPME/GC versus only 1 min for measurement of the PBN radical adducts by EPR with an autosampler. Second, hexanal analysis required sealing samples in individual headspace vials at the beginning of the study which is both time consuming and expensive, whereas samples could be stored in almost any container for EPR studies. This EPR method does require pre-incubation of the samples for 75 min (this can be done simultaneously for up to 25 samples). The GC must also be pre-heated before analysis and the samples must be equilibrated at 55 °C for 8 minutes prior to injection. These differences in time will obviously be increased with increasing sample numbers. Overall, monitoring of PBN radical adducts is a promising method to monitor lipid oxidation lag phase as it produces lag phases that are similar to hexanal formation, an important indicator of oil quality. However, PBN radical adduct formation was not similar to hexanal formation in sunflower oil for unknown

reasons suggesting that there are potential limitations to the method. Further research is needed to study other oils and food systems and to correlate this method to oil quality monitoring by sensory analysis.

CHAPTER 6

CONCLUSIONS

Many foods contain a wide variety of phospholipid combinations. In addition, the commercial phospholipids product, lecithin, is widely applied to many food products for their wide-ranging functional properties including antioxidant activity. However, due to their high degree of unsaturation and large surface area, phospholipids can readily react with prooxidants (e.g. transition metals), thus serving as a lipid oxidation substrate resulting in the development of off-flavors in food products like meats. This is also the case for food products with low lipid concentrations but concentrated phospholipid fractions such as the milk fat globule membrane in dried nonfat milk and whey products. Phospholipid can also promote lipid oxidation in bulk oils since its surface-active and thus can, alone or together with other polar components presented in bulk oil, form association colloids that increase interactions between oxidizable substrates and prooxidative metals. However, phospholipid can also act as antioxidants through one or any combinations of the following activities: chelating prooxidative metals, forming antioxidative Maillard reaction products, changing location of primary antioxidants or regenerating primary antioxidants. Thus, taking into account of all the possibilities of how phospholipid behaves in different food environments is essential to understand its antioxidant/prooxidant role before it can be utilized to inhibit lipid oxidation reactions.

The first research project indicated that DOPE can form association colloids and these physical structures increase lipid oxidation rates. The fact that DOPE had

higher CMCs than DOPC means that higher levels of DOPE are needed to form association colloids and thus promote lipid oxidation. Combination of DOPE and DOPC produced CMC values between the two individual phospholipids suggesting that they were acting as co-surfactants and forming mixed micelles. This data provide further evidence that surface active minor components of oil can form association colloids that impact lipid oxidation reactions. Gaining a better understanding of how these physical structures impact lipid oxidation could provide important information on how to produce novel antioxidant technologies by designing systems that concentrate the antioxidants at the site of the lipid oxidation reactions.

In the second project, the impact of two types of phospholipids, DOPC and DOPE, both individually and in combinations on the antioxidant activity of α-tocopherol or trolox in bulk oil was investigated. It was found that DOPC reverse micelles decreased the antioxidant activity of α-tocopherol and trolox. DOPE reverse micelles did not cause changes on the physical location of α-tocopherol, but increased its antioxidant activity through regeneration of oxidized α-tocopherol by its primary amine headgroup. The addition of DOPE to commercial soybean oil inhibited lipid oxidation while DOPC was ineffective. These results indicated that in bulk oils the primary amine headgroup of DOPE increase the antioxidant activity of α-tocopherol by regeneration of the α-tocopherol quinone. To get a better efficiency of natural antioxidants like tocopherols in bulk oil, decreasing PC reverse micelles and increasing PE could help stabilize the food oils.

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