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Antioxidant Combination of High Phosphatidylserine (PS) Lecithin with Mixed Tocopherol in Soybean Oil-in-Water Emulsion: Effect of pH and Salt

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ANTIOXIDANT COMBINATION OF HIGH PHOSPHATIDYLSERINE (PS) LECITHIN WITH MIXED TOCOPHEROL IN SOYBEAN OIL-IN-WATER EMULSION: EFFECT OF pH AND SALT

A Thesis Presented

By

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ABSTRACT

ANTIOXIDANT COMBINATION OF HIGH PHOSPHATIDYLSERINE (PS) LECITHIN WITH MIXED TOCOPHEROL IN SOYBEAN OIL-IN-WATER EMULSION: EFFECT OF pH AND SALT

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Lipid oxidation is one of the major challenges faced by the food industry as it contributes to the loss of nutritional quality and loss of flavor in food products. Studies have shown that naturally occurring phospholipids like phosphatidylserine (PS) and phosphatidylethanolamine (PE) can regenerate oxidized tocopherols and help delay the lipid oxidation in bulk oils and oil-in-water emulsions. Since consumers desire simpler and cleaner labels, without chemically synthesized antioxidants, this research is of great interest. The combination of PS and PE with tocopherols has already been studied. However, PS was a better antioxidant in combination with tocopherols in the oil-in-water emulsion system whereas PE was a better antioxidant in combination with tocopherols in bulk oils. But obtaining pure phospholipids is an expensive deal therefore, this study uses the more economical alternative, high phosphatidylserine (PS) lecithin in combination with mixed tocopherols in soybean oil-in-water system. PS (30 µmol/kg emulsion) along with mixed tocopherols (3 µmol/kg emulsion) were dissolved in oil and emulsions stabilized by

Tween20 were prepared. To determine the most effective concentration of mixed tocopherols, 0.5, 1.0 and 3 μmole of tocopherols/kg emulsion were used at pH of 3 and 7. Tocopherol with a concentration of 3 μmole/kg emulsion was found to be the most effective at pH 3. Tocopherols showed an extended lag phase at lower pH. The synergistic activities of authentic PS and high PS lecithin were compared with combination with tocopherol under similar conditions. They both had an almost similar lag phase. This combination was then tested for different pH of 3 and 7 and different salt concentrations of (0.5, 1 and 1.5 wt% of the emulsion) at pH 7 to determine the effects external factors on the synergistic antioxidant combination. It was observed that the combination had extended antioxidant ability at lower pH of 3 whereas salt had no effect on the combination. The results showed that high PS lecithin forms synergistic combination with mixed tocopherols to increase the lag phase in oil-in-water emulsions and can be used as a clean label antioxidant for oil-in-water emulsions.

KEYWORDS: *phospholipids, phosphatidylserine, antioxidant, lipid oxidation, tocopherol, lecithin, pH, salt, emulsion*

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CHAPTER 1

INTRODUCTION

Lipid oxidation is a serious problem in food industry as it decreases the shelf life and alters texture and appearance of food. Lipid oxidation also decreases the nutritional value of food as well as produces unpleasant rancid odor and oxidation end products that have potential ill effects on human health (E. N. Frankel, 1980; Joseph Kanner, 2007). Cancer, atherosclerosis, aging and several inflammatory diseases can be caused due to the consumption of potentially toxic lipid oxidation products (Ito et al., 1986). According to the dietary guidelines for Americans (DeSalvo et al., 2016), the replacement of saturated fats with polyunsaturated fats is a healthier option but it also leads to the risk of rancidity. Methods of preventing lipid oxidation are necessary for consumer health.

Lipids can be present in either the form of bulk oil or as heterogeneous emulsion system (D. J. McClements & Decker, 2000). Food products that are consumed by humans consists of many emulsions such as salad dressings, infant formula, mayonnaise, infant formula, beverages, soups, and creams. These are commonly present in our diet in lipid form or as lipid sources such as cooking oil, meat, baked goods like crackers (David Julian McClements, 2017; Okuda et al., 2005). Synthetically prepared antioxidants such as EDTA (ethylene diamine tetra acetic acid), BHA (tbutyl-4-hydroxyanisole), BHT (t-butyl-4-hydroxytoluene), and TBHQ (tert-butyl-hydroxyquinone) were used commercially but increase in demand of simpler and cleaner labels, i.e., without synthetic antioxidants, has led to researchers to look for naturally occurring antioxidants (Samdani et al., 2018; Xu et al., 2019)

Phospholipids have been shown to increase the antioxidant activity of mixed tocopherols. Phosphatidylserine (PS) works synergistically with mixed tocopherol in both bulk oil and oil-in-water emulsions. However, PS has increased antioxidant activity when used in combination with mixed tocopherols, in soybean oil-in-water emulsions (Samdani et al., 2018; Xu et al., 2019). Gautam used phosphatidylserine in combination with different homologues of tocopherol (α-, δ-, and mixed tocopherols) and concluded that mixed tocopherol is a more preferred homologue for the combination due to its commercial availability and synergistic interaction with phosphatidylserine (Samdani et al., 2018).

In this study, high PS lecithin is used in combination with mixed tocopherol to explore the antioxidant activity in oil-in-water emulsion system. High PS lecithin is more economical alternative of the authentic PS and hence is used in this study. For the first part of the study, antioxidant activity of mixed tocopherol is studied for two pH values (3 and 7) and for different mixed tocopherol concentrations (0.5, 1.0 and 3 μmole/kg emulsion). Mixed tocopherol concentrations are observed to have extended lag phase at lower pH of 3. This can be because of the reduced hydrogen donating ability of α-tocopherol at the oil-water interface due to the protonization of its phenolic hydrogen group at lower pH or because of slower depletion rate at lower pH (S.-W. Huang et al., 1996).

Comparative study was done between authentic PS and high PS lecithin and its combination with mixed tocopherol to understand the difference in their antioxidant activity at pH 7. This result showed that high PS lecithin can be used as an inexpensive alternative to authentic PS. The combination of high PS lecithin and mixed tocopherols was then studied under a lower pH of 3 to understand the effects of pH on the synergistic

antioxidant combination. The combination showed extended shelf life at lower pH of 3 which may be attributed to lower oxidation rates at lower pH. The combination was also tested for different salt concentrations of (0.5,1 and 1.5 wt% of the emulsion). It was observed that salt had no effect on the antioxidation ability of the combination at pH 7.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Unsaturated lipids when comes in contact with an initiator, gets oxidized to form alkyl radicals. These alkyl radicals, in the presence of oxygen, form peroxyl radicals which can further react with more lipids to produce lipid hydroperoxides (E. N. Frankel, 1984, 1996). This lipid hydroperoxide upon degradation produces numerous volatile compounds such as hexanal and propanal, which compromises the sensory quality of the food. Rapid lipid oxidation can be observed in oil in water emulsions owing to the sizable oil-water interface which also increases interfacial interaction between lipids and water soluble prooxidants such as transition metal ions (copper, iron) and facilitates increased accessibility of lipid droplets to the dissolved oxygen. Transition metals such as iron act as major pro-oxidants present commonly in foods for example 40µg iron/g present in egg yolk. The metal chelating complex molecules such as EDTA are very potent antioxidants and can delay lipid oxidation (Jacobsen et al., 2001).

2.2. Factors affecting lipid oxidation

The rate of lipid oxidation in emulsions can be affected by concentration of antioxidants and pro-oxidants and location of antioxidant, fatty acid composition, interfacial properties, droplet size, ionic concentration and aqueous phase pH (Waraho, Mcclements, et al., 2011).

2.2.1. Droplet interface properties

The interactions between lipids and pro-oxidants such as metal ions, the interface and related properties can affect rate of lipid oxidation (Waraho, Mcclements, et al., 2011). The interfacial layer has a very complex chemical composition. It contains minor lipid components, mineral ions, emulsifiers, and surface-active materials such as antioxidants. A larger dimensional molecular barrier can be formed by an emulsifier which can reduce the interactions between lipid phase and por-oxidants in aqueous phase. For example, salmon oil in water emulsions prepared with Brij 76 emulsions had higher lipid oxidation rates when compared to Brij 700 emulsions (W. Chaiyasit et al., 2000; D. J. McClements & Decker, 2000; Silvestre et al., 2000).

The surface area of lipid droplets in emulsion is higher than in bulk oil. The specific surface area of 65.2 652, 6522, 65220 m^2/Kg can be attributed to the droplet size of 100 µm, 10 µm, 1 µm and 100 nm respectively (E. A. Decker et al., 2017). The surface area of the droplet does not have a significant effect on lipid oxidation rate. This infers that surface does not affect the oxidation reaction rate and that oil-in-water emulsions usually have large surface area (Waraho, McClements, et al., 2011).

The charge (cationic, anionic and neutral) of the droplet is determined on the basis of pH or the type of emulsifier used (Djordjevic et al., 2007; Hu et al., 2003). The presence of charge can affect the repulsion and attraction ability of the droplet when cationic metal ions interact with the droplet (Haahr & Jacobsen, 2008).

2.2.2. Other factors

There are other minor components that can affect lipid oxidation in emulsions such as free fatty acids and phospholipids (Chen et al., 2011). In aqueous phase, small surfactant molecules otherwise known as Tweens form micelles after the threshold micelle concentration are achieved. Lipid oxidation can be influenced as the micelles change the interacting boundaries at molecular level between aqueous, interfacial and oil regions (Waraho, McClements, et al., 2011). Oil-in-water emulsion's antioxidant activity of α and δ-tocopherol was observed to increase by addition of Tween 20, which can be attributed to higher solubilization of tocopherol in aqueous phase (Kiralan et al., 2014b).

2.3. Mechanism of Lipid Oxidation

Lipid oxidation process is a free radical chain reaction that involves three steps: initiation – formation of free radicals, propagation – free radical chain reactions, and termination – formation of non-radical products (Wilailuk Chaiyasit et al., 2007; E. Decker, 2008; J. Kanner & Rosenthal, 1992; M. Laguerre et al., 2007; D. J. McClements & Decker, 2000; Pryor, 1976). It is an autoxidation reaction where organic compound reacts with molecular oxygen that behaves as a biradical due to its two unpaired electrons. However, certain prooxidant factors like transition metals (such as iron and copper), photosensitizers, and enzymes (such as lipoxygenases) can be present in food products that can initiate the lipid oxidation process. Additionally, harsh environmental, such as UV light or thermal processing can also initiate this process. Lipid oxidation pathway involves the following reactions: (Ahmed et al., 2016; Gray, 1978)

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

Propagation: $R\cdot + O_2 \rightarrow ROO\cdot$

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

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

$ROO\cdot + R\cdot \rightarrow ROOR$

$R\cdot + R\cdot \rightarrow RR$

Figure 2.1 The stages of the lipid oxidation reaction.

In the initiation stage, due to the presence of double bonds, hydrogen dissociates in the presence of heat, light, or metal ions, forming alkyl radicals and resulting in the exposure of unpaired electron. This is accompanied by the propagation step where oxygen reacts with the alkyl radicals to form peroxyl radicals. This peroxyl radical can then steal a hydrogen from another unoxidized lipid molecule, resulting in the formation of a hydroperoxide and new alkyl radical. The duration of the propagation step depends mainly upon the unsaturation degree of the lipid (Kubow, 1992). The primary oxidation products, hydroperoxides, are unstable and can further be cleaved into short-chain molecules via β-scission reaction. These short chain molecules include aldehydes, ketones, and hydrocarbons and it is because of these short chain, volatile compounds that food products get the off flavor of lipid oxidation. In the presence of transition metals, the peroxides decompose into peroxyl radicals and alkoxyl radicals, promoting chain reaction which can

propagate throughout the food product system until radicals polymerize with each other. This whole process is termed as autooxidation. (Ahmed et al., 2016)

2.4. Controlling Lipid Oxidation

Lipid oxidation control has been a focus of research and several methods have been proposed over the years to inhibit lipid oxidation in food products and packages. The use of nitrogen flushing, oxygen scavengers and use of antioxidants are some of the methods to limit lipid oxidation. In using antioxidants there can be different mechanisms such as pro-oxidant metal chelators, singlet oxygen quenchers, reducing agents and free radical scavenging (Reische et al., 2008).

2.4.1. Free radical scavengers

Lipid oxidation can be inhibited by free radical scavengers as they can obstruct the chain initiation or/and propagation steps by contributing a free hydrogen towards the lipid radical which can stabilize the radical (Choe & Min, 2006). The lipid oxidation inhibition potential of a free radical scavenger can be determined by chemical reactivity and physical location (interfacial region, oil phase or water phase). The theory of "The Polar Paradox" attempts to explain the significance of the physical location of an antioxidant's activity (Porter et al., 1989). It was suggested that polar antioxidants work better in bulk oil whereas non-polar antioxidants work better in lipid dispersion (oil in water emulsions). The higher potential of non-polar antioxidants in lipid dispersions can be because of the presence of non-polar antioxidants in oil droplet providing them access to scavenge the free radicals. While the polar antioxidants being present in aqueous phase within emulsion would not be able to perform free radical scavenging. The most efficient position for the non-polar

antioxidant is interfacial position which is a prime spot for oxidation (Edwin N. Frankel et al., 1994). There have been many studies where polar antioxidants such as ascorbic acid, carnosic acid, and Trolox were found to be less effective than the non-polar antioxidants such as ascorbyl palmitate, α-tocopherol, and carnosol (Waraho, Mcclements, et al., 2011). Research has been done on esterified antioxidants to check for the free radical scavenging ability when there was variation in length of the alkyl chain. The results showed that the highest free radical scavenging activity was observed in esters with chain length of 8-12 carbon atoms. The chain longer than 8-12 carbon range esterified antioxidants was less effective as they were more soluble in the lipid droplet thus located inside the lipid droplets and hence less surface active. The chain shorter than 8-12 carbon range esterified antioxidants were less effective due to higher solubility in aqueous phase therefore minimizing the interaction with lipid radicals (Mickaël Laguerre et al., 2009; Mickaël Laguerre et al., 2010). In the food industry a lot of synthetic strong free radical scavengers are used such as TBHQ, BHT, and PG which are being re-examined because of potential negative health concerns. These concerns have increased the demand for usage of natural free radical scavengers for the consumer acceptability and safety of the food products.

2.4.2. Metal chelators

Lipid oxidation activation energy can be reduced by the metals present in the matrix. Metal ions present can stimulate the initiation reactions and hydroperoxide decomposition resulting in secondary oxidation products such as aldehydes (E. Decker, 2008). Metal chelators have the ability to bind with the free metal ions reducing the effect of metal ions on lipid oxidation (Pokorný, 2007). Metal chelators can restrain reactions caused by metal catalysis by prevention of formation of insoluble metal complexes, redox

cycling, capturing metal coordination sites, and formation of oxidation intermediates such as hydroperoxides (steric hindrance in metal-lipid interactions) (Graf, 1990). In exception cases the metal chelator's antioxidant abilities can be concentration dependent. There are some conditions where metal chelators can increase the oxidation reactions by varying the redox potential or increasing the solubility of the metal ions (Cui & Decker, 2016a). The previous research has observed that EDTA can act as a pro-oxidant at an EDTA:iron ratio of less than equal to 1 while it acts as a strong metal chelator for the ratio greater than 1. Various studies have shown that phospholipids such as PS, PC, and PE can have antioxidant activity because of metal chelation while only a very small number of studies report pro-oxidant activity of the phospholipids. The phospholipid's pro-oxidant and antioxidant activities can be attributed to the metal concentration and types of metal used in different experiments (Cui & Decker, 2016a). The examples of metal chelators that can bind with metals to form stable complex compounds are tartaric acid, citric acid, phosphoric acid, and EDTA; EDTA being the most effective metal chelator and therefore hindering the decomposition of hydroperoxides and metal catalyzed initiation reactions.

2.4.3. Oxygen quenchers

Oxygen can react with the unsaturated fatty acids that can result in lipid degradation, thus being a significant factor to be considered to prevent lipid degradation. The two types of reactive oxygen present are triplet and singlet oxygen. The triplet oxygen is found more commonly, it is involved in the lipid oxidation caused by free radical chain reaction where it reacts rapidly with alkyl radical and forms peroxyl radical. The propagation of free radical chain reaction starts as the peroxyl radical extracts a hydrogen atom from lipids to produce hydroperoxides which in turn forms a free radical on another

lipid molecule and it goes on. The singlet oxygen is more reactive of the two species, and it can stimulate lipid oxidation in food that have photosensitizer.

2.5. Tocopherol

Tocopherols are naturally occurring antioxidants found majorly in vegetable oils. Tocopherols are fat soluble compounds that are known to possess high antioxidant activity against lipids. (Almeida et al., 2006). The structure of tocopherol is composed of a 6-chromanol ring with a saturated 16-carbon side chain. Tocopherols exist as four homologues (alpha, beta, gamma and delta), based on the location and number of methyl groups attached to the chromanol ring (Saini & Keum, 2016). Figure 2.2 shows the structures of the homologues of tocopherol.

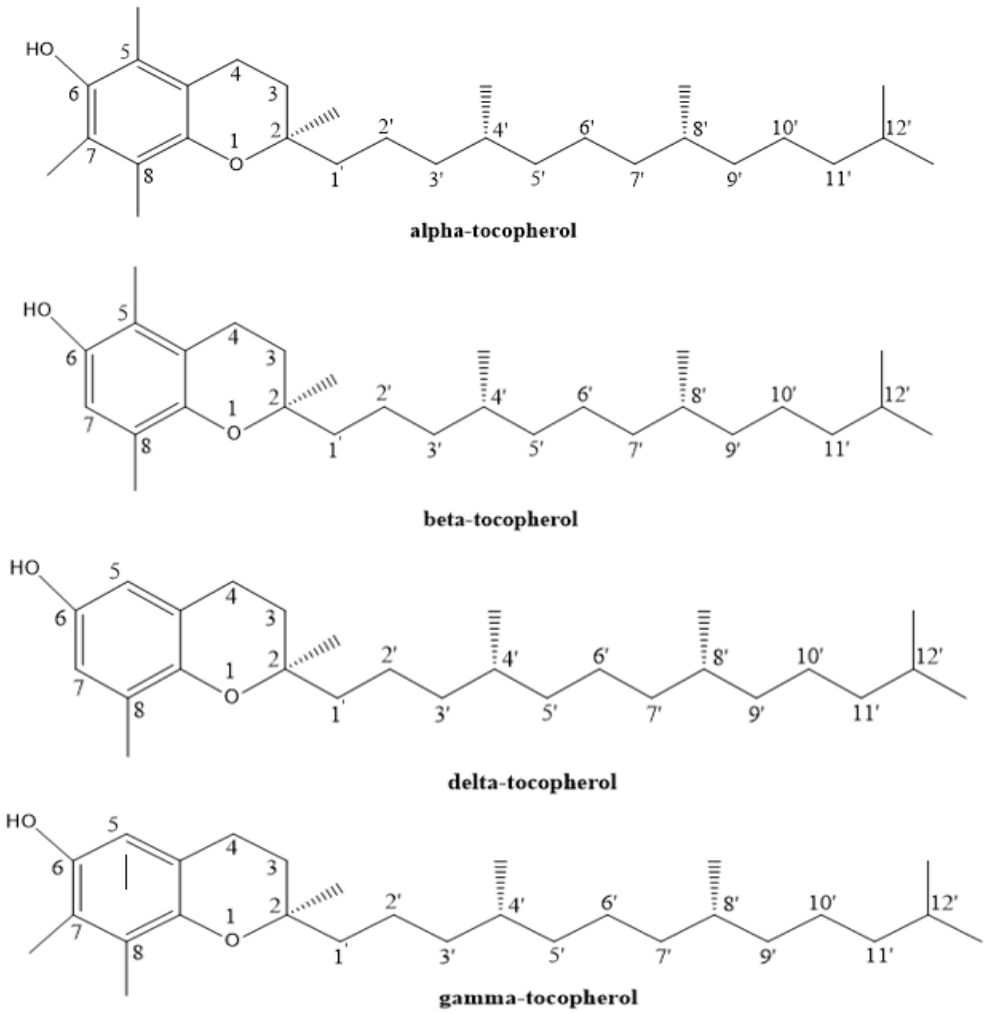

Figure 2.2. Structures of Tocopherol homologues

2.5.1. Tocopherol as an antioxidant or prooxidant

Tocopherols can prevent lipid oxidation by donating a phenolic hydrogen to the lipid peroxyl radicals, producing thereby inhibiting the formation of hydroperoxides. The oxidized tocopherol radical, called tocopheroxyl radical, is stable and does not take part in the oxidation process, rather it reacts with an another peroxyl radical to form a stable non-radical product (Yamauchi et al., 1996). The hydrogen donating capacity of the tocopherols varies with the oxidation condition as well as the model system (Ghazani & Marangoni,

2013; Seppanen et al., 2010). Although tocopherols have been shown to have strong antioxidant activity, there have been several reports where α-tocopherol has shown pro-oxidant activities. As reported by Kamal Eldin (Kamal-Eldin & Appelqvist, 1996) α-tocopherol behaves as a prooxidant suggestively due to the higher concentrations of α-tocopheroxyl radicals which causes many undesirable side reactions, initiate chain reactions and enhance the rate of peroxidation. The optimum concentration of α-tocopherol for inhibition of hydroperoxides in corn oil was determined to be 100ppm and in 10% corn oil-in-water emulsion, it was found to be in the range of 250–500 ppm. α-tocopherol was found to be behaving as a prooxidant at concentrations above 250ppm in corn oil and 500ppm in corn oil-in-water emulsions. For antioxidant activity of γ-tocopherol, the optimum concentration in corn oil was calculated to be 250-500ppm but it shows it's prooxidative activity at 5000ppm in vacuum distilled corn oil. δ-tocopherol exhibits antioxidant activity at 2000ppm in both the systems (S. W. Huang et al., 1994b, 1995). In purified soybean oil, α-, γ-, and δ- tocopherols have been reported to possess antioxidant properties at optimum concentration of 100, 250 and 500 ppm, respectively. Above these concentrations, these antioxidants had prooxidant effects under the same experimental setup (JUNG & MIN, 1990). Huang (S. W. Huang et al., 1994a) studied the formation of lipid hydroperoxides in vacuum distilled corn oil with α-, and γ-tocopherols. α-tocopherols worked best at 100ppm whereas the optimum concentration of γ-tocopherol ranged from 250 to 500ppm. Yoshida reported the optimum concentration for β- and γ-tocopherols to be 150-200ppm in vacuum distilled oils (Yoshida et al., 1993).

The antioxidant activity of α-tocopherol was found to be highest at pH 3 than at pH 7 in Tween20 stabilized corn oil-in-water emulsions by Huang et al.(S.-W. Huang et al.,

1996) This antioxidant activity of α-tocopherol is reported to be dependent on its hydrogen donating ability as well its depletion rate.

2.6. Phospholipids

Phospholipids are an integral part of all biological membranes and therefore are present in all living species from which foods are derived (Cui & Decker, 2016b).

2.6.1. Structures

Phospholipids have a backbone made of glycerol and a phosphate group attached to it at sn-3 position. Depending upon the group attached to the phosphate group, the phospholipids are named, for example, if serine group is attached to the phosphate group, the phospholipid is called phosphatidylserine. For other substitution groups such as choline, ethanolamine or inositol, phospholipids are named phosphatidylcholine (PC), phosphatidylethanolamine (PE), or phosphatidylinositol (PI). Phospholipids whose fatty acid chain has been removed from sn-2 position are categorized as lysophospholipids.(Fahy et al., 2005) Figure 2.3 shows the structures of phospholipids.

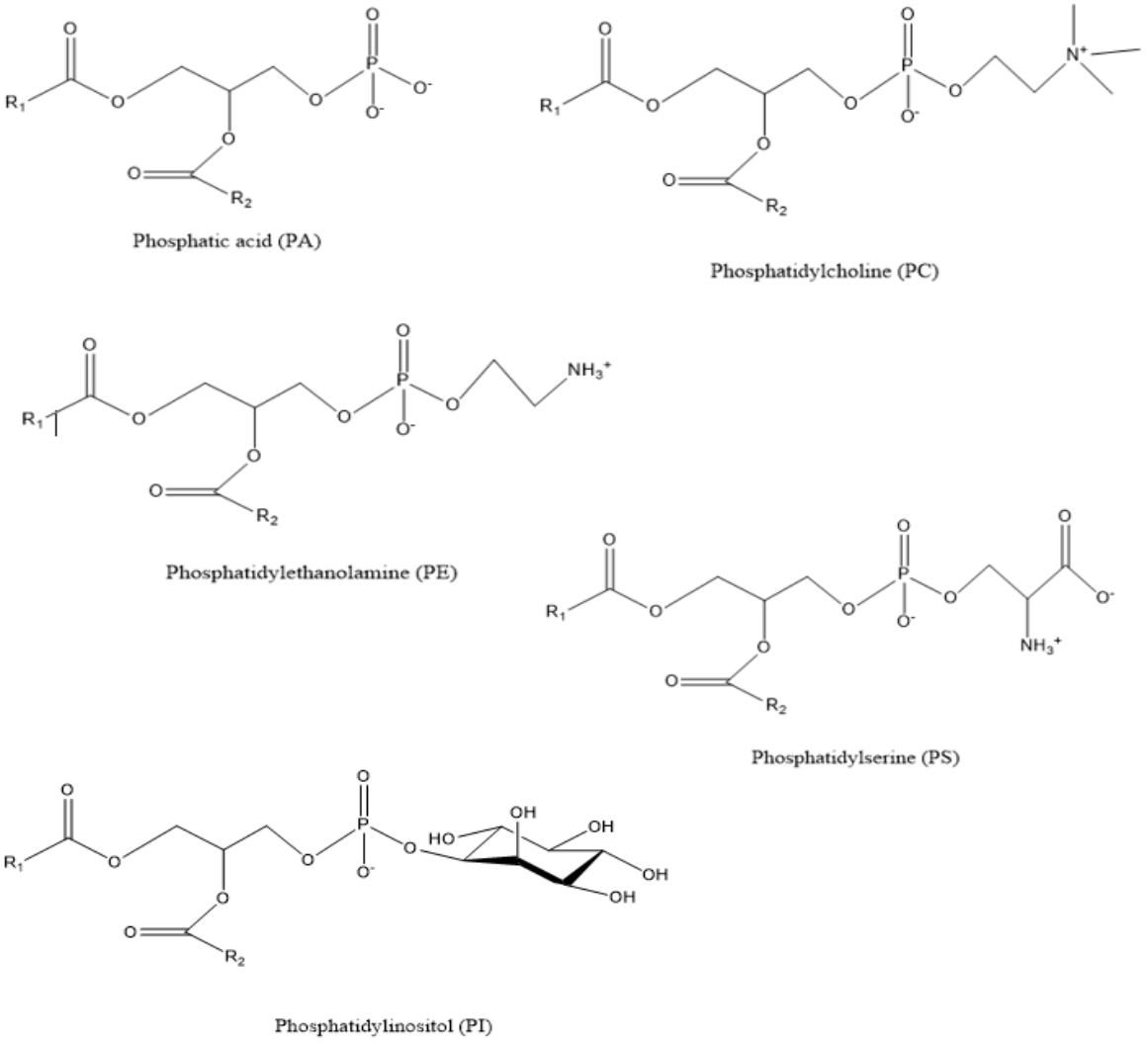

Figure 2.3. Structures of Phospholipids

2.6.2. Sources

Phospholipids can be found in vegetable seeds, cereal grains, and animal-based sources. Eggs, milk (Sánchez-Juanes et al., 2009), meats (Boselli et al., 2008; Ferioli & Caboni, 2010; Weihrauch & Son, 1983; Wood & Lister, 1973) and marine animals (Gbogouri et al., 2006; Medina et al., 1995; Weihrauch & Son, 1983) make up the major animal-based phospholipid sources. Egg yolks are rich sources of phospholipids, contributing to up to 10% of the total weight percentage, with PC and PE being the major

phospholipid, 66% and 19%, respectively (Weihrauch & Son, 1983). Raw meats contain large amounts of biological membranes and therefore contain around 0.5-1% of phospholipids. Vegetable seeds and cereal grains such as soybean (Chapman, 1980; Privett et al., 1973), sunflower (Chapman, 1980), corn (Tan & Morrison, 1979), cottonseed (), rapeseed (Vidal et al., 1983), oats and peanuts (Tan & Morrison, 1979; Weihrauch & Son, 1983) are rich sources of phospholipids. Also, vegetable, fruit and carbohydrate related food products have prevalent phospholipids content (Morrison et al., 1975; Vandercook et al., 1970; Weihrauch & Son, 1983).

2.6.3. Lecithin

Lecithin is described as the crude phospholipid mixture containing phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, and other phospholipids as well as other compounds such as fatty acids, carbohydrates, sterols, water, triglycerides, and glycolipids.(Szuhaj, 1983; Wendel, 2000). The lecithin composition can be modified by solvent extraction process as well as chemical and enzyme modification process. (Van Nieuwenhuyzen & Tomás, 2008). Also, every phospholipid has different solubility properties for different solvents, for example, PC dissolves in ethanol whereas PI and PA cannot (Wendel, 2000).

2.6.4. Prooxidative and antioxidative activities of phospholipids

Phospholipids are proven to show prooxidative or antioxidative properties or both depending up on the system they are used in. The different phospholipids have different susceptibility to oxidation because of their different degree of unsaturation and the polar

head groups (Cui & Decker, 2016a). The antioxidant effect may be attributed to one of the three mechanisms discussed below.

2.6.4.1. Metal chelation

The interaction between phospholipids and metals has been observed in bulk oil to inhibit the lipid oxidation**.** Phospholipids have the ability to bind with the positively charged prooxidant metals through the negative charge present on their phosphate group (Cui & Decker, 2016a). In stripped soybean oil-in-water emulsion at pH 7, PC was observed to possess antioxidant activity, possibly due to iron chelation, which disappeared at pH 3, which is near the pK_a of PC as a result there was no charge on the phosphate group.(Cardenia et al., 2011) Yoon (Yoon & Min, 1987) has showed that the phospholipids (PC, PE, PA, and PI) acted as antioxidants in soybean oil in the presence of 1 ppm of ferrous ion. Zago (Zago & Oteiza, 2001) also showed that ferrous has the ability to bind with PC/PS liposomes. As shown by Dacaranhe, iron-induced oxidation could be inhibited by PA and PS in sardine oil-in-water emulsion system (Dacaranhe & Terao, 2001). Although, the iron-binding ability of phospholipids does not always ensure that they can inhibit the lipid oxidation as increased solubility of iron may increase the oxidation. As shown by Gal (Gal et al., 2003)the increasing ratio of PS or PA to PC in liposomes produced more negative charge and resulted in more copper being bound to the membrane therefore resulting into more lipid oxidation.

2.6.4.2. Antioxidative properties of phospholipid Maillard reaction products

Maillard reaction is one of the important reactions in the food industry as they can impact the food aroma, taste, nutritional attributes, and color negatively or positively. This

reaction takes place in the presence of free amine groups (such as ethanolamine) and carbonyls (such as lipid oxidation aldehydes). Phospholipids that have amino group, such as PE, reacts with carbonyl groups produced during the lipid oxidation to form Maillard products (Cui & Decker, 2016a). Hidalgo (Hidalgo et al., 2004, 2005) showed that PE having a primary amine group reacted with 4,5-epoxy-2-heptenal to produce antioxidant Maillard products whereas PC, which has a tertiary amine group showed no effect on the lipid oxidation (Hidalgo et al., 2006). These results were in accordance with Alaiz (Alaiz et al., 1996), where primary and secondary amine showed inhibitory effects on lipid oxidation of soybean oil whereas tertiary amines showed no effect. As suggested by Bandarra (Bandarra et al., 1999), in sardine oil the synergism between PE or PC and α-tocopherol could be due to the Maillard products measures at 430 nm. The antioxidant activity of amine containing phospholipids (PC and PE) was because of the presence of α-tocopherol which was essential to produce antioxidative Maillard reaction products. (Shimajiri et al., 2013)

2.6.4.3. Synergism with tocopherols

Many of the antioxidant studies reported in this literature are related to their ability to increase lag phase synergistically with a primary antioxidant, mainly the tocopherols. PE and PS have been reported by Doert et al. (Doert et al., n.d.) to regenerate α-tocopherol by reacting with α-tocopherol quinone and thus increasing the antioxidant activity of tocopherol. The evidence of synergism of phospholipids and tocopherols can be demonstrated better in studies showing that phospholipids alone do not inhibit lipid oxidation but when they are in combination with tocopherols, a strong antioxidant effect is observed. Soybean lecithin was added to the virgin oil to check its effect on oxidative

stability of the oil. When the results were analyzed through Rancimat method (Koprivnjak et al., 2008), it was observed that lecithin was able to increase the tocopherol concentrations in the oil and hence increasing oxidative stability of the oil.

Phospholipids can alter the physical location of tocopherols and hence increase the effectiveness of the tocopherols. Physical location of antioxidants is known to influence their antioxidant activities. Huang et al.(S.-W. Huang et al., 1996) showed that the distribution of trolox and α-tocopherol was different for different lipid system and it resulted in the difference in antioxidant activities. Tocopherols as well as phospholipids have different surface activities and therefore their combination could influence the physical location of tocopherols as well as the other primary antioxidants, resulting in change in their location which could impact their antioxidant activity. (S.-W. Huang et al., 1996)

It was found that PE could regenerate α-tocopherol through α-tocopherol quinone in medium chain triglycerides however similar results were not observed with PC (Cui et al., 2015). Lambelet (Lambelet et al., 1994) observed that in the presence of phospholipids which contain primary amine such as PE and PS, the tocopherol in methyl linoleate degraded slowly. Synergism between PE, and PC with α-tocopherol was reported by Bandarra et al. (Bandarra et al., 1999). PE and PS were tested with different tocopherol homologues in bulk oil by (Xu et al., 2019). It was found that PE was a better synergistic combination with tocopherol than PS. A study done by Gautam (Samdani et al., 2018) showed that PS and PE can be used synergistically in soybean oil-in-water emulsions. However, PS showed increased antioxidant activity with tocopherol homologues as compared with PE.

CHAPTER 3

ANTIOXIDANT COMBINATION OF HIGH PHOSPHATIDYLSERINE LECITHIN WITH MIXED TOCOPHEROL IN SOYBEAN OIL-IN-WATER EMULSION: EFFECT OF pH AND SALT

3.1. Introduction

Lipids serve as an important determinant of food quality in terms of adding flavor, texture, nutritional value, and health benefits (Waraho, Mcclements, et al., 2011). However, lipids with double bonds are very susceptible to oxidative deterioration which is responsible for declining a food product's quality and thus shelf life (E. N. Frankel, 1980). Oxygen reacts with unsaturated fatty acids to produce unstable, colorless, odorless and tasteless lipid hydroperoxides as the primary products which further decompose to form complex, volatile and nonvolatile secondary oxidation products (E. N. Frankel, 1980). Secondary oxidation products are responsible for rancid odors and development of off flavors in food products (Gray, 1978).

Lipid oxidation can be delayed by using antioxidants which can both be naturally occurring or synthetically made. Synthetic antioxidants such as ethylenediaminetetraacetic acid (EDTA), tert-butyl-4- hydroxyanisole (BHA), tert-butyl-4-hydroxytoluene (BHT), and tert-butyl-hydroxyquinone (TBHQ), were used widely due to their higher antioxidant activity as compared to natural antioxidants. However, there are scattered reports of the deleterious health effects of these synthetic compounds which led to an increased demand to identify natural antioxidants for cleaner food labels (Samdani et al., 2018; Xu et al., 2019).

Tocopherols are one of the natural antioxidants that are found in abundance in vegetable oils, which protect fatty acids by getting oxidized preferentially and forming antioxidant radicals that are not strong prooxidants. Tocopherol exists in different forms, namely α-, β-, γ-, and δ- tocopherols (Wilailuk Chaiyasit et al., 2007). The combination of the homologues in mixed tocopherols have been seen to increase the oxidative stability of refined oil as compared to the individual homologues because the different physical and chemical properties of tocopherols allow them to partition into different locations, where they are able to inactivate multiple free radical species (E. N. Frankel, 1984).

The antioxidant activity of tocopherols can be enhanced by combining it with other antioxidants. The increased activity observed by antioxidant combinations can occur because the different antioxidants inhibit different pathways of lipid oxidation (e.g., free radical scavenging vs metal chelation (Naumov & Vasil'ev, 2003)), by scavenging different radicals by partitioning into different places in the food (Kiralan et al., 2014a) or by regenerating an oxidized free radical scavenger so that it is reactivated, (for example, oxidized tocopherols can be regenerated by carotenoids (Mortensen & Skibsted, 1997)).Phospholipids are important components of biological membranes of both animals and plants. Phospholipids have a phosphate group attached to the glycerol backbone, at sn-3 position (Cui & Decker, 2016a). According to Doert et al., phospholipids like PE and PS are reported to regenerate oxidized tocopherol by reacting with tocopherol quinone and hence increasing the antioxidant activity of tocopherol (Doert et al., 2012). Figure 3.1 shows the proposed mechanism of regeneration of tocopherol by phosphatidylserine.

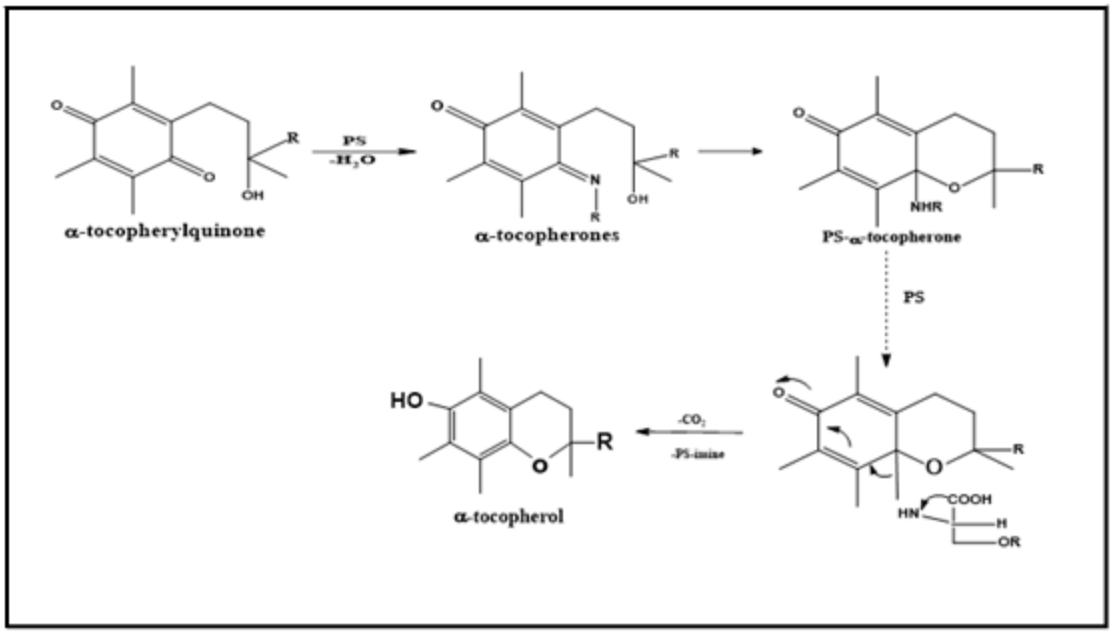

Figure 3.1: Proposed regeneration mechanism of α-tocopherol by phosphatidylserine (PS)

PS has been shown to increase the antioxidant activity of tocopherols in bulk oil (Xu et al., 2019) as well as in oil-in-water emulsions (Samdani et al., 2018). It was observed from these two studies that PS is a better antioxidant in combination with tocopherols for oil-in-water emulsions when compared with bulk oil.

The purpose of the current study is to evaluate the synergistic interactions between mixed tocopherol and a commercially available high phosphatidylserine (PS) lecithin in oil-in-water emulsion under various conditions. This combination could prove to be more viable option from industrial point of view because high PS lecithin is cheaper than high PE lecithin. Moreover, we investigated the effect of different pH and salt concentration on the ability of high PS lecithin and mixed tocopherol to inhibit lipid oxidation in oil-in-water emulsions.

3.2. Materials

Soybean oil for this study was purchased from a local store and stored at -20˚C until use. Lecithin derived phosphatidylserine (PS) was donated by Perimondo, NY. The high PS lecithin contained 73.3% PS, 2.6% lysophosphatidylserine, less than 2.5% water, less than 0.1% of phosphatidylcholine (PC), 0.3% of phosphatidylinositol (PI), 0.2% of phosphatidylethanolamine (PE), 0.3% of lysophosphatidylethanolamine, 1% of acyl-phosphatidylethanolamine, 0.4% of phosphatidylglycerol, 0.9% of diphosphatidylglycerol, 7.9% of phosphatidic acid, 2.2% of lysophosphatidic acid and 3.6% of other compounds as determined by the manufacturer.

Authentic PS (>99% purity) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Silicic Acid, activated charcoal, low α-type mixed tocopherol and Tween 20 were purchased from Sigma-Aldrich Co. (St. Louis MO, U.S.A.). All other reagents were of high-performance liquid chromatography (HPLC) grade or purer. Deionized and distilled water was used in all the experiments. All the glassware were soaked in 2N hydrochloric acid to remove metals followed by rinsing with distilled and deionized water, and then dried before use.

3.3. Preparation of Stripped Soybean Oil (SSO).

The soybean oil used in this study was stripped of minor components such as free fatty acids, phospholipids, mono- and diacylglycerols, and tocopherols as these compounds can alter oxidation pathways. The protocol followed for stripping of soybean oil is taken from the method described by Cui et al.(Cui et al., 2015) A chromatographic column (3.0 cm internal diameter and 35 cm long) was set up with three layers of activated charcoal and

silicic acid. Silicic acid was washed with distilled and deionized water and activated at 100˚C for 24h. The bottom layer consisted of 22.5 g of washed silicic acid, followed by a middle layer of 5.6 g of activated charcoal and then a top layer of 22.5 g of washed silicic acid. Thirty grams of commercial soybean oil was mixed with 30 mL of hexane and passed through this column which was then eluted using 270 mL of hexane. The hexane was removed from the stripped oil using a vacuum rotary evaporator (Rotovapor R100, Buchi, Flawil, Switzerland) at 27˚C. Residual solvent was evaporated by nitrogen flushing. Stripped soybean oil (SSO) was stored in the dark at -80˚C until the preparation of emulsions.

3.4. Emulsions Preparation and Storage Conditions.

Stripped soybean oil-in-water emulsions (1.0 wt% oil) were prepared with 10mM imidazole-acetate buffer containing Tween 20 as the emulsifier at a 1:10 emulsifier/oil ratio. Phosphatidylserine (PS) dissolved in chloroform and/or mixed tocopherols dissolved in ethanol were added to stripped soybean oil and stirred for 30 minutes at 4˚C prior to emulsion preparation. Imidazole-acetate buffer and Tween20 were added to the beaker containing stripped soybean oil. A handheld homogenizer (M133/1281-0, Biospec Products Inc., Bartlesville, OK) was used for 2 minutes to blend the mixture into a coarse emulsion, which was then homogenized using a microfluidizer (Microfluidics, Newton, MA, USA) for three passes at a pressure of 9 kbar. The microfluidizer coil and the beakers containing emulsions were submerged in ice during the homogenization process. The mouth of the beakers was also sealed using the paraffin. The pH of the emulsions was adjusted to pH 3.0 or pH 7.0, with 12N hydrochloric acid. Sodium chloride (0 wt%, 0.5 wt%, 1.0 wt%, and 1.5 wt%) was added after emulsion preparation. For storage studies, 1

mL of sample was pipetted into 10 mL headspace vials, sealed with aluminum caps containing polytetrafluoroethylene (PTFE)/silicone septa and then stored at a temperature of 37˚C or 45˚C in the dark.

3.5. Measurements of Particle Size Distributions and Zeta Potential.

Emulsions were diluted into acetate-imidazole buffer at an emulsion: buffer ratio of 1:250. The diluted samples were then vortexed. Both particle size and the ζ-potential of the emulsions were analyzed using ZetaSizer Nano-ZS (Malvern Instruments, Worcestershire, UK). All the measurements were taken in triplicates at room temperature.

3.6. Measurement of Lipid Oxidation.

The primary and secondary lipid oxidation products, lipid hydroperoxides and hexanal, respectively, were quantified to determine the oxidation stability of the oil-in-water emulsion samples. Both lipid hydroperoxides and headspace hexanal were determined every 24 hours starting from the day the emulsion samples were prepared (0th day).

3.6.1. Hydroperoxide analysis

Lipid hydroperoxides were measured using the method reported by Shantha and Decker (Shantha & Decker, 1994). This method includes mixing 0.3 mL of emulsion samples with 1.5 mL of isooctane-isopropanol (3:1, v/v) solution followed by vortexing for 10 s, 3 times. The mixture was then centrifuged (Centrific TM Centrifuge, Fisher Scientific, Fairlawn, NJ) at 3000 rpm for 2 minutes. The upper organic solvent layer (0.2 mL) was extracted from the centrifuged samples and 2.8 mL of methanol-butanol (2:1, v/v) was added along with 15 µL of 3.94 M ammonium thiocyanate and 15 µL of ferrous

solution (prepared from a 1:1 mixture of 0.14 M $FeSO_4$ and 0.13 M $BaCl_2$). The samples were kept in dark for 20 minutes and then absorbance was measured at 510 nm using a Genesys 20 spectrophotometer (ThermoSpectronic, Waltham, MA). Hydroperoxide concentrations were determined by using a standard calibration curve of cumene hydroperoxide.

3.6.2. Hexanal analysis

Hexanal was measured by solid-phase microextraction headspace gas chromatography with flame ionization detector (SPME-GC-FID) using a method described by Cardenia et al., (Cardenia et al., 2011) The gas chromatograph used in this study was a Shimadzu GC-2014 (Shimadzu, Kyoto, Japan) equipped with an AOC-5000 autosampler (Shimadzu) and a split−splitless injector. An Equity DB-1 column (30 m × 0.32 mm × 1 mm film thickness, Supelco, Bellefonte, PA) was used for separation of volatiles. Emulsion samples in headspace vials were shaken and heated for 13 minutes at 55˚C in the autosampler heating block before measurement. A 50/30 µm divinylbenzene (DVB)/Carboxen/polydimethylsiloxane (PDMS) solid phase microextraction (SPME) fiber needle from Supelco (Bellefonte, PA) was injected into the vial headspace for 2 minutes to absorb volatiles and then was transferred to the GC injector port (250 °C) for 3 min. The run time was 6 m at 65˚C. The detector was set at 250˚C. Helium was used as carrier gas at a flow rate of 1.0 mL/min with a split ratio of 1:7. Quantification of hexanal was done using a calibration curve made with hexanal standard solution.

3.7. Interaction Index.

Calculation of interaction index helped in determining if the interaction of the antioxidants was synergistic. It was calculated using the formula: (lag phase of the phosphatidylserine (PS)− tocopherol combination − lag phase of the control) / [(lag phase of tocopherol alone − lag phase of the control) + (lag phase of phosphatidylserine (PS) alone − lag phase of the control)]. An interaction index value of <1 indicates antagonistic interactions between the antioxidants, a value of 1 indicates an additive effect, and a value >1 indicates a synergistic interaction between the antioxidants.

3.8. Statistical Analysis.

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

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Effect of pH and mixed tocopherols concentration on oxidative stability of in oil-in-water emulsions:

Mixed tocopherols are commercially available and were shown to be more effective in combination with phosphatidylserine (PS) in oil- in-water emulsions by Samdani et al.,.(Samdani et al., 2018) To determine the most effective concentration of mixed tocopherol, different concentrations (0.5, 1.0 and 3 µmole/kg emulsion) of mixed tocopherols were tested at pH of 3 and 7. This study was conducted at 45˚C to shorten experimental times as the mixed tocopherols were expected to increase the lag phases.

At pH 3, (Figure 4.1) the control had a hydroperoxide lag phase of 0 days. Addition of 0.5, 1.0 and 3.0 µmole of mixed tocopherols/kg emulsion increased lag phases 1, 2 and 4 days, respectively. At pH 7, the control emulsion also had a lag phase of 0 days. Addition of 0.5 and 1.0 µmole of tocopherol/kg emulsion did not increase the lag phase compared to the control. Addition of 3 µmole of tocopherol/kg emulsion increased the lag phase to 1 day. Waraho et al., (Waraho et al., 2009) found that the oxidation rate of Tween 20 stabilized oil-in-water emulsions decreased with decreasing pH. We observed that mixed tocopherols were more effective at pH 3.0 than 7.0 which is likely due to the slower oxidation rates that would result in slower tocopherol consumption. Huang et al., (S.-W. Huang et al., 1996) also reported that α-tocopherol was also more effective at pH 3.0 than 7.0. Since 3.0 µmole of tocopherol/kg emulsion was effective at both pH 3.0 and 7.0, it was used for future studies.

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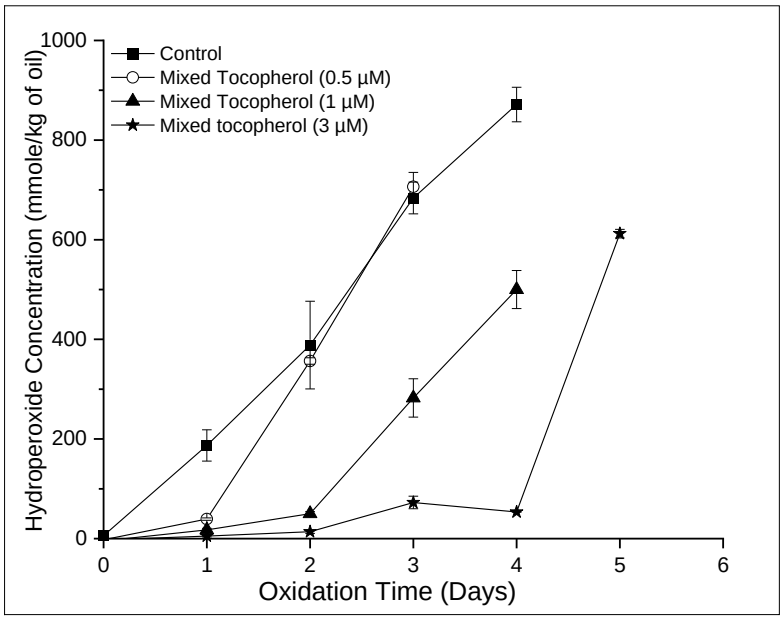

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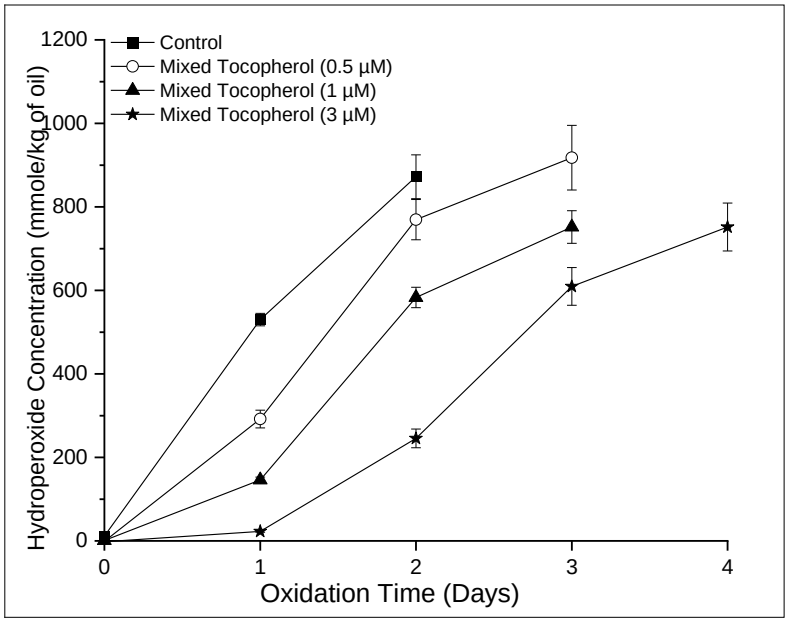

Figure 4.1: Formation of lipid hydroperoxides at pH 3 (A), and pH 7 (B) in 1% stripped soybean oil-in-water emulsion stabilized with Tween 20. Samples contained no mixed tocopherol (control) or 0.5, 1 or 3 µmole mixed tocopherols/kg emulsion at 45˚C. Each value represents the mean (n=3). Some error bars are within the data points.

4.2. Comparing Authentic PS and High PS Lecithin with and without mixed tocopherols.

The ability of high PS lecithin to enhance the activity of mixed tocopherols was compared to authentic PS at equal PS concentrations (Figure 4.2). Incubation temperature was decreased to 37˚C to increase the lag phase to be able to better see differences in oxidation rates between treatments. The hydroperoxide and hexanal lag phases for the control were 0 and 1 day, respectively, in the Tween20 stabilized 1% oil-in-water emulsion at pH 7.0. Addition of authentic PS (30 µmole/kg emulsion) and high PS lecithin (30 µmole/kg emulsion) did not extend the lag phase for either the hexanal and the hydroperoxide formation. Mixed tocopherols (3 µmole/kg emulsion) alone extended the hydroperoxide lag phase to 2 days and hexanal lag phase to 3 days. Combining authentic PS with mixed tocopherols extended the hydroperoxide and hexanal lag phases to 3 and 4 days, respectively, with an interactive index of 1.5 for both hydroperoxides and hexanal. High PS lecithin in combination with mixed tocopherols extended both hydroperoxide and hexanal lag phases to 4 days with an interactive index of 2 for hydroperoxide and 1.5 for hexanal. This study shows that high PS lecithin can be viable alternative to authentic, more expensive PS.

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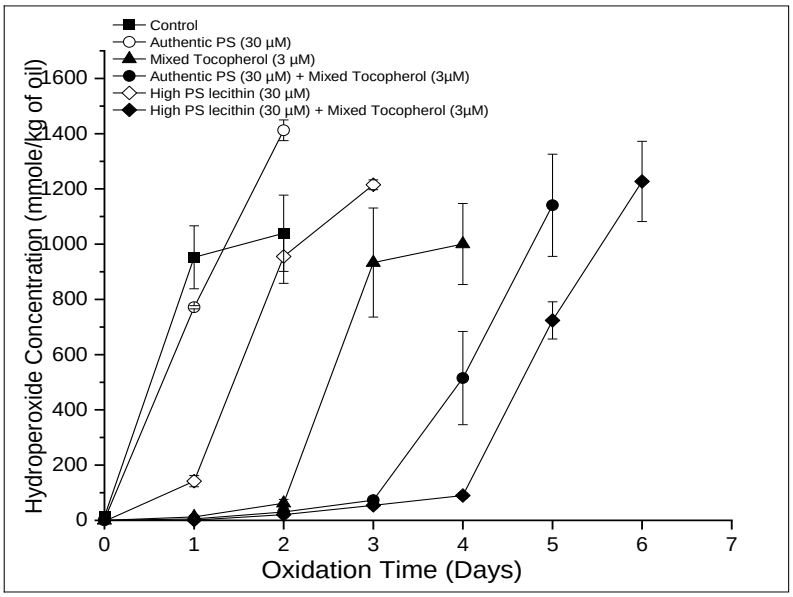

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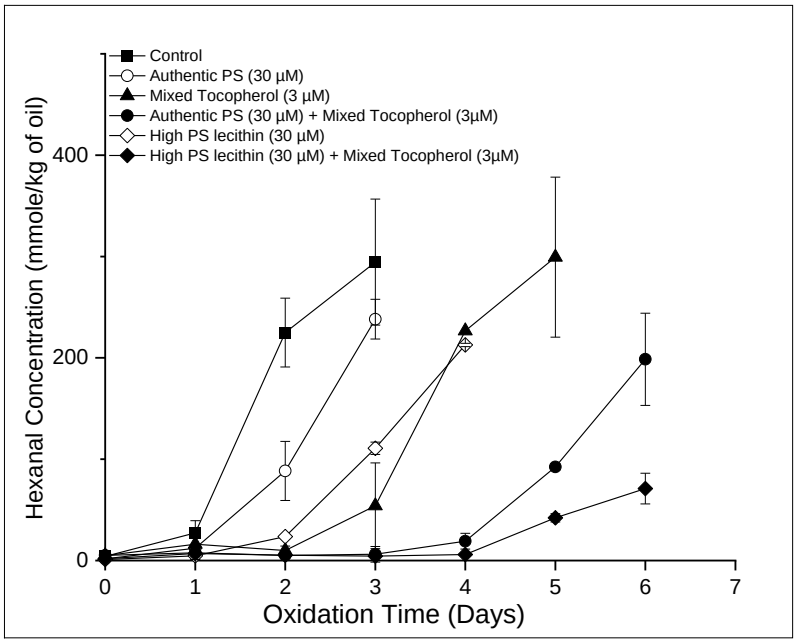

Figure 4.2: Formation of lipid hydroperoxides (A) and hexanal (B) in 1% stripped soybean oil-in-water emulsion stabilized with Tween 20 containing 3 µmoles of mixed tocopherol/kg of emulsion and/or 30 µmoles PS/kg of emulsion from either authentic PS or high PS lecithin at 37˚C and pH 7.0. Each value represents the mean (n=3). Some error bars are within the data points.

The activity of the high PS lecithin and mixed tocopherols was also determined at pH 3.0 (Figure 4.3). The hydroperoxide and hexanal lag phases of control were longer than at pH 7.0 i.e., 1 and 3 days, respectively, which is in agreement with Waraho et al., (Waraho et al., 2009) and Cardenia et al., (Cardenia et al., 2011). The addition of 3 µmole of mixed tocopherols/kg emulsion increased the hydroperoxide lag phase to 8 days and hexanal lag phase to 10 days. The high PS lecithin (30 µmole/kg emulsion) had similar hydroperoxide lag phase of 1 day whereas hexanal lag phase was 3 days. The combination of high PS lecithin and tocopherol extend the hydroperoxide lag phase to 10 days and hexanal lag phase to 13 days at pH 3.0. The interactive index for the combination for hydroperoxide was 1.29 and for hexanal was 1.43 which indicated the synergistic interaction of PS and tocopherol in oil-in-water emulsion of stripped soybean oil.

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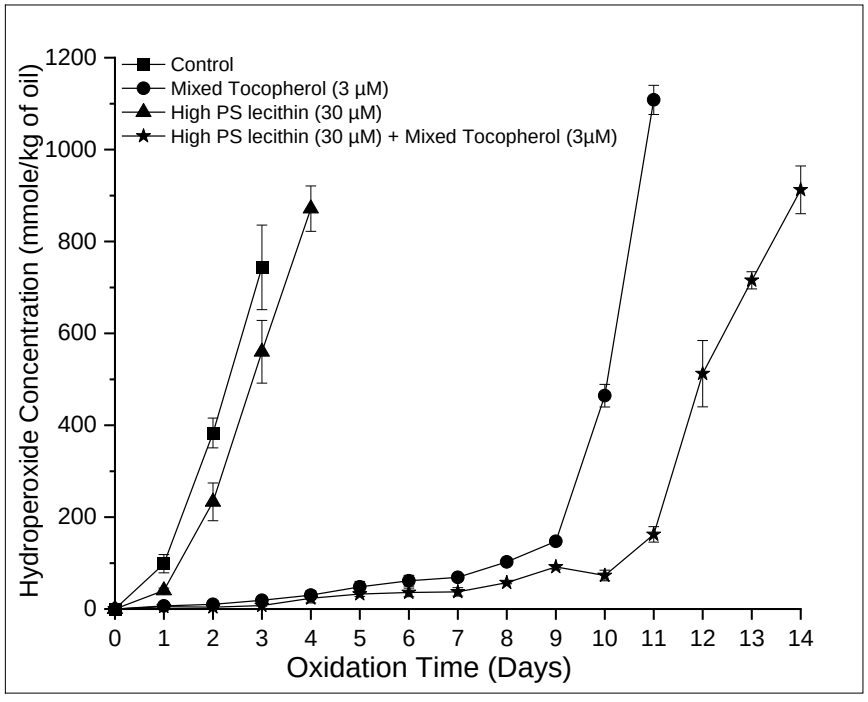

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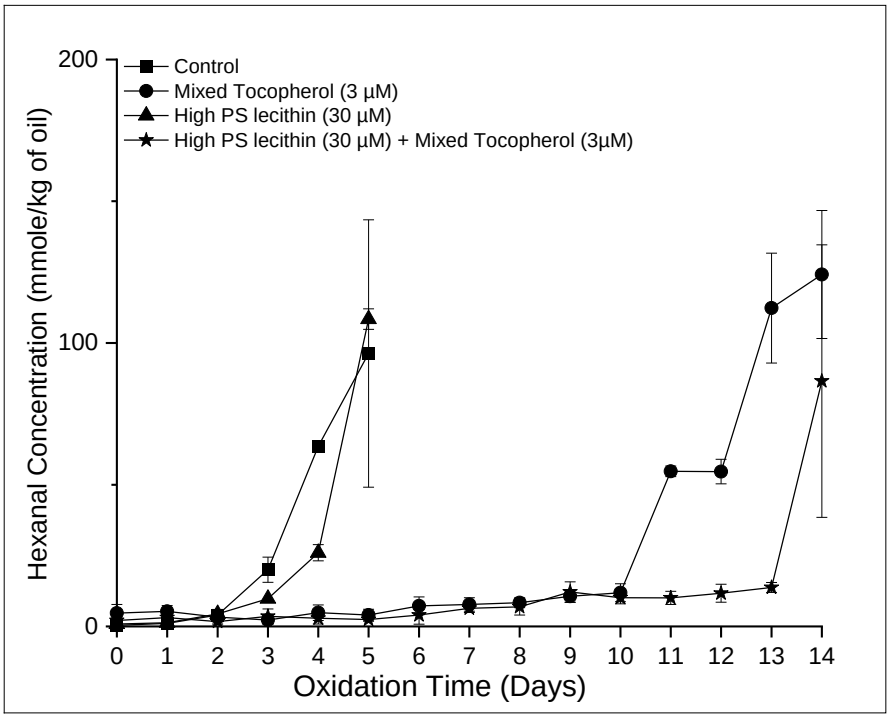

Figure 4.3: Formation of lipid hydroperoxides (A) and hexanal (B) in 1% stripped soybean oil-in-water emulsion stabilized with Tween 20 containing 3µM of mixed tocopherol and 30µM of high PS lecithin at 37˚C and a pH of 3.0. Each value represents the mean (n=3). Some error bars are within the data points.

4.3. Effect of sodium chloride salt on the combination of mixed tocopherol and high PS lecithin:

Salt is found to impart prooxidant properties in Tween 20 stabilized corn oil-in-water emulsions.(Cui et al., 2016) The prooxidant ability of sodium chloride has been postulated to be due to the ability of chloride to form prooxidative complexes with transition metals (Osinchak et al., 1992) and the ability of sodium to displace iron from macromolecules such as proteins (Joseph Kanner et al., 1991). This study was done to understand the effects of salt on the oxidative stability of the combination of 30 µmole/kg emulsion of high PS lecithin and 3 µmole/kg emulsion of mixed tocopherol in oil-in-water emulsions at 37˚C.

The concentrations of sodium chloride that are used were 0.5 wt%, 1 wt%, and 1.5 wt% of the soybean oil-in-water emulsions.

Control lag phases tended to be longer in the absence of salt although at some concentrations, differences could not be observed as lag phase for both the control and added salt were 0 days for hydroperoxide lag phases but hexanal lag phase of control without salt is extended to 1 day whereas control with salt has hexanal lag phase of 1 day (Figures 4.4 – 4.6). The lag phases for mixed tocopherol alone were 1 days and 2 days for both hydroperoxides and hexanal and these lag phases did not change with increasing salt concentrations. The hydroperoxide lag phases for mixed tocopherol and high PS lecithin combination were 2 days for all salt concentrations. Hexanal lag phases were 4-5 days in the presence of mixed tocopherols and high PS lecithin for all the three NaCl concentration. The difference in lag phase is less than 1 day. This study shows that sodium chloride has no effect on the ability of the high PS lecithin and mixed tocopherol combination to inhibit lipid oxidation in Tween 20 stabilized soybean oil-in-water emulsions.

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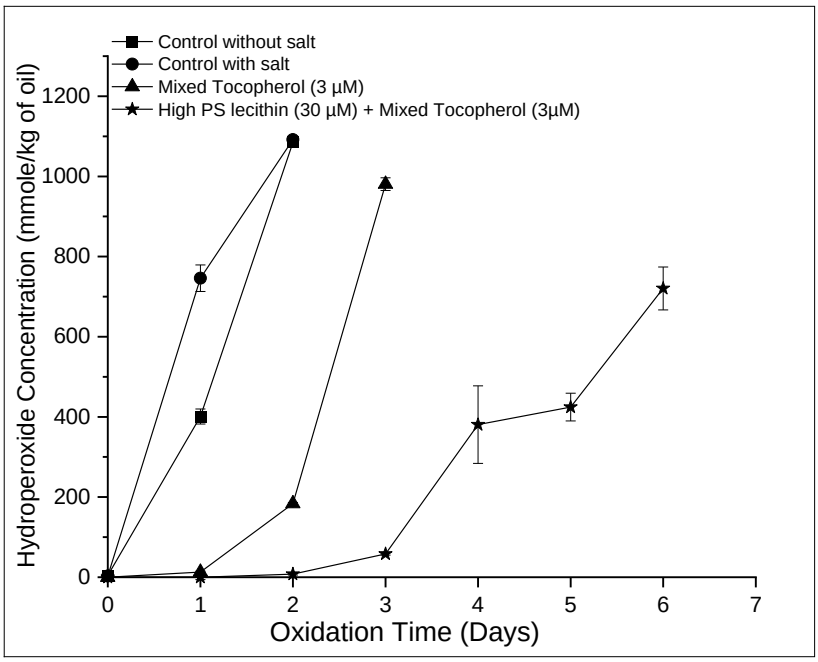

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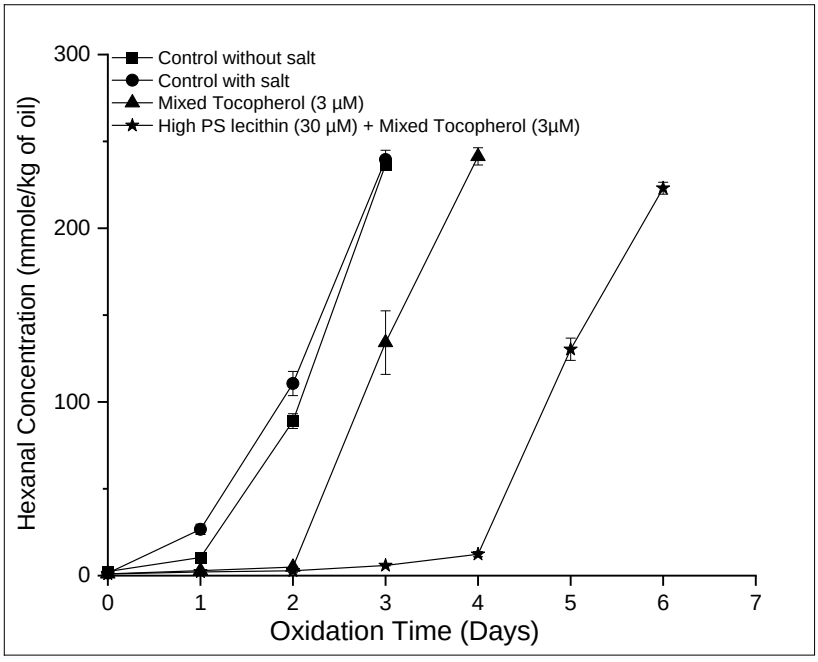

Figure 4.4: Formation of lipid hydroperoxides (A) and hexanal (B) in 1% stripped soybean oil-in-water emulsion stabilized with Tween 20 containing 3µM of mixed tocopherol/kg of emulsion and 30µM high PS lecithin/kg of emulsion at 37˚C and salt concentration of 0.5 wt% of emulsion. Each value represents the mean (n=3). Some error bars are within the data points.

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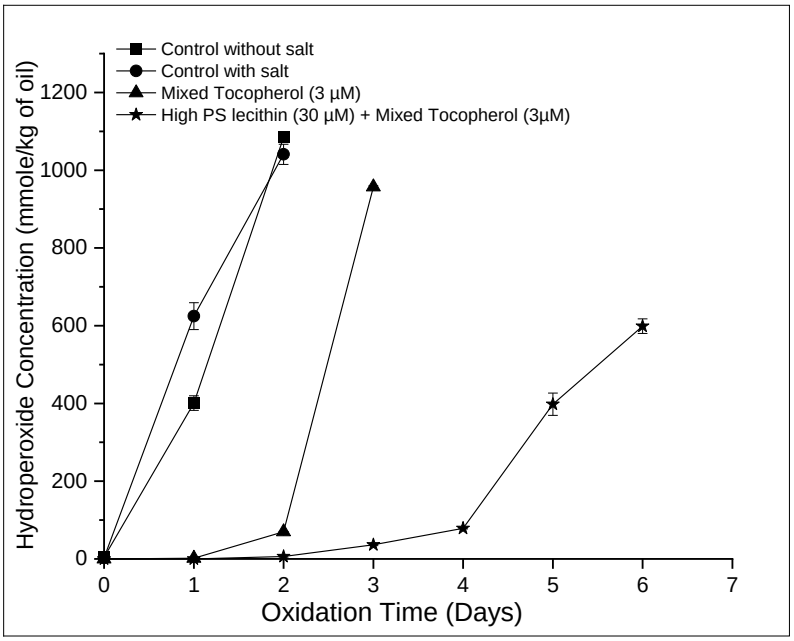

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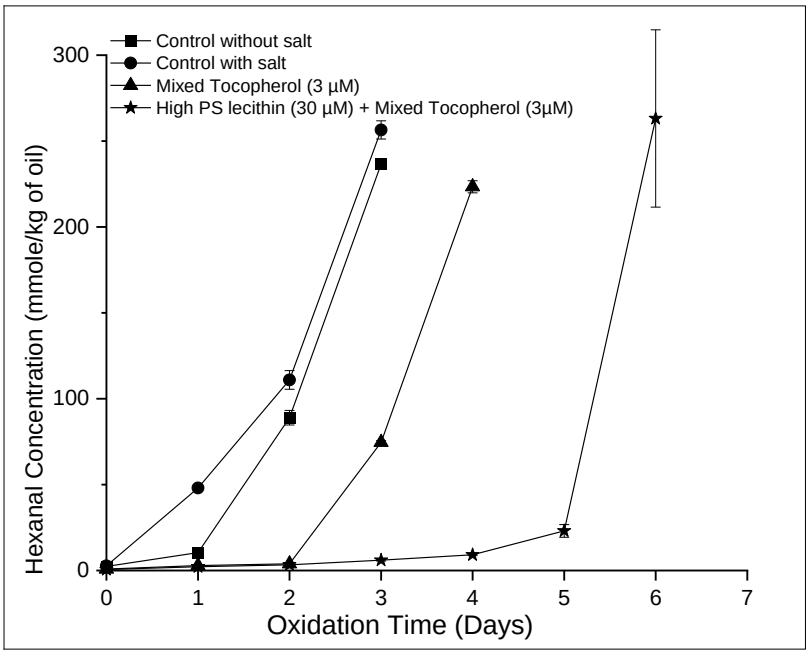

Figure 4.5: Formation of lipid hydroperoxides (A) and hexanal (B) in 1% stripped soybean oil-in-water emulsion stabilized with Tween 20 containing 3µM of mixed tocopherol/kg of emulsion and 30µM high PS lecithin/kg of emulsion at 37˚C and salt concentration of 1.0 wt% of emulsion. Each value represents the mean (n=3). Some error bars are within the data points.

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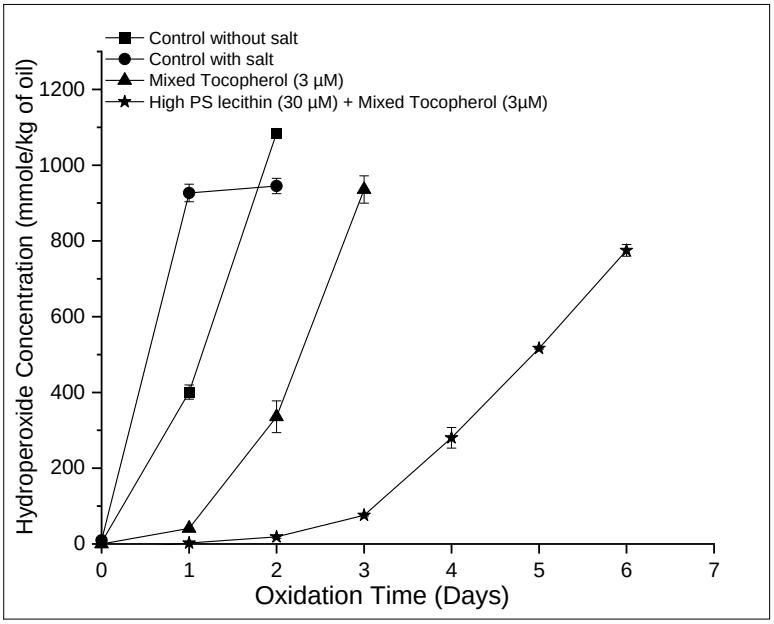

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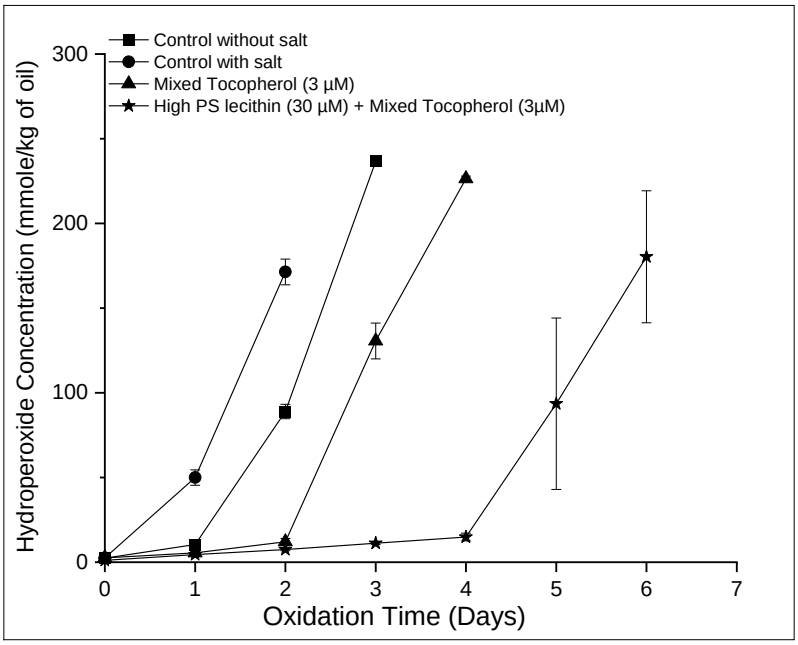

Figure 4.6: Formation of lipid hydroperoxides (A) and hexanal (B) in 1% stripped soybean oil-in-water emulsion stabilized with Tween 20 containing 3µM of mixed tocopherol/kg of emulsion and 30µM high PS lecithin/kg of emulsion PS at 37˚C and salt concentration of 1.5 wt% of emulsion. Each value represents the mean (n=3). Some error bars are within the data point

CHAPTER 5

DISCUSSION AND FUTURE WORK

This research shows that mixed tocopherol were more effective antioxidants at pH 3.0 than 7.0. Also, antioxidant ability increased with the increasing concentration of tocopherol i.e., 3 µmol/kg of emulsion. Authentic PS and high PS lecithin had almost similar lag phase days. Authentic PS extended the hydroperoxide and hexanal lag phase up to 3 and 4 days whereas high PS lecithin increased both the hydroperoxide and hexanal lag phase up to 4 days. It was also found that the combination of mixed tocopherols and high PS lecithin produced synergistic antioxidant activity in oil-in-water emulsions at both pH 3 and 7. However at pH 3, the antioxidant activity of the combination increases, which can be attributed to slower oxidation rate at the lower pH. The antioxidant activity of the combination is also not affected by adding different sodium chloride concentrations of 0.5, 1.0 and 1.5 wt% of sodium chloride salt in soybean oil-in-water emulsion. This shows that this synergistic combination has wide range of application and can be used as good clean label antioxidant strategy. More studies can be done on different concentrations of high PS lecithin along with mixed tocopherols. Effects of different surfactants can also be seen on this combination.

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