INVESTIGATING THE ANTIOXIDANT EFFECTS OF A COMMERCIAL SUNFLOWER DERIVED LECITHIN AND PHOSPHATIDYLSERINE

by

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TABLE OF CONTENTS

LIST OF TABLES v
LIST OF FIGURES vi
ABSTRACT ix
LIST OF ABBREVIATIONS AND SYMBOLS USED x
ACKNOWLEDGEMENTS xiii
CHAPTER 1 INTRODUCTION 1
1.1 BACKGROUND 1
1.2 LIPID OXIDATION AND OXIDATION PRODUCTS 5
1.3 Antioxidants 7
1.3.1 Phenolic Antioxidants – Tocopherols and Rosemary Extract 9
1.4 PHOSPHOLIPIDS AS ANTIOXIDANTS 12
1.4.1 Metal Chelation 13
1.4.2 Impact of Fatty Acid Moieties 15
1.4.3 Phospholipids as Synergists with Tocopherols 16
1.4.4 Amino-carbonyl Reactions 17
1.4.5 Maillard Browning Compounds 18
1.4.6 Reverse Micelles 20
1.5 OXIDATION MEASUREMENTS 21
1.6 OBJECTIVES 23
CHAPTER 2 CSL: DETERMINING POTENCY FOR OXIDATION STABILITY STUDIES 27
2.1 INTRODUCTION 27
2.2 MATERIALS AND METHODS 28
2.2.1 Sample Preparation and Storage Conditions 29
2.2.2 Oxidation Measurements 30
2.2.3 Statistical Analysis 32
2.3 RESULTS 32
2.4 DISCUSSION 37

2.5 CONCLUSION 43

CHAPTER 3 ANTIOXIDANT EFFECT OF PS AND CSL 44

3.1 INTRODUCTION 44

3.2 MATERIALS AND METHODS 46

3.2.1 Experimental Design 46

3.2.2 Preparation of Tocopherol Free Fish Oil 50

3.2.3 Sample Preparation and Storage Conditions 51

3.2.4 HPLC Tocopherol Analysis 52

3.2.5 Oxidation Measurements 53

3.2.6 Quantitative Estimation of Antioxidant Activity from PV 54

3.2.7 Maillard Browning Compounds 55

3.2.8 Separation of PL Classes by TLC and Analysis of Fatty Acid Moieties 55

3.2.9 Iron Chelating 57

3.2.10 Fatty Acid Analysis 59

3.2.11 Statistical Analysis 59

3.3 RESULTS 60

3.3.1 Effect of Tocopherols in the Presence of PS and CSL on the Formation of Primary and Secondary Oxidation Products 60

3.3.3 Effect of Stabilizing Antioxidants in CSL on Primary Oxidation.. 69

3.3.4 Effect of Stabilizing Antioxidants in CSL on Secondary Oxidation 73

3.3.5 Individual effects of RME and αT on Primary Oxidation 76

3.3.6 Individual effect of RME and αT on Secondary Oxidation 80

3.3.7 Development of Maillard Browning Compounds 81

3.3.8 Comparison of Fatty Acid Moieties on Soybean PS and CSL PS 84

3.3.9 Iron Chelating Ability of PS 85

3.4 DISCUSSION 86

3.4.1 Effect of PS on Primary and Secondary Oxidation of Fish Oil 87

3.4.2 Effect of Tocopherols on the Antioxidant Activity of PS 89

3.4.3 Effect of Tocopherols on the Antioxidant Effect of CSL 90

3.4.4 Effect of Tocopherols on Primary and Secondary Oxidation 90
3.4.5 Iron (II) Chelation of PS, CSL and RME 94
3.4.6 Effect of Fatty Acid Moieties 96
3.4.7 Effect of Maillard Browning Compounds 97
3.4.8 Antioxidant effect of RME and αT 99
3.4.9 Industrial Applications 100
3.4.10 Future Work 102
3.5 CONCLUSIONS 103
CHAPTER 4 CONCLUSION 105
4.1 CLOSING REMARKS 105
REFERENCES 108
APPENDIX I 117

LIST OF TABLES

Table 3.1 Comparison of the increase in PV over time for samples stored in open vials at ambient conditions. ... 64

Table 3.2 Total tocopherol content at the beginning and end of the 16-day stability study. ... 68

Table 3.3 Comparison of the increase in PV over time for samples stored in open vials at ambient conditions. ... 72

Table 3.4 Comparison of the increase in PV over time for samples stored in open vials at ambient conditions. ... 78

Table 3.5 Evolution of Maillard browning compounds over an eight-day period, investigating the combined effect of RME+αT on the antioxidant activity of PS.. ... 82

Table 3.6 Evolution of Maillard browning compounds over a seven-day period investigating the individual effect of RME and αT on the antioxidant activity of PS.. ... 83

Table 3.7 Mass percent of total fatty acid moiety present in the PS fraction of CSL, as well as the soybean PS. ... 85

LIST OF FIGURES

Figure 1.1 Chemical structure of: A) eicosapentaenoic acid and B) docosahexaenoic acid... 2

Figure 1.2 Chemical structures of: A) α-tocopherol, B) β-tocopherol, C) δ-tocopherol, D) γ-tocopherol.. 10

Figure 1.3 Chemical structures of: A) carnosol and B) carnosic acid....................... 12

Figure 1.4 Chemical structures of PL contained in CSL: A) Phosphatidylserine (PS), B) Phosphatidylcholine (PC), C) Phosphatidic acid (PA). 25

Figure 1.5 Chemical structures of PL contained CSL: A) Phosphatidylinositol (PI), B) Phosphatidylethanolamine (PE), C) N-acyl-phosphatidylethanolamine (APE), D) Phosphatidylglycerol (PG). 26

Figure 2.1 Typical composition of CSL in mass proportions……………………... 28

Figure 2.2 Experimental treatment and analytical measurements used to test the antioxidant activity of 1-10 mg g^{-1} CSL………………………………... 29

Figure 2.3 Effect of 1-10 mg g^{-1} CSL on the development of hydroperoxides in refined fish oil, measured by PV……………..………………………… 34

Figure 2.4 Effect of concentrations of CSL ranging from 1–10 mg g^{-1}on the development of secondary oxidation products in refined fish oil, measured by *p*AV…………..…………………………………………….. 36

Figure 3.1 Experimental treatments and analytical measurements used to compare the antioxidant effect of CSL and PS alone and in combination with mixed T…..…………………………………………………………….. 48

Figure 3.2 Experimental treatments and analytical measurements used to test for synergy between PS, RME and αT present in CSL…..………………… 49

Figure 3.3 Experimental treatments and analytical measurements used to test for synergy between RME and αT individually with PS…..………………… 50

Figure 3.4 Effect of added PS and CSL with and without added mixed T on the development of hydroperoxides in tocopherol stripped fish oil...……… 61

Figure 3.5 Effect of added PS and CSL with and without added mixed T on the development of CD in tocopherol stripped fish oil…..………………… 62

Figure 3.6 Estimated antioxidant activity, expressed in terms of ability to inhibit hydroperoxide formation, of PS and CSL with and without mixed T in tocopherol stripped fish oil 65

Figure 3.7 Effect of added PS and CSL with and without added mixed T on the development of secondary oxidation products, measured by pAV, in tocopherol stripped fish oil. 67

Figure 3.8 Effect of added PS, PS+RME+ αT, CSL and RME+αT on the development of hydroperoxides in tocopherol stripped fish oil 71

Figure 3.9 Estimated antioxidant activity, expressed in terms of ability to inhibit hydroperoxide formation, of PS, RME, αT and CSL in tocopherol stripped fish oil.. 73

Figure 3.10 Effect of added PS, RME, αT and CSL on the development of secondary oxidation products, as measured by pAV, in tocopherol stripped fish oil 75

Figure 3.11 Effect of added PS, RME, αT and CSL on the development of hydroperoxides in tocopherol stripped fish oil. 77

Figure 3.12 Antioxidant effects, expressed in terms of ability to inhibit formation of hydroperoxides, of PS, RME, αT and CSL in tocopherol stripped fish oil. 79

Figure 3.13 Effect of added PS, RME, αT and CSL on the development of secondary oxidation products, as measured by pAV, in tocopherol stripped fish oil.. 81

Figure 3.14 Mean (n=3) iron (II) chelating effect of CSL, soybean derived PS and RME. Error bars indicate the standard deviation. 86

ABSTRACT

The search for natural antioxidants for use in food and nutritional supplements led to an in-depth investigation into the antioxidant effect of a commercial sunflower derived lecithin (CSL), consisting of high levels of phosphatidlyserine (PS) and smaller quantities of other phospholipids (PL) in a medium chain triglyceride base, stabilized with rosemary extract (RME) and DL-α-tocopherol (αT). This ingredient was found to have an antioxidant effect in fish oil stored at 40 °C under accelerated oxidation conditions. Therefore, the antioxidant effects of the three main active components in CSL, PS, RME and αT, were assessed in fish oil by monitoring the formation of primary and secondary oxidation products during oxidation.

PS was found to chelate metal ions and form Maillard browning products, both of which may have contributed to a slight protection against formation of primary oxidation products; PS did not offer any protective effect against the formation of secondary oxidation product.

RME was able to chelate metal ions and, in combination with PS, had a synergistic protective effect against the formation of hydroperoxides, the major primary oxidation products. However, there was no evidence of synergism with respect to secondary oxidation and the overall antioxidant effect of RME alone and PS+RME was not as great as CSL.

αT seemed to provide much of the antioxidant effect of CSL with respect to secondary oxidation products. The combination of RME and αT provided a hydroperoxide protective effect similar to CSL during early stages of oxidation and overall, the combination of PS, RME and αT had an equivalent protective effect against the rate of hydroperoxide development compared to CSL. However, the high cost of a lecithin rich in PS, such as CSL, or pure PS limits their industrial usefulness. Further investigation is recommended on the combination of RME and αT and their ability to achieve an equivalent or greater antioxidant effect as CSL.

LIST OF ABBREVIATIONS AND SYMBOLS USED

AH	Chain breaking antioxidant
A•	Chain breaking antioxidant radical
ANOVA	Analysis of variance
AOCS	American Oil Chemists' Society
APE	Acyl-phosphatidylethanolamine
αT	DL-α-Tocopherol
BHA	butylated hydroxyanisole
BHT	Butylated hydroxytoluene
°C	Degrees Celsius
CD	Conjugated dienes
CMC	Critical micelle concentration
CSL	Commercial sunflower derived lecithin
DC_4PC	1,2-dibutyryl-*sn*-glycero-3-phosphatidylcholine
DHA	Docosahexaenoic acid
DOPC	Dioleoyl phosphatidylcholine
DPPA	Dipalmitoyl phosphatidic acid
DPPE	Dipalmitoyl phosphatidylethanolamine
DPPG	Dipalmitoyl phosphatidylglycerol
DPPS	Dipalmitoyl phosphatidylserine
EDTA	Ethylenediamine tetraacetic acid
EPA	Eicosapentaenoic acid
GC-FID	Gas chromatography-flame ionization detector

GOED	Global Organization for EPA and DHA
HPLC	High pressure liquid chromatography
MCT	Medium chain triglycerides
ME	Methyl esters
Mixed T	Mixed tocopherols
n-3	Omega-3
ND	Not detected
PA	Phosphatidic acid
*p*AV	*p*-Anisidine value
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidlyinositol
PL	Phospholipids
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
PV	Peroxide value
R•	Alkyl radical
RH	Unsaturated fatty acid
RME	Rosemary extract
RO•	Alkoxyl radical
ROO•	Peroxyl radical
ROH	Aldehyde

ROOH	Hydroperoxide
TBARS	thiobarbituric acid-reactive substances
TLC	Thin layer chromatography
α	Alpha
β	Beta
δ	Delta
γ	Gamma

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CHAPTER 1 INTRODUCTION

1.1 BACKGROUND

An observation made by researchers in the early 1970s sparked an interest in fatty acids within marine species, particularly long-chain polyunsaturated fatty acids (PUFA) (Bang *et al.*, 1971, Bang *et al.*, 1976). These researchers noted that the Inuit population who ate large amounts of marine species had lower incidences of mortality due to coronary heart disease, as well as the absence of diabetes mellitus, compared to the Danish population (Bang *et al.*, 1971). This observation was a critical starting point for the numerous studies that followed, leading to a substantial body of evidence indicating the health benefits of long-chain PUFA, specifically, the omega-3 (n-3) fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Figure 1.1). Today, EPA and DHA are recognized for their anti-inflammatory, cardio protective and anti-thrombotic effects, to name a few of their benefits (Siriwardhana *et al*., 2012, DiNicolantonio *et al*., 2014, Simpopoulos, 1991). Even though EPA and DHA are not in the strictest sense classified as essential fatty acids, as they can be synthesized from α-linolenic acid, their synthesis in humans is very inefficient (Simpopoulos, 1999, Anderson & Ma, 2009). Therefore, most of an individual's EPA and DHA must be derived from their diet. It is not surprising that EPA and DHA, generally from fish oil, are being added to food products. Fish oil nutritional supplements are also gaining popularity. However, the chemical structures of these fatty acids make them susceptible to oxidation. Unsaturated fatty acids are more vulnerable to oxidation than saturated fatty acids, and the rate of oxidation increases with

each additional double bond (Frankel, 2005, p. 21). Therefore, EPA and DHA with five and six double bonds, respectively, are highly vulnerable to degradation due to oxidation.

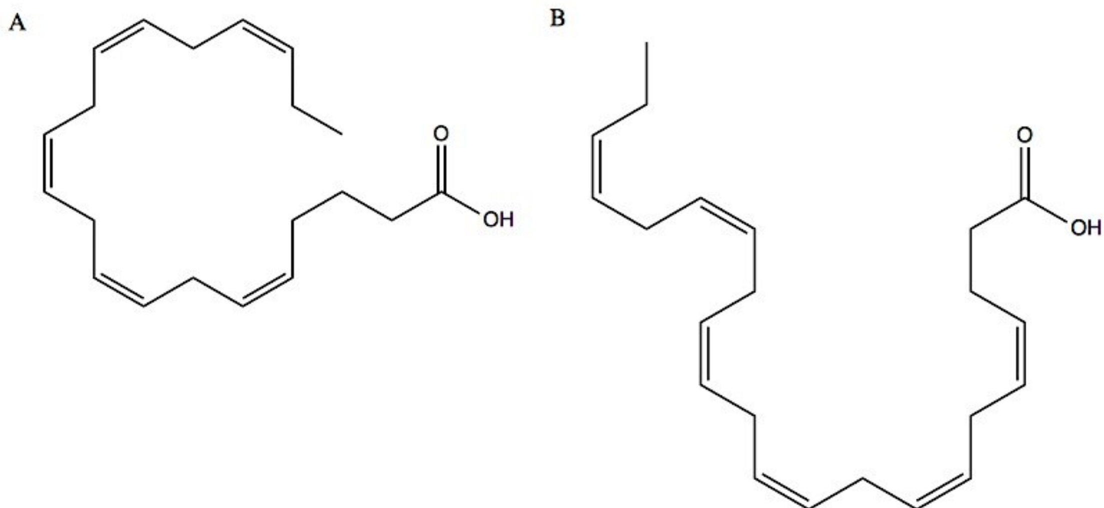


Figure 1.1 Chemical structure of: A) eicosapentaenoic acid and B) docosahexaenoic acid.

The use of antioxidants to create shelf stable products is a necessity; however, consumers are becoming increasing concerned about the ingredients in the products they purchase. Products containing "all natural" ingredients are perceived as being healthier and safer. In addition to consumer perception, the use of synthetic antioxidants has been restricted in several countries due to possible toxic or other undesirable effects on humans (Branen, 1975). This shift in thinking has led manufacturers to search for novel natural antioxidants or alternatively, re-investigation of natural antioxidants that have been used in years prior to the development of synthetic antioxidants.

Phospholipids (PL), in the form of lecithin (mixtures of PL produced as a by-product during processing of vegetable oils), were one of the earliest antioxidants used to protect

triacylglycerol oils and fats (Lea, 1957). Early research suggested that a fraction of lecithin, "kephalin," an acidic phosphate, possessed most of the antioxidant effect. Kephalin was also described as phosphatidylethanolamine (PE)-rich lecithin and Lea (1957) suggested that its activity arose from synergy with phenolic compounds such as tocopherols, through the inactivation of catalytic trace metals. Other studies have suggested that PLs' mechanism of antioxidant activity may be due to their metal chelation activity (Evans *et al*., 1954, Lunde *et al.*, 1976, Dacaranhe & Tero, 2001a), formation of Maillard browning compounds (Husain *et al*., 1986, King *et al*., 1992b) or synergistic activity with primary antioxidants (Hudson & Ghavami, 1984, Kashima *et al.*, 1991, Bandarra *et al*., 1999). The main PL in animal derived lecithin (from milk, eggs, and brain) are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) (van Nieuwenhuyzen & Tomas, 2008). PC is the most abundant PL in plants and animals as well as in plasma (AOCS Lipid library, 2014). Therefore it is generally the most plentiful PL in vegetable lecithin.

The production of vegetable lecithin begins with extraction of oil from seeds through solvent extraction or expulsion directly from the crushed seeds. The extracted oil is mixed with water in a process known as hydration. As the PL become "hydrated" they form a gum with a different density than the oil (van Nieuwenhuyzen & Tomas, 2008). Degumming of the oil then separates the PL, in the form of a wet gum, from the oil through centrifugation. This is an essential step in oil refining because removal of phospholipids reduces losses of oil due to emulsification, foaming during deodorization, and charring during heating, and reduces carry through of metal ions bound to the phospholipids (Weng & Gordon, 1993). Therefore, refined edible oils contain only very

small amounts of PL. The wet gum is dried, using a thin-film evaporator, to a moisture content of < 1% (van Nieuwenhuyzen & Tomas, 2008). Drying time is generally minimized to reduce darkening of the lecithin due to Maillard browning. However, the natural brown colour of lecithin may not be entirely due to Maillard browning compounds. Lecithin's colour may also result from the presence of carotenoids, melanoids and porphyrins (Scholfield & Dutton, 1954, van Nieuwenhuyzen & Tomas, 2008). The last step in lecithin processing is cooling of the lecithin in a heat exchanger to < 50 °C to reduce post-darkening (van Nieuwenhuyzen & Tomas, 2008).

The PL profile of lecithin can vary depending on the source from which is it derived. Unlike animal lecithin, vegetable lecithin does not generally contain measurable amounts of PS with PC, PE and PI making up the major PL in most vegetable lecithin (van Nieuwenhuyzen & Tomas, 2008).

Lecithin can be "refined" or modified to produce a product with a specific PL profile that can be used as a valued added ingredient. This can be desirable for delivery of a particular PL for medicinal or nutritional purposes (i.e. PS or PC) or to serve as emulsifiers or antioxidants. PS is found in highest quantities in animal brain and milk PL; however, market production of PS extracted from brain tissue ceased in the early 1990s with the bovine spongiform encephalopathy outbreak (van Nieuwenhuyzen & Tomas, 2008). PS is only found in very small quantities in vegetable lecithins (less than 1%) (van Nieuwenhuyzen & Tomas, 2008). Therefore, PS rich lecithins are commonly produced enzymatically from soy lecithin by the trans-phosphatidylation of PC with serine in the presence of D-phospholipase (de Ferra & Massardo, 2001).

1.2 LIPID OXIDATION AND OXIDATION PRODUCTS

The structure of the fatty acids comprising an oil is not the only factor affecting its stability. The presence of oxygen, trace metals, enzymes and hydroxy compounds as well as heat and light exposure all influence the rate of oxidation (Yanishlieva-Maslarova, 2001). Lipid oxidation is generally initiated by an external factor such as light, heat or oxygen. Briefly, oxidation is comprised of three phases: initiation, propagation and termination (Ingold, 1961). These phases are proceeded by the induction period, or lag phase, in which little oxidation occurs; many antioxidants act to extend this period. Initiation, as the name suggests, occurs when an initiator (such as singlet oxygen) reacts with the unsaturated fatty acid (RH) at the carbon alpha to a double bond in the hydrocarbon tail to remove a hydrogen atom. This in turn forms an alkyl radical (R•) (1). The alkyl radical (R•) can then react with additional oxygen to form a peroxyl radical oxidation product (ROO•) (2). Peroxyl radicals (ROO•) are quenched by abstracting hydrogen atoms from carbon atoms adjacent to double bonds in other unsaturated fatty acids to form primary oxidation products, hydroperoxides (ROOH) (3). The abstraction of a hydrogen atom creates another alkyl radical that is able to in turn react with oxygen; thus, propagation proceeds as a series of chain reactions. The decomposition of hydroperoxides can be accelerated with the addition of a free radical or reactive metal ions such as copper and iron. This chain reaction process continues until the free radicals begin reacting with each other to form non-radical compounds. The formation of non-reactive compounds marks the beginning of the termination phase. As the free radicals begin to decompose into non-reactive compounds, the oxidation cycle ends.

Hydroperoxides are unstable molecules that, over time, break down to form an alkoxyl radical (RO•) that can in turn form secondary oxidation products (aldehydes, ketones, alcohols and hydrocarbons). Aldehydes form through a reaction known as β-scission (Frankel, 2005, p. 10). In β-scission, the reactive alkoxyl radical can cleave the aliphatic chain of fatty acids, resulting in an aldehyde (ROH) and alkyl radical (R•) (Frankel, 2005, p. 10). The aldehydes that are formed are thought to give rise to off flavours and odours noted with rancid oils. Therefore, they are often monitored as a measurement of oil quality. The alkyl radical can react with a hydrogen atom, oxygen or a hydroxyl radical to form a hydrocarbon, hydroperoxide or an alcohol, respectively. Secondary oxidation products are also highly reactive and can initiate chain reactions *in vivo* that are thought to contribute to pathogenesis of cancer, atherosclerosis, heart and allergic diseases (Valko *et al*., 2007). Over time, the levels of hydroperoxides begin to drop as the formation of secondary oxidation products exceed the formation of hydroperoxides. Therefore, oxidative stability of food products and dietary supplements containing fish oil, or other oils rich in long-chain n-3 PUFA, is important economically as well as for preservation of health.

$$\mathrm{RH} \rightarrow \mathrm{R}\bullet + \mathrm{H}\bullet \qquad (1)$$

$$\mathrm{R}\bullet + \mathrm{O_2} \rightarrow \mathrm{ROO}\bullet \qquad (2)$$

$$\mathrm{ROO}\bullet + \mathrm{RH} \rightarrow \mathrm{ROOH} + \mathrm{R}\bullet \qquad (3)$$

1.3 Antioxidants

Antioxidants can be added to the system to prolong the induction period or interrupt the chain reaction of propagation, to slow oxidation of a substrate. Antioxidants are often classified according to their mechanism of action. Primary, or chain-breaking, antioxidants slow oxidation by interfering with the initiation or propagation of the oxidation chain reaction (Frankel, 2005, p. 209). Chain-breaking generally occurs with an antioxidant donating a hydrogen atom to a chain-carrying peroxyl (4) or alkyl radical (5), thereby "scavenging" the free radical. The radical formed from the chain-breaking antioxidant is generally stabilized by resonance and is not reactive enough to initiate a new chain reaction (Ingold, 1961). Secondary, or preventative antioxidants, slow the rate of oxidation by reacting with compounds that can initiate the oxidation reaction (i.e. metal chelators), destroying hydroperoxides, or reinforcing the activity of another antioxidant (Antolovich *et al.*, 2002; Frankel, 2005, p. 216-218).

Chain breaking antioxidant (AH)

$$ROO\bullet + AH \rightarrow ROOH + A\bullet \quad (4)$$

$$R\bullet + AH \rightarrow RH + A\bullet \quad (5)$$

Metal catalyzed oxidation can occur by two mechanisms (Frankel, 2005, p. 22-23): 1) electron transfer, where the reactive metal oxidizes the fatty acid by removing an electron, thus generating a lipid radical; or 2) redox reactions with hydroperoxides to form an alkoxyl radical or a peroxyl radical. However, prevalence of hydroperoxides (even trace levels) in oils reduces the likeliness of metals reacting directly with the fatty acids; decomposition of hydroperoxides occurs more often (Gordon, 2001, p. 16-17).

Metal chelators react with transition metals so that they cannot initiate the formation or decomposition of hydroperoxides. Common metal chelators are ethylenediamine tetraacetic acid (EDTA), citric acid and phosphoric acid.

Hydroperoxide destroyers inhibit oxidation through a non-radical reaction. Such antioxidants induce the decomposition of hydroperoxides into inactive products through hydrogen donation or reduction (Frankel, 2005, p. 218). This reaction results in the formation of stable lipid alcohols or other inactive compounds. Reducing agents are examples of hydroperoxide destroyers.

The combination of two or more types of antioxidants can often improve the substrate's resistance to oxidation in a way that is more than additive. Compounds that display this type of relationship are said to have a synergistic effect with one another. One of the compounds in the combination is generally less active or even inactive on its own; this compound is known as the synergist (Ingold, 1961). Synergists' activity may arise from their ability to regenerate the primary antioxidant via transfer of a hydrogen atom, decomposing hydroperoxides (thereby preventing their decomposition into radicals), or by chelating reactive metals. Polyvalent organic acids, amines and hydroxy acids, including phospholipids, are considered synergist (Pokorný, 2003a, p. 33). Synergists are commonly added to antioxidant blends containing phenolic compounds.

1.3.1 Phenolic Antioxidants – Tocopherols and Rosemary Extract

The chemical structure of an antioxidant determines its mechanism of action. Most natural antioxidants are phenolics with the hydroxyl groups in the *ortho* or *para* position (Frankel, 2005, p. 211). These structures are more active than phenolics with hydroxyl groups at the *meta* position because they are readily able to donate a hydrogen atom and form antioxidant radicals that are stabilized by resonance (Frankel, 2005, p. 211). Donation of a hydrogen atom breaks the chain reaction, thereby slowing the propagation phase of oxidation and increasing the induction period. Therefore, phenolic compounds are considered primary antioxidants. On their own, phenolic antioxidants provide limited oxidative protection, but when combined with a synergist they can be very efficacious (Yanishlieva & Marinova, 2001).

Tocopherols, an example of phenolic compounds, are endogenously present in most plants (Pokorný, 2003a, p. 32). Their high natural prevalence suggests their biological importance and they are commonly added to oils to increase stability. In nature, there are four tocopherol homologs, α, β, δ and γ, which differ in the substitution around the aromatic ring (Figure 1.2). α-Tocopherol, with three methyl groups around the aromatic ring, is the most biologically active (*in vivo*) antioxidant of the tocopherols; however, it is less active in bulk oils or food systems (Pokorný, 2003a, p. 33). β- and γ-tocopherols have double methyl substitution around the aromatic ring, and have been found to be the most active of the tocopherols in bulk oil systems. However, the antioxidant activity of the various homologs appears to differ depending on the oxidative medium and the storage conditions (Wagner & Elmadfa, 2000).

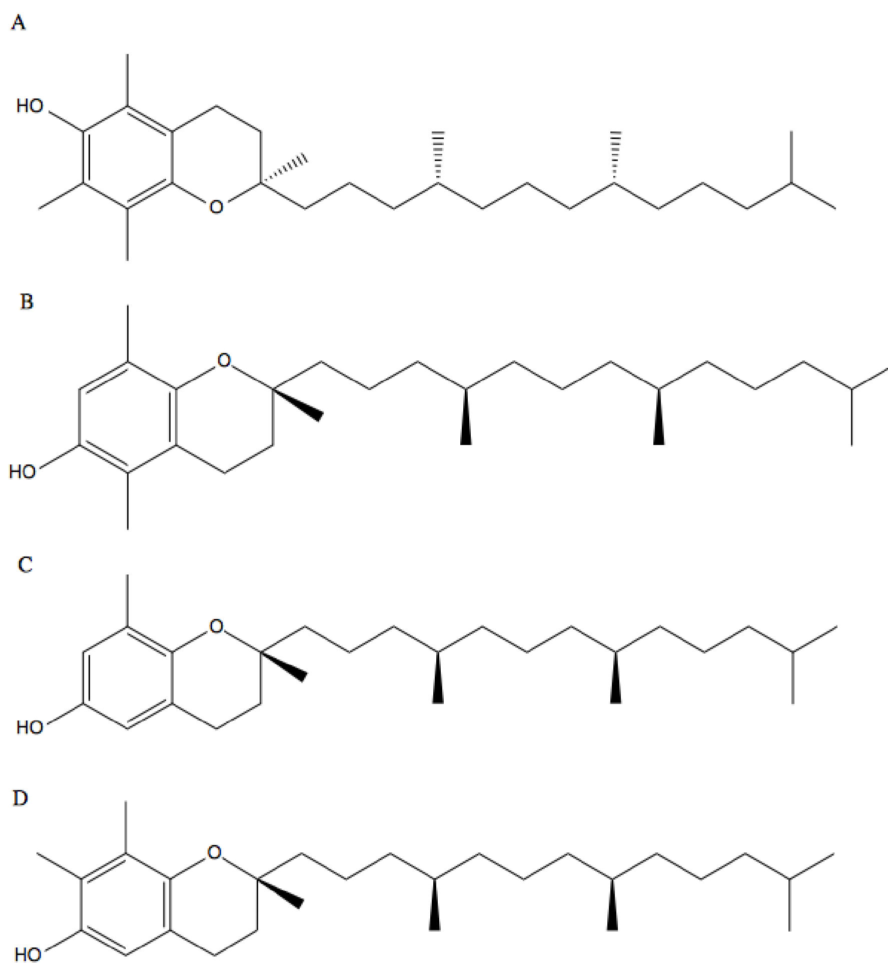


Figure 1.2 Chemical structures of: A) α-tocopherol, B) β-tocopherol, C) δ-tocopherol, D) γ-tocopherol.

Tocopherols act by competing with the substrate (i.e., fatty acids) in the system to donate a hydrogen atom to the chain-propagating peroxyl radical. Tocopherols generally have a faster reaction rate than lipids and they therefore react preferentially with the peroxyl radical. α-Tocopherol, the most common tocopherol naturally present in fish oil, reacts with the peroxyl radical to form a tocopherol radical that is stabilized by resonance. α-Tocopherol can alternatively be oxidized in the presence of air to form an electron acceptor (α-tocopheroquinone) (Frankel, 2005, p. 226). α-Tocopheroquinone can

compete with oxygen for alkyl radicals, slowing the formation of alkoxyl radicals. Tocopherols' activity is concentration dependent and at high concentrations they can act as pro-oxidants.

Tocopherols' efficacy is often improved with the presence of a synergist that is able to regenerate the tocopherol parent from the tocopherol radical. Ascorbic acid and rosemary extract have been found to have this regeneration effect in lipid systems (Frankel, 2005, p. 228; Wanda & Fang, 1992, Hopia *et al.*, 1996; Casarotti & Jorge, 2012). As well, rosemary extract's synergistic relationship has been suggested to be due to its ability to chelate metal ions (Wanda & Fang, 1992). Tocopherol homologs have also been found to act synergistically with one another; for instance, Wagner and Elmadfa (2000) found a concentration dependent, synergistic relationship between γ- and δ-tocopherols in thermal oxidation of olive and linseed oil. Tocopherols' activity can also be inhibited by metal ions; therefore, their activity can be enhanced in a system containing metal ions when metal chelators are also present.

Herbs and spices have been used as antioxidants in food products and folk medicine for centuries. Most of their activity is due to the presence of phenolic compounds. Of the herbs commonly used for their antioxidant activities, rosemary (*Rosmarinus officinalis L.*) is thought to contain the most active compounds and was the first natural extract from the *Lamiaceae* family marketed for its antioxidant effects (Yanishlieva & Marinova, 2001). Rosemary contains a number of phenolic diterpene compounds contributing to antioxidant activity, including carnosol, carnosic acid, rosmanol, epirosmanol and

isorosmanol (Erkan *et al.*, 2008; Richheimer *et al*., 1996). Of the many phenolic diterpenes in rosemary extract, carnosol and carnosic acid are thought to provide most of the oxidative protection (Figure 1.3) (Chen *et al.*, 1992). The antioxidant activity of carnosol and carnosic acid have been studied extensively; these compounds have been found to be radical scavengers, iron chelators and inhibitors of lipid peroxidation (Aruoma *et al*., 1992). They have been found to have a greater antioxidant effect, monitored by measuring the induction period with the Rancimat method, than the synthetic antioxidants, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Chen *et al.*, 1992).

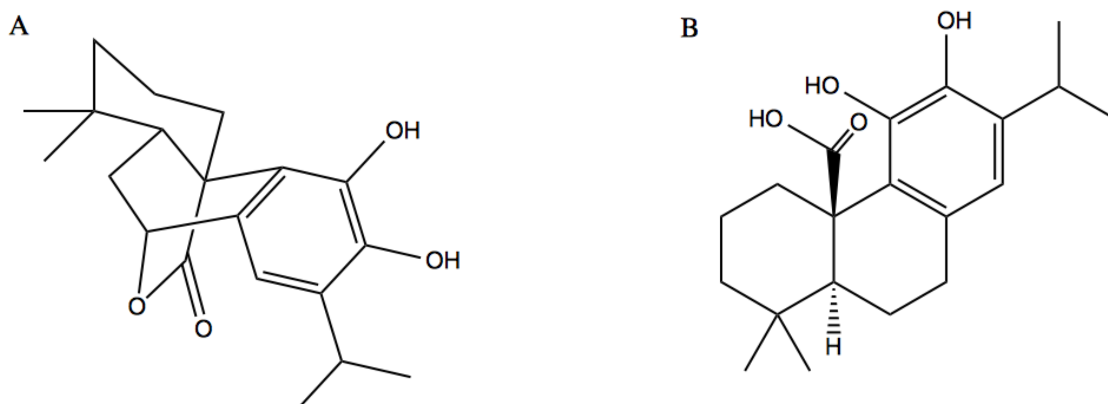


Figure 1.3 Chemical structures of: A) carnosol and B) carnosic acid

1.4 PHOSPHOLIPIDS AS ANTIOXIDANTS

Several researchers have investigated the antioxidant effects of a number of PL. However, these studies often used oils containing endogenous tocopherols as an oxidative medium or they failed to indicate if tocopherols were present. This omission makes it difficult to determine if PL function as independent antioxidants or if their activity arises

due to synergy. The fatty acid profile of the oxidative medium has been thought to play a role in the antioxidant activity of PL (Nwosu *et al*., 1997). Therefore, the information gathered for one oil type may not be transferrable to other oxidation mediums, and variations in oxidative mediums and conditions are likely responsible for the contradictory and inconclusive results in literature. Here, I summarize the potential mechanisms that may produce an antioxidant effect in PL and review the recent work in this area.

1.4.1 Metal Chelation

PL have been shown to sequester reactive metal ions and, therefore, their antioxidant activity may be due to metal chelation (Lunde *et al.*, 1976). Free iron can initiate oxidation by generating reactive oxygen species and promoting the breakdown of hydroperoxides, which feeds the free-radical chain reaction. Therefore, by binding reactive metal ions, such as iron, PL may be able to slow or reduce oxidation. Literature suggests that PL's chemical structure may impact their metal chelation activity; not all PL are able to bind metals to the same extent. Yoshida *et al.* (1991) found that PL with anionic polar head groups, such as PS, have a greater affinity for binding positively charged iron ions, compared to neutral or positively charged PL. Two separate studies (Yoshida *et al.*, 1991, Dacaranhe & Terao, 2001a) both found that PS greatly reduced ionic iron induced oxidation, whereas PE and PC showed significantly less oxidative protection (Yoshida *et al.*, 1991, Dacaranhe & Terao, 2001a). Yoshida *et al.* (1991) found that both PS with saturated and unsaturated fatty acid moieties protected against ionic iron induced oxidation, monitored by lipid peroxidation, indicating that the anionic PL

head group was responsible for the antioxidant activity. In contrast, Dacaranhe and Terao (2001a) found that the dipalmitoyl (saturated 16 carbon fatty acid moieties) form of PS (DPPS) inhibited iron induced oxidation, monitored by measuring thiobarbituric acid-reactive substances (TBARS) in PL bilayers, whereas dipalmitoyl phosphatidic acid (PA) (DPPA) and phosphatidylglycerol (PG) (DPPG), other anionic acidic PL, did not show such inhibition. Interestingly, DPPS and DPPA were both found to bind equivalent amounts of iron, but only DPPS showed a great reduction in iron induced oxidation (Dacaranhe & Terao, 2001a). Therefore, it has been suggested that PS's antioxidant activity is not only related to its anionic head group's ability to bind metal ions; it may also be related to PS's ability to prevent the decomposition of hydroperoxides, slowing the chain reaction (Dacaranhe & Terao, 2001a). This activity would be unique to PS as other anionic PL (PG and PA) were not as effective in protecting against iron induced oxidation.

However, the oxidation medium plays an important role in the antioxidant effects of PL. In a complex matrix (cooked beef homogenates) containing heme iron, DPPS and bovine PS did not reduce the formation of TBARS, and therefore did not have an antioxidant effect; bovine PS showed a slight pro-oxidant effect (Dacaranhe & Terao, 2001b). DPPS and bovine PS were also ineffective in reducing the formation of TBARS in a fish oil emulsion containing heme iron (Dacaranhe & Terao, 2001b). This lack of oxidation protection in the cooked beef homogenates cannot be directly linked to PS's ability or inability to chelate iron due to the complexity of the beef homogenate system and the structure of the heme iron. In contrast, bovine PS was found to provide an antioxidant effect in sardine oil emulsions containing non-heme iron ions (ferric nitrate/ascorbic acid)

(Dacaranhe & Terao, 2001b). This protective effect was suggested to be related to the anion head group's ability to bind iron ions, as was suggested by Yoshida *et al.* (1991) previously. Dacaranhe and Terao (2001b) did not indicate whether tocopherol levels were measured in the sardine oil emulsion; therefore, the complete matrix of this oxidative medium is unknown.

1.4.2 Impact of Fatty Acid Moieties

In contrast to Yoshida *et al.'s* (1991) findings that suggest that the fatty acids attached to the PL head group have little impact on the antioxidant effect due to metal chelation, other studies have suggested that they do affect the PL's antioxidant activity (Husain *et al*., 1986, Nwosu *et al*., 1997). Fatty acids present in the PL vary depending on the source material and location in the tissue from which they are derived. PL containing more saturated fatty acids provide greater stability compared to PL with unsaturated fatty acid moieties, despite similar head groups, as assessed by hydroperoxide formation and measurement of induction time (Ranicmat method) (Husain *et al*., 1986, Nwosu *et al*., 1997). Husain *et al*. (1986) found a pro-oxidant effect of highly unsaturated PC and PE, measured by hydroperoxide formation. This effect was thought to be due to oxidation of the unsaturated fatty acids (Husain *et al.*, 1986, Koga & Terao, 1994). In addition to the degree of unsaturation, the fatty acid chain length also appears to have an impact on the antioxidant activity of PL. By monitoring the induction time using the Ranicmat method, Nwosu *et al.* (1997) found distearoyl (C18:0) PC and dibehenoyl (C22:0) PC slowed oxidation of salmon oil more so than dipalmitoyl (C16:0) PC, suggesting that saturated fatty acids containing a longer chain may provide additional antioxidant protection.

However, this effect was not seen in menhaden oil, again indicating that the oxidation medium also plays a role in PL antioxidant activity (Nwosu *et al*., 1997). By monitoring the formation of methyl linoleate hydroperoxides, Koga and Terao (1995) also found that radical scavenging activity of vitamin E was increased in combination with PC with a longer fatty acid acyl chain. Increasing the acyl fatty acid chain length has been found to increase thermodynamic properties, such as phase transition temperature, change in enthalpy and change in entropy, of PC and these changes may be related to differences in antioxidant activity (Mason & Huang, 1981). Further, if the acyl chains are not of sufficient chain length, a bilayer will not form (Mason & Huang, 1981). PL with high surface energy influence the formation of bilayers and it has been suggested that PL may act synergistically with antioxidants in bulk oils by forming a barrier at the air oil interface (Porter, 1980).

1.4.3 Phospholipids as Synergists with Tocopherols

PS has been investigated for its antioxidant activity in a variety of mediums (oil, emulsions, meat) with conflicting results. Using the Rancimat method and FIRA-Astell equipment, Hudson and Ghavami (1984) found induction periods increased in soybean oil containing tocopherols with the addition of dipalmitoyl PE (DPPE), DPPC and bovine PS; this observation indicates synergy between these PL and tocopherols. Synergy, noted by monitoring the peroxide value, was observed when DPPE and α-tocopherol were added to lard that was free of other natural antioxidants (Hudson & Ghavami, 1984). Similarly, by measuring the peroxide and carbonyl values, Kashima *et al.* (1991) found PE and PS acted synergistically with endogenous tocopherols in perilla oil to delay

autoxidation. However, unlike Hudson and Ghavami (1984), Kashima *et al.* (1991) found that PC did not have a synergistic effect. The perilla oil used in this study was rich in endogenous γ-tocopherol. They suggested the synergistic activity was due to PE and PS decreasing the decomposition of endogenous γ-tocopherol. Bandarra *et al.* (1999) also found synergy existed between PE and α-tocopherol and to a lesser extent, PC and α-tocopherol, in sardine oil. Maillard browning compounds were formed in sardine oil samples containing PE and PC, suggesting that the synergy could be due to the formation of these compounds. Alternatively, PE's ability to reduce quinones formed from oxidized phenolic compounds offers another mechanism for synergy (Bandarra *et al.*, 1999). In contrast, Husain *et al*. (1986) found saturated PC and PE did not display any synergism with α-tocopherol, when investigating the oxidation of methyl linoleate, and suggested that unsaturated fatty acids in the PL could be necessary for synergy. The conflicting results of these studies point to the complexity concerning PLs' mechanism of antioxidant activity. Results indicating a synergistic relationship depend on the PL structure and can be confounded by the presence of endogenous compounds not accounted for in the oxidative medium.

1.4.4 Amino-carbonyl Reactions

During oxidation, the primary amino group of PL may react with the carbonyl group of oxidized fatty acids chains (within the PL or the oxidative medium) to form heterocyclic residues with antioxidant properties in an amino-carbonyl reaction (Hidalgo *et al.*, 2005, Hidalgo *et al*., 2006). Oxidized PE was found to have greater antioxidant activity than native PE; this antioxidant activity was attributed to the formation of pyrrolized PL by

amino-carbonyl reactions (Hidalgo *et al.*, 2005). Since PS also has a primary amino group, it is expected to form pyrrolized PL, which provide additional antioxidant effects. However, the antioxidant effect of oxidized PE, measured by formation of TBARS, begins to decrease as the unsaturated fatty acids of the PL are also oxidizing and creating a pro-oxidant effect (Hidalgo *et al*., 2005). Some of the pyrrole derivatives can further react to polymerize; this polymerization is related to non-enzymatic browning (Hidalgo & Zamora, 1993, Zamora *et al*., 2000). PL with amino groups, such as PE, have been shown to form not only pyrrolized PL, but also Maillard browning compounds during oxidation. Both compounds are thought to have antioxidant activity (Zamora *et al*., 2005, Hidalgo *et al*., 2005).

1.4.5 Maillard Browning Compounds

Maillard browning reactions are common in food systems containing sugars and amino acids. The reaction is initiated by the reactive carbonyl group of a sugar or a lipid oxidation product with the nucleophilic amino group of an amino acid or nitrogen containing compound (Zamora & Hidalgo, 2011). The browning compounds that result are responsible for desirable colour changes as well as flavour development. Carbonyl compounds can form as a result of complex reactions between lipids and oxygen (Zamora & Hidalgo, 2011).

PS's free amine group is thought to react with aldehydes forming during oxidation of oils to form melanoidins, Maillard browning compounds (Lea, 1957). Melanoidins have been found to possess moderate antioxidant activity (Elizalde *et al.*, 1991). The antioxidant

activity of Maillard browning compounds arises from their ability to chelate metal ions, as well as their formation of reductones (strong reducing agents) and imides (Pokorný, 2003b, p. 338). It is also thought that PL containing unsaturated fatty acids can undergo browning reactions (Pokorný, 1981).

PE has been found to form Maillard browning compounds during oxidation, which in turn are suspected to increase the oxidative stability of sardine oil (Bandarra *et al*., 1999). In that study, the presence of α-tocopherol was found to protect PE from oxidation, determined by measuring the formation of hydroperoxides, and conjugated dienes and trienes, and therefore also reduced the development of Maillard browning compounds. However, even with a reduction in Maillard browning compounds, the combination of PE with α-tocopherol greatly increased the oxidative stability of the sardine oil. This indicates that while Maillard browning compounds may function as an antioxidant, the synergistic combination of PE and α-tocopherol proved to have a stronger antioxidant effect.

King *et al.* (1992b) found that PC increased the oxidative stability of salmon oil held at 180 ^{o}C. This antioxidant effect, monitored by changes in TBARS, was suggested to be due to Maillard browning reactions, indicated by monitoring changes in the samples' absorbance at 430 nm, as the oil with PC experienced a colour change over time. Husain *et al.* (1986) reported an antioxidant effect, determined with hydroperoxide content, of PC and PE upon heating. At lower temperatures (50 ^{o}C) these unsaturated PL has a pro-oxidant effect. Husain *et al.* (1986) suggested this antioxidant activity with heating was related to the formation of browning products that have the ability to chelate metal ions.

1.4.6 Reverse Micelles

The amphiphilic structure of PL allows them to associate into colloidal structures in the presence of water. The formation of these structures depends on the PL head group and fatty acid tails, as well as the concentration present. The lipid monomer concentration above which micelles first begin to form is known as the critical micelle concentration (CMC). Therefore, the CMC must be reached in order for colloidal structures to form (IUPAC, 1997).

In emulsion chemistry, the oil-water interface plays an important role in overall oxidative stability, as it is the location where either pro- or anti-oxidation reactions occur (Waraho *et al.*, 2011). Bulk oils contain trace amounts of water, making PLs' ability to form reverse micelles relevant to their possible antioxidant activity. When present at concentrations greater than the CMC (1000 μM), dioleoyl PC (DOPC) is thought to increase the free radical scavenging activity of α-tocopherol and Trolox at low concentrations (10 μM) in stripped soybean oil containing trace levels of water (Chen *et al.*, 2011). It was suggested that the increased protective effect was due to the increased partitioning of α-tocopherol into the microenvironment of the water phase in the reverse micelles contained in the bulk oil; therefore, α-tocopherol is better able to react with radicals in water phase that initiate oxidation reactions (Koga & Terao, 1995). However, the same concentration of DOPC alone had a pro-oxidant effect in stripped soybean oil and reduced the effectiveness of 100 μM of both α-tocopherol and Trolox, as measured by hydroperoxide formation and hexanal, in the same medium (Chen *et al.*, 2011). The ability to form reverse micelles appears to influence the oxidative effect; 1,2-dibutyryl-

sn-glycero-3- PC (DC_4PC) does not form reverse micelles, but has the same head group as DOPC (Chen *et al.*, 2010). Equivalent concentrations of DC_4PC (1000 μM) alone had no effect on oxidation of stripped soybean oil, measured by hydroperoxide formation and propanal, while DOPC alone had a pro-oxidant effect (Chen *et al*., 2010). Therefore, it would be suspected that concentrations of PL below the CMC would have no effect on oxidation. The literature on the effect of PL reverse micelles on oil oxidation is limited. However, the ability of PL reverse micelles to increase the antioxidant activity of free radical scavengers at low concentrations may be another mechanism in which PL acts as synergists to increase oxidative stability of oils.

1.5 OXIDATION MEASUREMENTS

Antioxidants act in different ways to inhibit oxidation, especially in complex systems such as lipids. Therefore, when investigating antioxidant effects of an ingredient, it is important to determine the inhibition effects on both primary and secondary oxidation products. Primary and secondary oxidation products can be measured directly by titrimetric methods (to measure formation of hydroperoxides which are commonly referred to as peroxides) and spectrophotometric methods (to measure aldehydes with α- and β-unsaturation), respectively.

A common measure of primary oxidation is through an idometric titration known as the peroxide value (PV) test. This method is based on the ability of peroxides in an oil sample to oxidize iodide to iodine. The iodine content can be determined through a

titration with sodium thiosulfate. This provides an indication of the peroxide radicals present in the oil at a particular time point. PV is expresses as milliequivalents of peroxides per kilogram of oil (meq kg^{-1}). The formation and decomposition of peroxides is a dynamic process. In early stages of oxidation, the formation of peroxides will dominate over decomposition. However over time, hydroperoxides will decompose and form secondary oxidation products such as aldehydes and ketones. These are much better correlated with the sensory perception of rancidity so they are often measured along with PV to provide a clearer picture of the oxidative quality of an oil. *p*-Anisidine value (*p*AV) can be used to measure unsaturated aldehydes, one of the breakdown products of peroxides. This method is based on the reaction of *p*-anisidine with aldehydes, mainly 2-alkenals and 2,4-dienals, in an acidic environment (Tomkin & Perkins, 1999). This reaction forms yellow products that absorb at 350 nm, which can be measured spectrophotometrically. The *p*AV is expressed as the absorbance of 1 g of oil in 100 mL mixture of isooctane and the *p*-anisidine reagent. In this work, the effect of PS on the formation of hydroperoxides was monitored by the PV method (American Oil Chemist's Society (AOCS) official method CD 8-53, 1997) and the formation of secondary oxidation products were measured via the *p*AV method (AOCS official method Cd 18-90, 1997).

Measuring the development of conjugated dienes is another method for monitoring the progression of oxidation. The abstraction of a hydrogen atom from a PUFA during oxidation results in stabilization through delocalization of the double bonds in the fatty acid. This changes the configuration of the double bonds in the PUFA to *cis* and *trans*

forms and creates conjugated double bonds. Conjugated dienes absorb at 233 nm and conjugated trienes absorb at 268 nm. Both conjugated dienes and trienes can therefore be measured spectrophotometrically to indicate the progression of primary oxidation.

The development of non-enzymatic browning compounds, such as Maillard browning compounds, can also indicate oxidation reactions are progressing. These compounds may form in the presence of PL containing an amine group and they are measured spectrophotometrically. The oil sample is dissolved in an organic solvent and the absorption is measured at 430 nm.

Oxidation of PL can also lead to the formation of pyrroles. These pyrrolized PL can be measured according to Hidalgo *et al.* (2004). Briefly, the oil sample is combined with 150 mM sodium phosphate (pH 7.0), containing 3 % sodium dodecyl sulfate. The resulting solution is treated with Ehrlich reagent (*p*–dimethylaminobenzaldehyde dissolved in an ethanol and hydrochloric acid solution) and is incubated at 45 °C for 15 min. The absorbance is measured at the maximum of 570 nm.

1.6 Objectives

A commercial sunflower derived lecithin (CSL) rich in PS was found to protect fish oil from oxidation. This CSL contains approximately 200 mg g^{-1} PS and lesser amounts of PC, phosphatidic acid (PA) (Figure 1.4), PI, PE, *N*-acyl-phosphatidylethanolamine (APE) and PG (Figure 1.5) in a medium chain triglycerides (MCT) base. The product is also

stabilized with rosemary extract (RME) and DL-α-tocopherol (αT). Please note that αT refers to the racemic stereoisomer mix used in these studies, while α-tocopherol refers to what was described in literature, the D-stereoisomer. Literature surrounding PS's antioxidant activity is conflicting. Therefore, this research sets out to determine if PS, being the major active ingredient in the CSL, is the source of the antioxidant activity. This knowledge will lead to better understanding of the applications for a potentially under-utilized antioxidant.

Specific project objectives were as follows:

1. Determine the concentration of PS to investigate based on CSL's antioxidant effect.
2. Determine if PS has an independent antioxidant effect in fish oil.
3. Determine if PS has a synergistic relationship with tocopherols typically present in fish oil or other primary antioxidants used to stabilize CSL.

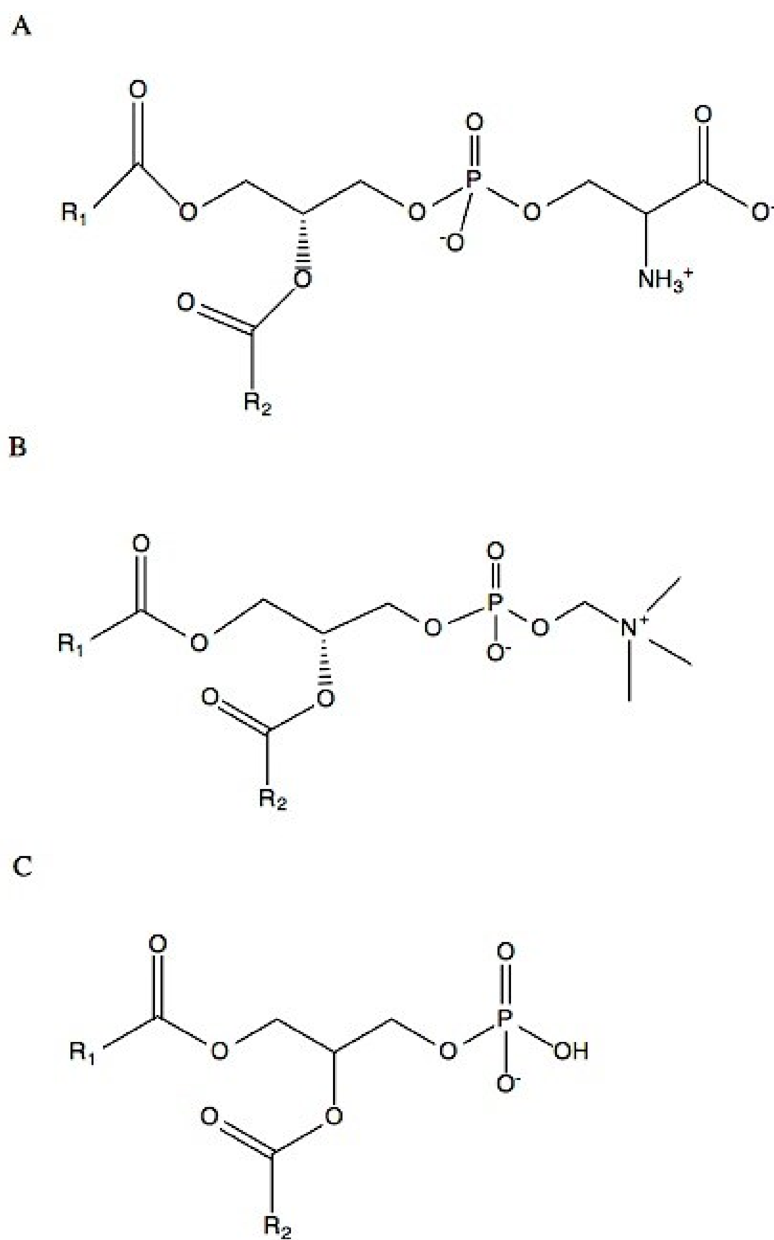


Figure 1.4 Chemical structures of PL contained in CSL: A) Phosphatidylserine (PS), B) Phosphatidylcholine (PC), C) Phosphatidic acid (PA).

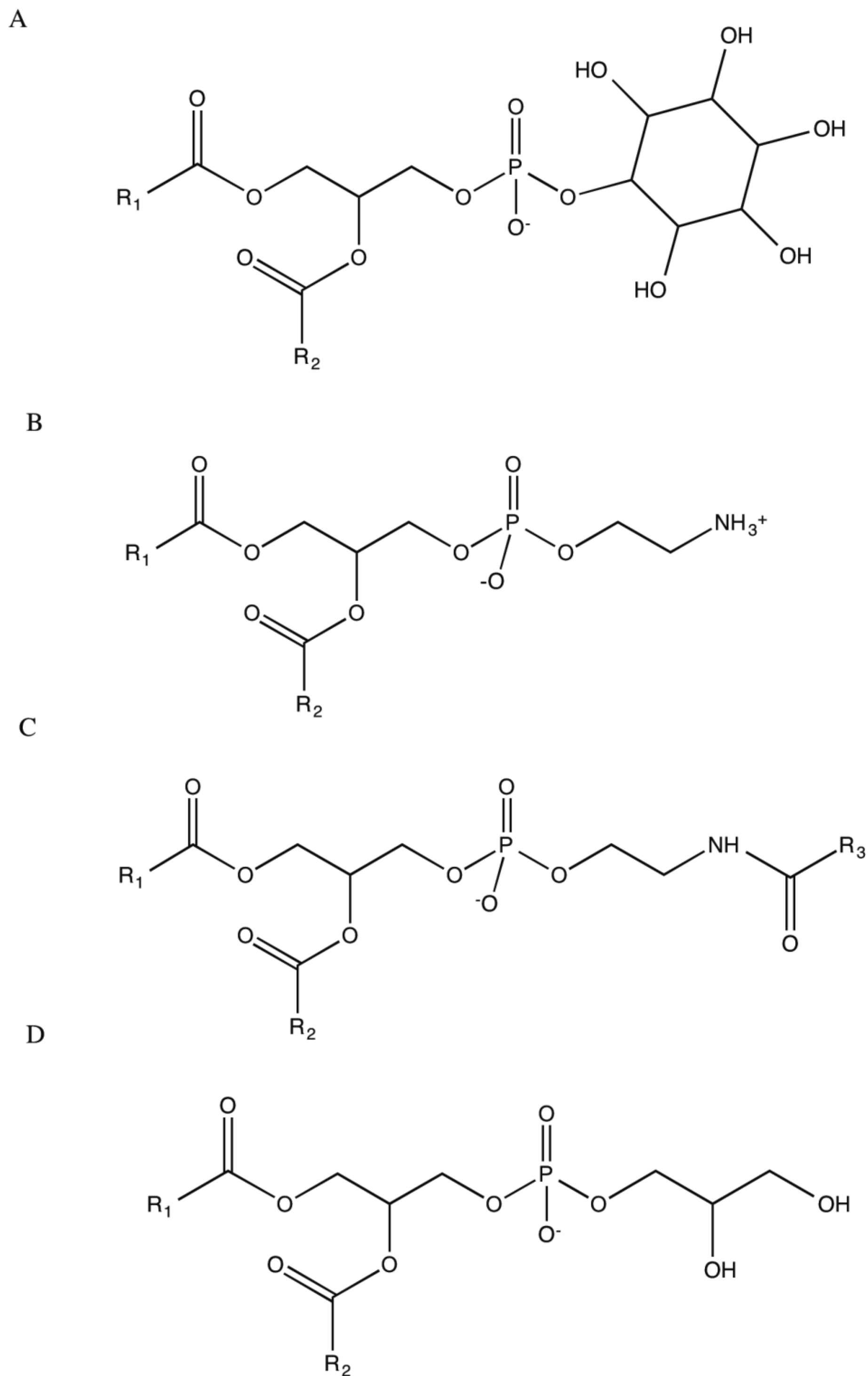


Figure 1.5 Chemical structures of PL contained CSL: A) Phosphatidylinositol (PI), B) Phosphatidylethanolamine (PE), C) N-acyl-phosphatidylethanolamine (APE), D) Phosphatidylglycerol (PG).

CHAPTER 2 CSL: DETERMINING POTENCY FOR OXIDATION STABILITY STUDIES

2.1 INTRODUCTION

Informal stability studies in our lab found CSL had greater antioxidant activity compared to more traditionally used RME, as analyzed by PV. However, CSL differs from typical plant derived lecithin in that it is rich in PS. Typically, PS is only found in quantities less than 1% in vegetable lecithins (van Nieuwenhuyzen & Tomas, 2008). CSL is derived from sunflower seeds, but it is standardized to contain a minimum of 200 mg g^{-1} PS. The lecithin is then diluted with palm derived MCT and stabilized with approximately 3 mg g^{-1} αT and 1 mg g^{-1} RME, containing a minimum of 5 % carnosic acid (Figure 2.1). Since this is a natural product it also contains smaller amounts of naturally occurring PL, including PC, PI, PA and minor amounts of PE, APE and PG.

CSL is oil soluble at low concentrations and it imparts minimal flavour at these levels. However, it is less soluble in oils containing high levels of long chain PUFA. It is golden to dark brown in colour, but at low concentrations it does not affect the colour of fish oil stored at ambient and 40 °C conditions. These properties make it a suitable candidate for industrial antioxidant applications. The goal of this initial study was to determine the concentration of CSL that provides the greatest antioxidant effect in refined fish oil.

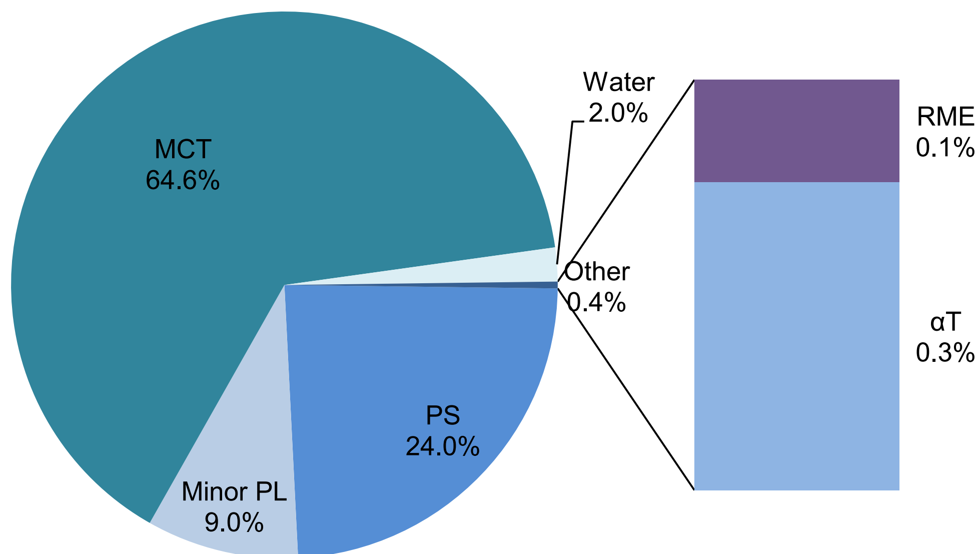


Figure 2.1 Typical composition of CSL in mass proportions.

2.2 Materials and Methods

Concentrations of CSL screened were selected to be within a range determined to be soluble with minimal or unperceivable effects on the fish oil's sensory properties. CSL, marketed as a PS rich ingredient, was added to a refined bulk fish oil (anchovy and sardine) at levels of 1 mg g^{-1}, 2.5 mg g^{-1}, 5 mg g^{-1}, 7.5 mg g^{-1} and 10 mg g^{-1} to determine the effect that varying levels of CSL has on oxidation. These concentrations provide approximately 0.2 mg g^{-1} to 2 mg g^{-1} PS, 1 µg g^{-1} to 10 µg g^{-1} RME and 3 µg g^{-1} to 30 µg g^{-1} αT along with minor PL. Some antioxidants, such as αT, while having an antioxidant effect at low concentrations, have a known pro-oxidant effect at high concentrations. Therefore, a range of concentrations of CSL were tested to determine the concentration

that had the greatest protective effect. Primary and secondary oxidation products were monitored by measuring the PV and *p*AV, respectively.

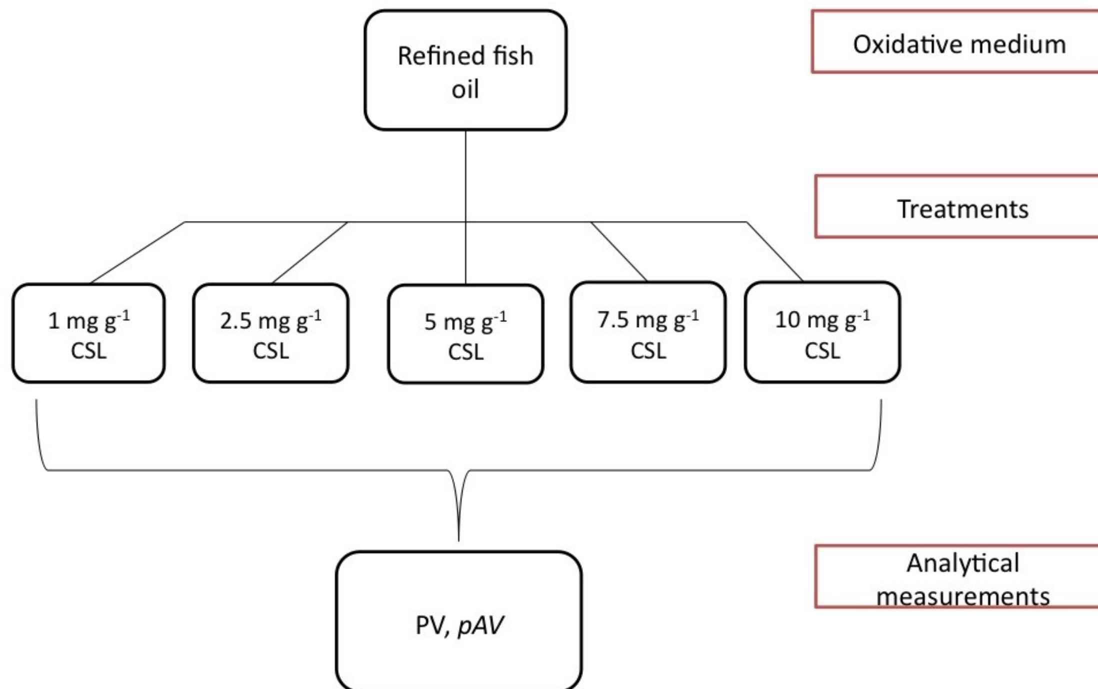


Figure 2.2 Experimental treatment and analytical measurements used to test the antioxidant activity of 1-10 mg g^{-1} CSL. Samples were stored in the dark, in open vials at 40 °C. A control of refined fish oil was tested concurrently.

2.2.1 Sample Preparation and Storage Conditions

Bulk fish oil, containing approximately 1 mg g^{-1} mixed tocopherols and 30% EPA+DHA, was combined with the appropriate amount of CSL by stirring for 10 min with a magnetic stir bar on a stir plate. Samples were then aliquoted in 7 ± 0.5 g quantities in 22 mL scintillation vials. Each treatment was prepared in triplicate. The control, refined fish oil, was handled in the same manner as CSL treatments.

Uncapped scintillation vials of each treatment and control (n=15) were stored in the dark under accelerated oxidation conditions (40 ± 3 °C). Three separate vials were used for each replicate measurement per time point. Sampling occurred over a range of time points from initial to day 8. Primary and secondary oxidation measurements were taken at six time points over the 8-day period. Sample remaining after PV analysis was nitrogen purged and frozen for *p*AV analysis when *p*AV measurements could not be completed on the same day.

2.2.2 Oxidation Measurements

Primary oxidation products were measured in triplicate using a modified version of the AOCS official method CD 8-53 (1997) for PV. The method was modified to measure the formation of peroxides in approximately 1 to 5 g samples depending on the expected level of oxidation. The Official method requires a sample of 5 g; however, it is common practice to test less sample when the PV is expected to be high (Wrolstad *et al*., 2005, p. D2.1.4). Aliquots of known mass were added to 250 mL stoppered flasks and dissolved in 30 mL of 3:2 (v/v) glacial acetic acid: chloroform. The samples were mixed with 500 µL of saturated potassium iodide and after exactly 1 min the reaction was stopped with the addition of 30 mL deionized water. The PV was determined by titration using 0.01 N standardized sodium thiosulfate in the presence of a starch indicator. The Official method uses sodium thiosulfate titrant with a normality of 0.1 N. A more dilute titrant

was used to achieve more accurate measurements for the earlier time points when the samples were expected to have low PV. The PV was calculated as follows:

$$PV = \frac{(S - B) \times N \times 1000}{W}$$

where S is the titration of the sample, B is the titration of the blank, N is the normality of the titrant and W is sample weight.

Secondary oxidation products were measured in triplicate using the AOCS official method Cd 18-90 (1997) for *p*AV. Briefly, aliquots of 0.5 g of sample were weighed into 25 mL volumetric flasks and were filled to the mark with isooctane. Absorbance measurements were recorded at 350 nm before and after 1.00 mL of *p*-anisidine solution was added to 5 mL of each test solution. The *p*AV was calculated as follows:

$$pAV = \frac{25(1.2 \times A_a - A_b)}{W}$$

where A_a is the absorbance of the solution containing the sample and *p*-anisidine solution, A_b is the absorbance of the solution containing the sample, and W is the sample weight. All solvents and reagents were purchased from Fisher Scientific (Guelph, ON) unless otherwise specified.

2.2.3 Statistical Analysis

All analyses were conducted in triplicate and results were analyzed using one-way analysis of variance (ANOVA) with a significance level of $p < 0.05$ (MiniTab 16.2.4). When significance was found, Tukey's multiple comparison test was used to determine which means were different from each other (significance was determined at a level of $p < 0.05$). All errors are reported as standard deviations.

2.3 RESULTS

All concentrations began showing a protective effect against the development of hydroperoxides after one day of storage under accelerated conditions and this protective effect remained throughout the 8 day study (Figure 2.3; ANOVAs were significant at a level of $p < 0.05$ for every time point). The concentrations of CSL had an equivalent antioxidant effect up to day 2. By day 6, the control had a PV of 119.4 ± 5.8 meq kg^{-1} and thus, it was removed from all further one-way ANOVA to avoid skewing results comparing the treatments. One-way ANOVA of only the treatments on day 6 found that the 5, 7.5 and 10 mg g^{-1} CSL samples had an equivalent antioxidant effect; only the 10 mg g^{-1} CSL sample had a significantly greater antioxidant effect than the 1 and 2.5 mg g^{-1} CSL samples (Tukey's test: $p < 0.05$). On day 7 the 10 mg g^{-1} CSL sample showed a significantly greater antioxidant effect than all other samples (Tukey's test: $p<0.05$) and it maintained this superior protection on the final day of the study, day 8 (Tukey's test: $p < 0.05$). The least protective effect was found with the 1 mg g^{-1} and 2.5 mg g^{-1} CSL samples over days 7 and 8. These samples had equivalent protection against hydroperoxide

formation (Tukey's test: $p > 0.05$). The 5 mg g^{-1} CSL sample had a lower PV than the 2.5 mg g^{-1} samples; however, the difference was not statistically significant on day 7, but by day 8, the 5 mg g^{-1} had a greater protective effect against the formation of hydroperoxides than the 2.5 mg g^{-1} sample (Tukey's test: $p < 0.05$). Similarly, the 7.5 mg g^{-1} CSL sample provide equivalent protection as the 5 mg g^{-1} CSL sample on day 7, but by day 8 the 7.5 mg g^{-1} CSL sample provided a statistically greater antioxidant effect than the 5 mg g^{-1} CSL sample (Tukey's test: $p < 0.05$).

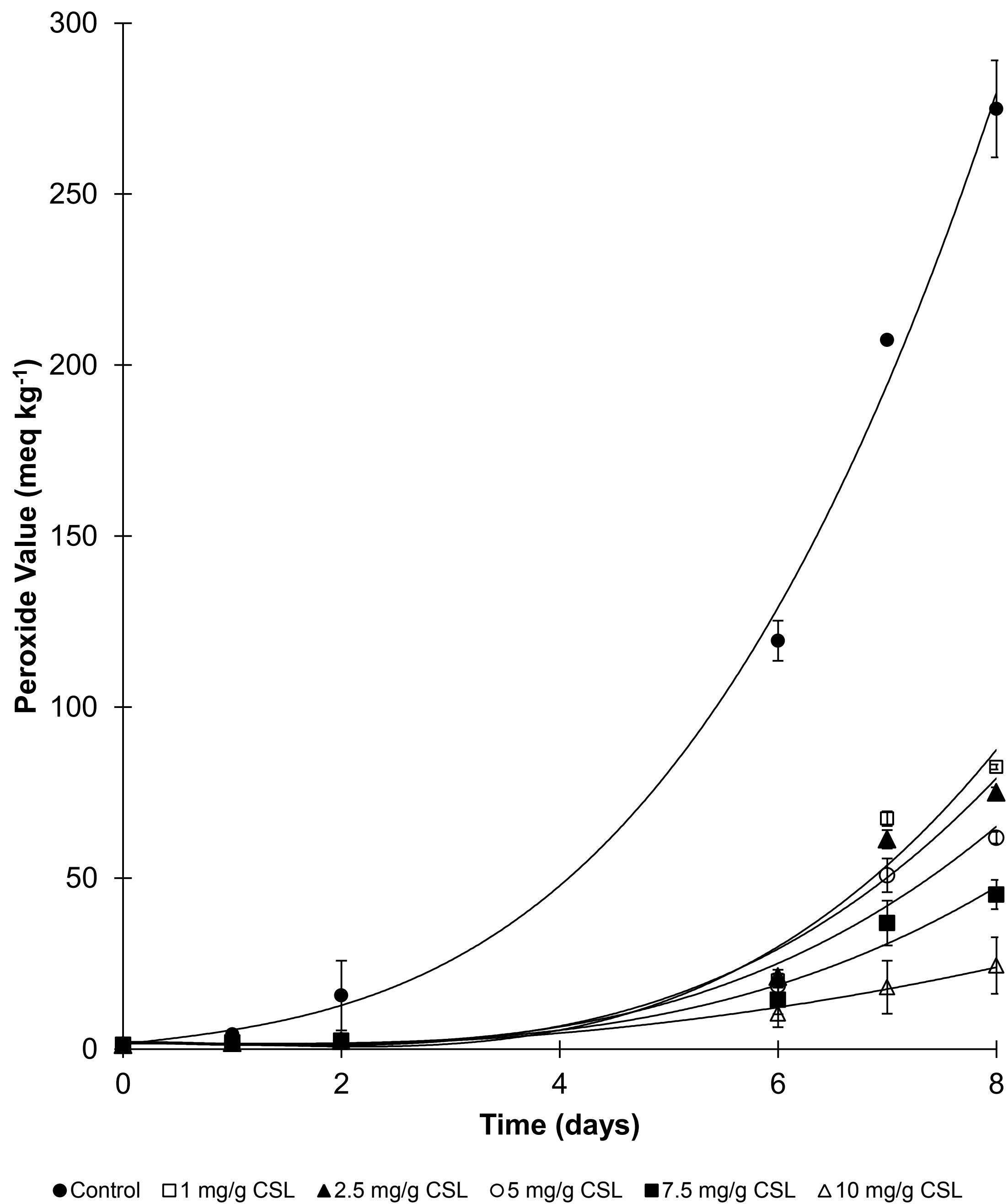


Figure 2.3 Effect of 1-10 mg g^{-1} CSL on the development of hydroperoxides in refined fish oil, measured by PV. For each sampling point, n=3 with error bars indicating standard deviation. Trend lines are added to guide the eye; they do not indicate a function.

The addition of CSL protected against the formation of secondary oxidation products, as measured by *p*AV, compared to the control (Figure 2.4; ANOVAs were significant at a significance level of $p < 0.05$ for every time point). After one day of storage, CSL began having a protective effect, with 10 mg g^{-1} CSL providing greater protection than 1 mg g^{-1}, but equivalent protection to all other inputs (Tukey's test: $p < 0.05$); however, by day 2 all treatments provided equivalent antioxidant effect. As in the analysis of the protection against hydroperoxides, the control was removed from day 6 to 8 one-way ANOVA as its *p*AV was much greater than all of the treatments (control *p*AV on day 6 = 29.8 ± 2.6). One-way ANOVA of only the treatments on day 6 found the same trend as the protection against hydroperoxides; the 5, 7.5 and 10 mg g^{-1} CSL samples had an equivalent antioxidant effect, and once again, only the 10 mg g^{-1} CSL sample had a significantly greater antioxidant effect than the 1 and 2.5 mg g^{-1} CSL samples (Tukey's test: $p < 0.05$). On the final two days of the study (day 7 and 8) the 10 mg g^{-1} CSL sample began to more clearly show its superior ability to protect against the development of secondary oxidation products as measured by *p*AV; the 10 mg g^{-1} sample provided significantly greater protection than all other treatments (Tukey's test: $p < 0.05$). The 1 and 2.5 mg g^{-1} CSL samples continued to display equivalent antioxidant effect over days 7 and 8 (Tukey's test: $p > 0.05$). The 5 and 7.5 mg g^{-1} CSL samples also had statistically equivalent antioxidant effect on day 7 (Tukey's test: $p > 0.05$). By the end of the study the order of greatest protection against the development of secondary oxidation products, as measured by *p*AV, was: 10 mg g^{-1} CSL > 7.5 mg g^{-1} CSL > 5 mg g^{-1} CSL > 2.5 mg g^{-1} CSL = 1 mg g^{-1} CSL. This was essentially the same trend that was found in with the protection against hydroperoxides.

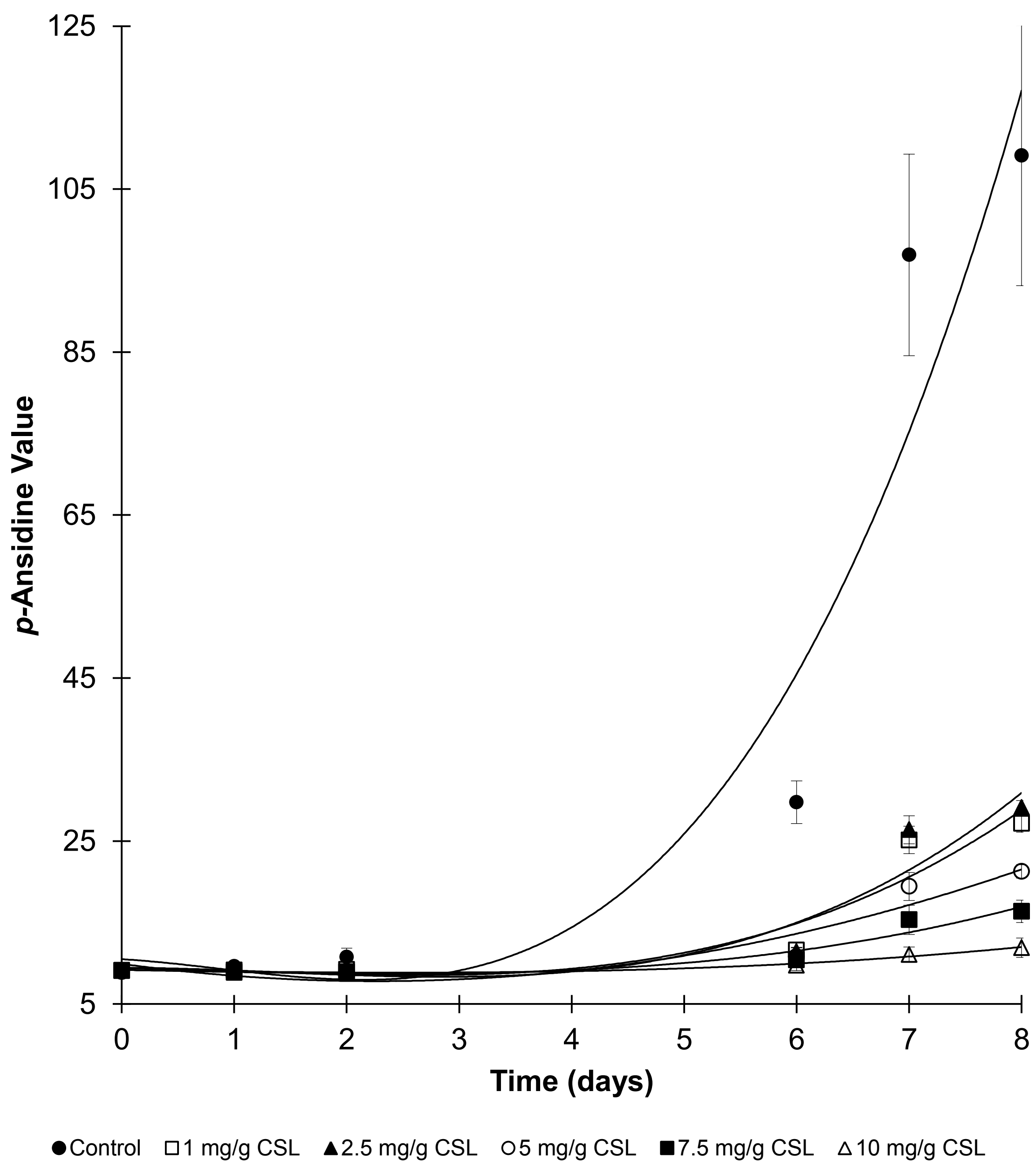


Figure 2.4 Effect of concentrations of CSL ranging from 1–10 mg g^{-1} on the development of secondary oxidation products in refined fish oil, measured by *p*AV. For each sampling point, n=3 with error bars indicating standard deviation. Trend lines are added to guide the eye; they do not indicate a function.

2.4 DISCUSSION

The purpose of this study was to determine if CSL has varying antioxidant activity with different levels of inclusion to decide the concentration of PS to be investigated in further stability studies. Not only is it more costly to add higher amounts of CSL to oil, there are also solubility, sensory and oxidative stability concerns. Antioxidants may have an equivalent protective effect over a range of inputs. Therefore, it would be wasteful to include more CSL than is necessary and the minimum concentration providing the greatest antioxidant effect had to be determined. Also, PL have been found to have an antioxidant effect at lower concentrations, but at higher inputs they can have a pro-oxidant effect (Yoon & Min, 1987). Thus screening a range of concentrations of CSL (1-10 mg g^{-1} containing approximately 0.2-2 mg g^{-1} PS) was necessary to establish CSL's antioxidant effect in refined, tocopherol enriched fish oil.

Yoon and Min (1987) found PC added at 0.3 mg g^{-1} increased oxygen consumption in soybean oil, accelerating oxidation (Yoon & Min, 1987). This pro-oxidant activity was thought to result from PL's ability to decrease the surface tension of the oil due to their amphiphilic structure, increasing the rate of oxygen diffusion (Yoon & Min, 1987). However contrary to Yoon and Min's (1987) findings, here CSL had a protective effect against the development of hydroperoxides and secondary oxidation products, as measured by PV and *p*AV, respectively, at all levels tested (1-10 mg g^{-1}, approximately equivalent to 0.2-2 mg g^{-1} PS). These results agree with Judde *et al.*'s (2003) study that found 10 to 50 mg g^{-1} lecithin had an antioxidant effect in refined rapeseed oil, as measured by PV and Rancimat method. However, the authors noted it was difficult to

achieve a homogenous dispersion of lecithin and oil at higher inputs and, therefore, they focused most of their research on 10 mg g^{-1} lecithin.

There are a number of possible reasons for the difference in results observed by Yoon and Min (1987) and this study. Firstly, Yoon and Min (1987) found the pro-oxidant effect was reversed in the presence of 1 μg g^{-1} ferrous iron. Metal analysis (analyzed by Research and Productivity Council (RPC), Fredericton, NB, Canada) of multiple batches of CSL consistently found 1 μg g^{-1} iron present. It was suggested that if PL are not chelating metals, their hydrophilic groups associate with the surface of the oil lowering the surface tension which results in enhanced oxygen solubility and leads to their pro-oxidant effect (Yoon & Min, 1987). Therefore, in this case, the presence of iron in CSL could prevent the PL from increasing the solubility of oxygen in the oil, and thus avoided a pro-oxidant effect. Secondly, this stability study was carried out in refined, but not tocopherol stripped fish oil. Therefore, minor components in the oil or the added tocopherols present in the refined fish oil used in this study could have an effect on the antioxidant activity of CSL. Yoon and Min (1987) used stripped soybean oil to study the effects of PC. The fish oil used in this study contained a mixture of tocopherols (mainly α-,γ- and δ-tocopherols), and it has been suggested that PL donate hydrogen atoms to tocopherols thereby extending the antioxidant activity of primary antioxidants (Hildebrand *et al*., 1984, Kashima *et al.*, 1991, King *et al*., 1992b). Miyazawa *et al*., (1984) also suggested that the nitrogen moiety of choline and ethanolamine PL head groups are involved in the peroxide decomposition ability of PC and PE (Miyazawa *et al*., 1984). While it is difficult to determine whether these results indicate hydroperoxide

decomposition or hydroperoxide inhibition, as there was an increase in hydroperoxide content of each treatment over time, it is possible that the nitrogen moiety of PS could act in a similar manner to decompose hydroperoxides as they are forming, rather than slowing their formation. Lastly, CSL also contains low levels of stabilizing antioxidants (RME and αT) that could be interacting with the PL in CSL or imparting their own antioxidant effect, influencing the overall protection. Thus, this study did not find a pro-oxidant effect of concentrations of CSL contributing up to 2 mg g^{-1} PS as found in the Yoon and Min (1987) study. Therefore, in an industrial setting where the goal is to extend the shelf life of refined, tocopherol enriched fish oil, CSL would be expected to reduce the formation of hydroperoxides and aldehydes, as measured by PV and *p*AV, respectively.

All concentrations of CSL provided essentially equivalent protection against the formation of hydroperoxides for the first 6 days of the stability study. All concentrations provided a significantly greater antioxidant effect compared to the control; on day 6, the sample containing 10 mg g^{-1} of CSL had an average PV of 10.4 meq kg^{-1} compared to the control with an average PV of 119.4 meq kg^{-1}. This indicates CSL has a potent ability to reduce the development of hydroperoxides. Saito and Ishihara (1997) found that the choline and ethanolamine moieties of PL inhibited the development of lipid hydroperoxides, which they attributed to their hydroperoxide decomposition activity. This activity was thought to be mainly related to the side-chain amine moiety but also might be aided by the intramolecular hydroxyl group associated with the amine moiety of ethanolamine and thus, it is reasonable to think that PS, being a phosphatidylamino

alcohol, would have similar hydroperoxide inhibition/decomposition activity (Saito & Ishihara, 1997; Miyazawa *et al*., 1984). However, these results are confounded by the presence of approximately 1 mg g^{-1} added mixed tocopherols in the refined fish oil and the minor components, RME, αT and additional PL, in CSL; some of the effect could be due to synergy with the mixed tocopherols (Hildebrand *et al*., 1984, Kashima *et al.*, 1991, King *et al*., 1992b, Judde *et al*., 2003). Once the rate of hydroperoxide formation in the control began to increase rapidly (days 7 and 8), the higher inputs (7.5 and 10 mg g^{-1}) of CSL began to show more protective effect than 1 and 2.5 mg g^{-1} CSL (Figure 2.3). It may be that once the added mixed tocopherols are consumed in the fish oil, the rate of hydroperoxide formation increases rapidly. Therefore, these results could indicate that higher concentrations of the PS in CSL are better able to extend the protective effect of the mixed tocopherols through synergy by suppressing their oxidative decomposition as suggested by Kashima *et al.*, 1991. Alternatively, the greater protective effect noted with the higher concentrations of CSL could be due to there being greater amounts of RME, αT and minor PL, and it is these ingredients alone or in combination with the PS in CSL that is producing the antioxidant effect.

The *p*AV results followed a similar trend as the protection against hydroperoxides (Figure 2.4). During early stages of oxidation 1 to 10 mg g^{-1} CSL provided equivalent antioxidant effect, measured by PV and *p*AV. Since 7.5 and 10 mg g^{-1} CSL samples had a greater protective effect against the development of hydroperoxides on days 7 and 8 it was not surprising that the *p*AV of these samples were also significantly lower than all other treatments; fewer primary oxidation products are forming to be decomposed into

secondary oxidation products such as aldehydes. This suggests that CSL, a component of CSL, or the combination of CSL and minor components added to the refined fish oil are very effective at slowing the development of secondary oxidation products (in the form of aldehydes), and at later phases of oxidation, this effect is concentration dependent. Therefore, the antioxidant activity of CSL could be of particular interest for industrial application where sensory qualities are very important as secondary oxidation products are more closely related to off-flavour and odour development. Saito and Ishihara (1997) suggested that the amine moiety of PE and PC may act to decompose hydroperoxides in a nucleophilic substitution reaction to form an alcohol. Alcohols are not detected in the *p*AV method. Therefore, if the PS in CSL is decomposing hydroperoxides into alcohols, it would be expected that the *p*AV remain low as was found in this study.

Others have found that a PL's ability to form association colloids as reverse micelles affects the antioxidant activity of α-tocopherol (Koga & Terao, 1995, Chen *et al*., 2011). Chen *et al*. (2011) found that the presence of 1,2-dioleoyl-*sn*-glycero-3-PC (DOPC) at 1000 μM (greater than the CMC) increased the antioxidant effect of 10 μM α-tocopherol (equal to 4.3 μg g^{-1}), as measured by hydroperoxides and hexanal, in stripped soybean oil. However, DOPC decreased the antioxidant effect of 100 μM (equal to 0.043 mg g^{-1}) α-tocopherol and had a pro-oxidant effect on its own, measured by hydroperoxide formation and hexanal (Chen *et al*., 2011). Samples in this study had a greater tocopherol content than 100 μM (supplier documentation indicates not less than 1 mg g^{-1} mixed tocopherols), but an antioxidant effect was noted with CSL at all inputs, measured by PV and *p*AV. A PL's CMC can be affected by the PL composition and solvent medium, and

since samples were not analyzed for the presence of reverse micelles, it is not known whether they were present in the samples containing CSL (Kittipongpittaya *et al*., 2014). However, the CMC of 1,2-didecanoyl-*sn*-glycero-3-phospho-L-serine (10:0 PS) is 0.096 mM and the approximate concentration of PS in 1 mg g^{-1} of CSL was 0.26 mM (Avanti Polar Lipids Inc., n.d.). Therefore, even at the minimum input, the CMC of PS was likely met and it was expected that reverse micelles may have formed with trace amounts of water present in the oils and CSL; supplier documentation indicated that 1 mg g^{-1} water was present in CSL. Therefore, the possible formation of reverse micelles by CSL could be another potential mechanism in which it is acting as an antioxidant to enhance the antioxidant activity of the mixed tocopherols in the bulk fish oil or the αT in CSL.

The exact mechanism behind the antioxidant effect noted at each concentration input of CSL cannot be determined from this study. However, 10 mg g^{-1} was found to provide the greatest protection against the formation of hydroperoxides and aldehydes, as measured by PV and *p*AV, respectively, compared to lower inputs. Since 10 mg g^{-1} CSL contains approximately 2.0 mg g^{-1} PS and it is suspected that PS is the major contributor to the antioxidant effect of CSL, similar concentrations of this PL will be examined further for its effect on the formation of primary and secondary oxidation products. Tocopherol stripped fish oil will be used to eliminate their possible influence.

2.5 CONCLUSION

Even at low concentrations, CSL had a protective effect against the development of hydroperoxides and secondary oxidation products (α- and β-unsaturated aldehydes). The development of hydroperoxides and secondary oxidation products, as measured by *p*AV, were more greatly suppressed with higher inputs of CSL, with an inclusion of 10 mg g^{-1} CSL having the greatest protective effect at later stages of oxidation. At this input, CSL was soluble in refined fish oil and thus, it may prove to be an industrially useful antioxidant. Further research will be conducted using an input of 10 mg g^{-1} CSL and the corresponding concentrations of PS and stabilizing antioxidants (RME and αT) to identify the components of CSL that are contributing to its antioxidant effect, and to better elucidate the associated mechanisms.

CHAPTER 3 ANTIOXIDANT EFFECT OF PS AND CSL

3.1 INTRODUCTION

Typically, fish oil used in dietary supplements undergoes a number of refining processes to remove oxidation products and contaminants, including hydrocarbons and other non-triacylglycerol compounds (Bimbo, 1998). The most common refining procedures involve degumming, alkali refining, bleaching, deodorization, winterization and molecular distillation and/or steam deodorization (Bimbo, 2007, p. 94). These processes reduce or completely remove the natural antioxidants, such as tocopherols and carotenoids, as well as other endogenous compounds, such as PL, within the oil (Bimbo, 2007, p. 93).

Without sufficient antioxidants, the n-3 PUFA within the oil are oxidized, generating unpleasant flavours and odours, (Turner *et al*., 2006). Therefore, to maintain the quality and stability of these oils, they are often stabilized with antioxidants. Tocopherols are commonly added back to bulk fish oil after or during the refining process to preserve oil quality. Thus, most fish oil supplements contain tocopherols. However, tocopherols alone do not provide sufficient inhibition for long-term storage and additional antioxidants are often used (Kamal-Eldin & Yanishlieva, 2002). Common antioxidants added to fish oil used for nutritional supplements include RME and ascorbyl palmitate, in addition to tocopherols either naturally present or added by the refiner. Lecithin, often from a plant based source, is rarely used as an antioxidant on its own. However, lecithin can be found in commercial antioxidant blends and some studies have investigated its oxidative

protective effects (Hamilton *et al*., 1998). For example, Hamilton *et al.* (1998) found the ternary blend of δ-tocopherol, ascorbyl palmitate and lecithin had a strong antioxidant effect.

In its unrefined state, fish oil contains a PL profile similar to mammals; that is, the typical profile consists of mainly PC and PE, with smaller amounts of PS and in some cases, PI (Weihrauch & Son, 1983). However, refining removes PL that may stabilize the crude oil. King *et al*. (1992a) demonstrated that the addition of PL fractions from bluefish (*Pomatomus saltatrix*) to refined salmon oil increased the oil's oxidative stability, measured by TBARS and polyene index. Although PL have been shown to have an antioxidant effect, their mechanism of action is unclear. The most commonly suggested mechanisms are metal chelation by the phosphate groups (Lunde *et al.*, 1976, Yoshida *et al.*, 1991, Dacaranhe and Terao, 2001a), synergy with tocopherols (Hudson & Ghavami, 1984, Kashima *et al.*, 1991, Bandarra *et al*., 1999) and formation of Maillard-type or other browning compounds (King *et al*., 1992b, Bandarra *et al*., 1999, Hidalgo *et al*., 2005).

CSL studied in this research contains approximately 200 mg g^{-1} PS as well as approximately 3 mg g^{-1} αT and 1 mg g^{-1} RME in a MCT base. RME and αT also have antioxidant properties and are thought to maintain the potency and stability of CSL. Here, I tested the hypotheses that: 1) soybean PS has an antioxidant effect in fish oil; and 2) that there is a synergistic relationship between soybean PS and αT and/or RME.

3.2 Materials and Methods

3.2.1 Experimental Design

Oxidative stability studies using tocopherol stripped refined fish oil were completed to test these hypotheses. The refined fish oil was stripped of all tocopherols, allowing any antioxidant effect noted to be attributed to the treatment or combination of treatments added to the system. A control of tocopherol stripped fish oil, handled in the same as all other samples, without any additives was consistently run with each stability study.

A number of methods to assess the extent of oxidation as well as to gain understanding into possible mechanisms for antioxidant effects were used in this work. Primary (hydroperoxides) and secondary (unsaturated aldehydes) oxidation products were measured over the course of the stability studies using PV and *p*AV, respectively, to determine if the treatments had an antioxidant effect. In one study, conjugated dienes (CD), another primary oxidation product, were measured in addition to PV. These measurements were used to compare the antioxidant activity of CSL and PS with and without mixed T, as well as PS with RME and/or αT. Both primary and secondary oxidation products were monitored to investigate the role of each treatment during oxidation. Tocopherol content was quantified by high pressure liquid chromatography (HPLC) for the initial and final time points in one study to determine whether CSL or PS have a tocopherol sparing effect on mixed T in fish oil enriched with tocopherols. The formation of browning compounds was measured spectrophotometrically. Measurements were taken periodically throughout most stability studies and trends were compared for

each treatment at those common time points. A modified spectrophotometric method was developed to assess the iron ion binding ability of CSL, soybean PS and RME. Iron is a known catalyst of oxidation reactions; therefore, the ability to bind iron ions prevents the initiation of oxidation.

Three different stability studies were conducted in tocopherol stripped fish oil at ambient temperatures. The first comparison investigated the antioxidant activity of approximately 3 mg g^{-1} mixed T (Sigma Aldrich, Oakville, ON), 2.1 mg g^{-1} soybean PS (≥ 97% pure, Sigma Aldrich, Oakville, ON) and 10 mg g^{-1} of CSL alone and the combination of PS+mixed T and CSL+mixed T (Figure 3.1). The concentration of 3 mg g^{-1} was chosen for this comparison because refined fish oil typically contains between 1 and 3 mg g^{-1} tocopherols. Additional in-depth comparisons were made to investigate the individual and combined antioxidant effects of the main components in CSL. To investigate the hypothesis that PS has a synergistic antioxidant effect with the combination of RME and αT (both provided by CSL manufacturer), the antioxidant effects of 10 mg g^{-1} of CSL was compared to the combination of 2.1 mg g^{-1} PS + 0.01 mg g^{-1} RME + 0.03 mg g^{-1} αT, as well as 2.1 mg g^{-1} PS alone and 0.01 mg g^{-1} RME + 0.03 mg g^{-1} αT (Figure 3.2). Lastly, the individual antioxidant effects of 0.01 mg g^{-1} RME and 0.03 mg g^{-1} αT and their possible synergistic relationship with 2.1 mg g^{-1} PS were compared with 10 mg g^{-1} of CSL to determine their contribution to CSL's antioxidant effect (Figure 3.3). PS was the only PL examined as it was the major PL species in CSL and was thus suspected to be imparting the greatest antioxidant effect. The concentrations of PS, RME and αT were chosen to match the levels that are present in the concentration of CSL that was found to

have the greatest antioxidant effect in refined fish oil established in Chapter 2. The average composition of the approximate 3 mg g^{-1} mixed T used in this research was approximately 0.6 mg g^{-1} α-tocopherol, 1.4 mg g^{-1} γ-tocopherol and 0.8 mg g^{-1} δ-tocopherol.

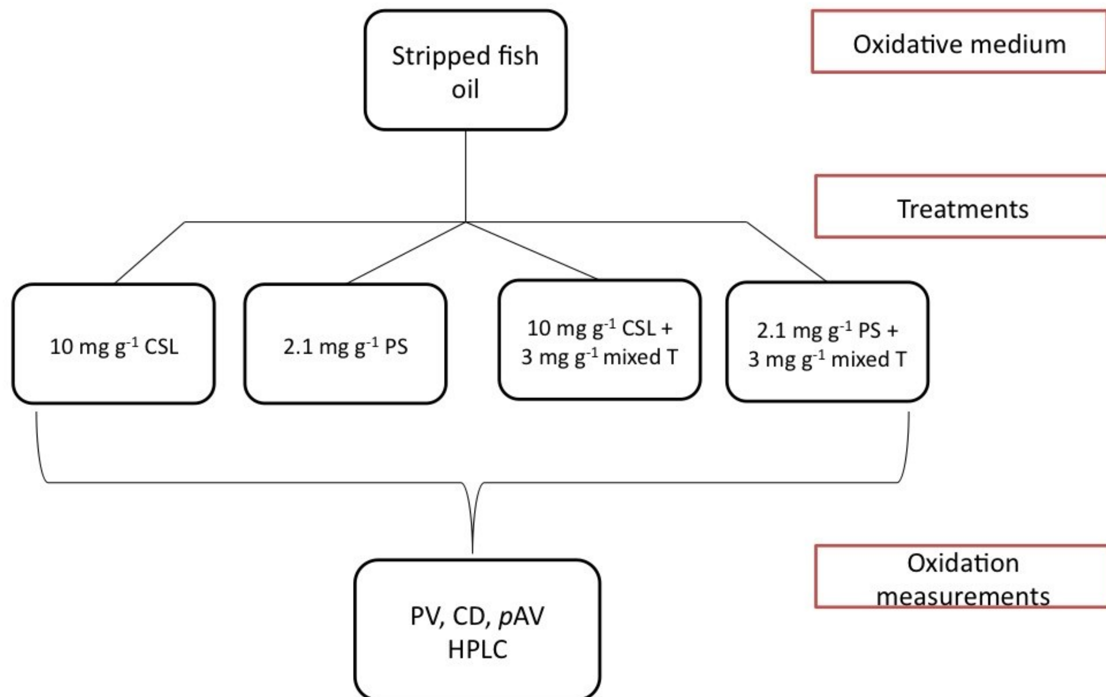


Figure 3.1 Experimental treatments and analytical measurements used to compare the antioxidant effect of CSL and PS alone and in combination with mixed T. Samples were stored in the dark, in open vials at ambient temperatures. A control of tocopherol stripped fish oil was tested concurrently.

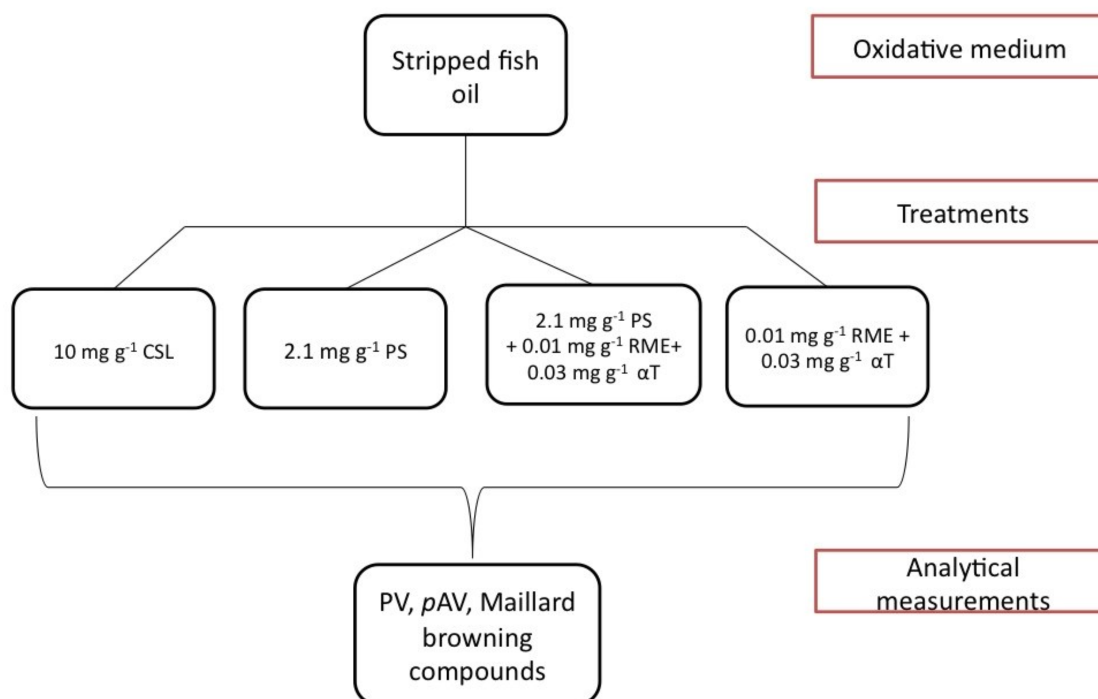


Figure 3.2 Experimental treatments and analytical measurements used to test for synergy between PS, RME and αT present in CSL. Samples were stored in the dark, in open vials at ambient temperatures. A control of tocopherol stripped fish oil was tested concurrently.

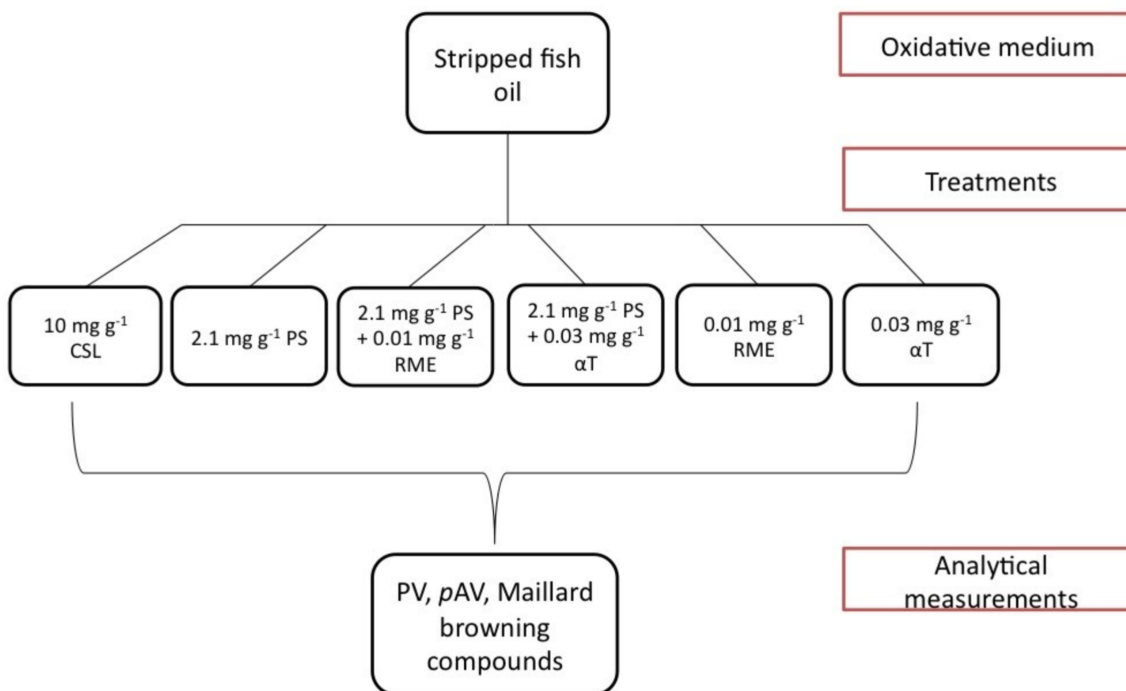


Figure 3.3 Experimental treatments and analytical measurements used to test for synergy between RME and αT individually with PS. Samples were stored in the dark, in open vials at ambient temperatures. A control of tocopherol stripped fish oil was tested concurrently.

3.2.2 Preparation of Tocopherol Free Fish Oil

Commercially available refined sardine, anchovy and mackerel fish oil stabilized with mixed tocopherols was stripped of its tocopherols using an adsorption chromatography method by Lampi *et al*. (1999). Aluminum oxide (Sigma-Aldrich, Oakville, ON) was activated for a minimum of 3 hrs at approximately 210 °C before use. A glass column (40 cm x 3 cm i.d.) was wrapped in aluminum foil and packed with 100 g of activated aluminum oxide that had been mixed with 275 mL hexane (Fisher Scientific, Guelph,

ON). Fish oil (50 g) was dissolved in 50 mL of hexane and added to the column. The oil was eluted with 200 - 300 mL of hexane. The oil and hexane solution was collected in a 500 mL round bottom flask wrapped in aluminum foil. To reduce oxidation, the round bottom collection flask remained in an ice bath with a gentle stream of nitrogen flowing into the neck of the flask. The hexane was removed from the stripped oil with a vacuum rotary evaporator (Heidolph, Germay) with a water bath set at 40-45 °C (WB 2000, Caframo, Ontario, Canada). Traces of hexane were removed using a vacuum pump. Before stripping a second batch of fish oil, the activated aluminum oxide in the column was washed with 200 mL of hexane. After two batches of fish oil were stripped, the column was repacked with fresh, activated aluminum oxide. To ensure tocopherols were removed from the fish oil, the stripped fish oil was analyzed for tocopherols using HPLC (section 3.2.4). The EPA and DHA composition of the oil used for each stability study is found in Appendix I. All solvents and reagents used in these studies were purchased from Fisher Scientific (Guelph, ON) unless otherwise stated.

3.2.3 Sample Preparation and Storage Conditions

The stripped fish oil was combined with the appropriate additive by stirring for 5 to 10 min with a magnetic stir bar on a stir plate, followed by sonication in a water bath sonicator (Model 2510, Branson Ultrasonics, Danbury, CT, USA) for 5 min. The sonicated samples were stirred for 5 minutes and then aliquoted in 3.2 ± 0.2 g samples into 4 mL screw thread clear glass vials (45 mm x 15 mm i.d.) (Thermo Scientific,

Rockwood, TN, USA). Each treatment was prepared in triplicate. The control samples for each trial underwent the same mixing procedure as the samples.

A minimum of 21 vials of each treatment and control samples were stored in the dark at ambient conditions (20 ± 3 °C) in uncapped vials. Three separate vials were used for each replicate measurement per time point. Sampling occurred over a range of time points from initial to day16. Primary and secondary oxidation measurements were taken for a minimum of seven and six time points, respectively, over the 16-day period. Sample remaining after PV analysis was nitrogen purged and frozen at -18 °C for *p*AV analysis when *p*AV measurements could not be completed on the same day.

3.2.4 HPLC Tocopherol Analysis

Stripped fish oil and samples were analyzed in triplicate for tocopherols using normal phase HPLC with a Thermo Finnigan Surveyor HPLC coupled to a Finnigan Surveyor UV-Vis plus detector (Thermo Fisher Scientific, Mississauga, ON) according to Carpenter (1979). Briefly, samples were diluted in 1.5% isopropyl alcohol (IPA) in hexane to a final concentration of approximately 0.2 g $ml^{-1}$and filtered through 0.2 µm filters (4 mm RC, Thermo Scientific, Waltham, MA, USA). Prepared samples were injected, at a level of 10 µL, on an YMC-Pack PVA-Sil column (5 µm particles, 250 mm x 4.6 mm i.d.; Sigma Aldrich, Supelco, Bellefonte, PA, USA) with a matching guard column. A mobile phase of 1.5 % IPA in hexane with a flow rate of 2 mL min^{-1} was used for the 10 min run. Tocopherols were detected at 295 nm. Tocopherol concentrations in

each sample were determined using calibration curves of α-, β-, δ- and γ-tocopherol standards (Sigma Aldrich, Oakville, ON). Standard purity was 93 % or greater.

3.2.5 Oxidation Measurements

Primary oxidation products were measured in triplicate using a modified version of the AOCS official method CD 8-53 (1997) for PV as described in Section 2.2.2, with following modifications to accommodate the reduced sample sizes. Aliquots of known mass (approximately 0.1 g) were added to 50 mL or 125 mL Erlenmeyer flasks, depending on the expected level of oxidation, and dissolved in 10 mL of 3:2 glacial acetic acid: chloroform. The samples were mixed with 100 μL of saturated potassium iodide and after exactly 1 min the reaction was stopped with the addition of 10 mL deionized water. The PV was determined by titration using 0.01 N standardized sodium thiosulfate in the presence of a starch indicator.

*p*AV were determined following the procedure described in Section 2.2.

Conjugated dienes were measured for one stability study. Measurements were taken in triplicate based on the AOCS official method Ti 1a-64 (1989). Approximately 0.5 g of sample was accurately weighed into a 25 mL volumetric flask. The flask was made up to the mark with isooctane. Absorbance was measured at 233 nm. Percent CD was determined as follows:

$$\% \text{ Conjugated dienoic acid} = 0.84 \left(\frac{A_s}{bc} - k_o\right)$$

where A_s is the absorbance of the sample at 233 nm, b is the cell path length in cm, c is the concentration of the sample in g L^{-1} and k_o is the absorptivity of the triacylglycerol group.

3.2.6 Quantitative Estimation of Antioxidant Activity from PV

Antioxidant activity was calculated as percent inhibition of lipid oxidation compared to the control oil. Linear regression of PV plots during the 16 day oxidation period was used to calculate the slope for each sample. Percent inhibition and percent synergistic effect were calculated following the equations by Saito and Ishihara (1997):

$$\% \text{ inhibition} = \frac{(S_{control} - S_{treatment})}{S_{control}} \text{ x } 100$$

$$\% \text{ synergistic effect} = \frac{(S_{antioxidant} - S_{antioxidant+PS})}{S_{antioxidant}} \text{ x } 100$$

where $S_{control}$, $S_{treatment}$ and $S_{antioxidant}$ are the slopes of the PV vs. time plot for the control (tocopherol stripped fish oil), each treatment and medium with added antioxidants (RME or αT), respectively. $S_{antioxidant+PS}$ is the slope of the sample containing an added antioxidant (RME or αT) and PS.

3.2.7 Maillard Browning Compounds

Maillard browning compounds were detected spectrophotometrically based on the method described by Husain *et al*. (1986). A portion of each sample was dissolved in isooctane to a concentration of 20 mg mL^{-1}. Absorbance was measured using a UV spectrophotometer (Novaspec II, LKB Biochrom, Holliston, MA, USA) at 430 nm.

3.2.8 Separation of PL Classes by TLC and Analysis of Fatty Acid Moieties

Thin layer chromatography (TLC) was used to separate phospholipids in CSL. PL standards (PS, PC, PI, and PE; Sigma Aldrich, Oakville, ON) were prepared in chloroform, to a final concentration of 10 mg mL^{-1}. CSL (10 mg mL^{-1}) was prepared in the same way as the phospholipid standards. Silica gel 60 plates (20 x 20 cm, 210-270 µm layer thickness) (EMD Chemicals, Billerica, MA, USA) were washed in a developing tank containing 200 mL of 1:1 chloroform: methanol (v/v). Plates were then activated at 100 °C for 1 hr. PL standards were spotted on the plates along with a streak of CSL. Plates were developed in a solvent system containing chloroform: methanol: acetic acid: acetone: water (35:25:4:14:2 (v/v)) according Xu *et al*. (1996). Detection of phospholipids was performed by spraying with 0.2% 2',7'-dichlorofluorescein in ethanol (Kodak, Rochester, NY, USA) and visualizing under UV light.

The PS band was scraped from the silica plate and the lipids were extracted twice with 5 mL of chloroform: methanol: water (5:5:1 (v/v)). The combined supernatants were washed with 5 mL of deionized water. Centrifugation at 1000 rpm provided a clear

separation between the organic and the water phase. The lower cholorform: methanol phase containing the lipid was collected and evaporated to dryness under nitrogen (N-Evap 112 Nitrogen evaporator with OA-SYS heating system, Orgnomation Associates Inc, Berlin, MA, USA). The collected lipids were then dissolved in 2:1 cholorform: methanol with 0.01 % butylated hydroxytoluene (BHT) for storage.

The lipids were converted to methyl-esters (ME) using the Hilditch method. Briefly, the extracted PS band was evaporated to dryness. Then both the recovered CSL PS and the pure soybean PS were combined with 1.5 mL dichloromethane with 0.01% BHT and 3 mL of Hilditch reagent (0.5 N H_2SO_4 in methanol). The solutions were allowed to react at 100 °C for 1 hr. Once cooled, the reaction was stopped with the addition of water and the ME were extracted with hexane. Extracts were dried with anhydrous Na_2SO_4. Samples were made to a final concentration of approximately 20 mg mL^{-1}. Fatty acid analysis was performed on a gas chromatograph (GC) (Bruker) with a DB-23 column (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID). Split injection was used with an injector temperature of 250 °C. The following temperature program was used: initial temperature of 153 °C held for 2 min then ramped to 174 °C at 2.3 °C min^{-1} and held for 0.2 min This was followed by another ramp to 210 °C at 2.5 °C min^{-1} and held for 3.27 min for a total run time of 29 min. The FID was set to 270 °C. Samples were analyzed in triplicate.

3.2.9 Iron Chelating

Metal chelation was examined as a possible mechanism in which PS may act as an antioxidant. Metal chelators act as secondary antioxidants by either precipitating metal ions or by activating all of its coordinate sites (Mahoney & Graf, 1986). If the complexed iron has at least one free coordinate site, it is able to catalyze oxidation. Phosphate and pyrophosphate can precipitate transition metals, reducing oxidation by lowering the amount of soluble metals present in the system (Mahoney & Graf, 1986). Therefore, phospholipids may act as antioxidants depending on the solubility of the iron complex formed (Brandt *et al*., 1973, as cited in Mahoney & Graf, 1986). However, a pro-oxidant effect may be possible if the iron complex formed is oil-soluble (Brandt *et al*., 1973, as cited in Mahoney & Graf, 1986**).**

In 1970, Lawrence Stookey developed ferrozine, a water-soluble reagent that is able to complex with iron (II) to form a coloured complex that has a maximum absorbance at 562 nm. The ability of a compound to compete with ferrozine is determined by the decrease in absorbance as the compound binds iron (II), thus reducing the amount of iron (II) available to complex with ferrozine. Carter (1971) used ferrozine to develop a method for quantifying iron in serum. This method was adapted over the years to be used to measure the iron chelating ability of various compounds including fruit extracts. In this research, the method was modified to be used for compounds that are in-soluble in water, such as PS, to measure their iron (II) binding capabilities.

The iron (II) chelating ability of pure soybean PS, CSL and RME were determined using a modified version of Dinis *et al*. (1994). This method was designed for compounds that are water soluble since the ferrozine and $Fe(II)Cl_2$ solutions used are aqueous. Because CSL, PS and RME are not water soluble, method modifications were necessary. Constant agitation and the addition of a small, but concentrated portion of the test compound in an organic solvent was used to ensure that the ferrozine and $Fe(II)Cl_2$ were interacting with the test solution. To confirm this interaction, 2,2'-bipyridine, a known metal chelator with low water-solubility, was carried through this method. This trial found that 2,2'-bipyridine (11 mg g^{-1} in chloroform) (Eastman Organic Chemicals, Rochester, NY, USA) was able to chelate 45% of the iron (II). Therefore, agitation was allowing the iron (II) ions to come into contact and be complexed by 2,2'-bipyridine; this modified method was applied to test the chelating ability of PS, CSL and RME. Specifically, 100 µL of each treatment, 310 mg mL^{-1} CSL (providing equivalent to 65 mg mL^{-1} PS and approximately 0.31 mg mL^{-1} RME), 0.31 mg mL^{-1} RME dissolved in chloroform, and 65 mg mL^{-1} soybean PS dissolved in 95:5 chloroform: methanol, according to each treatment's solubility, were combined with 1 mL of 0.0344 mM $Fe(II)Cl_2$ and 2 mL of 3.44 mM ferrozine (ACROS Organics, Geel, Belgium) in water. Samples were mixed, with constant agitation, for 10 min, centrifuged and the aqueous phase was removed. The absorbance of the aqueous phase was measured at 562 nm. A control was prepared in the same manner as the samples except 100 µL of the solvent in which the antioxidant of interest was dissolved was added in place of the sample. A blank was prepared to eliminate the influence of traces of iron in the test samples, PS, CSL and RME. This

blank was prepared in the same manner as the samples, except 1 mL of distilled water was added in place of $Fe(II)Cl_2$.

The percent chelating effect was determined accordingly:

$$\% \text{ chelating effect} = \frac{A_{control} - (A_{sample} - A_{blank})}{A_{control}} \times 100$$

where $A_{control}$, A_{sample} and A_{blank} are the absorbance of the control, sample and blank, respectively.

3.2.10 Fatty Acid Analysis

Tocopherol stripped fish oils were analyzed for EPA and DHA using GC-FID. Lipids were converted to ME following the modified Global Organization for EPA and DHA Omega-3s (GOED) Voluntary Monograph for Omega-3 (2012), using methyl tricosonate as an internal standard, as well as external standards for EPA and DHA (Nu-Chek Prep, Elysian, MN, USA). ME were analyzed using the same column and instrumental conditions described in Section 3.2.8.

3.2.11 Statistical Analysis

All analyses were conducted in triplicate and results were analyzed using one-way ANOVA at a significance level of $p < 0.05$ (MiniTab 16.2.4), except for estimates of antioxidant activity from PV, which were analyzed with Prism (GraphPad Software Inc., Version 4.0c). When significance was found, Tukey's multiple comparison test was used

to determine which means were different from each other. A paired t-test was used to determine significance for HPLC tocopherol results where initial and final tocopherol concentrations were individually compared for each treatment at a significance level of $p < 0.05$ (Prism, GraphPad Software Inc., Version 4.0c). Errors for each test were expressed as standard deviations of the triplicate measurements except for the error associated with the linear regression slopes where standard errors are reported.

3.3 RESULTS

3.3.1 Effect of Tocopherols in the Presence of PS and CSL on the Formation of Primary and Secondary Oxidation Products

All of the treatments except PS alone showed an antioxidant effect, monitored by the evolution of hydroperoxides, throughout the entire 16-day period (Figure 3.4; ANOVAs were significant at the $p < 0.05$ level at all time points). It was noted that the presence of mixed T or the αT present in CSL had an immediate effect on the samples' PV; post hoc analysis found samples containing mixed T or αT had a significantly lower initial PV than the control and PS sample (Tukey's test: $p < 0.05$). PS only began to show an antioxidant effect on day 4, with the PV of the control and PS sample equal to 40.4 and 36.9 meq/kg, respectively (Tukey's test: $p < 0.05$). From day 4 onward, the PS sample continued to have a significantly lower PV than the control, and therefore continued to display a slight antioxidant effect in preventing the formation of hydroperoxides. However, the PS sample displayed the least amount of antioxidant effect compared to all other treatments (Tukey's test: $p < 0.05$).

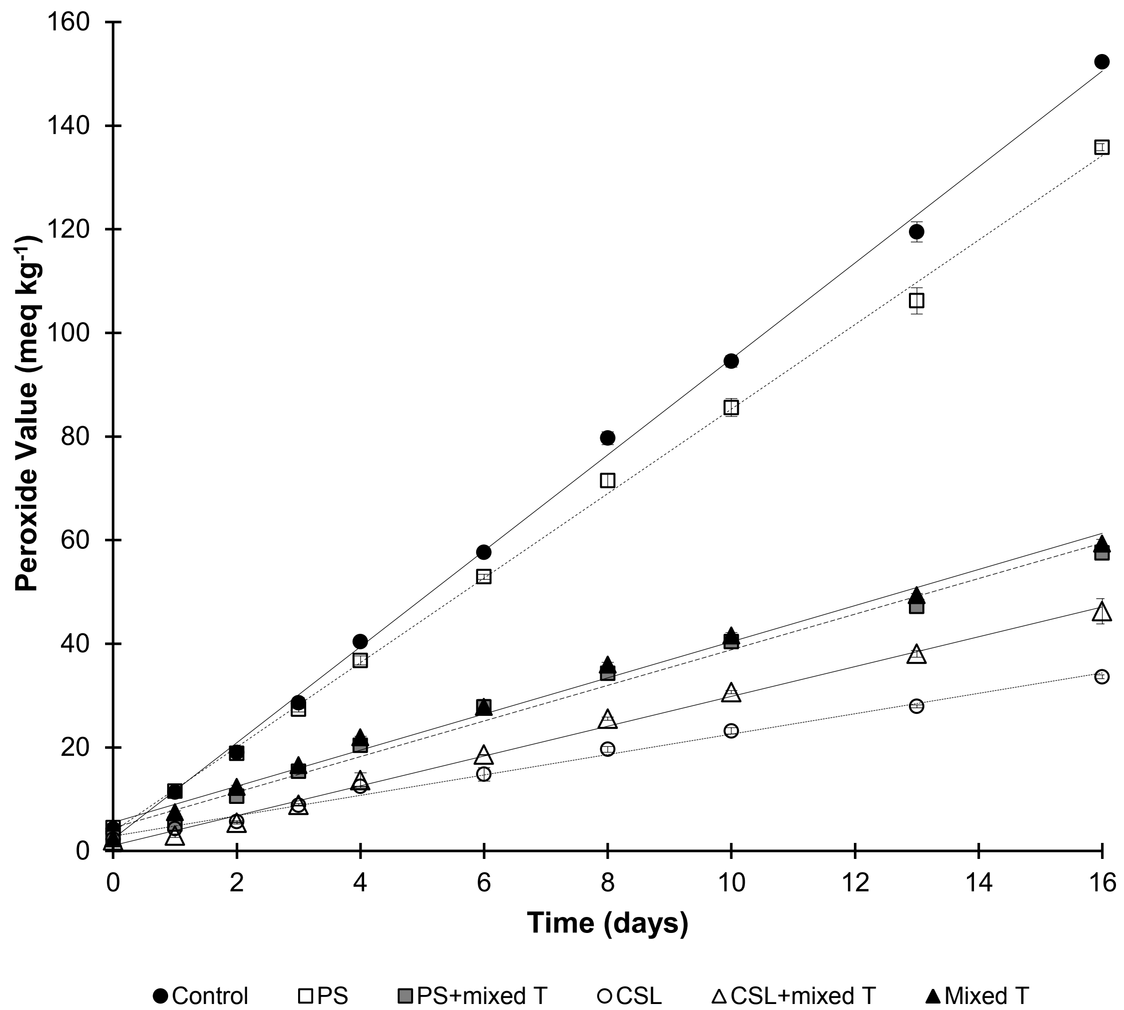


Figure 3.4 Effect of added PS and CSL with and without added mixed T on the development of hydroperoxides in tocopherol stripped fish oil. For each sampling point, n=3 with error bars indicating standard deviation.

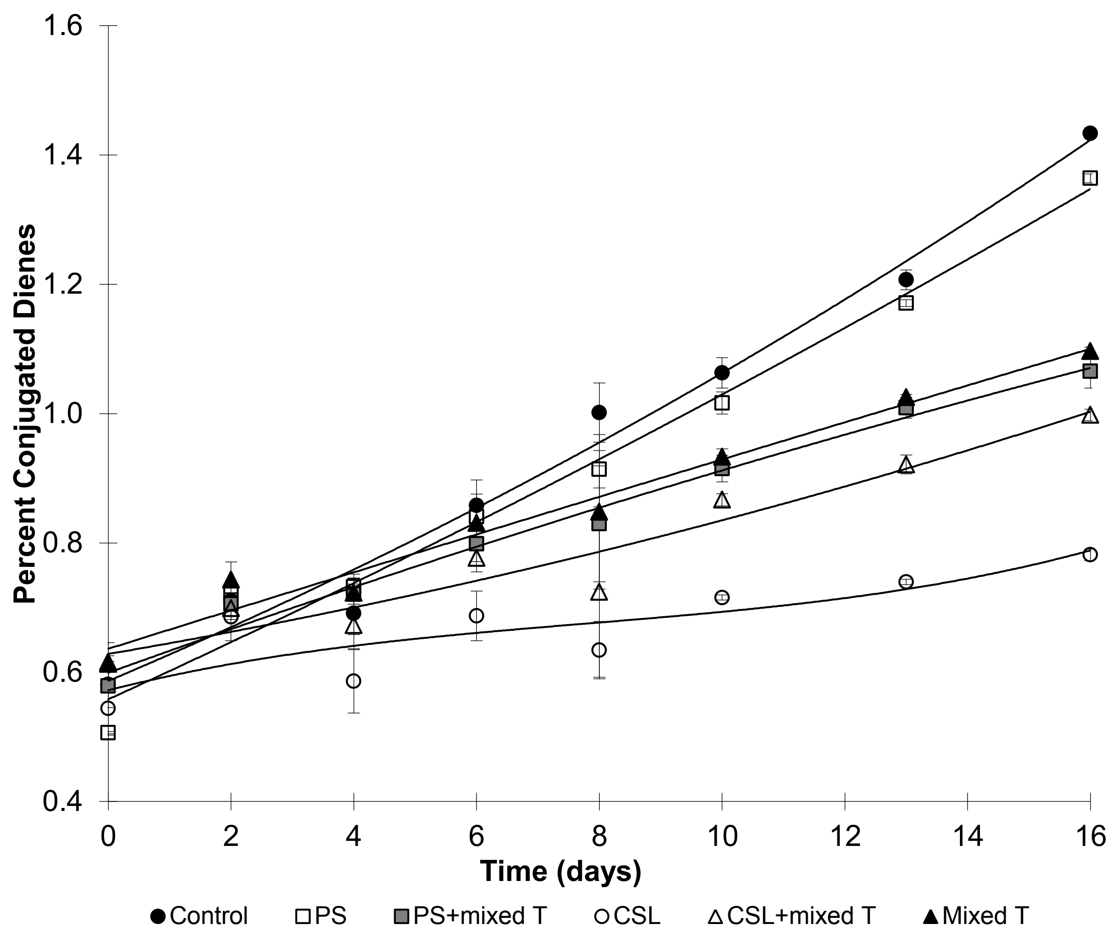


Figure 3.5 Effect of added PS and CSL with and without added mixed T on the development of CD in tocopherol stripped fish oil. For each sampling point, n=3 with error bars indicating standard deviation. Trend lines are added to guide the eye; they do not indicate a function.

A short-lived synergistic effect was noted for the combination of PS+mixed T; early in the stability study (days 1 and 2), the combination of PS+mixed T had a significantly greater protective effect, as monitored by PV, compared to mixed T or PS alone (Tukey's test: $p < 0.05$; Appendix I). This antioxidant effect was synergistic, not additive, because during days 1 and 2, while ANOVA indicated an overall significant effect of treatment of PS+mixed T, PS itself did not impart any protective effect; its PV was the same as the control, and the PV of PS+mixed T was significantly lower than mixed T alone (Tukey's test: $p < 0.05$; Appendix I). After this point, PS+mixed T had equivalent protection against hydroperoxides as mixed T alone, indicating that there was no benefit in adding PS to a system containing 3 mg g^{-1} mixed T as the oil becomes more oxidized.

Interestingly, CSL did not show synergy with mixed T; it provided either greater or equivalent protection against the formation of hydroperoxides compared to CSL+mixed T. Day 1 of the stability study was the only time point where CSL had a greater PV than CSL+mixed T (Tukey's test: $p < 0.05$; Appendix I). From day 6 to day 16, CSL had a lower PV and, therefore, provided greater protection against the formation of hydroperoxides than CSL+mixed T (Tukey's test: $p < 0.05$). It also provided the greatest protection against the development of hydroperoxides compared to all other treatments and the control from day 6 to the end of the study (Tukey's test: $p < 0.05$). Therefore, CSL was able to prevent the formation of hydroperoxides to a greater extent than all other treatments as the oil became highly oxidized.

Although the distribution of PS+mixed T, CSL and mixed T samples were better fitted by a non-linear model, they were analyzed by linear regression so their slopes could be compared to all other treatments and the control. Comparing the slopes of the linear regression lines of each treatment provides a measure of the rate of oxidation (increase in PV over time) in each system (Table 3.1). The more effective the antioxidant is at preventing the formation of hydroperoxides, the smaller the slope. These results, along with the expression of antioxidant activity, more clearly demonstrate the slight antioxidant effect of PS alone and clearly show there is no overall synergy between PS and mixed T; their slopes were equivalent (Table 3.1, Figure 3.6). CSL reduced the development of hydroperoxides significantly more than CSL+mixed T (Tukey's test: $p < 0.05$), as indicated by CSL's smaller slope, once again supporting the idea that CSL is more effective at protecting against the development of hydroperoxides than CSL+mixed T. CSL provided the most protection against hydroperoxide formation compared to all other treatments.

Table 3.1 Comparison of the increase in PV over time for samples stored in open vials at ambient conditions. Slopes are the mean values from the linear regression (meq PV kg^{-1} d^{-1}). Means that do not share the same letter are significantly different (Tukey's test: $p < 0.05$).

Sample	Slope
Control	9.26 (0.08)^{A}
PS	8.16 (0.07)^{B}
PS+mixed T	3.44 (0.08)^{C}
CSL	1.97 (0.04)^{D}
CSL+mixed T	2.88 (0.05)^{E}
Mixed T	3.49 (0.07)^{C}

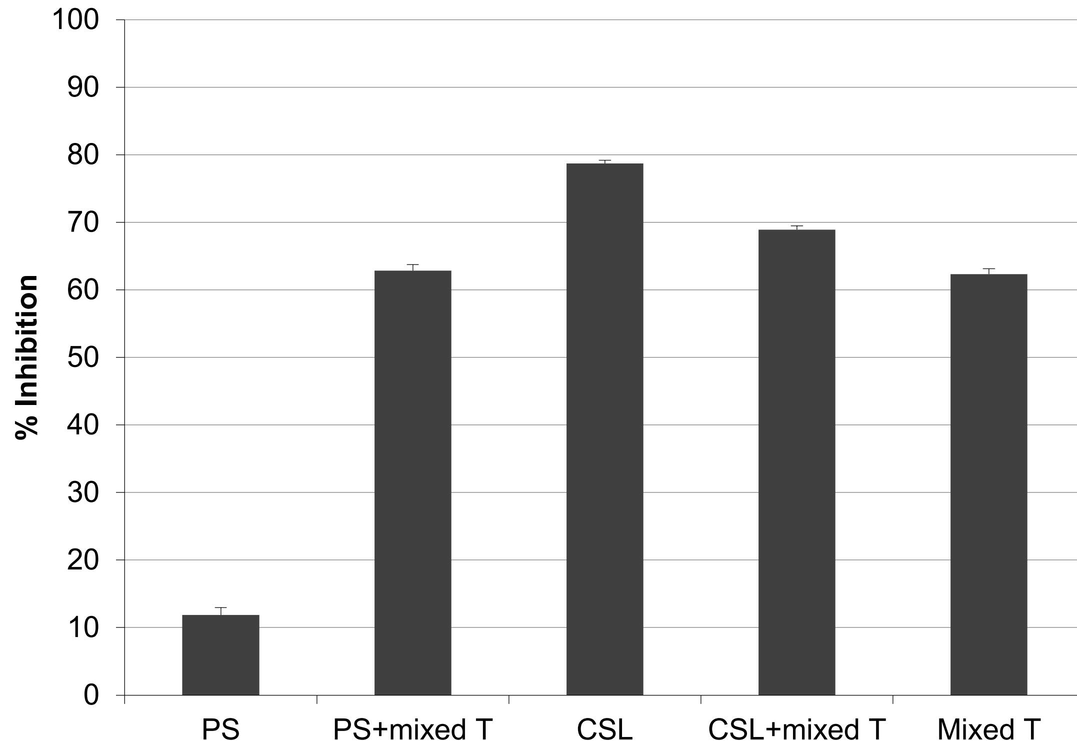


Figure 3.6 Estimated antioxidant activity, expressed in terms of ability to inhibit hydroperoxide formation, of PS and CSL with and without mixed T in tocopherol stripped fish oil. Values are expressed as means (n=3) with standard error indicated by error bars.

The evolution of CD was slower than the formation of PV (Figure 3.5; ANOVAs were significant at $p < 0.05$ for every time point). However, similar to the PV results, synergy between PS, CSL and mixed T was not found for the protection against CD. CSL began to show a significant protective effect at day 4, earlier than the other treatments, and provided significantly greater protection throughout the later phases of oxidation (Tukey's test: $p < 0.05$). Throughout the study, PS+mixed T and mixed T showed the same level of protection against CD formation (Tukey's test: $p > 0.05$). CSL+mixed T, while providing the second greatest protection against CD, was not as effective as CSL alone (Tukey's test: $p < 0.05$).

Similar trends were noted in the formation of secondary oxidation products, measured by *p*AV, as were observed with the primary oxidation products (hydroperoxides and CD) (Figure 3.7; ANOVAs were significant at a significance level of $p < 0.05$ for every time point). However, unlike its protection against hydroperoxide formation, CSL on its own did not provide as great an antioxidant effect as CSL+mixed T (Tukey's test: $p < 0.05$) and PS alone did not have an antioxidant effect.

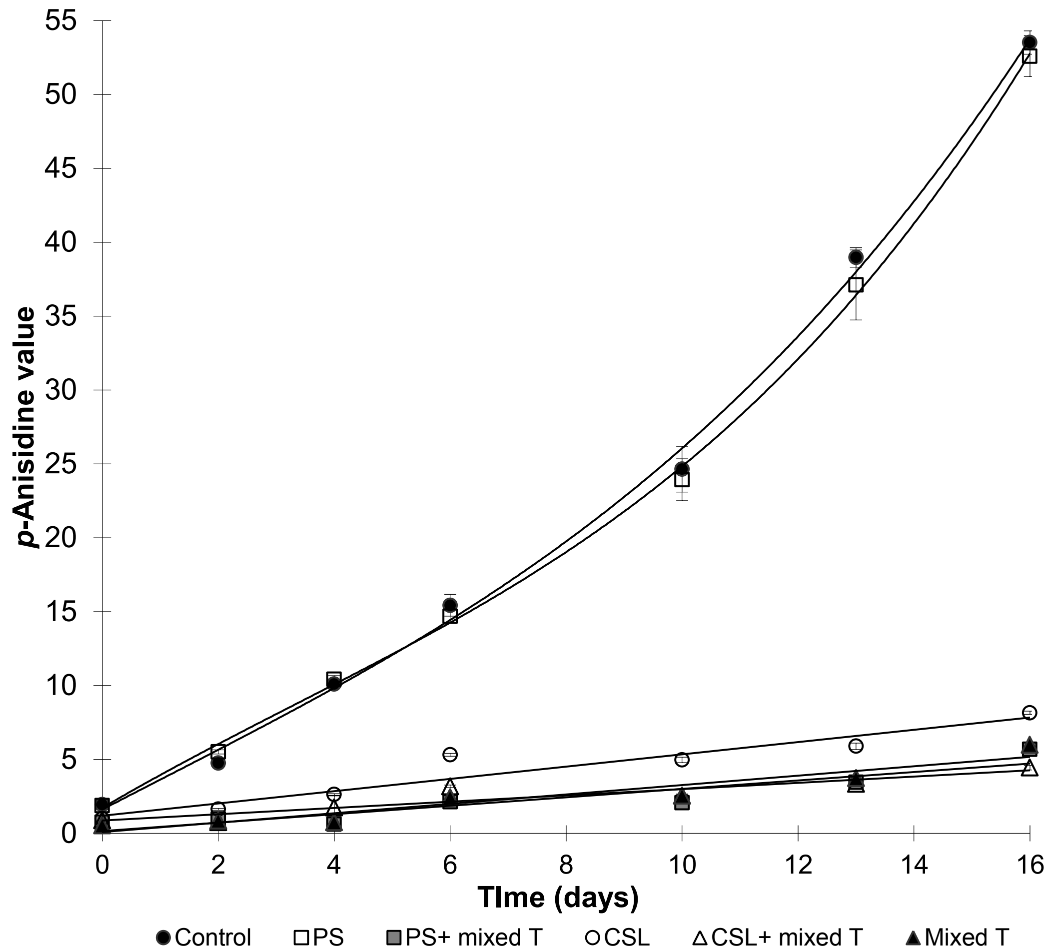


Figure 3.7 Effect of added PS and CSL with and without added mixed T on the development of secondary oxidation products, measured by pAV, in tocopherol stripped fish oil. For each sampling point, n=3 with error bars indicating standard deviation. PS+mixed T, CSL+mixed T and mixed T samples overlap. Trend lines are added to guide the eye; they do not indicate a function.

Initially, the control and PS sample had significantly greater *p*AV than the other treatments (Tukey's test: $p < 0.05$). By day 2, PS+mixed T, CSL+mixed T and mixed T had lower *p*AV compared to PS, CSL and the control (Tukey's test: $p < 0.05$; Appendix I). The trend of samples containing mixed T providing greater protection against the development of secondary oxidation products continued throughout the 16-day study.

For the most part, PS+mixed T, CSL+mixed T and mixed T provided greatest protective effect against the development of secondary oxidation products; this protection was equal among all three of these treatments (from days 2-16, excluding day 6 where PS+mixed T had greater protection than CSL+mixed T (Tukey's test: $p < 0.05$)). By the end of the study, day 16, CSL provided less protection than PS+mixed T, CSL+mixed T and mixed T (Tukey's test: $p < 0.05$).

Initial and final tocopherol levels were measured by HPLC as a final test to investigate the possibility that synergy with tocopherols was the reason for the antioxidant effect of CSL. α-, γ- and δ-Tocopherols did not change over the course of the study for CSL+mixed T, PS+mixed T and mixed T (Table 3.2; paired t-tests showed no significant differences at a significance level of $p < 0.05$). This indicates that the degree of oxidation displayed in this study was not great enough to show a significant decrease in tocopherols.

Table 3.2 Total tocopherol content at the beginning and end of the 16-day stability study. Values are mean sums (n=3) of α-, γ- and δ-tocopherols (mg g^{-1}), with standard deviations indicated in parentheses. The amount of α-tocopherol in CSL was below the detection limit of the method. ND = not detected.

	Time Point	
Sample	Initial	Day 16
Control	ND	ND
PS	ND	ND
PS+mixed T	2.59 (0.17)	2.76 (0.42)
CSL	ND	ND
CSL+mixed T	2.63 (0.23)	2.41(0.42)
Mixed T	2.88 (0.38)	2.92 (0.27)

3.3.3 Effect of Stabilizing Antioxidants in CSL on Primary Oxidation

The previous experiment found that PS had antioxidant activity only after the fish oil medium had undergone a large amount of oxidation, and therefore it was not the main component contributing to CSL's antioxidant activity; it also demonstrated that PS's activity was not increased by the presence of mixed T. Since mixed T were not found to act synergistically with PS as literature had suggested, the antioxidant activity of CSL must be attributed to the presence of another component of CSL. Therefore, the stabilizing antioxidants in CSL were investigated for their effect on the antioxidant activity of PS.

RME and αT are added to CSL as antioxidants to maintain the stability and potency of PS in CSL; they are not considered active ingredients according to supplier documentation. The combined antioxidant effect (assessed by PV) of PS+RME+αT at all time points was significantly greater than PS alone (ANOVAs were significant at a level of $p < 0.05$ for every time point; Tukey's test: $p < 0.05$; Figure 3.8). However, overall there was no added benefit in having PS present in the system; RME+αT had equivalent antioxidant effect compared to PS+RME+αT for the majority of the 16-day stability study (Tukey's test: $p > 0.05$). During the first third of the stability study, CSL, PS+RME+αT, and RME+αT had equal protective effect against the formation of hydroperoxides (Tukey's test: $p > 0.05$). As the tocopherol stripped fish oil medium became more oxidized later in the study, CSL began to show a significantly greater protective effect than PS+RME+αT and RME+αT, making it the most effective antioxidant compared to the other treatments (Tukey's test: $p < 0.05$). By the end of the study, day 16, the control had an average PV

of 180.7 meq kg^{-1}, and while this value was not statistically different from the PS sample, its presence skewed the post hoc analysis of the other treatments. Therefore, it was removed from the one-way ANOVA analysis at this time point to achieve a more accurate view of the effect of each treatment. On day 16, the CSL sample continued to have the lowest PV; however, for the first time in the study, the PS+RME+αT had a significantly lower PV than the RME+αT sample (Tukey's test: $p < 0.05$).

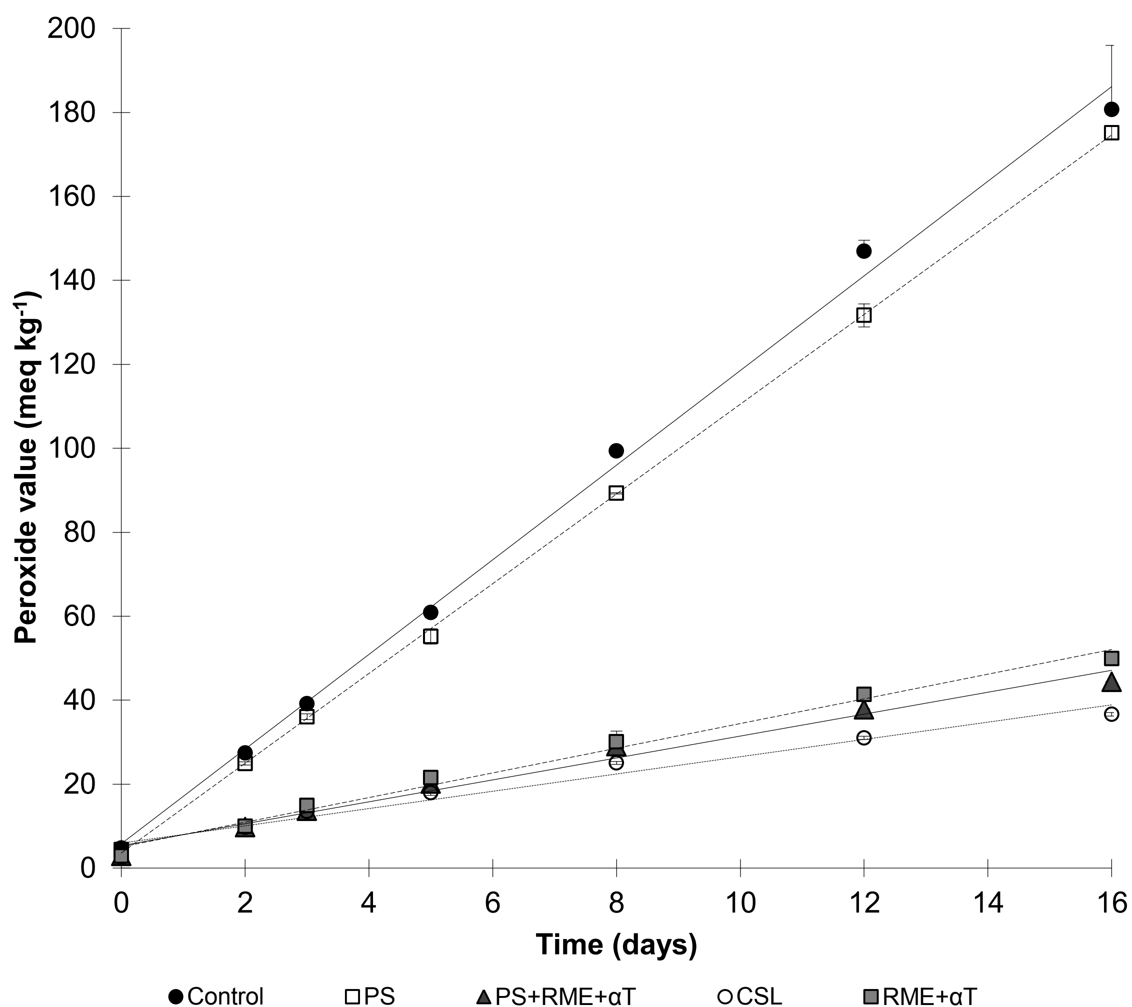


Figure 3.8 Effect of added PS, PS+RME+ αT, CSL and RME+αT on the development of hydroperoxides in tocopherol stripped fish oil. For each sampling point, n=3 with error bars indicating standard deviation. Lines indicate the linear regression.

When slopes of the regression are used to assess the rate of oxidation, it is obvious PS did not slow the development of hydroperoxides compared to the control (Tukey's test: $p > 0.05$; Table 3.3). However, PS+RME+αT and CSL had the same rate of increase in PV over the 16-day oxidation period (Tukey's test: $p > 0.05$). RME+αT had a slightly faster, and significant, rate of increase in PV compared to CSL (Tukey's test: $p < 0.05$), but this

rate was not significantly different than PS+RME+ αT (Tukey's test: $p > 0.05$). Antioxidant activity estimated as percent inhibition also supports this, with percent inhibition increasing in the following trend: PS << RME+ αT ≅ PS+RME+ αT < CSL (Figure 3.9, Table 3.3). Percent synergy between PS and RME+αT can also be estimated in a similar manner as percent inhibition by comparing the slopes of RME+αT to PS+RME+αT, relative to the control. Using this calculation, slight synergy (11.2 ± 5.5 %) was found to exist between PS and RME+αT. However, ANOVA showed no difference between the individual treatment slopes used in this calculation, suggesting that synergy did not exist (Tukey's test: $p > 0.05$; Table 3.3).

Table 3.3 Comparison of the increase in PV over time for samples stored in open vials at ambient conditions. Slopes are the mean values from the linear regression (meq PV kg^{-1} d^{-1}) with standard errors in parenthesis. Means that do not share the same letter are significantly different (Tukey's test: $p < 0.05$).

Sample	Slope
Control	11.27(0.25)^{A}
PS	10.69 (0.06)^{A}
PS+RME+αT	2.61 (0.09)^{B,C}
CSL	2.06 (0.09)^{B}
RME+αT	2.94 (0.08)^{C}

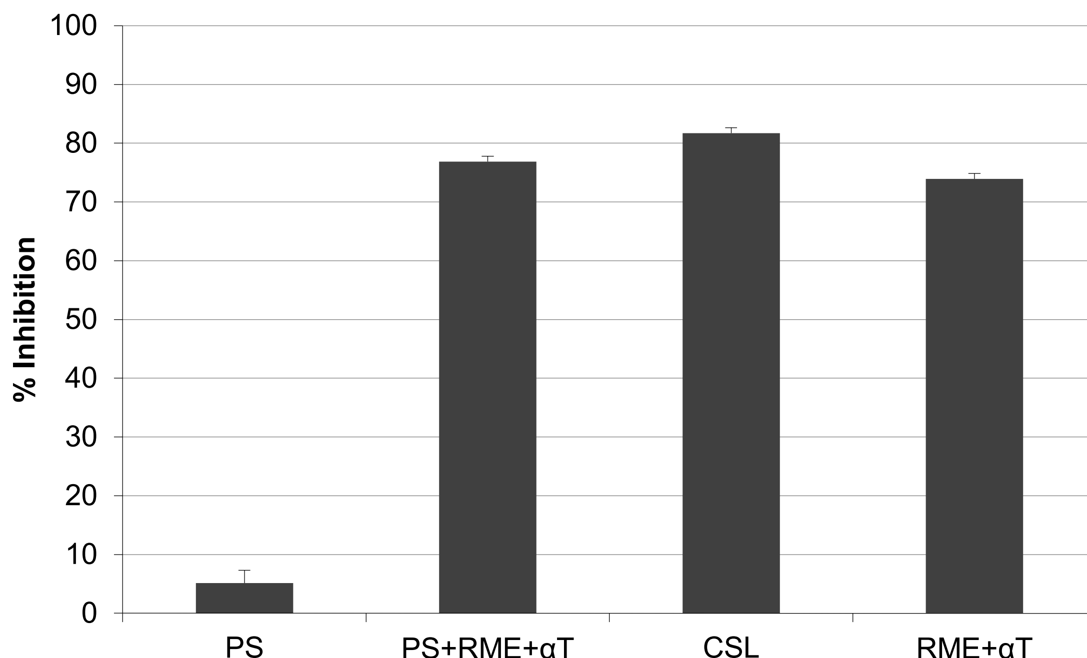


Figure 3.9 Estimated antioxidant activity, expressed in terms of ability to inhibit hydroperoxide formation, of PS, RME, αT and CSL in tocopherol stripped fish oil. Values are expressed as means (n=3) with standard error indicated by error bars.

3.3.4 Effect of Stabilizing Antioxidants in CSL on Secondary Oxidation

Throughout the entire oxidation period, the sample containing PS had equivalent or greater *p*AV compared to the control, indicating that PS did not provide any protection against secondary oxidation products (Figure 3.10; ANOVAs were significant at a significance level of $p < 0.05$ for every time point). PS+RME+αT and RME+αT provided equal protection against secondary oxidation products as measured by monitoring the *p*AV, throughout the entire study (Tukey's test: $p > 0.05$). Therefore, just as synergy did not exist between PS and the combination of RME+αT in the evolution of hydroperoxides (determined by linear regression slopes), there was no evidence of synergy in the protection against secondary oxidation products. Unlike the protection

against hydroperoxides, in the early stages of oxidation, PS+RME+αT had a slightly, albeit significantly, lower *p*AV than CSL (Tukey's test: $p < 0.05$). For the remainder of the 16-day trial PS+RME+αT, RME+αT and CSL had an equivalent protective effect against formation of secondary oxidation products (Tukey's test: $p > 0.05$). These results indicate that CSL has no added benefit over RME+αT in regards to protection against the formation of secondary oxidation products.

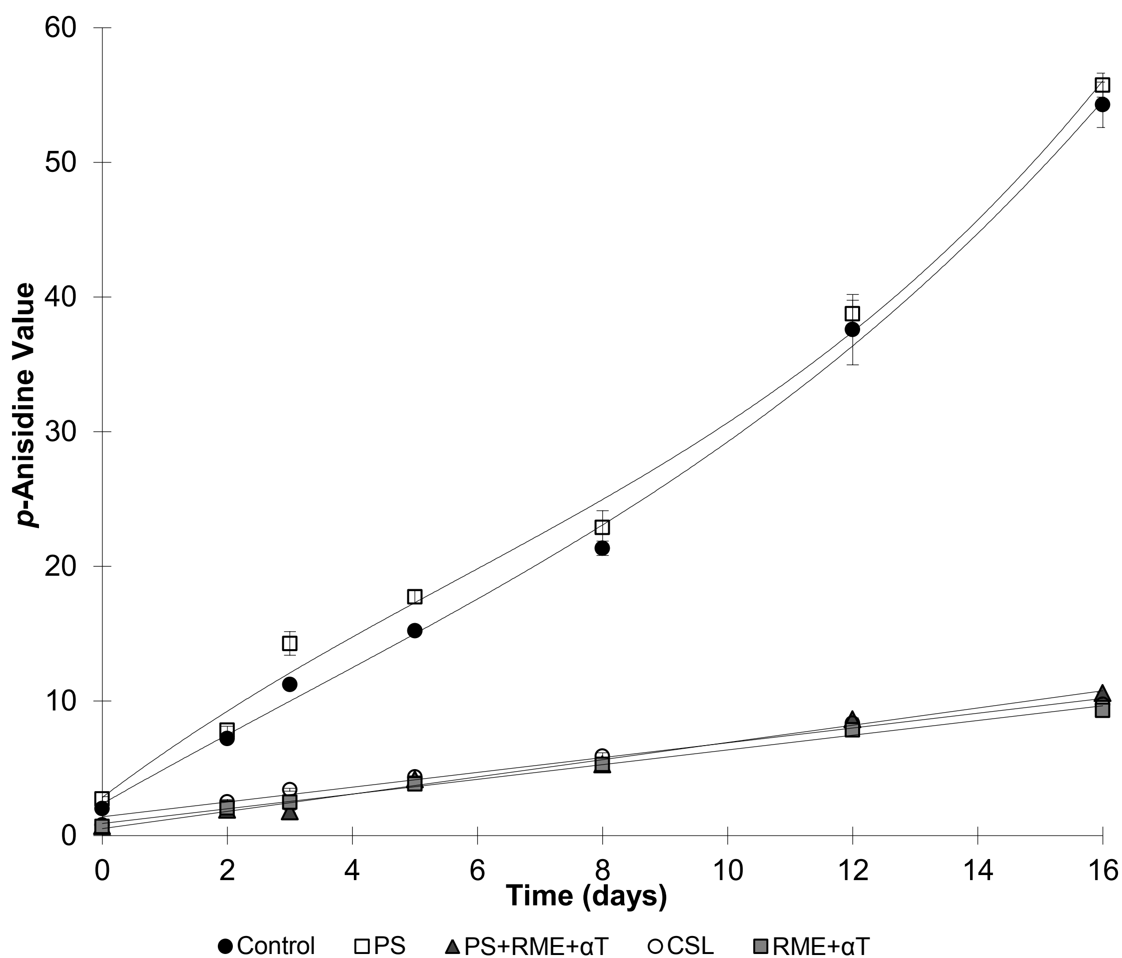


Figure 3.10 Effect of added PS, RME, αT and CSL on the development of secondary oxidation products, as measured by pAV, in tocopherol stripped fish oil. For each sampling point, n=3 with error bars indicating standard deviation. PS+RME+ αT, CSL and RME+αT samples overlap. Trend lines are added to guide the eye; they do not indicate a function.

3.3.5 Individual effects of RME and αT on Primary Oxidation

To gain more insight into the potential interactions between RME, αT and PS, the individual effects of RME and αT with and without PS on the evolution of hydroperoxides were investigated (Figure 3.11; ANOVAs were significant at a significance level of $p < 0.05$ for every time point). CSL had the greatest antioxidant effect of all the treatments examined as measured by PV (Tukey's test: $p < 0.05$). This protection was significantly greater than either PS+αT or PS+RME (Tukey's test: $p < 0.05$). Therefore, the combination of PS+RME+αT appears to be important in providing CSL's ability to protect against hydroperoxide formation. PS+αT and αT alone provided the second greatest protection against hydroperoxide development; this protection did not differ significantly throughout the entire oxidation period (Tukey's test: $p > 0.05$), indicating that synergy does not exist between PS and αT. Just as the initial stability study found the addition of PS to mixed T had no added benefit in the protection against hydroperoxides (Figure 3.4), PS combined with αT offered no additional benefit. In early stages of oxidation, RME had a greater protective effect against hydroperoxides than PS; however, near the end of the 16-day study, RME became a less effective antioxidant than PS (Tukey's test: $p < 0.05$).

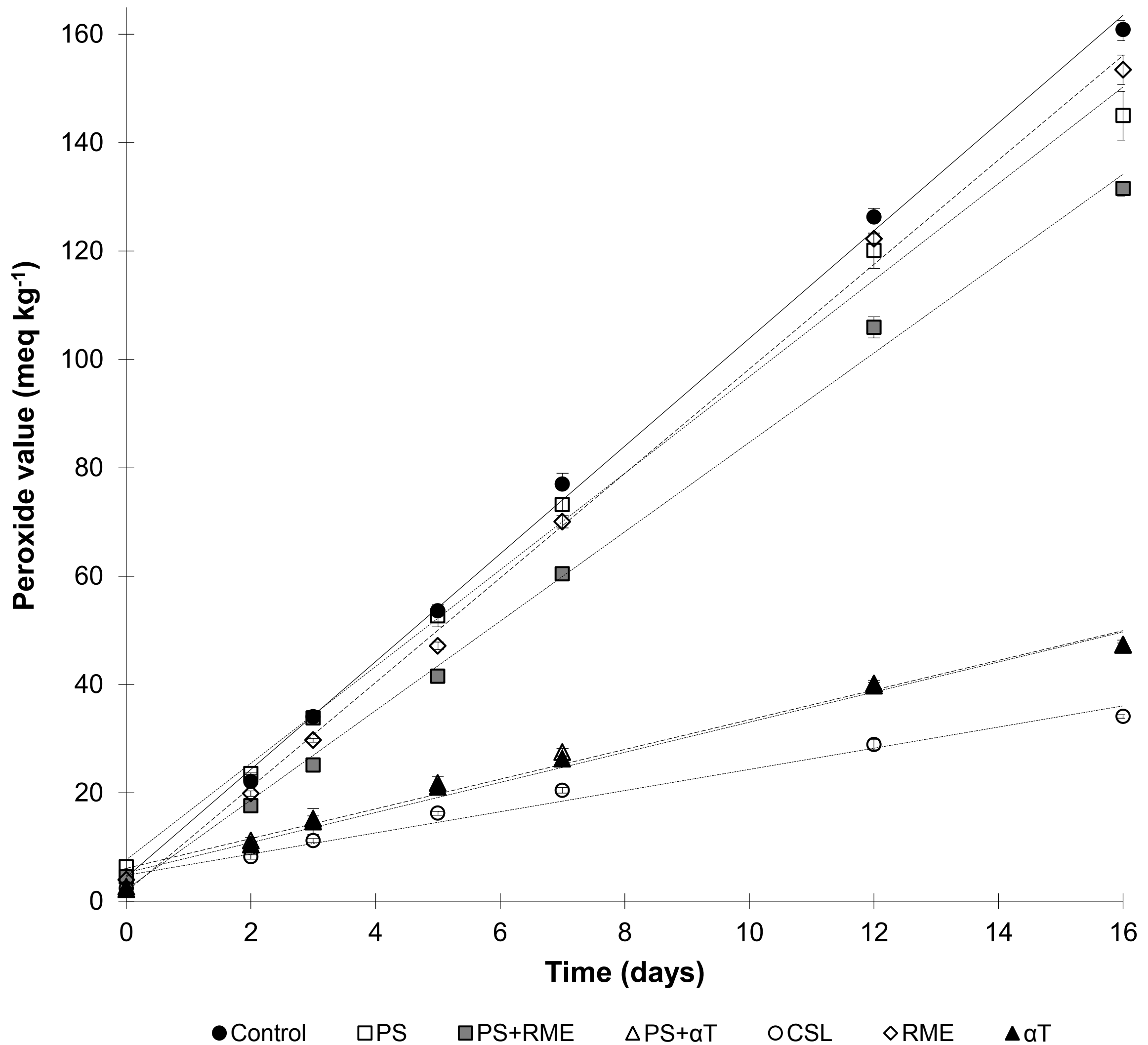


Figure 3.11 Effect of added PS, RME, αT and CSL on the development of hydroperoxides in tocopherol stripped fish oil. For each sampling point, n=3 with error bars indicating standard deviation. PS and RME samples overlap for much of the study, as well as PS+αT and αT. Lines indicate the linear regression.

The rate of hydroperoxide formation, as determined by the linear regression slopes of the PV vs. time graph, was significantly slower in CSL sample compared to all other treatments and the control (Table 3.4; Tukey's test: $p < 0.05$). This agrees with the

analysis of PV at each time point. The only sample that did not show an antioxidant effect according to the rate of hydroperoxide formation was RME; its slope did not differ significantly from the control. The rate of PV increase was statistically equivalent for PS+αT and αT, supporting the previous data (Tukey's test: $p > 0.05$; Figure 3.4) that synergy does not exist between PS and αT. The rate of hydroperoxide formation also indicated that there was a synergistic relationship between PS and RME; the rate of PV increase was significantly lower in the PS+RME samples compared to PS and RME alone (Tukey's test: $p < 0.05$). PS alone was found to slow the rate of PV increase over time (Tukey's test: $p < 0.05$), indicating a small antioxidant effect.

Table 3.4 Comparison of the increase in PV over time for samples stored in open vials at ambient conditions. Slopes are the mean values of linear regression with standard errors in parenthesis. Means that do not share the same letter are significantly different (Tukey's test: $p < 0.05$).

Sample	Slope
Control	9.95 (0.10)^{A}
PS	8.92 (0.16)^{B}
PS+RME	8.25 (0.11)^{C}
PS+αT	2.74 (0.09)^{D}
CSL	1.95 (0.07)^{E}
RME	9.65 (0.12)^{A}
αT	2.78 (0.16)^{D}

The estimated antioxidant effect measured as percent inhibition found RME had the least protective effect against production of hydroperoxides (Figure 3.12). CSL again provided the greatest inhibition of hydroperoxide formation, with PS+αT and αT providing second greatest levels of inhibition. Results of the estimated antioxidant effect for each treatment indicated a slight synergistic relationship between PS and RME; PS and RME alone provided 10.4 ± 1.8 % and 3.0 ± 1.6 % inhibition of hydroperoxide formation compared

to the control, respectively, whereas PS+RME inhibited development of hydroperoxides by 17.1 ± 1.4 % compared to the control.

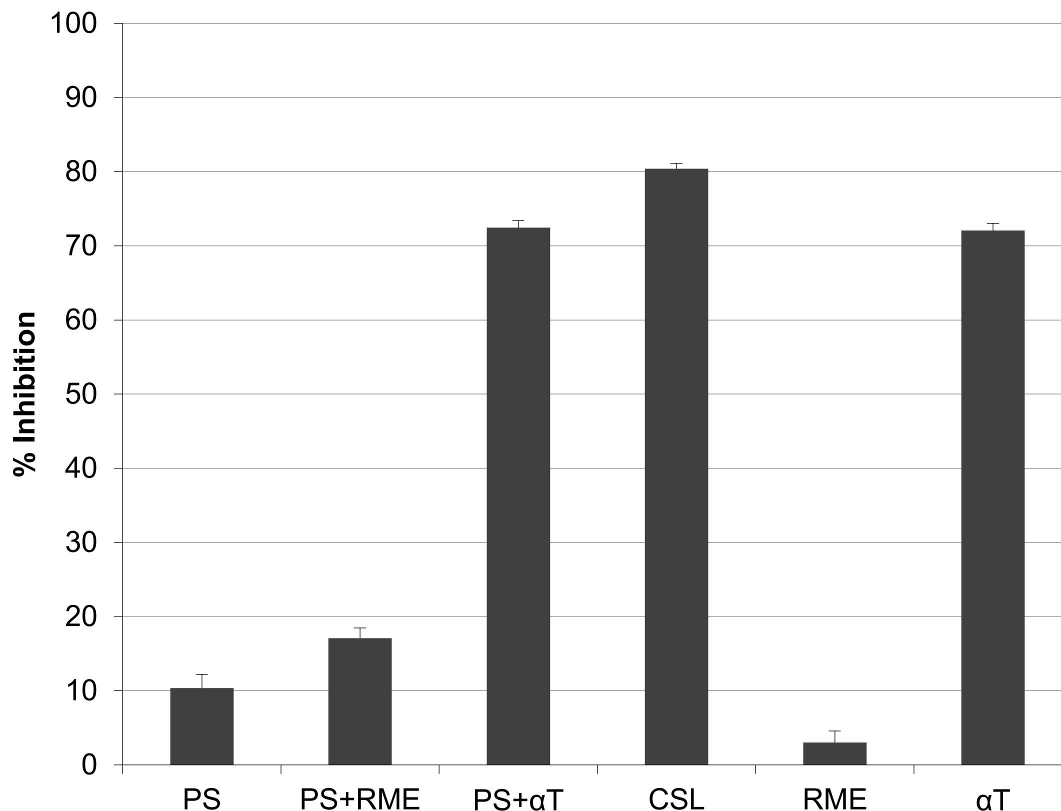


Figure 3.12 Antioxidant effects, expressed in terms of ability to inhibit formation of hydroperoxides, of PS, RME, αT and CSL in tocopherol stripped fish oil. Values are expressed as means (n=3) with standard error indicated by error bars.

There was a slight synergistic relationship (14.5% ± 1.6%) between PS and RME with respect to hydroperoxide formation. However, there is essentially no synergy (1.4% ± 4.5%) between PS and αT.

3.3.6 Individual effect of RME and αT on Secondary Oxidation

In contrast to the development of hydroperoxides, there was no indication of synergy in the reduction of the formation of secondary oxidation products between PS and RME (Figure 3.13; ANOVAs were significant at a significance level of $p < 0.05$ for every time point); both the sample containing PS+RME and RME individually had statistically equivalent antioxidant effects according to the development of secondary oxidation products (Tukey's test: $p > 0.05$). PS was the only treatment that did not have an antioxidant effect with regards to the formation of secondary oxidation products, consistent with the earlier stability studies. Therefore, it is not surprising that the combination of PS with either RME or αT did not provide any added benefit compared to RME or αT alone. CSL, PS+αT and αT had the greatest protective effect against the development of secondary oxidation products, according to *p*AV. The antioxidant effect of these three treatments was statistically equivalent throughout the 16-day study (Tukey's test: $p > 0.05$).

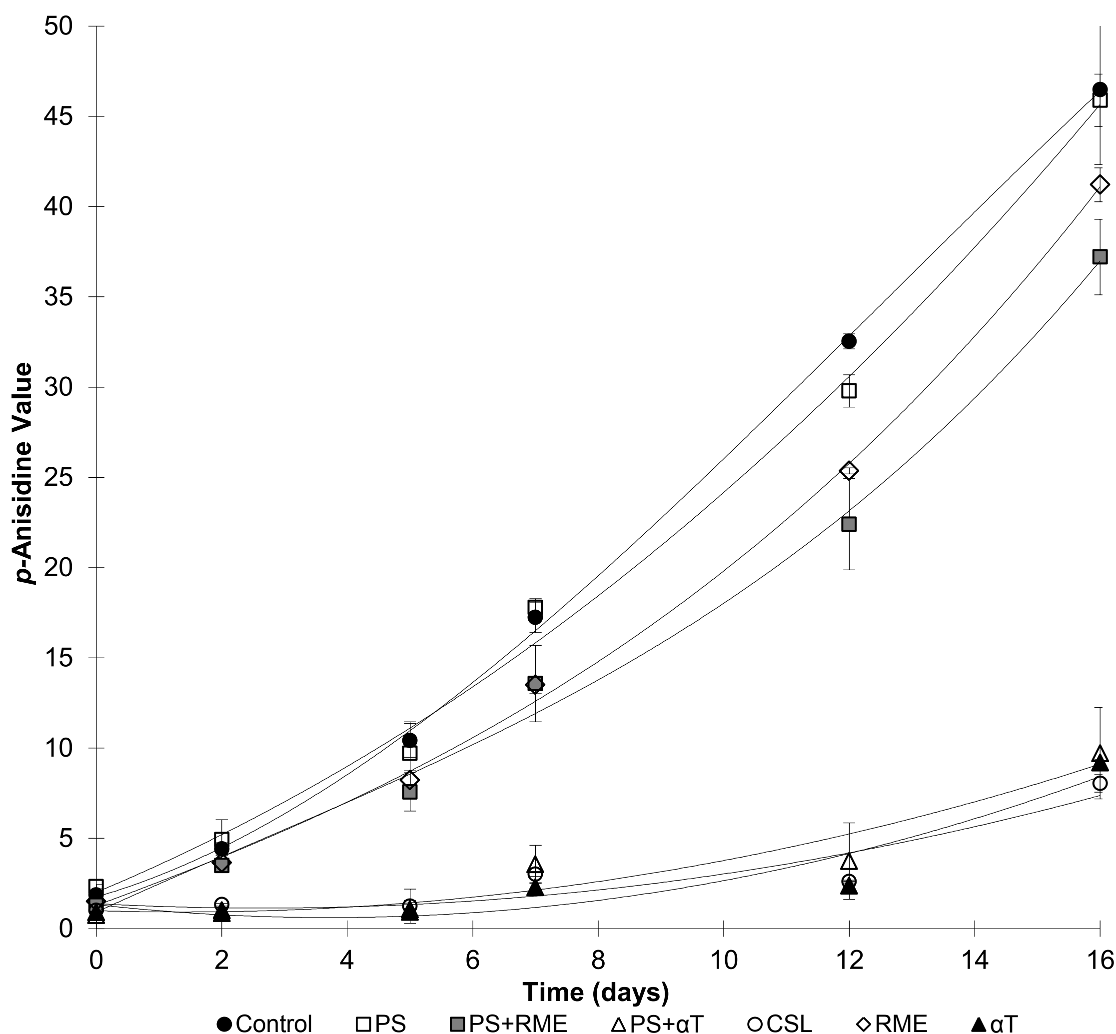


Figure 3.13 Effect of added PS, RME, αT and CSL on the development of secondary oxidation products, as measured by pAV, in tocopherol stripped fish oil. For each sampling point, n=3 with error bars indicating standard deviation. PS+αT, CSL and αT samples overlap. Trend lines are added to guide the eye; they do not indicate a function.

3.3.7 Development of Maillard Browning Compounds

Literature has indicated that PL with a free amine group can react with aldehydes to form Maillard browning compounds. Thus, Maillard browning compounds were measured

spectrophotometerically in experiments that examined the effect of stabilizing antioxidants (RME and αT) in combination (Table 3.5) and individually (Table 3.6) on the antioxidant effect of PS.

The PS sample showed significant formation of Maillard browning compounds on days 3 and 5 (Tukey's test: $p < 0.05$) (Table 3.5; ANOVAs were significant at a level of $p < 0.05$ on days 2, 3, 5 and 8). By day 8, the last time point measured, the control and PS sample had significantly greater amounts of browning compounds present than PS+RME+αT, CSL and RME+αT (Tukey's test: $p < 0.05$). However, the browning at this point was equivalent for both the control and PS sample (Tukey's test: $p > 0.05$). The measurement of Maillard browning compounds was stopped after day 8 because the control and PS samples became turbid after this time point. The turbidity was only noted in the control and PS samples; it was likely related to the great extent of lipid oxidation experienced in these samples compared to PS+RME+αT, CSL and RME+αT.

Table 3.5 Evolution of Maillard browning compounds over an eight-day period, investigating the combined effect of RME+αT on the antioxidant activity of PS. Values represent the absorbance at 430 nm expressed as the mean (n=3) with standard deviations in parenthesis. Values with different superscript letters within the same time point are significantly different (Tukey's test: $p < 0.05$).

	Absorbance at 430 nm Time (days)				
Sample	0	2	3	5	8
Control	$0.00(0.00)^{A}$	$0.03(0.01)^{A,B}$	$0.02(0.00)^{A}$	$0.05(0.02)^{A}$	$0.18(0.02)^{A}$
PS	$0.00(0.00)^{A}$	$0.04(0.00)^{A}$	$0.06(0.02)^{B}$	$0.08(0.01)^{B}$	$0.17(0.01)^{A}$
PS+RME+αT	$0.00(0.00)^{A}$	$0.03(0.00)^{B}$	$0.02(0.00)^{A}$	$0.04(0.00)^{A}$	$0.09(0.01)^{B}$
CSL	$0.00(0.00)^{A}$	$0.03(0.00)^{A,B}$	$0.03(0.01)^{A}$	$0.05(0.01)^{A}$	$0.10(0.00)^{B}$
RME+αT	$0.00(0.00)^{A}$	$0.02(0.00)^{B}$	$0.01(0.00)^{A}$	$0.03(0.00)^{A}$	$0.08(0.00)^{B}$

There was no particular trend in the development of browning compounds in the study investigating the individual effects of RME and αT in combination with PS (Table 3.6; ANOVAs were significant at a level of $p < 0.05$ on days 5 and 7). Absorbance measurements ceased after day 7 due to turbidity in the control, PS, PS+RME and RME samples. All samples experienced equivalent development of browning compounds until day 5 when the control and PS samples had a greater amount of browning compared to PS+αT, αT alone and CSL, which in turn displayed the same level of browning (Tukey's test: $p < 0.05$). The PS+RME and RME samples had statistically equivalent development of browning compared to all samples (control, PS, PS+αT, αT and CSL) (Tukey's test: $p > 0.05$). By the end of the measured time points, PS and PS+RME had the most browning compounds, closely followed by RME (Tukey's test: $p < 0.05$).

Table 3.6 Evolution of Maillard browning compounds over a seven-day period investigating the individual effect of RME and αT on the antioxidant activity of PS. Values represent the absorbance at 430 nm expressed as the mean (n=3) with standard deviations in parenthesis. Values with different superscript letters within the same time point are significantly different (Tukey's test: $p < 0.05$).

	Absorbance at 430 nm Time (days)			
Sample	0	2	5	7
Control	$0.00(0.00)^{A}$	$0.19(0.09)^{A}$	$0.20(0.05)^{A}$	$0.15(0.04)^{A,B,C}$
PS	$0.01(0.00)^{A}$	$0.24(0.03)^{A}$	$0.21(0.01)^{A}$	$0.24(0.02)^{D}$
PS+RME	$0.00(0.00)^{A}$	$0.21(0.01)^{A}$	$0.15(0.01)^{A,B}$	$0.20(0.01)^{A,D}$
PS+αT	$0.01(0.01)^{A}$	$0.20(0.00)^{A}$	$0.12(0.01)^{B}$	$0.15(0.01)^{A,B,C}$
CSL	$0.01(0.00)^{A}$	$0.20(0.00)^{A}$	$0.11(0.00)^{B}$	$0.14(0.01)^{B,C}$
RME	$0.00(0.00)^{A}$	$0.23(0.01)^{A}$	$0.17(0.01)^{A,B}$	$0.19(0.00)^{A,B}$
αT	$0.00(0.00)^{A}$	$0.22(0.00)^{A}$	$0.12(0.01)^{B}$	$0.13(0.01)^{C}$

CSL is a brown viscous liquid. Pure soybean PS is white to pale yellow; therefore, it was thought that the brown colour of CSL might be due to processing. Correspondence with the supplier of CSL confirmed that the product is heated to 40 °C during production. To determine if heating at 40 °C is sufficient to cause browning, a 2.1 mg g^{-1} mixture of soybean PS and MCT from palm kernel was prepared and heated in a 40 °C water bath for 90 min. PS was diluted in MCT because it is the carrier oil used in CSL to create a fluid lecithin with a standardized concentration of PS. Observations were noted after 30 min of heating and then every 15 min until 90 min was reached. No observable colour changes were noted during the 90 min period. Therefore, it does not appear that heating of the PS during manufacture of CSL is the cause of its brown colour.

3.3.8 Comparison of Fatty Acid Moieties on Soybean PS and CSL PS

Although both sources of PS had different proportions of fatty acids, they both contained 16:0, 18:0, 18:1n-9, 18:1n-7, 18:2n-6, 18:3n-3 (Table 3.7). CSL PS also contained trace amounts of 20:0 and 22:0, which were not present in the soybean PS, while the soybean PS contained trace amounts of 17:0 (Table 3.7). The soybean PS contained a greater amount of saturated fatty acid moieties, with 22.6% saturated and 77.4% unsaturated fatty acid moieties (Table 3.7). In comparison, CSL PS contained 14.1% saturated and 85.9% unsaturated fatty acid moieties. CSL PS also contained more polyunsaturated fatty acids, with 69.6% compared to 61.0% in the soybean PS.

Table 3.7 Mass percent of total fatty acid moiety present in the PS fraction of CSL, as well as the soybean PS. Values are means (n=3) with standard deviations shown in parenthesis.

	PS Source	
Fatty Acid	CSL	Soybean
16:0	10.15 (0.59)	17.42 (0.73)
17:0	-	0.10 (0.09)
18:0	3.37 (0.10)	5.02 (0.19)
20:0	0.14 (0.08)	-
22:0	0.44 (0.02)	-
18:1n-9	15.58 (0.16)	14.51 (0.07)
18:1n-7	0.73 (0.01)	1.94 (0.01)
18:2n-6	69.43 (0.36)	57.07 (0.77)
18:3n-3	0.16 (0.01)	3.91 (0.08)
% Saturated	14.10	22.57
% Unsaturated	85.90	77.43

3.3.9 Iron Chelating Ability of PS

This research indicates that soybean derived PS, CSL and RME are able to chelate iron (II) (Figure 3.14). CSL had a greater ability to chelate iron (II) compared to the pure soybean derived PS, with 36.5% ± 2.5% chelation compared to ferrozine, in contrast to PS with 16.4% ± 4.4%. The RME, at 0.31 mg mL^{-1}, was found to bind 4.4% ± 2.4% of the iron (II). However, in the process of this analysis, it was noted that the blank of CSL and RME had measurable absorption; therefore, since there is no added iron (II) in the blank, the ferrozine was complexing iron that was present in CSL and RME. Thus, the presence of RME, as well as other minor PL, in CSL may be providing the additional iron (II) chelating effect compared to pure soybean PS.

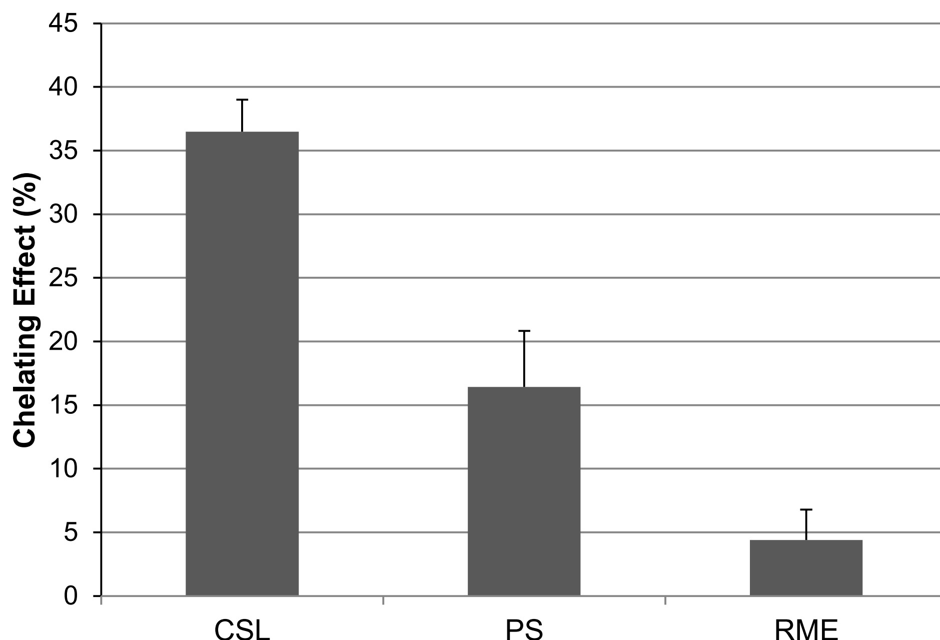


Figure 3.14 Mean (n=3) iron (II) chelating effect of CSL, soybean derived PS and RME. Error bars indicate the standard deviation.

3.4 Discussion

This research set out to answer two specific questions: 1) Does PS have an independent antioxidant effect in fish oil? and 2) Does PS have a synergistic relationship with tocopherols or primary antioxidants (RME and αT) used to stabilize CSL? Both primary and secondary oxidation products were monitored to assess how these ingredients affected the stability of fish oil at different stages of oxidation. While secondary oxidation products are more closely linked to changes to the oil's sensory profile, monitoring both is important to give a better indication of antioxidant mechanism. For instance, in the experiments investigating the individual effect of RME and αT with PS on development of primary and secondary oxidation, it was found that while PS+αT did

not protect against the formation of hydroperoxides as well as CSL, it did provide equivalent protection against the development of secondary oxidation products.

3.4.1 Effect of PS on Primary and Secondary Oxidation of Fish Oil

Monitoring the development of hydroperoxides over time clearly showed that PS has minimal protective effect against formation of primary oxidation products (Tables 3.1, 3.3 and 3.4), only slightly slowing the development of PV in two studies (Tables 3.1 and 3.4). This slight decrease in development of PV represents the reduction of PV, compared to the control, at later stages of oxidation once the oil was highly oxidized, that was maintained until the end of the study (Figures 3.4 and 3.11). A possible explanation for the antioxidant activity of PS at later stages is that through oxidation the serine moiety is cleaved from the phosphate group and it is the serine moiety that possesses the antioxidant activity. Saito and Ishihara (1997) suggested that it is the separated ethanolamine or choline moiety of PE or PC that has the antioxidant effect. They indicate that the ethanolamine or choline moiety may be easily removed from the PL in lecithins through decomposition. Therefore crude lecithin, which has likely gone through more oxidation/decomposition reactions than pure PL, would be expected to have a greater antioxidant effect. While CSL is not a crude lecithin, the pure PS in these studies likely did not undergo as much oxidation as CSL. Thus, the reduced antioxidant activity of pure PS may also be in part due to its level of decomposition compared to CSL. The hydroperoxide protective effect is not of industrial importance as the oil was well beyond acceptable quality limits for fish oil when this antioxidant effect was noted. Health

Canada has set the maximum allowable PV as 5 meq kg^{-1} for fish oil natural health products (Health Canada, 2013).

As mentioned in the previous chapter it is suspected, but not confirmed, that the CMC of PS was met in these experiments. If it were to behave similarly to DOPC, one would expect that PS alone would have had a pro-oxidant effect (Chen *et al*., 2011). However, 2.1 mg g^{-1} PS, overall, had either no effect or a slight antioxidant effect compared to the control, as measured by the development of hydroperoxides (Tables 3.1, 3.3 and 3.4). Also, the presence of PS had no effect on the antioxidant activity of 0.03 mg g^{-1} αT, whereas Chen *et al*., (2011) found the antioxidant effect of 10 μM (equal to 4.3 μg g^{-1}) α-tocopherol was increased in combination with DOPC at a concentration greater than the CMC. Therefore, either reverse micelles were not formed, or reverse micelles of PS behave differently than DOPC, especially in differing oxidative mediums.

PS alone did not have an effect on secondary oxidation products, monitored by *p*AV. This, in combination with the PV results, indicates that PS has minimal antioxidant effect on its own. Any antioxidant effect it may have appears to be related to an ability to reduce hydroperoxide formation or act as a hydroperoxide destroyer at later stages of oxidation. The slight antioxidant activity noted could also be due to PS's ability to act as an iron (II) chelator (Figure 3.14). While its chelation ability was not as strong as CSL, this research found it was able to bind some iron (II), potentially preventing at least a portion of metal ions from catalyzing oxidation reactions.

3.4.2 Effect of Tocopherols on the Antioxidant Activity of PS

A number of studies have suggested that the antioxidant activity of PS is due to synergy with tocopherols or α-tocopherol (Hudson & Ghavami, 1984, Kashima, 1991); however, the results of this research saw little evidence to support that theory. Synergy with αT was not noted in the stability study investigating the individual effect of RME and αT on the antioxidant effect of PS (Figures 3.11 and 3.12), although there was evidence of synergy with mixed T over the course of two days in a 16-day stability study (Figure 3.4). Since the concentration of mixed T did not change over the course of that study, it is unlikely that the synergy displayed between PS+mixed T on days 1 and 2 was due to PS regenerating tocopherols as was suggested by Kashima *et al.* (1991). This synergy could be due to PS chelating iron, preventing the inactivation of mixed T, through oxidation. However, the overall rate of hydroperoxide formation was equivalent for both PS+mixed T and mixed T alone, indicating there is no synergy between PS and mixed T (Table 3.1). Therefore, it was the tocopherols (mixed T or αT) that were providing the bulk of the antioxidant effect in studies in which PS was combined with mixed T or αT. This is especially true in the development of secondary oxidation products. The reduction in *p*AV in PS+mixed T or PS+αT was due to the presence of mixed T or αT, respectively; PS did not reduce the development of secondary oxidation products (Figures 3.7 and 3.13).

3.4.3 Effect of Tocopherols on the Antioxidant Effect of CSL

The antioxidant effect of CSL, according to hydroperoxide and CD formation and the rate of hyrdoperoxide formation, was not improved by the addition of mixed T (Figures 3.4 and 3.5, Table 3.1). In fact, at later stages of the stability study, the addition of mixed T was found to increase the formation of hydroperoxides and CD compared to the CSL sample alone. The overall rate of hydroperoxide formation was greater in the CSL+mixed T sample compared to CSL alone (Table 3.1); therefore, synergy did not exist between CSL and mixed T in the protection against the development of primary oxidation products.

However, the CSL+mixed T sample had more protective effect against the formation of secondary oxidation products, as measured by *p*AV, than CSL alone. The mixed T sample alone, for the most part, provided equivalent or greater protection from the formation of α- and β- unsaturated aldehydes. Therefore, it seems that mixed T, and possibly αT in particular, are providing the bulk of the antioxidant effect against the formation of secondary oxidation products.

3.4.4 Effect of Tocopherols on Primary and Secondary Oxidation

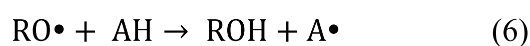
These stability studies found samples containing αT (CSL, RME+αT, PS+RME+αT, PS+αT and αT alone) had the greatest decrease in the formation of secondary oxidation products compared to the control and other treatments. Similarly, samples containing added mixed T generally had reduced *p*AV compared to samples without added

tocopherols (Figure 3.7). Therefore, as the initial experiments suggested, it seems that αT at a concentration of 0.03 mg g^{-1}, was providing the antioxidant effect against the formation of secondary oxidation products in CSL. The ability of αT to inhibit the development of secondary oxidation products has been noted by others (Frankel *et al.*, 1994, Huang *et al.*, 1994). Frankel *et al.* (1994) found 0.1 mg g^{-1} and 0.5 mg g^{-1} α-tocopherol inhibited the formation of hexanal, a volatile secondary oxidation product, in tocopherol stripped corn oil. This inhibition was concentration dependent with samples containing 0.5 mg g^{-1} α-tocopherol providing a greater reduction in hexanal formation than 0.1 mg g^{-1}α-tocopherol; however, the higher concentration of α-tocopherol increased the formation of hydroperoxides (Frankel *et al.*, 1994). Other studies have also found hexanal to be correlated with *p*AV (Tompkins & Perkins, 1999). The suggested mechanism by which α-tocopherol reduces hexanal formation is due to α-tocopherol's strong ability to act as a hydrogen donor making hydrogen abstraction from α-tocopherol by alkoxyl radicals more favorable than β-scission of the radical (Hopia *et al.*, 1996). Bypassing β-scission reduces the formation of reactive secondary oxidation products such as aldehydes and, instead, hydrogen donation to the alkoxyl radical forms more stable alcohols and tocopheroxyl radicals (Hopia *et al.*, 1996, Huang *et al.*, 1994).

Despite having equivalent protection against the formation of secondary oxidation products, as measured by *p*AV, neither PS+αT nor αT alone had as great protection against hydroperoxide formation as CSL. PS+αT and αT did reduce the development of hydroperoxides compared to the control, which is expected due to tocopherols' ability to scavenge free radicals. However, these results indicate that CSL's ability to reduce

hydroperoxide formation is due to more than the presence of PS and αT. Hopia *et al*. (1996) found higher concentrations of α-tocopherol (40 mM or approximately 17 mg g^{-1}) reduced the decomposition of methyl linoleate hydroperoxides while, simultaneously, methyl linoleate hydroxy compounds were formed. The exact mechanism by which α-tocopherol reduces or prevents the decomposition of hydroperoxides remains unclear. However, it has been suggested that hydrogen donating antioxidants can slow radical-mediated hydroperoxide decomposition by scavenging intermediate radicals and reducing hydroperoxides to more stable hydroxy compounds, while metal chelators slow decomposition by chelating metal ions (Frankel, 2005, p. 216 & 226). If Hopia *et al*. (1996) are correct in that tocopherols are good hydrogen donators and promote the protonation of alkoxyl radicals, it may be that α-tocopherol can also donate a hydrogen atom to peroxyl radicals, an intermediate in the decomposition of hydroperoxides, to reform hydroperoxides, thus slowing hydroperoxide decomposition into secondary oxidation products (4) (Mäkinen & Hopia, 2000). At the same time, if any alkoxyl radicals are formed, α-tocopherol may donate a hydrogen atom to form more stable hydroxy compounds, preventing formation of aldehydes (6) (Hopia *et al*., 1996). If the proposed mechanism in which hydrogen abstraction is favoured over β-scission, then alcohols that are formed would not be detected by the *p*AV method and α and β-unsaturated aldehydes that would be detected are not being formed as readily.

$$\mathrm{ROO\bullet + AH \rightarrow ROOH + A\bullet} \qquad (4)$$



$$\mathrm{RO\bullet + AH \rightarrow ROH + A\bullet} \qquad (6)$$

This antioxidant activity of αT was also demonstrated in the stability study investigating the effect of mixed tocopherols on the antioxidant activity of CSL and PS (Figures 3.4 and 3.7). Even though CSL had the lowest PV at most time points compared to CSL+mixed T and PS+mixed T treatments, its protection against secondary oxidation products was not as great as those antioxidant combinations. Just as Frankel *et al*. (1994) found protection against hexanal formation by α-tocopherol was concentration dependent, the additional α-tocopherol in the mixed T, or perhaps the other tocopherol isomers in the mixed T, reduced the formation of α and β-unsaturated aldehydes, as measured by *p*AV, to a greater extent than CSL alone, which contained only the αT isomer. Therefore, even though the PV of CSL was lower than CSL+mixed T, the greater *p*AV may have been because less α-tocopherol was present to drive the formation of alcohols over aldehydes. The higher PV in CSL+mixed T sample could be due to a slight pro-oxidant effect of the higher tocopherol concentration. While the concentration of approximately 3 mg g^{-1} mixed T, which consisted of about 0.6 mg g^{-1}α-tocopherol, used in this study has an antioxidant effect compared to the control, even slight increases in αT (such as the 0.03 mg g^{-1} in CSL) may have a pro-oxidant effect or be less effective at preventing hydroperoxide formation as noted by a greater increase in PV. For instance, Frankel *et al*. (2005, p. 232) found hexanal formation was inhibited even at 1 mg g^{-1} α-tocopherol, while hydroperoxide formation was increased at the same α-tocopherol concentration during early stages of oxidation.

3.4.5 Iron (II) Chelation of PS, CSL and RME

Reduction of free iron (II) in food and biological systems is important as it decreases the occurrence of the Fenton reaction. Briefly, the Fenton reaction refers to the reaction between hydrogen peroxide and iron (II) to form a radical that can react with a variety of organic species (Winterbourn, 1995). In biological systems, this reaction is thought to be related to numerous disease states (Winterbourn, 1995). In food systems, the formation of hydroxyl radicals via the Fenton reaction leads to oxidation of constituents, including lipids, in turn, leading to physical and chemical changes.

PS and RME were chosen to investigate their iron (II) chelating ability because literature suggested that PL and RME may possess some metal chelating abilities (Lunde *et al.*, 1976, Yoshida *et al.*, 1991, Dacaranhe and Terao, 2001a, Aruoma *et al*., 1992). Results from the oxidative stability studies completed also indicated that PS had some additional antioxidant benefit at later stages of oxidation (Figures 3.4, 3.8 and 3.11). While CSL had a greater protective effect against the formation of hydroperoxides compared to the PS+RME+αT and RME+αT, the PS+RME+αT had an equivalent effect with regards to rate of hydroperoxide formation (Figure 3.8 and Table 3.3). The added benefit of PL, particularly PS, may be due to iron chelating abilities. This study found CSL and RME were also able to chelate iron (II) but the chelation ability of CSL was much stronger than PS or RME alone. There are two possible explanations for the additional chelation ability of CSL, compared to PS: 1) the minor PL within the mixture may have contributed an additional chelation effect, possibly showing synergism with RME; and 2) there may have been a synergistic relationship between RME and αT. Wada and Fang (1992) found

the depletion of α-tocopherol in sardine oil was slowed and oxidative stability increased when RME was added to the system. The authors suggested this effect could be due to RME acting synergistically with α-tocopherol by chelating metal ions or donating a hydrogen atom to regenerate tocopherols, thus preventing α-tocopherol's inactivation (Wada and Fang, 1992). In the case of CSL, the presence of both PS and RME, which were found to have chelation abilities, may be more effective at preventing αT's inactivation than RME alone. The rate of hydroperoxide formation data supports this hypothesis as the combination of PS+RME+αT had an equivalent increase in hydroperoxides over time compared to CSL (Table 3.3). However, RME+αT had a slightly faster rate of hydroperoxide formation than CSL (Table 3.3). A trend pointing towards slight synergy, measured by comparing the slopes of RME+αT compared to PS+RME+αT, was suggested between PS and RME+αT. This synergy could be a result of the combined metal chelation ability of PS and RME or just due to PS. However, additional studies, investigating synergy between RME and αT compared to PS+RME+αT, would have to be completed to validate the hypothesis that the combination of PS+RME is protecting the inactivation of αT by metal ions.

Metal analysis of CSL revealed that it did contain measurable amounts of iron (1 $\mu g\ g^{-1}$, analyzed by Research and Productivity Council (RPC), Fredericton, NB, Canada). The presence of iron is not entirely unexpected as PL have been described as metal chelators; therefore, metals present in the sunflower oil may have been bound by the PL which were extracted to form the resulting lecithin. The analysis of RME indicated some iron was also pre-bound, as there was a slight absorbance measured in the blank for this sample.

Therefore, some of the binding sites in both CSL and RME were already occupied with iron and thus this method may not have accurately evaluated their actual iron (II) ion chelating ability. These ingredients may have shown increased chelating ability if all of their binding sites were available to react with the $Fe(II)Cl_2$ added to the system in the Ferrozine method.

3.4.6 Effect of Fatty Acid Moieties

The fatty acid moieties of CSL and soybean PS were typical of plant oils with elevated proportions of 16 and 18 carbon saturated, monounsaturated and di-unsaturated fatty acids (Chowdhury *et al*., 2007). The soybean derived PS contained approximately 4% 18:3n-3, while CSL PS only contained trace amounts. This was expected as only trace amounts of 18:3n-3 are present in sunflower oil (Chowdhury *et al*., 2007). While the phosphate and serine functionalities did not differ between soybean PS and that in CSL, the differences in concentration of the fatty acid structures within the PS may affect the PL's antioxidant effects (Husain *et al*., 1986, Nwosu *et al*., 1997), particularly at later stages of oxidation. According to Husain *et al*. (1986) and Nwosu *et al*. (1997), the greater level of unsaturated fatty acid moieties esterified to CSL's PS should lead to reduced oxidative stability. However, the opposite was found; CSL provided superior protection against the formation of hydroperoxides compared to the soybean PS. The PV results for the closest recreation of CSL, PS+RME+αT, were equivalent to CSL for the first five days of the study (Figure 3.8). Therefore, it appears as though the action of the minor stabilizing antioxidants, RME and αT, prevented any possible pro-oxidant effect

due to fatty acid moieties of the PL. It is also possible that the RME and αT may have simply prevented the oxidation of the unsaturated fatty acid moieties.

Hidalgo *et al*. (2005) offer another potential explanation for the greater hydroperoxide protective effect of CSL compared to PS+RME+αT on days 8 and 12 (Figure 3.8, Appendix I); oxidation of primary amino groups could lead to the formation of pyrrolized PL with suggested antioxidant activity. If the PS in CSL was more likely to oxidize compared to the soybean derived PS because of its greater amount of unsaturated fatty acids, particularly PUFA (Husain *et al*., 1986), this might lead to the formation of higher concentrations of these amino derived pyrrolized PL. Quantifying these compounds may have given more insight into the oxidation of PS and CSL and their antioxidant effect. Quantification involves reacting the sample with Ehrlich reagent and measuring the absorbance at the maximum of 570 nm (Hidalgo *et al.*, 2004). This optimized method could have been used to monitor the development of pyrrolized PL in CSL, PS, and PS+RME+αT samples.

3.4.7 Effect of Maillard Browning Compounds

When these experiments were initiated, the hypothesis was that any browning was a result of Maillard browning. Therefore, it was expected that only samples containing PS, with its free amino group, would develop Maillard browning compounds and show an increase in absorbance over time. However, the extensive oxidation achieved in these studies may have resulted in polymerization, leading to the development of other browning compounds in not only samples containing PS or CSL, but also the control.

Maillard browning compounds are one type of non-enzymatic browning compound that can form during lipid oxidation when sugars or amino group in PL (PS or PE), react with secondary oxidation products (Hidalgo & Zamora, 2000). However, other non-enzymatic brown-coloured oxypolymers can form from the polymerization of lipid oxidation products (Hidalgo & Zamora, 2000; Zamora & Hidalgo, 2005). Therefore, the browning noted in the control and RME samples at later stages of the study may have been due to polymerization. This is likely why the more highly oxidized samples became turbid before analysis, preventing measurement of absorbance and browning during the second half of the stability studies. With some of the browning occurring at later stages of oxidation being due to oxidation, it becomes difficult to draw conclusions from the Maillard browning data collected. However, it is interesting to note that at approximately the same time point that the PS sample began showing an antioxidant effect against the formation of hydroperoxides, it also showed significantly greater browning than the control (Tables 3.5 and 3.6). It cannot be clearly determined if these browning compounds were Maillard browning compounds but they could be contributing to the small antioxidant effect of PS as indicated by the slower rate of hydroperoxide formation (Tables 3.1 and 3.4).

As mentioned in the previous section, the formation of pyrroles (pyrrolized PL) during oxidation may contribute to the antioxidant effect; however, they are also products of non-enzymatic browning reactions and their polymerization contribute to browning (Zamora & Hidalgo, 2003). To determine if pyrroles were being formed in reactions of PS with lipid oxidation products, the disappearance of PS and formation of pyrrolized PL

could have been monitored. This would have avoided the interference of polymerization noted in the spectrophotometric Maillard browning compound method. HPLC methods have been developed to quantify PL, and pyrrolized PL can be quantified using a modified method employing the Ehrlich reagent as described above (Hidalgo *et al.*, 2004). Since degumming would remove most endogenous PL and fish oil is not expected to contain amino acids, monitoring the formation of pyrroles would indicate if PS is interacting with secondary or tertiary oxidation products.

3.4.8 Antioxidant effect of RME and αT

The combination of RME+αT for the first 5 days of the 16 day stability study (Figure 3.8) had increased the oxidative stability of tocopherol stripped fish oil, compared to the control, to the same extent as CSL, whereas individually, RME or αT had less antioxidant activity than CSL, measured by the formation of hydroperoxides (Figure 3.11). This indicates that there could be a synergistic relationship between RME and αT. Components of RME have been suggested to have a protective effect on α-tocopherol. The combination of α-tocopherol and carnosic acid, one of the major contributors to RME's antioxidant effect, was found to increase oxidative stability of tocopherol stripped corn oil, determined by monitoring the formation of CD, hydroperoxides and hexanal (Hopia *et al.*, 1996). It has been suggested that carnosic acid increases the stability of α-tocopherol by hydrogen donation to the oxidized tocopheroxyl radical to regenerate α-tocopherol (Hopia *et al*., 1996). Hopia *et al.*'s (1996) observation supports the added benefit of RME+αT noted in this research. This effect could be due to tocopherol-sparing activities of compounds in the RME as suggested by Hopia *et al.* (1996). Carnosic acid

has been reported to have a lower half-wave potential than other phenolic diterpenes in RME, making it more likely to be oxidized (Schwarz & Ternes, 1992). Therefore, the carnosic acid in RME may be imparting a tocopherol-sparing effect by being preferentially oxidized, resulting in an increased antioxidant effect (Hopia *et al*., 1996, Wanda & Fang, 1992, Gordon & Kourimská, 1995). Unlike Hopia *et al.* (1996), this research did not clearly indicate that RME inhibited the decomposition of hydroperoxides. The formation of secondary oxidation products in the sample containing only RME, as measured by *p*AV, was equivalent to the control for half of the stability study, indicating minimal, if any, protection against hydroperoxide decomposition. This discrepancy to Hopia *et al.*'s (1996) work could be due to the differences in the oxidative medium. An oxidative medium of tocopherol stripped fish oil used in this research, with its high PUFA content, compared to the tocopherol stripped corn oil, used by Hopia *et al*. (1996) may provide a more applicable indicator of how a RME could act in a system with a large proportion of PUFA.

3.4.9 Industrial Applications

The early stages of oxidation are most industrially relevant for dietary supplements and food applications, because a high PV and *p*AV indicate highly degraded oil that likely has poor sensory characteristics and may have negative health implications. Therefore, it is important to choose an antioxidant or mixture of antioxidants that are able to protect the lipids immediately (i.e., increase the lag phase) and then slow the progression of oxidation after it is initiated. PS on its own did not meet these criteria, and would not be

recommended for use as an antioxidant. Pure, plant derived, PS is very expensive. The added benefit of PS in the protection against development of hydroperoxides at later stages of oxidation is not likely worth the added cost to the product as the product would be outside of industry acceptable PV limits when the antioxidant effect is initiated. PS may be useful in stabilizing PUFA including EPA and DHA in another manner; it is possible that EPA and DHA esterified to PS or another PL could be more resistant to oxidation compared to their triacylglycerol form, as lipids in the PL form tend to be more stable than in the triacylglycerol form (Cho *et al*., 2001).

This research indicates that RME+αT in CSL are providing the bulk of the antioxidant effect during early stages of oxidation; therefore, the combination of these phenolic compounds should be examined further to optimize their effect for more efficient use in industry applications. While CSL showed effective, and greater, antioxidant activity in tocopherol stripped fish oil than RME+αT, combining it with mixed T decreased its effectiveness to reduce the formation of hydroperoxides. Most bulk oils contain, at a minimum, trace amounts of endogenous α-tocopherol or added mixed T; thus, use of CSL as antioxidant is not likely warranted in bulk oils. The cost benefit of a possible slightly longer product shelf life is likely not great enough to justify the added cost of purchasing this premium ingredient with high levels of PS.

There will always be alternate uses for CSL. It is a safe, vegetarian source of PS, which has been suggested to have cognitive benefits (Schreiber *et al*., 2000, Crook *et al*., 1991). In addition to the suggested medicinal benefit of PS, the antioxidants in CSL indicate that

it is a stable ingredient that could contribute additional stability to lipids in the product it is to enrich.

3.4.10 Future Work

The focus of this research was PS; however, much more antioxidant activity was noted with the combination of RME+αT. Therefore, continuation of this work would involve closer investigation of these ingredients. Neither the technique used to extract RME used here, nor its exact composition is known. In order to optimize the use of RME+αT as an antioxidant, it is important to know the chemical breakdown of the RME. Different extraction methods can selectively recover particular compounds and thus a RME obtained by solvent extraction may vary in its composition compared to a supercritical fluid CO_2 extract. Different antioxidant effects are expected depending on the method by which the RME was extracted. Therefore, to make this work more relevant to others, additional information regarding the RME is needed.

This work was carried out in tocopherol stripped fish oil to eliminate interference due to endogenous and added tocopherols. However, before these recommendations can be applied to industry, the effectiveness of RME+αT must be examined in bulk fish oils to ensure the additional α-tocopherol does not have a pro-oxidant effect. The results suggest there may be a synergistic relationship between RME+αT. However, none of the experiments measured this relationship. Therefore, further work is also necessary to confirm there is a synergistic relationship between RME+αT.

Lastly, the method selected to measure Maillard browning compounds did not provide usable data. In the future, it would be recommended to look at the disappearance of the amino PL of interest and the formation of pyrrolized PL. This is suspected to eliminate the interference of polymerization.

3.5 CONCLUSIONS

PS was found to have a small antioxidant effect in its ability to reduce the rate at which hydroperoxides form. The mechanism in which it has this effect was not clearly determined, but this research indicates PS may act as a hydroperoxide destroyer at later stages of oxidation. Alternatively, as PS is being oxidized it could be forming pyrrolized PL that reduce the formation of hydroperoxides, and may contribute to the browning noted in these experiments. The formation of Maillard browning through PS's interaction with oxidation products may also contribute to PS's antioxidant effect. PS was found to be a metal chelator. This ability may have also resulted in PS's slight protective effect against the development of hydroperoxides at later stages of oxidation. Therefore, while PS is not a superior antioxidant to CSL, its presence may contribute slightly to CSL's antioxidant effect by the mechanisms mentioned.

CSL is a complex mixture of PL, with PS as the major PL, as well as RME and αT in a MCT base. Since it is a naturally derived ingredient extracted from sunflower seeds, it is very likely that it also contains endogenous compounds, such as carotenoids, that may impart an antioxidant effect and colour. This is an acknowledged limitation of these

studies; without knowledge of the minor components of CSL, it is difficult to speculate further on the cause of its antioxidant effect. These stability studies indicate that the combination of PS+RME+αT is able to provide equivalent protection as CSL against the rate of hydroperoxide formation, as well as equivalent protection against the development of secondary oxidation products (Table 3.3 and Figure 3.10). However, individual PV results suggest that during early stages of oxidation most of the antioxidant effect is due to RME+αT (Figure 3.8). The increase in hydroperoxide protective effect noted in late stages of oxidation may be due to the minor PL in CSL that were not accounted for in these studies or CSL's ability to chelate ferrous iron to a greater extent than PS and RME individually. Tocopherols were not found to have a synergistic relationship with CSL or PS, which is contrary to what has been suggested in literature. Both PV and *p*AV results indicate that PS plays a minimal role in CSL's antioxidant ability. Since the combination of RME and αT appear to be responsible for the antioxidant effect of CSL, further work is needed to learn more about the relationship between RME and αT to optimize this relationship and determine concentrations that may be beneficial for food or nutraceutical applications.

CHAPTER 4 CONCLUSION

4.1 CLOSING REMARKS

CSL rich in PS was found to reduce the formation of hydroperoxides and secondary oxidation products, measured by PV and *p*AV, respectively, in refined tocopherol enriched fish oil. An inclusion of 10 mg g^{-1} CSL provided the greatest antioxidant effect in this medium. This work lead to the investigation of the antioxidant effects of individual components of CSL to better understand its antioxidant effect and enable better utilization of this ingredient or its constituents.

The oxidative effect of a sunflower derived CSL and soybean derived PS alone and in the presence of primary antioxidants, mixed T, RME and αT, were examined in tocopherol stripped refined fish oil. The effect of each treatment was monitored by measuring the formation of primary and secondary oxidation products and Maillard browning compounds.

In general, PS alone slowed the rate in which hydroperoxides were formed compared to the control. However, the hydroperoxide protective effect was only apparent at later stages of oxidation when the samples' PVs were no longer at a level that would be considered acceptable by commercial standards. The exact mechanism in which PS had this protective effect was not determined, although it was found to have a slight metal chelating ability. It also appears as though PS was acting as a hydroperoxide destroyer as it only had a reducing effect on the PV and not secondary oxidation products as measured by *p*AV. Results also loosely suggest that as the oil medium becomes increasingly

oxidized, PS is forming browning compounds that may have a protective effect on hydroperoxide formation. However, PS alone is not responsible for the antioxidant effect noted with CSL.

Neither PS nor CSL had a synergistic effect with mixed T or αT, as has commonly been suggested in literature. PS did have a slight synergistic relationship with RME; however, this combination was not as effective an antioxidant as CSL, as measured by PV and *p*AV.

These findings suggest that CSL's protective effect against the rate of hydroperoxide formation was due to the combination of PS+RME+αT and likely the majority of the protection against secondary oxidation products was related to the presence of αT. Also, during early stages of oxidation, RME+αT appear to be providing the protective effect against hydroperoxide formation in CSL, as measured by PV. However, at later stages of oxidation, the presence of PS and/or PS plus the minor PL in CSL began to impart an additional antioxidant effect. This suggests that PS and/or the minor PL in CSL have an antioxidant effect as they become oxidized or they are acting as hydroperoxide destroyers at later stages of oxidation. CSL and RME were also found to be metal chelators, which likely contributed to their antioxidant effects as well.

Overall, the high cost of CSL and pure soybean PS limit their use in industrial settings. This research indicates that RME+αT provide equivalent protection as CSL against hydroperoxides and secondary oxidation products, as measured by PV and *p*AV,

respectively, during early stages of oxidation. Therefore, further research into the antioxidant effect of different concentrations of these antioxidants may prove to have equivalent or greater efficacy than CSL or PS+RME+αT in the long term.

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APPENDIX I

Table A1 PV (meq kg^{-1}) of tocopherol stripped fish oil stored in open vials at ambient conditions. Values expressed as the mean (n=3) with standard deviations in parenthesis. Values with different superscript letters within the same time point are significantly different (Tukey's test: $p < 0.05$).

	Time (days)				
Sample	0	1	2	3	4
Control	4.26(0.20)[a]	11.34 (0.41)[a]	19.04 (0.85)[a]	28.58 (1.04)[a]	40.39 (0.79)[a]
PS	4.51 (0.51)[a]	11.56 (0.14)[a]	18.83 (0.35)[a]	27.38 (0.55)[a]	36.78 (0.87)[b]
PS+mixed T	2.59 (0.18)[b]	5.15 (0.31)[b]	10.57 (0.28)[b]	15.38 (0.31)[b]	20.39 (0.29)[c]
CSL	2.27 (0.08)[b]	4.30 (0.13)[c]	5.65 (0.17)[c]	8.79(0.06)[c]	12.47 (0.20)[d]
CSL+mixed T	2.05 (0.11)[b]	2.94 (0.33)[d]	5.41(0.20)[c]	8.91(0.23)[c]	13.63 (1.44)[d]
Mixed T	2.37 (0.40)[b]	7.48 (0.28)[e]	12.34 (0.33)[d]	16.54 (0.08)[b]	21.91 (0.22)[c]

	Time (days)				
Sample	6	8	10	13	16
Control	57.64 (0.77)[a]	79.68 (1.23)[a]	94.51 (1.12)[a]	119.47 (1.95)[a]	152.31 (0.93)[a]
PS	52.92 (0.51)[b]	71.48 (1.18)[b]	85.58 (1.73)[b]	106.18 (2.54)[b]	135.83 (0.68)[b]
PS+ mixed T	27.82 (1.40)[c]	34.27 (0.49)[c]	40.40 (0.18)[c]	47.21 (0.96)[c]	57.49 (0.28)[c]
CSL	14.80 (1.30)[d]	19.61 (0.54)[d]	23.18 (0.63)[d]	27.91 (0.31)[d]	33.62 (0.38)[d]
CSL+mixed T	18.57 (0.05)[e]	25.52 (0.32)[e]	30.64 (0.31)[e]	38.04 (0.67)[e]	46.25 (2.44)[e]
Mixed T	27.79 (0.77)[c]	35.94 (0.50)[c]	41.57 (0.57)[c]	49.38 (0.31)[c]	59.31 (0.83)[c]

Table A2 *p*AV of tocopherol stripped fish oil stored in open vials at ambient conditions. Values expressed as the mean (n=3) with standard deviations in parenthesis. Values with different superscript letters within the same time point are significantly different (Tukey's test: $p < 0.05$).

	Time (days)						
Sample	0	2	4	6	10	13	16
Control	1.93 $(0.32)^{a}$	4.76 $(0.24)^{a}$	10.10 $(0.25)^{a}$	15.43 $(0.73)^{a}$	24.64 $(1.54)^{a}$	38.96 $(0.66)^{a}$	53.52 $(0.79)^{a}$
PS	1.88 $(0.05)^{a}$	5.51 $(0.15)^{b}$	10.42 $(0.26)^{a}$	14.70 $(0.50)^{a}$	23.93 $(1.41)^{a}$	37.11 $(2.38)^{a}$	52.59 $(1.38)^{a}$
PS+mixed T	0.75 $(0.01)^{b}$	0.96 $(0.02)^{c}$	0.62 $(0.04)^{b}$	2.13 $(0.09)^{b}$	2.08 $(0.25)^{b}$	3.46 $(0.08)^{b}$	5.68 $(0.13)^{b}$
CSL	0.99 $(0.10)^{b,c}$	1.63 $(0.069)^{d}$	2.63 $(0.05)^{c}$	5.31 $(0.10)^{c}$	4.97 $(0.18)^{c}$	5.89 $(0.21)^{b}$	8.16 $(0.11)^{c}$
CSL+mixed T	0.89 $(0.17)^{b}$	0.75 $(0.04)^{c}$	1.74 $(0.80)^{c}$	3.18 $(0.09)^{d}$	2.55 $(0.09)^{b}$	3.35 $(0.15)^{b}$	4.44 $(0.16)^{b}$
Mixed T	0.54 $(0.03)^{b,d}$	0.85 $(0.13)^{c}$	0.74 $(0.04)^{b}$	2.43 $(0.23)^{b,d}$	2.54 $(0.12)^{b}$	3.73 $(0.10)^{b}$	5.99 $(0.17)^{b}$

Table A3 CD value of tocopherol stripped fish oil stored in open vials at ambient conditions. Values expressed as the mean (n=3) with standard deviations in parenthesis. Values with different superscript letters within the same time point are significantly different (Tukey's test: $p < 0.05$).

Sample	Time (days)			
	0	2	4	6
Control	0.58 (0.04)^{a}	0.71 (0.01)^{a,b}	0.69 (0.06)^{a}	0.86 (0.04)^{a}
PS	0.51 (0.00)^{b}	0.72 (0.00)^{a,b}	0.73 (0.01)^{a}	0.84 (0.04)^{a}
PS+mixed T	0.58 (0.01)^{a}	0.71 (0.01)^{a,b}	0.72 (0.02)^{a}	0.80 (0.03)^{a}
CSL	0.54 (0.04)^{a,b}	0.69 (0.04)^{b}	0.59 (0.05)^{b}	0.69 (0.04)^{b}
CSL+mixed T	0.61 (0.01)^{a}	0.70 (0.02)^{a,b}	0.67 (0.02)^{a}	0.78 (0.02)^{a}
Mixed T	0.62 (0.03)^{a}	0.74 (0.03)^{a}	0.72 (0.03)^{a}	0.83 (0.02)^{a}

Sample	Time (days)			
	8	10	13	16
Control	1.00 (0.05)^{a}	1.06 (0.02)^{a}	1.21 (0.02)^{a}	1.43 (0.01)^{a}
PS	0.91 (0.03)^{a,c}	1.02 (0.02)^{a}	1.17 (0.01)^{b}	1.36 (0.01)^{b}
PS+mixed T	0.83 (0.09)^{a}	0.91 (0.02)^{b}	1.01 (0.02)^{c}	1.07 (0.03)^{c}
CSL	0.63 (0.04)^{b}	0.72 (0.00)^{c}	0.74 (0.00)^{d}	0.78 (0.01)^{d}
CSL+mixed T	0.72 (0.13)^{b,c}	0.87 (0.01)^{d}	0.92 (0.02)^{e}	1.00 (0.01)^{e}
Mixed T	0.85(0.12)^{a,b}	0.93(0.01)^{b}	1.03(0.00)^{c}	1.10(0.01)^{c}

Table A4 PV (meq kg^{-1}) of tocopherol stripped fish oil stored in open vials at ambient conditions. Values expressed as the mean (n=3) with standard deviations in parenthesis. Values with different superscript letters within the same time point are significantly different (Tukey's test: $p < 0.05$).

	Time (days)						
Sample	0	2	3	5	8	12	16*
Control	4.76	27.44	39.17	60.88	99.35	146.96	180.68
	(0.24)^{a}	(0.57)^{a}	(0.57)^{a}	(1.1)^{a}	(1.1)^{a}	(2.6)^{a}	(15.3)
PS	4.43	24.92	36.06	55.19	89.28	131.65	175.15
	(0.45)^{a}	(0.40)^{b}	(0.69)^{b}	(1.8)^{b}	(0.17)^{b}	(2.7)^{b}	(1.5)^{a}
PS+RME	2.89	9.72	13.61	20.08	29.00	37.81	44.36
+ αT	(0.18)^{b}	(0.48)^{c}	(1.1)^{c}	(0.20)$^{c. d}$	(0.79)^{c}	(0.14)^{c}	(0.11)^{b}
CSL	2.59	9.74	13.50	17.96	25.04	31.00	36.63
	(0.31)^{b}	(0.70)^{c}	(0.43)^{c}	(0.66)^{d}	(0.34)^{d}	(0.37)^{d}	(0.41)^{c}
RME+αT	2.85	9.98	14.93	21.54	30.06	41.39	49.89
	(0.42)^{b}	(0.04)^{c}	(0.28)^{c}	(0.21)^{c}	(2.6)^{c}	(0.44)^{c}	(0.76)^{d}

*The control sample was omitted from the day 16 ANOVA due to its large value reducing the sensitivity of Tukey's post hoc analysis to detect difference between the treatments.

Table A5 *p*AV of tocopherol stripped fish oil stored in open vials at ambient conditions. Values expressed as the mean (n=3) with standard deviations in parenthesis. Values with different superscript letters within the same time point are significantly different (Tukey's test: $p < 0.05$).

	Time (days)						
Sample	0	2	3	5	8	12	16
Control	2.01	7.22	11.23	15.21	21.34	37.58	54.27
	(0.17)^{a}	(0.08)^{a}	(0.32)^{a}	(0.28)^{a}	(0.53)^{a}	(2.6)^{a}	(1.7)^{a}
PS	2.72	7.82	14.27	17.72	22.89	38.75	55.73
	(0.17)^{b}	(0.32)^{b}	(0.88)^{b}	(0.48)^{b}	(1.2)^{a}	(1.0)^{a}	(0.88)^{a}
PS+RME	0.71	1.92	1.80	4.17	5.28	8.65	10.58
+ αT	(0.23)^{c}	(0.16)^{c}	(0.11)^{c}	(0.07)^{c}	(0.24)^{b}	(0.09)^{b}	(0.06)^{b}
CSL	0.81	2.51	3.41	4.37	5.91	8.34	9.74
	(0.12)^{c}	(0.30)^{d}	(0.21)^{d}	(0.19)^{c}	(0.21)^{b}	(0.24)^{b}	(0.14)^{b}
RME+ αT	0.67	2.05	2.49	3.84	5.29	7.84	9.31
	(0.15)^{c}	(0.11)^{c,d}	(0.24)^{c,d}	(0.08)^{c}	(0.04)^{b}	(0.36)^{b}	(0.07)^{b}

Table A6 PV (meq kg^{-1}) of tocopherol stripped fish oil stored in open vials at ambient conditions. Values expressed as the mean (n=3) with standard deviations in parenthesis. Values with different superscript letters within the same time point are significantly different (Tukey's test: $p < 0.05$).

	Time (days)			
Sample	0	2	3	5
Control	4.66 (0.28)^{a,b}	22.08(0.28)^{a}	34.05(1.16)^{a}	53.60(0.51)^{a}
PS	6.33 (1.41)^{a}	23.54(0.14)^{b}	33.80(1.18)^{a}	52.67(2.05)^{a}
PS+RME	4.48 (0.13) ^{b,c}	17.61(0.21)^{c}	25.13(0.38)^{b}	41.50(0.83)^{b}
PS+ αT	2.68 (0.98)^{c,d}	11.17(0.63)^{d}	15.22(0.57)^{c}	21.80(1.24)^{c}
CSL	2.37 (0.19)^{d}	8.21(0.40)^{e}	11.18(0.38)^{d}	16.24(0.36)^{d}
RME	3.93(0.12)^{b,c,d}	19.87(0.49)^{f}	29.72(0.36)^{e}	47.15(0.71)^{e}
αT	2.21 (0.02)^{d}	10.42(0.53)^{d}	14.70(2.39)^{c}	21.10(0.28)^{c}

	Time (days)		
Sample	7	12	16
Control	76.97(2.02)^{a}	126.26(1.63)^{a}	160.90(1.62)^{a}
PS	73.22(1.28)^{b}	120.08(3.25)^{b}	144.99(4.48)^{c}
PS+RME	60.43(0.28)^{c}	105.95(1.95)^{c}	131.52(1.37)^{d}
PS+ αT	27.54(0.67)^{d}	40.19(0.60)^{d}	47.30(0.92)^{e}
CSL	20.47(0.53)^{e}	28.90(0.92)^{e}	34.11(0.30)^{f}
RME	70.04(1.14)^{f}	122.23(0.94)^{a,b}	153.46(2.72)^{b}
αT	26.29(0.44)^{d}	39.78(0.61)^{d}	47.36(0.33)^{e}

Table A7 *p*AV of tocopherol stripped fish oil stored in open vials at ambient conditions. Values expressed as the mean (n=3) with standard deviations in parenthesis. Values with different superscript letters within the same time point are significantly different (Tukey's test: $p < 0.05$).

	Time (days)					
Sample	0	2	5	7	12	16
Control	1.86 (0.19)^{a}	4.41 (0.35)^{a,b}	10.43 (0.94)^{a}	17.24 (0.84)^{a}	32.54 (0.42)^{a}	46.48 (4.16)^{a}
PS	2.32 (0.12)^{b}	4.92 (1.11)^{a}	9.72 (1.74)^{a,b}	17.79 (0.50)^{a}	29.79 (0.90)^{a}	45.89 (1.45)^{a}
PS+RME	1.29 (0.08)^{c,d}	3.49 (0.16)^{b}	7.58 (1.06)^{b}	13.57 (2.12)^{b}	22.41 (2.53)^{b}	37.21 (2.09)^{b}
PS+αT	0.75 (0.04)^{e}	1.01 (0.32)^{c}	1.07 (0.51)^{c}	3.56 (0.24)^{c}	3.74 (0.36)^{c}	9.72 (0.55)^{c}
CSL	1.01 (0.00)^{d,e}	1.32 (0.08)^{c}	1.25 (0.12)^{c}	3.01 (0.07)^{c}	2.60 (0.48)^{c}	8.05 (0.19)^{c}
RME	1.51 (0.16)^{c}	3.65 (0.25)^{a,b}	8.22 (0.52)^{a,b}	13.50 (0.48)^{b}	25.36 (0.17)^{b}	41.22 (0.94)^{a,b}
αT	0.91 (0.09)^{e}	0.85 (0.19)^{c}	0.93 (0.17)^{c}	2.29 (0.26)^{c}	2.38 (0.23)^{c}	9.20 (0.17)^{c}

Table A8 EPA and DHA composition of the tocopherol stripped fish oil used as the oxidative medium in these stability studies. Oil A was the medium for the study investigating the effect of mixed T on the antioxidant effect of PS and the CSL; Oil B was the medium for the study investigating the combined effect of RME+αT on the antioxidant effect of PS; Oil C was the medium for the study investigating the individual effect of RME and αT on the antioxidant effect of PS. Values are expressed as the mean (n=3) with standard deviations in parenthesis.

	Fatty Acid (mg g^{-1} as TG)	
Oxidative Medium	EPA	DHA
Oil A	184.51 (1.09)	90.41 (0.86)
Oil B	216.75 (0.49)	69.24 (0.12)
Oil C	202.44 (1.44)	75.89 (0.18)