Minor Components and Their Roles on Lipid Oxidation in Bulk Oil That Contains Association Colloids

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MINOR COMPONENTS AND THEIR ROLES ON LIPID OXIDATION IN BULK OIL THAT CONTAINS ASSOCIATION COLLOIDS

A Dissertation Presented
by
Bingcan Chen

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY
MAY 2012
The Department of Food Science
MINOR COMPONENTS AND THEIR ROLES ON LIPID OXIDATION IN BULK OIL THAT CONTAINS ASSOCIATION COLLOIDS

A Dissertation Presented

by

BINGCAN CHEN

Approved as to style and content by:

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Department of Food Science
DEDICATIONS

To my parents and my wife,

who made all of this possible, for their endless encouragement and patience.
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ABSTRACT
MINOR COMPONENTS AND THEIR ROLES ON LIPID OXIDATION IN BULK OIL THAT CONTAINS ASSOCIATION COLLOIDS
MAY 2012
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The combination of water and surface active compounds found naturally in commercially refined vegetable oils have been postulated to form physical structures known as association colloids. This research studied the ability of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and water to form physical structures in stripped soybean oil. Interfacial tension and fluorescence spectrometry results showed the critical micelle concentration (CMC) of DOPC in stripped soybean oil was 650 and 950 μM, respectively. Light scattering attenuation results indicated that the structure formed by DOPC was reverse micelles. The physical properties of DOPC reverse micelles were determined using small-angle X-ray scattering (SAXS) and fluorescence probes. These studies showed that increasing the water concentration altered the size and shape of the reverse micelles formed by DOPC.

The impact of DOPC reverse micelles on the lipid oxidation of stripped soybean oil was investigated by following the formation of primary and secondary lipid oxidation products. DOPC reverse micelles had a prooxidant effect, shortening the oxidation lag
phase of SSO at 55 °C. It also was not able to change the lipid oxidation of stripped soybean oil compared with DOPC reverse micelles at same concentration (i.e., 950 μM). 1,2-dibutyl-sn-glycero-3-phosphocholine (DC_{4}PC) which has the shorter fatty acid than DOPC was not able to form association colloids and did not impact lipid oxidation rates. This indicated that the choline group of the phospholipid was not responsible for the increased oxidation rates and suggested that the physical structure formed by DOPC was responsible for the prooxidant effect.

The impact of the DOPC reverse micelles on the effectiveness and physical location of the antioxidants, α-tocopherol and Trolox was also studied. Both non-polar (α-tocopherol) and polar (Trolox) were able to inhibit lipid oxidation in stripped soybean oil in the presence of DOPC reverse micelles. Trolox was a more effective antioxidant than α-tocopherol. Fluorescence steady state and lifetime decay studies suggested that both α-tocopherol and Trolox were associated with DOPC reverse micelle in bulk oil. Trolox primarily concentrated in the water pool of reverse micelle since it quenched NBD-PE fluorescence intensity with increasing concentrations. A portion of α-tocopherol was also associated with the aqueous phase of the DOPC reverse micelles but this was likely at the oil-water interface since α-tocopherol is not water soluble.

The addition of ferric chelator, deferoxamine (DFO) to stripped soybean oil significantly prevented the lipid oxidation caused by DOPC reverse micelles as the lag phase was extended from 2 to 7 days. DFO was also found to increase the antioxidant activity of both Trolox and α-tocopherol. Trolox and α-tocopherol were found to be rapidly decomposed by high-valence Fe(III) while low-valence-state (Fe (II) was much less reactive. Fe(III) was also consumed by both hydrophilic Trolox and lipophilic α-
tocopherol presumably though reduction to Fe (II). DOPC reverse micelles were able to decrease antioxidants-iron interactions as evidence by a decrease in antioxidant depletion by iron and a decrease in iron reduction by the antioxidants. These results suggested that the ability of DFO to increase the antioxidant activity of α-tocopherol and Trolox was due to its ability to decrease free radical production and not its ability to decrease direct iron-antioxidant interactions.

Overall, the results presented in this dissertation show phospholipids and water can form reverse micelles in edible oils. These reverse micelles increase lipid oxidation rates by increasing the prooxidant activity of iron. Free radical scavenging antioxidants can inhibit oxidation promoted by the reverse micelles with polar Trolox being more effective than non-polar α–tocopherol presumably because Trolox is more highly associated with the reverse micelle. The reverse micelles produced by DOPC protected α–tocopherol and Trolox from direct degradation by iron. The knowledge gained from this study will improve our understanding of the mechanism of lipid oxidation in bulk oils which will hopefully provide new technologies to improve the oxidation stability of edible oils. For example, it may be able to use oil refining technologies to remove prooxidative minor components that for physical structure in bulk oils.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
</tr>
<tr>
<td>ABSTRACT</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
</tr>
</tbody>
</table>

## 1. INTRODUCTION

1.1 Oil refining procedure

1.1.1 Storage

1.1.2 Preparation

1.1.3 Solvent extraction

1.1.4 Oil refining

1.2 Minor components in bulk oil

1.2.1 Free fatty acid

1.2.2 Monoacylglycerols and Diacylglycerols

1.2.3 Phospholipids

1.2.4 Tocopherol

1.2.5 Pigments

1.2.6 Miscellaneous compounds

1.2.6.1 Nonsaponifiable minor components

1.2.6.2 Polar triacylglycerol polymers

1.2.6.3 Oxidized triacylglycerols

1.2.7 Water

1.2.8 Oxygen

1.3 The effect of minor components on bulk oil oxidation

1.3.1 The effect of oxygen on the chemical stability of bulk oil

1.3.2 The effect of water on the chemical stability of bulk oil

1.3.3 The effect of free fatty acid on the chemical stability of bulk oil

1.3.4 The effect of MAG and DAG on chemical stability of bulk oil
oil ....................................................................................................... 24

2.3.5 The effect of phospholipids on the chemical stability of bulk oil .................................................. 24

2.3.6 The effect of tocopherols on the chemical stability of bulk oil .................................................. 25

2.3.7 The effect of pigments on the chemical stability of bulk oil .................................................... 26

2.3.8 The effect of miscellaneous compounds on the chemical stability of bulk oil ................................. 28

2.4 Association colloids and lipid oxidation in bulk oils ................................................................. 29

2.4.1 Evidence for the presence of association colloids in bulk oil .................................................. 29

2.4.2 Do association colloids impact lipid oxidation? ........................................................................ 30

2.4.3 The perspective researches on the mechanism of lipid oxidation in bulk oil ............................... 32

2.5 Methodologies to study the impact of minor components on the physicochemical properties of bulk oil .................................................................................................. 33

2.5.1 Chemical analysis .................................................................................................................. 34

2.5.1.1 Primary lipid oxidation products ........................................................................... 34

2.5.1.2 Secondary lipid oxidation products ........................................................................ 34

2.5.2 Physical analysis .................................................................................................................. 35

2.5.2.1 Small/wide angle x-ray scattering (SAXS/WAXS) .................................................. 35

2.5.2.2 Steady-state and time resolved fluorescence measurements ....................................... 36

2.5.2.3 Interfacial tension analysis .................................................................................. 37

3. THE IMPACT OF PHYSICAL STRUCTURES IN SOYBEAN OIL ON LIPID OXIDATION ................................................................. 38

3.1 Introduction .......................................................................................................................... 38

3.2 Materials and Methods ......................................................................................................... 39

3.2.1 Materials .......................................................................................................................... 40

3.2.2 Preparation of stripped soybean oil .................................................................................... 40

3.2.3 Light scattering properties of oil samples ........................................................................... 41

3.2.4 Determination of critical micelle concentration (CMC) in stripped soybean oil .................. 42

3.2.5 Small angle X-ray scattering (SAXS) .................................................................................. 43

3.2.6 Front-Face (FF) flurometric measurements ............................................................................ 44

3.2.7 Measurement of oxidation parameters .............................................................................. 45

3.2.8 Statistical analysis ........................................................................................................... 46

3.3 Results and discussion .......................................................................................................... 46
3.3.1 Attenuation of the water/phospholipid/MCT system ............................ 46
3.3.2 Determination of the CMC of phospholipids in SSO and MCT ................................................................. 47
3.3.3 Detection and characterization of phospholipids physical structures by small angle x-ray scattering .............................................. 51
3.3.4 Effect of water/phospholipid molar ratio on the physical properties of DOPC association colloids ........................................ 54
3.3.5 Oxidation of stripped soybean oil containing association colloids ...................................................................................... 58

4. ROLE OF REVERSE MICELLES ON LIPID OXIDATION IN BULK OILS: IMPACT OF PHOSPHOLIPIDS ON ANTIOXIDANT ACTIVITY OF α-TOCOPHEROL AND TROLOX ........................................ 61

4.1 Introduction .................................................................................................................. 61
4.2 Materials and Methods ................................................................................................. 63
  4.2.1 Materials ................................................................................................................. 63
  4.2.2 Preparation of stripped soybean oil ......................................................................... 64
  4.2.3 Formation of DOPC reverse micelles in stripped soybean oil ........................... 64
  4.2.4 Measurement of oxidation parameters ................................................................ 65
  4.2.5 Small angle x-ray scattering (SAXS) study of bulk oil with DOPC and antioxidants ............................................................. 66
  4.2.6 Fluorescence measurement of bulk oil with DOPC and antioxidants ......... 67
  4.2.7 Statistical analysis .................................................................................................. 68

4.3 Results ......................................................................................................................... 69
  4.3.1 Impact of phospholipids on oxidative stability of SSO ................................. 69
  4.3.2 The impact of phospholipids on the oxidative stability of SSO in the presence of α-tocopherol ...................................................... 71
  4.3.3 The impact of phospholipids on the oxidative stability of SSO in the presence of Trolox .................................................................... 75
  4.3.4 The impact of antioxidants on the properties of DOPC reverse micelles in SSO ............................................................................. 79

4.4 Discussion ................................................................................................................... 83

5. IRON/ANTIOXIDANTS RELATIONSHIP IN BULK OIL OXIDATION ................................................................. 87

5.1 Introduction .................................................................................................................. 87
5.2 Materials and Methods ................................................................................................. 89
  5.2.1 Materials ................................................................................................................. 89
5.2.2 Formation of DOPC association colloids in stripped soybean oil or MCT
5.2.3 Measurement of oxidation parameters
5.2.4 Determination of Trolox and α-tocopherol concentrations
5.2.5 Determination the concentration of ferric iron in MCT
5.2.6 EPR spectroscopy
5.2.7 Statistical analysis

5.3 Results

5.3.1 Effects of the metal chelator, Deferoxamine (DFO) on the oxidative stability of stripped soybean oil
5.3.2 Effects of Fe(III) and Fe(II) on the depletion of α-tocopherol and Trolox in MCT
5.3.3 Effects of Fe(III) and Fe(II) on the depletion of α-tocopherol or Trolox in the presence of DOPC reverse micelles
5.3.4 Measurement of Fe(III) loss by α-tocopherol and Trolox
5.3.5 Effects of Fe(III) and Fe(II) on the formation of PBN spin adducts in MCT

5.4 Discussion

6. CONCLUSION

BIBLIOGRAPHY
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.</td>
<td>Free fatty acid content in crude soybean oil as the function of bean storage time</td>
<td>9</td>
</tr>
<tr>
<td>2.2.</td>
<td>Free fatty acid content in soybean oil as a function of processing step</td>
<td>9</td>
</tr>
<tr>
<td>2.3.</td>
<td>Free fatty acid content in retail oil</td>
<td>11</td>
</tr>
<tr>
<td>2.4.</td>
<td>Concentration of MAG and DAG in various oils as a function of refining</td>
<td>12</td>
</tr>
<tr>
<td>2.5.</td>
<td>Phospholipid composition (%) as extracted from commercial seeds</td>
<td>13</td>
</tr>
<tr>
<td>2.6.</td>
<td>Phosphorous content (ppm) of soybean oil as a function of refining step</td>
<td>14</td>
</tr>
<tr>
<td>2.7.</td>
<td>Water content in freshly-opened, commercially available vegetable oils as determined by Karl Fischer Coulometer</td>
<td>18</td>
</tr>
<tr>
<td>2.8.</td>
<td>Solubility of oxygen (ppm) in Marine oil</td>
<td>20</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>2.1</td>
<td>The schematic of oil refining process.</td>
<td>6</td>
</tr>
<tr>
<td>2.2</td>
<td>Schematic representation of the association colloids formed by minor constituents in bulk oil (A) a reverse micelle formed by oleic acid; (B) a reverse micelle formed by phospholipids; (C) Inverse lamella structure formed by monoacylglycerol; (D) a mixed reverse micelle formed by free fatty acids and phospholipids.</td>
<td>30</td>
</tr>
<tr>
<td>2.3</td>
<td>The essential parts of a small angle scattering system. The drawing shows the X-ray source X, the sample S, the scattering angle θ, the slits used to define the incident and scattered beams, and the detector D.</td>
<td>35</td>
</tr>
<tr>
<td>3.1</td>
<td>Molecular structures of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dibutryl-sn-glycero-3-phosphocholine (DC₄PC).</td>
<td>39</td>
</tr>
<tr>
<td>3.2</td>
<td>Attenuation of medium chain triacylglycerols (MCT) samples containing 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and varying concentrations of water.</td>
<td>47</td>
</tr>
<tr>
<td>3.3</td>
<td>Determination of critical micelle concentration (CMC) of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) in medium chain triacylglycerols (A) and stripped soybean oil (B) as determined by the absorbance (480 nm) of 7,7,8,8-tetracyano-quinodimethane.</td>
<td>49</td>
</tr>
<tr>
<td>3.4</td>
<td>Determination of critical micelle concentration of 1,2-dioleoyl-sn-glycero-3-phosphocholine in stripped soybean oil using interfacial tension.</td>
<td>51</td>
</tr>
<tr>
<td>3.5</td>
<td>Small angle x-ray scattering profiles of stripped soybean oil (SSO) and SSO + 950 μM 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or 1,2-dibutryl-sn-glycero-3-phosphocholine (DC₄PC).</td>
<td>52</td>
</tr>
<tr>
<td>3.6</td>
<td>The pair distribution functions, p(r), calculated from the scattering profiles of stripped soybean oil (SSO) and SSO + 950 μM 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or 1,2-dibutryl-sn-glycero-3-phosphocholine (DC₄PC) using GNOM analysis of the three small angle x-ray scattering profiles indicated in Figure 5.</td>
<td>53</td>
</tr>
<tr>
<td>3.7</td>
<td>Small angle x-ray scattering profiles of stripped soybean oil with different water/1,2-dioleoyl-sn-glycero-3-phosphocholine molar ratios (Wₒ). Inserted figures are the 2 dimensional small angle x-ray scattering pattern of each sample.</td>
<td>55</td>
</tr>
</tbody>
</table>
3.8. A postulated structural change of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as phospholipid concentrations are increased to above the critical micelle concentrations (CMC) form a reverse micelle and then the water:molar ratio is increased to form an anisotropic structure with a non-spherical shape .................................................. 57

3.9. The fluorescence intensity of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE) in stripped soybean oil with different water/1,2-dioleoyl-sn-glycero-3-phosphocholine molar ratios (W0). Inserted figure is the molecular structure of (NBD-PE) ....................... 58

3.10. Formation of lipid hydroperoxides (A), propanal (B) in stripped soybean oil containing 1000 μM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC4PC) and 200 ppm water at 37 °C. Data represent means (n =3) ± standard deviations. Some error bars are within data points .................................................................................................................... 59

4.1. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil containing 1000 μM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC4PC) during storage at 55 °C. Some of the error bars are within data points .................................................................................................................. 70

4.2. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil containing 1000 μM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) with or with 10 or 100 μM α-tocopherol during storage at 55 °C. Some of the error bars are within data points .......... 74

4.3. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil containing 1000 μM of 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC4PC) with or with 10 or 100 μM α-tocopherol during storage at 55 °C. Some of the error bars are within data points ............ 75

4.4. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil containing 1000 μM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) with or with 10 or 100 μM Trolox during storage at 55 °C. Some of the error bars are within data points ................... 77

4.5. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil containing 1000 μM of 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC4PC) with or with 10 or 100 μM Trolox during storage at 55 °C. Some of the error bars are within data points ....................... 78
4.6. The neutralized fluorescence intensity of NBD-PE at stripped soybean oil containing 1000 µM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) with varying concentrations (0-100 µM) of α-tocopherol and Trolox ........................................................................................................................................81

4.7. The fluorescence lifetime decay of NBD-PE at stripped soybean oil containing 1000 µM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) with varying concentrations (0-100 µM) of α-tocopherol (A) and Trolox (B) concentrations ........................................................................................................82

5.1. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil containing 2 mM deferoxamine (DFO) and/or 1000 µM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) during storage at 55 °C. Some of the error bars are within data points ..........................................................................................................................95

5.2. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil containing 100 µM α-tocopherol, 2 mM deferoxamine (DFO) and/or 1000 µM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) during storage at 55 °C. Some of the error bars are within data points ..........................................................................................................................97

5.3. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil containing 100 µM Trolox, 2 mM deferoxamine (DFO) and/or 1000 µM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) during storage at 55 °C. Some of the error bars are within data points ..........98

5.4. Depletion of α-tocopherol and Trolox (100 µM) in medium chain triacylglycerols (MCT) in the presence of (A) Fe(III); and (B)Fe(II).............101

5.5. Depletion of α-tocopherol and Trolox (100 µM) in medium chain triacylglycerols (MCT) containing 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC4PC) in the presence of (A) 40 ppm Fe(III); and (B) 100 ppm Fe(III) .................................................................................................................................103

5.6. Depletion of α-tocopherol and Trolox (100 µM) in medium chain triacylglycerols (MCT) containing 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC4PC) in the presence of (A) 40 ppm Fe(II); and (B)100 ppm Fe(II) .................................................................................................................................105
5.7. Fe(III) development in medium chain triacylglycerols (MCT) containing 
(A) α-tocopherol or Trolox; (B) 1000 µM of 1,2-dioleoyl-sn-glycero-
3-phosphocholine (DOPC); (C) 1,2-dibutyryl-sn-glycero-3-
phosphocholine (DC4PC) after 24 h storage at room temperature. For 
each group, different letters on the top of columns represent significant 
differences (p < 0.05)..........................................................................................................................109

5.8. EPR detection of radical formations in the reaction consisting of (A) 0.1 
M PBN in stripped soybean oil; (B) 0.1 M PBN in MCT; (C) 0.1 M 
PBN+100 µM α-tocopherol in MCT; (D) 0.1 M PBN+100 µM Trolox 
in MCT; (E) 0.1 M PBN+10 mM cumene hydroperoxides+40 ppm 
Fe(II) in MCT; (F) 0.1 M PBN+cumene hydroperoxides+40 ppm 
Fe(III) in MCT after 24 h storage at 55 °C .................................................................110

6.1. Proposed mechanism of bulk oil oxidation that contains reverse micelles ......... 115

LIST OF ABBREVIATIONS

xvii
BHA: Butylated hydroxyanisole
BHT: Butylated hydroxytoluene
CA: Calcein
CMC: Critical micelle concentration
DAG: Diacylglycerols
DC₄PC: 1,2-dibutyl-sn-glycerol-3-phosphocholine
DFO: Deferoxamine
DOPC: 1,2-dioleoyl-sn-glycerol-3-phosphocholine
EPR: Electron paramagnetic resonance
FFA: Free fatty acid
MAG: Monoacylglycerols
MCT: Medium chain triacylglycerols
NBD-PE: N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt
PAHs: Polycyclic aromatic hydrocarbons
PBN: N-t-butyl-phenylnitrone
PC: Phosphatidylcholine
PE: Phosphatidylethanolamine
PI: Phosphatidylinositol
PLs: Phospholipids
PS: Phosphatidylserine
SAXS: Small angle X-ray scattering
SSO: Stripped soybean oils
TAG: Triacylglycerols
TBHQ: tert-Butylhydroquinone

TCNQ: 7,7,8,8-tetracyano-quinodimethane

TGP: Triacylglycerol polymers

TROLOX: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
CHAPTER 1
INTRODUCTION

Food oils from a wide variety of sources are important to human health and the food industry since they are important components in a large variety of food products and since they are an important cooking medium (1). However, most oils are susceptible to oxidation as a result of their unsaturated fatty acids (2). The products formed in oxidized oil include numerous free radical species, primary oxidation products like lipid hydroperoxides and secondary oxidation products like aldehydes, hydrocarbons, ketones and epoxides that negatively impact aroma (3). In addition, lipid oxidation can produce toxic products that can adversely impact biological tissues. For instance, linoleic acid hydroperoxides are toxic to wild type *Saccharomyces cerevisiae* at a low levels (0.2 mM) (4). Oxidized palm oil has been shown to induce reproductive toxicity and organotoxicity of the kidneys, lungs, livers, and heart of rats (5). High intake of a mixture of oxidized lard and cod liver oil caused impaired fertility in female rats and an increased incidence of morphologically abnormal spermatozoa in male rats (6). Oxidized lard, soybean oil, and particularly sardine oil increased spontaneous liver tumor development and the formation of 8-hydroxy-deoxyguanosine (8-OH-dG) in the liver DNA of mice (7). These results along with others suggest that consumption of oxidized lipids should be avoided whenever possible.

How to prevent or retard lipid oxidation in food oil is a major focus of lipid research. In the past six decades, a lot of attention has been drawn on finding effective ways to extend the shelf life of oils. The addition of antioxidants is one effective way to retard lipid oxidation (8). The most widely used antioxidants are free radical scavengers that
inactive free radicals formed in the initiation and propagation steps of lipid oxidation. A number of natural or synthetic phenols can interact with hydroperoxyl (LOO•) and alkoxyl (LO•) radicals, producing hydroperoxides and alcohols, respectively, and low energy antioxidant radicals that do not readily promote the oxidation of unsaturated fatty acids (3).

Among the free radical scavenging antioxidants, synthetic phenolic antioxidants [e.g., butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-Butylhydroquinone (TBHQ) normally exhibit higher antioxidative activity than natural antioxidants. However, the utilization of many effective synthetic antioxidants is being limited by consumer concerns of their negative impact on health. Research showed that large doses of both BHA and BHT (500 mg/kg body weight/day) result in certain pathological, enzyme, and lipid alterations in both rodents and monkeys, and in some cases BHT has been reported to have teratogenic and carcinogenic effects in rodents (9).

Over the past several decades, food scientists have been mostly unsuccessful in their search for natural antioxidants which could possess similar antioxidant activity as synthetic antioxidants (10). The most common natural antioxidants in foods are tocopherol and rosemary extracts. Interestingly, rosemary extracts are not actually approved by the FDA for use as an antioxidant but instead as a flavor extract (FDA 21 CFR 182.(II)). Since the number of antioxidant options has not significantly increased in the past 30 years and in fact may be decreasing since food companies are reluctant to utilize synthetic antioxidants, new approaches are needed to fulfill the goal of retarding oil and foods from oxidation. The development of novel approaches to inhibiting lipid oxidation is highly reliant on our understanding of lipid oxidation mechanisms in variable
lipid systems.

The mechanism of lipid oxidation in bulk oil has been studied for many decades (12). However, research has revealed that this reaction is extremely complex being a balance of the activity of numerous prooxidative and antioxidative factors (12). On top of these chemical effects, lipid oxidation is also impacted by physical properties of food and food components that influence factors such as partitioning of antioxidants, diffusion of oxygen and interaction of prooxidants with lipid substrates. The impact of physical properties on lipid oxidation can be seen in heterogeneous food systems such as emulsions where the characteristics of the emulsion droplet interface are an important determinant in oxidative stability (13). Even bulk oils, which are often assumed to be a homogenous liquid, have numerous physical structures that impact lipid oxidation. This is because refined bulk oils contain numerous minor components that are amphiphilic, such as monoacylglycerols (MAG), diacylglycerols (DAG), phospholipids, sterols, free fatty acids (FFA), and polar products arising from lipid oxidation, such as lipid hydroperoxides, aldehydes, ketones, and epoxides. These surface active compounds in combination with water can form physical structures known as association colloids that can physically impact lipid oxidation (14).

The objective of this research is therefore to better understand how these association colloids impact the lipid oxidation in bulk oil. Besides, how the interactions between the minor components naturally from bulk oils and association colloids could impact the lipid oxidation of bulk oil will be investigated. By doing so, the results can not only enhance the oxidative stability of bulk oil by avoiding the prooxidative minor components during refining process, but also improve the antioxidative ability of antioxidants in the bulk oil.
CHAPTER 2
LITERATURE REVIEW

2.1 Oil refining procedure

The progress of oil refining has been progressed from emphasizing one operation independently in the early days toward the current integrating manufacturing facilities to produce a more complete range of value-added products from the raw plant seeds to the final products. Before getting the final oils, there are several procedures need to be carried out, i.e., storage, preparation, mechanical extraction, solvent extraction, degumming, deodorization, bleaching, etc.

2.1.1 Storage

The typical operation associated with storage includes receiving, sampling, drying, storage, and cleaning prior to processing. All these procedures are to make sure the seeds are suitable to extract oil. The following description is based on the industrial soybean oil extraction and refining processing. Initially, soybeans received are sampled and analyzed for moisture content, foreign matter, and damaged seeds. Then the seeds are weighed and conveyed to large silo or metal tanks for storage prior to processing. When the facility is ready to process the seeds, the seeds are removed from the containers and cleaned of foreign materials and loose hulls if necessary. Screens typically are used to remove foreign materials such as sticks, stems, pods, tramp metal, etc. An aspiration system is used to remove loose hulls from the seeds. The seeds are passed through dryers to reduce their moisture content to approximately 10 to 11 wt % and then are conveyed to process bins for temporary storage and tempering for 1 to 5 days in order to facilitate dehulling.
2.1.2 Preparation

The preparation processing is fairly well standardized consisting four principal operations: cracking, dehulling/hull removal, conditioning, and flaking. Soybeans are conveyed from the process bins to the mill by means of belts or mass flow conveyors and bucket elevators. In the mill, the beans may be aspirated again, weighed, cleaned of tramp metal by magnets, and fed into corrugated cracking rolls. The cracking rolls “crack” each bean into four to six particles, which are passed through aspirators to remove the hulls. Then, the cracked beans and bean chips are conveyed to the conditioning area, where they are put either into a rotary steam tubed device or into a stacked cooker and are heated to “condition” them (i.e., make them pliable and keep them hydrated). Conditioning is necessary to permit the flaking of the chips and to prevent their being broken into smaller particles. Finally, the heated, cracked beans are conveyed and fed to smooth, cylindrical rolls that press the particles into smooth “flakes”, which vary in thickness from approximately 0.25 to 0.51 mm. Soybean flakes allow the soybean oil cells to be exposed and the oil to be more easily extracted.

2.1.3 Solvent extraction

The extraction process consists of “washing” the oil from the soybean flakes with hexane solvent in a countercurrent extractor. Then the solvent is evaporated from both the solvent/oil mixture and the solvent-laden, defatted flakes. The solvent in oil is removed by exposing the solvent/oil mixture to steam (contact and noncontact). Then the solvent is condensed, separated from the steam condensate, and reused. Residual hexane not
condensed is removed with mineral oil scrubbers. The solvent removed oil, called “crude” soybean oil, is stored for further processing or transportation.

2.1.4 Oil refining

Crude vegetable oils predominantly contain triacylglycerols and small amounts of minor components that naturally occur in the plant component of their origin, such as proteins, free fatty acids, phospholipids, metals, tocopherols, pigments and sterols. The effective removal some of those minor components without sacrificing the loss of antioxidants is necessary to achieve a finished oil quality with acceptable standards for flavor, appearance and stability. The oil refining process which includes degumming, neutralization, bleaching, and deodorization is therefore performed to produce oils suitable for use by the food industry and the consumer (Figure 2.1).

![Figure 2.1. The schematic of oil refining process](image)

Oil seed extraction will produce a crude oil that contains lipids such as phospholipids and sterols in addition to triacylglycerols. However, the extraction process will also produce conditions where triacylglycerols can react with enzymes such as lipase
and lipoxygenase to form hydrolytic products of triacylglycerols (e.g. monoacylglycerols, diacylglycerols and free fatty acids) and lipid oxidation products such as hydroperoxides. Oil refining is performed to reduce the concentration of these minor components as they can negatively impact the quality of the oil. For instance, caustic alkali is used to remove free fatty acids in neutralization step since free fatty acids cause foaming and decreases the smoke point of oils (15). Phosphoric or citric acid is used to aid the removal of nonhydratable and hydratable phospholipids during the degumming step since phospholipids cause cloudiness and can form brown colors during heating (16).

Bleaching is performed with activated bleaching earth at relatively high temperatures around 100°C to decrease the concentration of pigments (e.g. chlorophyll), trace metals and other polar compounds that can accelerate lipid oxidation. Finally, since the harsh conditions of these steps can produce off-flavors due to oxidation and since the oil can contain pesticides from the original oilseeds, the oils undergoes a deodorization step at high temperatures (200-260°C) under reduced pressure. Deodorization processing under high temperature not only removes volatile off-flavors and pesticides but also decomposes lipid hydroperoxides, a potential lipid oxidation substrate. Overall, refining is designed to remove minor components that negatively impact oil quality. However, steps such as deodorization can also remove factors that improve oil quality such as tocopherols and can cause the formation of trans fatty acids. While refining significantly decreases the concentration of the minor components that impact the organoleptic quality of oil, one should realize that even at these lower concentrations, minor components can still impact oil chemistry such as lipid oxidation.
2.2 Minor components in bulk oil

2.2.1 Free Fatty Acid

The reaction between water and triacylglycerols (TAG) results in the formation of free fatty acid (FFA) and diacylglycerol (DAG). TAG hydrolysis is accelerated by lipases, extremes in pH and heat (17). The presence of FFA produces undesirable flavors, foaming during mixing and heating and causes a decrease in the smoke/flash point of oil thus reducing the maximum temperature to which they can be heated. FFAs in crude vegetable oils will increase with the age of the oilseeds and can be abnormally high if the oilseeds have been damaged or improperly stored. This is due to damage to cells in the seeds allowing lipase to interact with TAG. For instance, in oilseeds stored from 0 to 47 days, the FFA content in the extracted crude oil increased from 0.88 to 1.80 wt % (18). Recent research from Alencar and coworkers showed that FFA concentrations will also increase during crude oil storage (19).

There are several factors which can impact FFA concentrations in crude oils and during the refining process, such as oilseeds storage time before extraction (Table 2.1), the initial FFA content, water content and processing temperatures (Table 2.2), oil type, etc. The presence of FFA can catalyze the further hydrolysis of TAG, thereby increasing the total FFA concentration in oil.
Table 2.1 Free fatty acid content in crude soybean oil as the function of bean storage time

<table>
<thead>
<tr>
<th>Bean storage time(d)</th>
<th>FFA(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.88</td>
</tr>
<tr>
<td>11</td>
<td>0.60</td>
</tr>
<tr>
<td>22</td>
<td>1.28</td>
</tr>
<tr>
<td>35</td>
<td>1.40</td>
</tr>
<tr>
<td>47</td>
<td>1.80</td>
</tr>
</tbody>
</table>

The moisture in soybean seeds is 13-15 %

Table 2.2 Free fatty acid content in soybean oil as a function of processing step

<table>
<thead>
<tr>
<th>Processing step</th>
<th>FFA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>0.61</td>
</tr>
<tr>
<td>Degummed</td>
<td>0.31</td>
</tr>
<tr>
<td>Refined</td>
<td>0.05</td>
</tr>
<tr>
<td>Deodorized</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Some earlier results showed the rate of TAG hydrolysis is proportional to the initial level of FFA (20). Another important factor is the water concentration since this is one of the required substrates for the hydrolytic reaction. Sarkadi and coworkers showed that increasing of water content as a result of steam deodorization of peanut oil at 180°C increased FFA formation (21). Considering that both FFA and water are important factors in TAG hydrolysis, the following equation was used to estimate the formation of FFA over time ($t$) in the bulk oils containing water (20).

$$\frac{d[FFA]}{dt} = k', \frac{[H_2O]}{[H_2O]_{Sat}}[FFA]$$

(1)

Here, $k'$ is the rate constant, [FFA] is the free fatty acid concentration, [H$_2$O] is the water concentration, [H$_2$O]$_{Sat}$ is the water concentration at saturation.

The FFA content in deodorized food oils is required to less than 0.05 % in the United States (22, 23). FFA are typically removed from oils by chemical neutralization
which normally involves the conventional alkali (sodium hydroxide) neutralization process, precipitating the FFA as soap stock and being removed by mechanical separation from the oil. Recently, some oil refineries are using physical refining. Physical refining is a high temperature, vacuum process that can remove both FFA and phospholipids at the same time thus effectively combining the neutralization and degumming steps. The choice of physical or chemical refining is highly correlated to the initial FFA concentration of the crude oil. When the FFA content is lower than 2.5 % in the crude oil, physical refining can be employed without the neutralization step. In some cases physical refining is used on oils with > 2.5 % FFA but neutralization must be performed first (12). A major limitation of physical refining is that the high temperatures required can cause the formation of trans fatty acids (24).

FFA are produced at almost each step of refining since they often involve high temperatures and water (25). Despite this FFA concentrations as low as 0.005 wt % can be obtained (26). However, one should realize that these FFA concentrations cannot be maintained as even low FFA concentration can promote TAG hydrolysis. Therefore, the FFA content of retail bottled edible oil is higher than the concentrations measured immediately after refining process (Table 2.3). For example, retail canola oil has a FFA concentration of approximately 0.1 wt%. Finally, free fatty acid concentrations can increase in refined oils during operations such as frying where the oils are exposed to high temperatures and water (27).
Table 2.3 Free fatty acid content in retail oil

<table>
<thead>
<tr>
<th>Oil type</th>
<th>Free Fat acids (wt %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canola</td>
<td>0.1</td>
</tr>
<tr>
<td>Coconut</td>
<td>3.93</td>
</tr>
<tr>
<td>Corn</td>
<td>0.12</td>
</tr>
<tr>
<td>Olive</td>
<td>0.30</td>
</tr>
<tr>
<td>Extra virgin olive</td>
<td>0.28</td>
</tr>
<tr>
<td>Peanut</td>
<td>0.32</td>
</tr>
<tr>
<td>Palm kernel</td>
<td>2.49</td>
</tr>
<tr>
<td>Palm olein</td>
<td>0.15</td>
</tr>
<tr>
<td>Rapeseed</td>
<td>0.04</td>
</tr>
<tr>
<td>Rice bran</td>
<td>0.08</td>
</tr>
<tr>
<td>Safflower</td>
<td>0.03</td>
</tr>
<tr>
<td>Sesame</td>
<td>2.37</td>
</tr>
<tr>
<td>Soybean</td>
<td>0.05</td>
</tr>
<tr>
<td>Sunflower</td>
<td>0.09</td>
</tr>
</tbody>
</table>

2.2.2 Monoacylglycerols and Diacylglycerols

Monoacylglycerols (MAG) are monoester of glycerol in which one of the hydroxyl groups is esterified with fatty acids. There are two isomeric forms of MAG, i.e., 1-MAG and 2-MAG, depending on the position of the ester bond on the glycerol group. Diacylglycerols (DAG) are esters of the glycerol in which two of the hydroxyl groups are esterified with fatty acids. They can exist in three structural isomers namely, 1,2-DAG, 2,3-DAG and 1,3-DAG (28). DAG is a common precursor for the synthesis of both TAG and phospholipids in the oilseeds and oil bodies (28). Therefore, DAG that exists naturally in crude vegetable oils can be derived from the incomplete biosynthesis of TAG.
and phospholipids. Knowledge of the amounts of DAG naturally found in oilseeds is scare due to the lack of in situ measurements.

MAG and DAG are also formed from the partial hydrolysis of TAG (29). The amount of DAG and MAG reported in the crude oil are therefore composed by two parts, inherent concentrations in the seeds and those formed after crushing the seeds and during refining. The formation of DAG and MAG depends on oilseeds storage conditions, temperature, and moisture as was discussed previously for FFA.

**Table 2.4.** Concentration of MAG and DAG in various oils as a function of refining (30, 31).

<table>
<thead>
<tr>
<th>Refining step</th>
<th>Soybean oil</th>
<th>Palm oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAG(%)</td>
<td>DAG(%)</td>
</tr>
<tr>
<td>Crude oil</td>
<td>0.11</td>
<td>1.10</td>
</tr>
<tr>
<td>Degummed</td>
<td>0.10</td>
<td>1.44</td>
</tr>
<tr>
<td>Bleached</td>
<td>0.06</td>
<td>1.25</td>
</tr>
<tr>
<td>Deodorized</td>
<td>0.07</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Table 2.4 lists the amount of DAG and MAG in crude and refined soybean oil and palm oil. After bleaching and deodorization, MAG concentrations decease while DAG concentrations remain similar to crude oil or even increase (30-32). Farhoosh and coworkers recently showed the DAG concentration in crude soybean oil (1.66 %) decreased to 1.35 % after the neutralization step. However, after deodorization, the DAG concentrations (1.74 %) increased and reached around the initial amount in the crude soybean oils (25). They also observed the similar trend in the canola oil. Possible reasons for the increased in DAG in these reports is the existence of lipase in the crude oil which could form DAG or from TAG hydrolysis by high temperatures and water in the deodorization step (33).
2.2.3 Phospholipids

Phospholipids (PLs) are integral elements of all cellular membranes in living organism and possess unique chemical structures containing both lipophilic and hydrophilic groups. Crude oil contains substantially high amount of PLs due to the solvent extraction of cell membranes. Phospholipids in oils include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI). Different species of seeds have different compositions of phospholipids, but in general PC is predominant (Table 2.5).

Table 2.5 Phospholipid composition (%) as extracted from commercial seeds

<table>
<thead>
<tr>
<th></th>
<th>Soybean</th>
<th>Sunflower</th>
<th>Corn</th>
<th>Rapeseed</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>21.9</td>
<td>25.4</td>
<td>30.4</td>
<td>24.6</td>
</tr>
<tr>
<td>PE</td>
<td>13.6</td>
<td>11.0</td>
<td>3.2</td>
<td>22.1</td>
</tr>
<tr>
<td>PI</td>
<td>12.0</td>
<td>19.4</td>
<td>16.3</td>
<td>14.7</td>
</tr>
</tbody>
</table>

The amount of phospholipids in the oilseeds is small compared to animal tissues (34). PLs in food oils cause foaming, cloudiness and darkening during thermal processing. Normally, there are two types of PLs in crude oil, hydratable and non-hydratable. The degumming step removes hydratable PLs by washing them into the water phase. Non-hydratable PLs are not affected by water washing alone so citric or phosphoric acid are often added to the wash water to bind divalent cations thus making non-hydratable PLs hydratable. Physical refining can also be used to remove phospholipids as discussed above. Like other minor components, only trace amount of PLs will remain in the final refined oil (Table 2.6) with concentrations ranging from 20-2000 ppm with variations among different oilseeds species and refining methods (35, 36).

Table 2.6 phosphorous content (ppm) of soybean oil as a function of refining step
<table>
<thead>
<tr>
<th>Refining step</th>
<th>Soybean</th>
<th>Soybean palm</th>
<th>Soybean</th>
<th>com</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Physical</td>
<td>Chemical</td>
<td>Physical</td>
<td>Chemical</td>
</tr>
<tr>
<td>Crude</td>
<td>600</td>
<td>510</td>
<td>9.1</td>
<td>327.8</td>
</tr>
<tr>
<td>Degummed</td>
<td>58</td>
<td>120</td>
<td>0.7</td>
<td>5.7</td>
</tr>
<tr>
<td>Refined</td>
<td>13.1</td>
<td>1</td>
<td>0.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Deodorized</td>
<td>0.16</td>
<td>1</td>
<td>0.5</td>
<td>0.2</td>
</tr>
</tbody>
</table>

2.2.4 Tocopherols

Tocopherols are the major oil soluble vitamin in crude oil. When the oilseeds are crushed, the tocopherols are extracted along with the oil. Natural tocopherols are mixtures consisting of α-, β-, γ-, δ- tocopherols. The volatility of tocopherols is above free fatty acids but below triacylglycerols. Thus, during the refining processing, tocopherols are partially removed during deodorization thus decreasing their concentrations in the refined oil. Gogolewski and coworkers found that after the refining of rapeseed oil the tocopherol losses amounted to 30 %. Two thirds of the loss resulted from distillation and thermal degradation during deodorization, while one third was caused by the combined effects of neutralization and bleaching (37). Rossi and coworkers reported that steam deodorization removed 200-300 ppm of tocopherols out of the original ~ 1000 ppm in crude palm oil (38).

The initial concentration and type of tocopherols in bulk oil vary with different oilseeds species. For instance, soybean oil has a high tocopherol concentrations with more than 65% γ-tocopherols and 20% δ-tocopherols while there is almost no δ-tocopherols in grape seeds oil (39). During storage tocopherol concentrations decrease which is accompanied by the generation of oxidized tocopherols. This is because tocopherols act as hydrogen donors and react with free radicals.
2.2.5 Pigments

The intensity of the bulk oils color mainly depends on the presence of coloring pigments such as carotenoids and chlorophyll. Vegetable oils with a minimum color index are considered to be more suitable for edible and industrial purposes (40). Thus, pigments in crude oil are partially removed during the degumming, refining, and bleaching steps of the refining process.

Carotenoids are pigments in many vegetable oils and particularly in palm oil. They contain a long conjugated polyene chains and are yellow/orange/red in color. Crude palm oil normally contains 500-700 ppm of carotenes. These are mainly $\alpha$-carotene (24-42 % of total carotene) and $\beta$-carotene (50-60 %) along with low levels of several other carotenes (41).

Chlorophylls are also found in vegetable oils. Chlorophyll includes chlorophyll a, chlorophyll b, and the Mg-free chlorophyll derivatives pheophytin a and pheophytin b. Some studies have reported no detectable chlorophylls in soybean oil after deodorization (42). However, other groups showed the existence of chlorophyll and pheophytins in refined oils (43). Pheophytin was reported to be the predominant chlorophyll, being about 0.06 and 0.10 ppm in refined soybean oil and corn oil, respectively (43).

2.2.6 Miscellaneous compounds

2.2.6.1 Nonsaponifiable minor components

Nonsaponifiable minor components in food oils include sterols, polycyclic aromatic hydrocarbons (PAHs) and hydrocarbons (e.g. alkanes, alkenes and squalene)(44). Most vegetable oils contain 700-1100 mg/100g of sterols, partly as free and partly as esterified
sterol. High levels are present in rapeseed oil and in corn oil (45). Sitosterol is generally the major phytosterol (50-80% of total sterol) with campesterol, stigmasterol, and Δ5-avenasterol also frequently attaining significant levels. Brassicasterol is virtually absent from the major seed oils except for rapeseed oil where it comprises 10% of the total sterols.

Food oils can be contaminated with PAHs due to the wide distribution of PAHs in the environment, their lipophilic nature, and the migration from contaminated packaging materials (46). Sekeroglu and coworkers tested 40 vegetable oil samples from Turkey for PAHs and found the total PAHs were above 25 ppb in most of the oils samples (47). More significantly, total PAHs levels in virgin olive oil always were 54.4-110.8 ppb (48). However, the refined food oils only occasionally exceeded the 25 ppb limit. Recently, Guillen and coworkers confirmed the occurrence of PAHs during the sunflower oil oxidation stored in the close container at room temperature for 112 months (49, 50).

Squalene (C$_{30}$H$_{50}$) is a highly unsaturated open-chain triterpene. Olive oil is the major commercial oil with significant amounts of squalene (51). In refined food oils, new nonsaponifiable compounds are also formed as a consequence of the reactions occurring during the refining process (52). These compounds include steroidal, arising from the dehydration of sterols, terpenic from terpenic alcohols, and other compounds deriving from squalene isomerization.

### 2.2.6.2 Polar triacylglycerol polymers

Polar triacylglycerol polymers (TGP) are formed during vegetable oil refining (53, 54). TGP are generally formed during the high temperature refining steps, e.g. bleaching
and especially deodorization. An early study on the evolution of TGP in vegetable oils during physical refining reported a 1-2 wt % increase of TGP concentrations at physical refining temperatures less than 240 °C. When physical refining temperatures were increased to 270 °C, a sharp increase in TGP concentration to 3.0 wt % was observed. TGP formation also depends on the degree of saturation of the oils. For more saturated oils, such as palm oil, TGP increases were restricted to 2 wt %, even at temperatures of 270 °C (55). Gomes used high-performance size exclusion chromatography to quantify the amount of oligopolymer in refined oils and found that refined olive oil had 0.7 wt % of oligopolymer twice of that in refined olive pomace oil (56). The final amounts of TGP found in food oils are generally ~1 wt % depending on the fatty acid composition, initial quality and refining technology used (57).

2.2.6.3 Oxidized triacylglycerols

Oxidized triacylglycerols (ox-TG), sometimes referred to as core aldehydes, are generally nonvolatile oxidation products left in the oil after deodorization. Initial reports suggested that ox-TG concentrations ranged from 4.55 wt % in crude oil to 9.29 wt % after oil refining (57). However, more recently Hopia investigated the impact of oil refining on the ox-TG contents in three vegetable oils, i.e., sunflower oil, soybean oil and low erucic acid rapeseed oil (58). The results showed that the concentration of ox-TG ranged from 0.53 to 0.90 % oil in the crude oils. After refining, ox-TG concentrations were decreased in bulk oil and ranged from 0.29 % for sunflower oil to 0.78 wt % for soybean oil. The loss was attributed to the adsorption of ox-TG by the activated earth during bleaching. Farhoosh and coworkers found ox-TG concentrations in crude soybean
oil and canola oil at 4.2 and 2.8 wt %, respectively (25). After oil refining, ox-TG decreased to 2.8 and 2.0 wt % in soybean oil and canola oil, respectively. They also noticed the level of the ox-TG in the refined oils was proportional to its original level in the crude oils.

### 2.2.7 Water

Since water and oil have opposite polarities and they tend to spontaneously phase separate because of the hydrophobic effect one might not expect to find water in bulk oil (59). However, as discussed in the proceeding sections, refined oils contain many surface active compounds such as free fatty acids (FFA), monoacylglycerols (MAG), diacylglycerols (DAG) and phospholipids (PLs). These surface active compounds have the ability to emulsify water into bulk oils. The water content in freshly-opened, commercially available vegetable oils range from 200-2000 ppm (Table 2.7) (14). Water concentrations in oil can change after opening since oil can absorb water from atmosphere or water in the oil can evaporate.

**Table 2.7.** Water content in freshly-opened, commercially available vegetable oils as determined by Karl Fischer Coulometer

<table>
<thead>
<tr>
<th>Oil</th>
<th>Water Content (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunflower</td>
<td>203</td>
</tr>
<tr>
<td>Canola</td>
<td>236</td>
</tr>
<tr>
<td>Peanut</td>
<td>221</td>
</tr>
<tr>
<td>Sesame</td>
<td>197</td>
</tr>
<tr>
<td>Extra virgin olive</td>
<td>865</td>
</tr>
</tbody>
</table>

Water in refined oil is derived in two possible ways. The first is from the original water in the oilseeds. Sorption isotherms experiments showed water with three levels of
affinity in soybean seeds: (i) a region of strongly bound water at moisture concentrations below 8%; (ii) a region of weakly bound water at moisture concentrations between 8 and 24%; and (iii) a region of very loosely bound water at concentrations greater than 24% (60). When soybeans are dried for storage, the moisture content is ~ 10 wt %. That water is likely the strongly bound water in the oilseed but it is possible that some of this water ends up in the crude oil. The second possible source of water is the water used to wash oils and remove the free fatty acids and phospholipids during the neutralization and degumming steps. The majority of this water is removed by centrifugation. The remaining water is removed by a vacuum dryer that controls the moisture content of the washed oil to below 1000 ppm, most often in the range of 500 ppm (61).

2.2.8 Oxygen

Oxygen is a primary reactant in lipid oxidation reaction which fuels the fatty acid decomposition pathway that causes rancidity. There are two types of oxygen in bulk oil systems, dissolved oxygen (DO) and non dissolved oxygen. Dissolved oxygen is incorporated into food while non dissolved oxygen stays in the headspace (62). The solubility of oxygen and the rate of its diffusion in the bulk oil are relevant to the rate and extent of lipid oxidation. Oxygen is as about 3-10 times more soluble in bulk oil than water (saturation occurs at 5-10 ppm in pure water at 20 °C) (63, 64). Schrader and coworkers determined that the diffusion coefficient of oxygen in soybean oil was $0.6 \times 10^{-9} \text{ m}^2 \text{s}^{-1}$ at 20 °C which is lower than that of olive oil (65). The impact of temperature on the solubility of oxygen in different types of oil is listed in Table 2.8. Some researchers suggested that oxygen solubility was related to fatty acid chain length with oxygen
solubility decreasing with increasing the hydrocarbon chain length (66).

Table 2.8. Solubility of oxygen (ppm) in Marine oil

<table>
<thead>
<tr>
<th>Oil type</th>
<th>Temperature(°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Redfish oil</td>
<td>20.4</td>
</tr>
<tr>
<td>Capelin oil</td>
<td>19.5</td>
</tr>
<tr>
<td>Herring oil</td>
<td>35.8</td>
</tr>
<tr>
<td>Mackerel oil</td>
<td>13.6</td>
</tr>
<tr>
<td>Harp seal oil</td>
<td>29.1</td>
</tr>
<tr>
<td>Flounder oil</td>
<td>23.2</td>
</tr>
<tr>
<td>Cod liver oil</td>
<td>39.3</td>
</tr>
</tbody>
</table>

The pressure is 101.325 Kpa

Another form of oxygen associated with bulk oil is non dissolved oxygen or headspace oxygen. The air space above the oil, for example in the oil tank or commercial oil bottle, is where headspace oxygen located. The headspace oxygen content in olive oil and marine oil at temperature lower than 60 °C increases with the increase of temperature, which following the rule of the Henry’s Law, i.e., the solubility is proportional to the partial pressure of headspace gas (67). In other words, as temperatures increase, oxygen in the oil moves into the headspace. At temperatures higher than 60 °C there is a large variation in the solubility of oil, especially in marine oil presumably due to heat accelerated oxidation which consumes oxygen (Table 2.8.). The mass transfer between headspace oxygen and oxygen in the oil is influenced by: (i) the rate of absorption; (ii) the oxygen concentration in the headspace; (iii) the headspace surface area to volume ratio; and (iv) agitation. With those factors being considered, an equation was developed to describe the absorption rate of oxygen between bulk oil and oxygen in the headspace.
Here, $C_0$ is the equivalent solubility of oxygen in the bulk oil; $C$ is the oxygen content in bulk oil after $t$ times; $K$ is the mass transfer coefficient; $A/V$ is the area to volume ratio.

2.3 The effect of minor components on bulk oil oxidation

2.3.1 The effect of oxygen on the chemical stability of bulk oil

Lipid oxidation is not a spontaneous reaction. Thermodynamically, oxygen cannot react directly with double bonds because the spin states are different. Ground state, atmospheric oxygen is in a triplet state, whereas the double bond of unsaturated fatty acids is in singlet state. Quantum mechanics requires that spin angular momentum be conserved in reactions. Therefore, interactions between triplet oxygen and unsaturated fatty acids demands that either the double bond be excited into a triplet state or oxygen is converted to a singlet state. The former seems impossible due to the requirement of prohibitive amounts of energy. The triplet oxygen also cannot convert to singlet states itself thus direct reaction occurs between the oxygen and double bonds in oil do not occur unaided (68). However, under the assistance of other minor components in bulk oil, several reactions can happen. For example, photosensitizers such as chlorophylls can produce singlet oxidation by mechanisms 3-5 outlined below.

\[
\text{Sen} + h\nu \rightarrow \text{Sen}^* \quad (3)
\]

\[
\text{Sen}^* + {^3}\text{O}_2 \rightarrow {^1}\text{O}_2^* \quad (4)
\]

\[
{^1}\text{O}_2^* + \text{LH} \rightarrow \text{LOOH} \quad (5)
\]

In addition, oxygen along with iron can generate alkyl radicals ($\text{L}^•$) as shown in reactions 6 and 7. Schafer and coworkers proposed that iron-oxygen complexes ($[\text{Fe}^{2+}$-
O$_2$]) are one route by which iron can promote lipid oxidation in cell membrane (69).

$$ Fe^{2+} + O_2 \rightarrow [Fe^{2+}O_2] \quad (6) $$

$$ [Fe^{2+}O_2] + LH \rightarrow L\bullet \quad (7) $$

Triplet oxygen is directly involved in lipid oxidation propagation through its ability to react with alkyl radicals in diffusion controlled radical-radical reactions to form peroxyl radicals as shown in reactions 8 and 9 (70):

$$ L\bullet + O_2 \leftrightarrow LOO\bullet \quad (8) $$

$$ LOO\bullet + RH \rightarrow LOOH + R\bullet \quad (9) $$

Because of these multiple pathways that oxygen can be involved in lipid oxidation, it is not surprising that many studies have shown that reduction of oxygen in packaged bulk oil can extend shelf life (71, 72). An 18-months shelf life test, performed on virgin olive oil indicated that the slowest oxidation rates occurred at the lowest initial dissolved oxygen concentration (71). The same group also reported that removal of oxygen from extra virgin olive oil by nitrogen purging lowered peroxide values (72). Packaging technologies such as oxygen scavenging film can also improve the oxidative stability of bulk oil (73, 74).

### 2.3.2 The effect of water on the chemical stability of bulk oil

Despite being present at relatively low levels, water could have an impact of the rate, extent and mechanism of lipid oxidation in bulk oils since it may act as a solvent for hydrophilic or amphiphilic antioxidants and prooxidants (such as transition metals, free fatty acids, or lipid hydroperoxides)
2.3.3 The effect of free fatty acid on the chemical stability of bulk oil

Generally, FFA with unsaturated bonds showed a lower oxidative stability than their corresponding methyl esters and triacylglycerols (75). In addition, FFA themselves have been reported to accelerate the oxidation of triacylglycerols in vegetable oils. FFA have been shown to have several different prooxidative tendencies. Yoshida and coworkers demonstrated that FFA (i.e., caprylic, capric, lauric, myristic, palmitic or stearic acid) in microwaved stripped soybean oil increased oxidation with shorter the chain length and the higher the levels of FFA being more prooxidative (76, 77). Aubourg and coworkers later showed similar results in marine oil with a higher degree of oxidation seen in the presence of short chain (lauric and myristic) than long chain (stearic and arachidic) fatty acids at 30 °C (78). Paradiso and coworkers recently reported that low amounts of FFA caused an increase in the formation of oxidized triacylglycerol and triacylglycerol oligopolymers again showing the prooxidant activity of FFA (79). The prooxidant activity of FFA is associated with its free acid group since methyl esters of fatty acids do not accelerate oxidation. Proposed mechanisms of FFA include their ability to accelerate the decomposition of hydroperoxides and bind metals to make them more prooxidative (75). Obviously, it is very critical to control the levels and formation of FFA in crude, refined and stored oils. While most commercial oils have FFA concentrations less than 0.05 wt %, there may be addition benefits if these levels could be even lower. Minimizing FFA formation during storage can be accomplished by decreased water exposure, minimizing temperatures, preventing contact with lipases and avoiding exposure to extremes in pH.

2.3.4 The effect of MAG and DAG on chemical stability of bulk oil
Different impacts of MAG and DAG on the oxidative stability of bulk oils has been reported. Min and coworkers found that 0-0.5 wt % of monostearin, distearin, monolinolein, or dilinolein acted as prooxidants in soybean oil (80, 81). Colakoglu and coworkers found that soybean oil containing 1 wt % monoolein increased the rate of oxygen consumption (82). Wang and coworkers observed that randomized corn oil contained higher levels of MAG and DAG (0.3 and 5.1 %, respectively) oxidized much faster than natural corn oil with no detectable MAG and 1.4% of DAG (83). On the contrary, Gomes et al found that 1-3 wt % MAG decreased oxidation in stripped olive oil at 60 °C (84). The effect of combinations of MAG or DAG with antioxidants in the bulk oil has also been studied. MAG or DAG in combination with citric acid resulted in greater antioxidant activity than the MAG or DAG itself (85). In addition, as the chain length of the fatty acids on MAG or DAG was increased, the more effective citric acid became.

2.3.5 The effect of phospholipids on the chemical stability of bulk oil

The impact of phospholipids (PLs) on bulk oil oxidation is also controversial. Dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidylethanolamine (DPPE) were reported to have poor antioxidant activity at 50°C and showed no synergistic effect with α-tocopherol in methyl linoleate (86). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from egg yolk accelerated the oxidation of methyl linoleate (86). Takenaka and coworkers also found that PC and PE promoted bonito oil oxidation in the absence of α-tocopherol. However, they found that PE showed synergistic antioxidant activity with α-tocopherol, while PC did not (87).
Alternatively, Bandarra and coworkers showed that 0.5 % PC was an effective antioxidant in sardine oil at 40°C. In addition, a high synergistic effect was observed in the same system with a mixture of α-tocopherol and PE (88). Others have reported similar results that PLs can increase the activity of tocopherols in bulk oils (89-92). For the researchers who observed the antioxidant activity of PLs in bulk oil, metal chelating, free radical scavenging and formation of Maillard reaction products were given as the mechanisms by which PLs inhibit oxidation (93-95).

2.3.6 The effect of tocopherols on the chemical stability of bulk oil

Tocopherols are the most common free radical scavenging antioxidants found in vegetable oils. Vegetable oils normally contain tocopherols concentrations in the range of 200-1000 ppm which originate from the seeds. After refining, almost 70 % of the tocopherols remain in the bulk oil with 30 % being removed during deodorization. Overall, tocopherols are probably the most important antioxidants in vegetable oils (for review see refs (96, 97)). The antioxidant mechanisms of tocopherols (TOC) are shown below.

\[
\text{LOO}^\cdot + \text{TOC} \rightarrow \text{LOOH} + \text{TOC}^\cdot \quad (10)
\]

\[
\text{LOO}^\cdot + \text{TOC}^\cdot \rightarrow \text{LOO-TOC} \quad (11)
\]

However, under certain conditions tocopherols can also act as prooxidants. This is thought to be due to their ability to reduce endogenous transition metals naturally occurring in the oil or metals resulting from contamination during processing. These reduced metals can then promote the decomposition of pre-existing lipid hydroperoxides as shown in pathways 12 and 13. Yoshida and coworkers found that both α-tocopherol
(100 M) and α-tocotrienol (100 M) reduced cupric iron (300 M) and the concomitant formation of α-tocopheryl quinone and α-tocotrienyl quinone was observed by UV absorption spectrum and also by HPLC analysis in methyl linoleate. Interestingly, the βδ-, and γ-forms of tocopherols were not found to reduce cupric iron in the same system (98)

\[
\text{TOC} + \text{Mn}^{2+} \rightarrow \text{TOC}^+ + \text{Mn}^+ \quad (12)
\]

\[
\text{LOOH} + \text{Mn}^+ \rightarrow \text{LO} + \text{HO}^- + \text{Mn}^{2+} \quad (13)
\]

The prooxidant activity of tocopherols has also been proposed to be due to the ability of the tocopherol radical to promote fatty acid oxidation especially when tocopherol radical concentrations are high (98-100).

\[
\text{LOOH} + \text{TOC}^\bullet \rightarrow \text{LOO}^\bullet + \text{TOC} \quad (14)
\]

\[
\text{LH} + \text{TOC}^\bullet \rightarrow \text{L}^\bullet + \text{TOC} \quad (15)
\]

Trace amounts of oxidized tocopherol exist in bulk oil after refining. Min and coworkers reported that oxidized α-, γ- and δ-tocopherols promote the oxidation of purified soybean oil in the dark at 55 °C (101, 102). Since it is possible that oxidized tocopherols occur naturally in bulk oil and that tocopherol ingredients could contain oxidized tocopherols, this could be a source of prooxidants in oils. More work is need to determine if and how oxidize tocopherol could impact the antioxidant/prooxidant activity of tocopherols.

2.3.7 The effect of pigments on the chemical stability of bulk oil

The two major pigments that can impact lipid oxidation in oils are chlorophyll and carotenoids. Chlorophyll, a photosensitizer, is prooxidative when exposed to light due to
its ability to promote the formation of singlet oxygen (103). As described previously, singlet oxygen is formed by chlorophyll photosensitization by energy transfer from light to a sensitizer and then to triplet oxygen (104). Singlet oxygen can then initiate lipid oxidation because it can directly react with the double bonds of unsaturated fatty acids to form lipid hydroperoxides (105). In the bulk oil, it is important to remove as much chlorophyll as possible during refining since most retail oils are stored in transparent plastic packages.

Unexpectedly, chlorophyll and its degradation product, pheophytin, have also been reported to inhibit lipid oxidation in the rapeseed and soybean oils at 30 °C during storage in the dark. This has been proposed to be due to their ability to scavenge peroxyl and other free radicals (106).

Carotenoids can be efficient singlet oxygen and excited photosensitizers quenchers that reduce oxidation by converting excited photosensitizers or singlet oxygen to their less reactive states (107). Burton also proposed that under low oxygen partial pressure conditions, β-carotene can act as a lipid soluble chain breaking antioxidant (108). They also found that at oxygen pressures of 20 kpa or higher, β-carotene and related compounds are prooxidants in a methyl linoleate model system possibly due to the formation of carotenoids oxidation products (109). During high temperature oil refining, carotenoids can also become thermally degraded. Thermally degraded carotenoids were reported to accelerate lipid oxidation in soybean oil at concentration of 50 ppm (110). More research is needed to elucidate how chemical changes of carotenoids impact the oxidative stability of oils.
2.3.8 The effect of miscellaneous compounds on the chemical stability of bulk oil

Small amounts of squalene (200 ppm) were reported to have a limited impact on the oxidative stability of stripped olive oil at 40 and 62 °C in the dark, whereas higher concentration (7000 ppm) of squalene showed an antioxidative effect at the same system (111). In rape seed oil, Malecka found that squalene (4000ppm) increased the oxidative stability of oil heated at 170 °C for 10 h (112). No significant impact of squalene (0-8000ppm) on stripped sunflower oxidation was observed by Mateos and coworkers (113).

Oppositely, squalene was found to accelerate oxidation of stripped olive oil in a Rancimat apparatus at 100 °C (114).

The effect of sterols on the oxidation of olive oil was studied at 180°C by Gordon (115). They found that Δ5-avenasterol and fucosterol were effective as antioxidants at concentration of 0.1 wt %, while other sterols, including cholesterol and stigmasterol, were ineffective. The proposed mechanism was that lipid free radicals could react rapidly with sterols at unhindered allylic carbon atoms (115). Phytosterols have also been reported to act as antioxidants in soybean, rice bran and sunflower (116-118).

Yoon and coworkers found that the oxidized triacylglycerol fractions obtained by thermal oxidation of soybean oil acted as prooxidant when it was added to stripped soybean oil (119). Gomes and coworkers found that oxidized triacylglycerol oligopolymers, a class of oxidation compounds present in refined vegetable oils, were the most prooxidative in vegetable oil amongst all the oxidized triacylglycerol fractions tested (120).

2.4. Association colloids and lipid oxidation in bulk oils
2.4.1 Evidence for the presence of association colloids in bulk oil

As discussed briefly above, one of the major issues that is often overlooked in bulk oil oxidation is the physical properties of minor components (121). Bulk oil minor components, such as monoacylglycerols (MAG) and diacylglycerols (DAG), phospholipids (PLs), sterols, free fatty acids (FFA), and polar products arising from lipid oxidation are surface active compounds. These surface active molecules have the ability to form physical structures in the bulk oils in the presence of the small quantities of water (~300 ppm) typically found in refined oils (14, 121). These structures are known as association colloids. Recent research suggests that association colloids could be lipid oxidation reaction sites in oils.

Previous studies have demonstrated that surface active molecules can self-assemble in non-polar solvents (e.g. benzene, isoctane, cyclohexane, toluene, free fatty acid and triacylglycerols) in the presence of water to form a variety of association colloids such as reverse micelles, micro-emulsions, lamella structures, and cylindrical aggregates (Figure 3) (122-125). For instance, Sosaku and coworkers formed reverse micelle systems using soybean phospholipids as a surfactant with fatty acids or fatty acid ethyl esters as the organic solvent (126). Sinoj and coworkers recently formed nutrient delivery systems consisting of water-in-oil nanoemulsions using oleic acid embedded in canola oil (127). Chen and coworkers identified ordered lamellar structures in hazelnut oil in the presence of monoglycerols (MAG) (128, 129). These studies suggest that the amphiphilic molecules in refined vegetable oils have the ability to form association colloids (130).
There are several lines of evidence that association colloids occur naturally in refined and crude oils. The complete removal of water from refined oil is very difficult even at temperatures up to 200 °C. This suggests that some of the water in oil is bound to polar compounds possibly in association colloids. We have indirectly seen this in our research since water content in oil is reduced by the removal of polar compounds. In addition, it was observed by research groups using ultrafiltration (UF) that only a small portion of the phospholipids in oils penetrates through UF membranes with a pore diameter of the order of 4 nm (131-133). The proposed reason for the rejection of phospholipids by these non-porous membranes was suggested to be due to the formation of phospholipids reverse micelles, swollen in the presence of small quantities of water plus containing other minor components such as pigments, which are then rejected by size exclusion. The amount of rejection phospholipids closely depended on the amount and the size of reverse micelles formed during solvent extraction (134-136).

2.4.2 Do association colloids impact lipid oxidation?
Many of the compounds involved in lipid oxidation reactions such as lipid hydroperoxides, free fatty acids and antioxidants are surface active. Previous research with oil-in-water emulsions has shown that the physiochemical property of the water-oil interface plays a very important role in lipid oxidation chemistry since it can impact the location and reactivity of both prooxidants and antioxidants (137). This suggests that the water-oil interface of association colloids could also impact lipid oxidation chemistry by acting as nano-reactors. Some of the first research in this area was by Koga and Terao who observed that the presence of phospholipids enhanced the antioxidant activity of α-tocopherol in stripped oil containing a trace amount of water (1%) (92). This study found that the presence of phospholipid increased the degradation of α-tocopherol in the presence of the water soluble free radical generator, 2,2-azobis(2-amidinopropyl) dihydrochloride (AAPH) suggesting that the phospholipids increased the exposure of α-tocopherol to the water phase. This could also be due to the fact that the reduction potential of α-tocopherol is lower in polar environments which could make tocopherol a more efficient free radical scavenger (138). Degradation of α-tocopherol by the water-soluble free radicals decreased as the phospholipid’s hydrocarbon tail group size was decreased, and thus the ability of the phospholipid to form association colloids was lost. Koga and Terao also found that the antioxidant activity of α-tocopherol could be increased in bulk oil when it was conjugated to the polar head group of phosphatidylcholine (PC) (95). This increase in activity was again thought to be due to the increased partitioning of the reactive portion of α-tocopherol into the water phase of the association colloids in bulk oil (95). This pioneering work was the first to suggest that the presence of physical structure in bulk oils could impact lipid oxidation chemistry by
altering the activity of free radical scavenging antioxidants.

Wilailuk and coworkers evaluated the ability of water, cumene hydroperoxide, oleic acid, and phosphatidylcholine to influence the structure of reverse micelles in a model oil (n-hexadecane) system containing sodium bis(2-ethylhexyl) sulfosuccinate (AOT) reverse micelles. This study found that water, cumene hydroperoxide, oleic acid, and phosphatidylcholine can alter reverse micelle size and lipid oxidation rates (139, 140). Kasaikina and coworkers found the decomposition of cumene hydroperoxides into free radicals was accelerated by the existence of reserve micelle formed by cationic surfactants in organic media (130, 141-144). In addition, they found the oxidation stability of sunflower oil at 80°C in the presence of 0.1 mM 2,6-di-tetra-tert-butyl-4-methylphenol (BHT) was decreased in the presence of fatty alcohols, such as 1-tetradecanol, 1-octadecanol, and the MAG, 1-monopalmitoylglycerol. This result was explained by the so-called "micellar effect": the relatively higher concentration of polar species such as hydroperoxide and peroxyl radicals within and nearby the reverse micelles formed in the presence of these fatty alcohols and MAG, leading to an increase of the rate of chain initiation via an acceleration of the hydroperoxides decomposition (145).

2.4.3 The perspective researches on the mechanism of lipid oxidation in bulk oil

Currently in the food industry, the trends of changing lipid profiles to contain more polyunsaturated fatty acids and less hydrogenated fats result in food products with an increased susceptibility to rancidity. In the meantime, the efficacy antioxidants available in FDA “direct contact with food” category are limited. To overcome the challenge of
developing low trans fatty acids products with nutritionally significant amounts of unsaturated fatty acids, the food industry must develop new antioxidant technologies. The overall objective of this research will be to gain a better understanding of how the chemical and physical properties of edible oils impact the deterioration of polyunsaturated lipids. In particular, focus will be on how nano-sized structures in bulk oil (association colloids) impact the oxidative stability of lipids since above mentioned evidences indicate that these physical structures are the site of oxidation reactions. Understanding how the physical nature of edible oils impacts rancidity could lead to the development of new antioxidant technologies and to the more efficient use of existing antioxidant ingredients.

The framework of this research include:(1) producing association colloids in stripped soybean oil using inherent minor component(s) in bulk oil. The well-defined structure will be characterized by all sorts of techniques; (2) how the physical properties of association colloids impact the chemical properties of bulk oil in a more complex system after the addition of minor oil components, including different phospholipids, antioxidants and prooxidant (i.e.,transition metals); (3) elucidate the physical location of these minor components in the association colloids. The relationship of the physical location and chemical reactivity of the minor components will then be compared to determine their relative overall ability to inhibit or promote lipid oxidation in bulk oil. By better understanding how physical structures in bulk oils impact reactions that cause rancidity, it is hoped that new antioxidant technologies can be developed.

2.5 Methodologies to study the impact of minor components on the physicochemical
properties of bulk oil

2.5.1 Chemical analysis

2.5.1.1 Primary lipid oxidation products

Lipid hydroperoxide in stripped soybean oil with or without association colloids will be determined (146). Precisely weighted of oil will be mixed with 2.8 mL of methanol/1-butanol (2:1). The reaction will be started by added 15 μL of 3.94 M ammonium thiocyanate and 15 μL of ferrous iron solution. After 20 min of incubation at room temperature, the absorbance will be measured at 510 nm using an UV-vis spectrophotometer. Hydroperoxides concentrations will be determined using a standard curve prepared from hydrogen peroxide.

2.5.1.2 Secondary lipid oxidation products

Headspace hexanal will be determined using a gas chromatograph (147). The headspace conditions will be as follows: Sample (1 mL) in 10 mL glass vials capped with aluminum caps with PTFE/silicone septa were pre-heated at 55 °C for 15 min in an autosampler heating block. A 50/30 μmDVB/ Carboxen/PDMS solid-phase microextraction (SPME) fiber needle from Supelco (Bellefonte, PA, USA) was injected into the vial for 2 min to absorb volatiles and then was transferred to the injector port (250 °C) for 3 min. The injection port was operated in split mode, and the split ratio was set at 1:5. Volatiles were separated on a Supelco 30 m × 0.32 mm Equity DB-1 column with a 1 μm film thickness at 65 °C for 10 min. A flame ionization detector was used at a temperature of 250 °C. Concentrations will be determined using a standard curve made from hexanal.
2.5.2 Physical analysis

2.5.2.1 Small/wide angle x-ray scattering (SAXS/WAXS)

X-ray scattering is a powerful method for unraveling structural details and molecular shapes \((148)\). The physical principles of SAXS/WAXS are similar. Firstly, the electric field of the incoming wave induces dipole oscillations in the atoms, then the accelerated charges generate secondary waves that add at large distances (far field approach) to the overall scattering amplitude. All secondary waves have the same frequency but may have different phases caused by different path lengths.

![Diagram of small angle scattering system]

**Figure. 2.3.** The essential parts of a small angle scattering system. The drawing shows the X-ray source \(X\), the sample \(S\), the scattering angle \(\theta\), the slits used to define the incident and scattered beams, and the detector \(D\).

X-ray from the source is formed into a fine beam, often by slits, and strike the samples. A small fraction of this beam is scattered in other directions which forms an angle with the direction of the incoming beam. A detector is used to record the scattering intensity which depends on the scattering angle. The ordered structure information of the sample can often be obtained from the analysis of the scattering intensity at a sequence of scattering angles.

\[
q = 4\pi \frac{\sin \theta}{\lambda} \tag{16}
\]
Also, from the relation between the diffraction peaks, the nature of ordered materials, such as liquid crystalline phase may be identified. When the composition of the sample is known together with the nature of materials, X-ray diffraction data can be used to extract information about the characteristic dimensions of materials. Also for systems without long-range order, SAXS may provide useful information for micelles, liposomes and other disperse systems, as well as on gel structure (149).

2.5.2.2 Steady-state and time resolved fluorescence measurement

Luminescence is the emission of light that can occur when a molecule excited to a higher order electronic state relaxes back to the ground state. Depending on the electronic nature of the excited state, singlet ($S_1$) versus triplet ($T_1$), luminescence is divided into two categories, fluorescence and phosphorescence, respectively (150).

Fluorescence measurements can be typically made in two regimes, i.e., steady state and time resolved measurements. Steady-state measurements are measured with constant illumination and observation. Due to the nanosecond timescale of fluorescence, the steady-state condition for a system is normally reached almost immediately after sample is illuminated. In contrast, time-resolved measurements involve monitoring the temporal dependence of a given fluorescence parameter, like emission intensity or anisotropy. For these experiments the sample is exposed to a pulse of light that is shorter than the decay time of the sample and measurements are then made using a high-speed detection system, capable of making discrete observations within a nanosecond time regime. Although time-resolved experiments are more complex, these measurements can provide novel information that may not be observed in steady-state measurements due to averaging.
processes. For example, anisotropy decays of fluorescent macromolecules are frequently more complex than single exponential. The precise shape of the anisotropy decay contains information about the shape of the macromolecule and its flexibility. Time-resolved measurements of intensity decays may also contain information that is obscured in steady-state measurements due to averaging processes (151, 152).

2.5.2.3 Interfacial tension analysis

Interactions occur between the molecules of a liquid and those of any liquid which is not soluble in the liquid. These result in the formation of an interface. Energy is required to change the form of this interface. The work required to change the shape of a given surface is known as the interfacial tension.

Surfactants consist of a hydrophilic head and a hydrophobic tail. If a surfactant is added to oil then it will initially enrich itself at the surface; the hydrophilic head projects from the surface. Only when the surface has no more room for further surfactant molecules will the surfactant molecules start to form agglomerates inside the liquid. These agglomerates are known as reverse micelles. The interfacial tension of system will decline as the increasing of surfactant concentration. The steady lowest interfacial tension of the system will reach when reverse micelle formed after which will not change. The surfactant concentrations at which reverse micelle formation is known as the reverse critical micelle formation concentration (CMC). By doing interfacial tension analysis, the surface active properties of minor components are able to be investigated.
CHAPTER 3
THE IMPACT OF PHYSICAL STRUCTURES IN SOYBEAN OIL ON LIPID OXIDATION

3.1 Introduction

Bulk oil is often assumed to be a homogenous liquid, in contrast to more complex multiphase systems, such as emulsions, which are regarded as heterogeneous liquids. However refined oil, which is processed to remove many of the non-triacylglycerol fractions, still contains numerous minor components that are amphiphilic, such as monoacylglycerols, diacylglycerols, phospholipids, sterols, free fatty acids, and polar products arising from lipid oxidation, such as lipid hydroperoxides, aldehydes, ketones, and epoxides (121, 153, 154). In addition, refined oils also contain small but significant amounts of water. Previous studies have demonstrated that surface active molecules can self-assemble in the presence of water to form a variety of association colloids in non-polar solvents (e.g. benzene, isooctane, cyclohexane, toluene, free fatty acid and triacylglycerols), such as reverse micelles, micro-emulsions, lamella structures, and cylindrical aggregates (122-124). For instance, Sosaku and coworkers formed reverse micelle systems using soybean phospholipids as a surfactant in fatty acid or fatty acid ethyl esters as the organic solvent (126). Sinoj and coworkers recently formed nutrient delivery systems consisting of water-in-oil nanoemulsions using oleic acid embedded in canola oil (127). These studies suggest that the amphiphilic molecules in refined vegetable oils have the ability to form association colloids (130).

The mechanism of lipid oxidation in bulk oil has been studied for decades (155). Research over the past two decades has shown that in emulsion systems, lipid oxidation
is strongly influenced by the physical properties of the emulsion droplets and their interfaces, which can impact pro-oxidant/lipid interactions and the location of antioxidants (156). Most previous research has treated bulk oil as a homogeneous system without considering how lipid oxidation reactions could be influenced by their physical structure. If bulk oils are actually micro-heterogeneous systems containing association colloids, it is highly likely that these physical structures will impact lipid oxidation as they do in emulsion systems.

To better understand how microstructures impact the oxidation of bulk oil, it will be necessary to develop model association colloid systems that reflect the types of physical structures that would be expected to exist in refined vegetable oils. Therefore, in this study, a model system consisting of different concentrations and types of phospholipids [1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC₄PC), Figure 3.1] and water was used to characterize association colloids in stripped soybean oil and to evaluate their impact on lipid oxidation.

![Figure 3.1](image-url) Molecular structures of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC₄PC).

**3.2 Materials and Methods**
3.2.1 Materials

Avanti Polar Lipids, Inc (Alabaster, AL) was the source of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC4PC). N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE) was acquired from Invitrogen (Carlsbad, CA). Soybean oil was purchased from a local store and stored at 4 °C. Silicic acid (100-200 mesh), activated charcoal (100-400 mesh), 7,7,8,8-TCNQ (7,7,8,8-tetracyano-quinodimethane) and hexane were purchased from Sigma-Aldrich Co. (St. Louis, MO). Medium chain triacylglycerols (MCT, Miglyol®) were purchased from Sasol North America Inc (Houston, TX). All other reagents were of HPLC grade or purer. Distilled and deionized water was used in all experiments.

3.2.2 Preparation of stripped soybean oil

Stripped soybean oil was prepared according to the method of Boon et al (157). In short, silicic acid (100 g) was washed three times with a total of 3 L of distilled water and activated at 110 °C for 20 h. Activated silicic acid (22.5 g) and activated charcoal (5.625 g) were suspended in 100 and 70 mL of n-hexane, respectively. A chromatographic column (3.0 cm internal diameter × 35 cm height) was then packed sequentially with 22.5 g of silicic acid followed by 5.625 g of activated charcoal and then another 22.5 g of silicic acid. Thirty grams of soybean oil was dissolved in 30 mL of hexane, which was then loaded onto the column and eluted with 270 mL of n-hexane. To retard lipid oxidation during stripping, the collected soybean oil was held on ice and covered with aluminum foil to eliminate the impact of light. The solvent in the stripped oil was
removed with a vacuum rotary evaporator (RE 111 Buchi, Flawil, Switzerland) at 37 °C, and the remaining trace solvent was removed by flushing with nitrogen for at least one hour. The colorless oil was kept at -80 °C for subsequent studies. Removal of minor components was verified by spotting a sample on a silica gel G thin layer chromatography (TLC) plate and developed in a tank with hexane/diethyl ether/acetic acid (70:30:1 v/v/v) (158). Water content of the stripped oils was determined by Karl Fisher (159).

3.2.3 Light Scattering Properties of Oil Samples

A series of samples were prepared to determine the DOPC and water concentrations that produced association colloids without causing cloudiness in the oil. A stock solution of DOPC was prepared at a concentration of 127 μmoles DOPC/mL chloroform, which was blended into MCT over the concentration range of 0 to 1270 μmole/kg lipid which is in the range of phospholipid concentrations found in refined bulk oils (~ 2000 μmoles/kg lipid). The mixture of MCT and DOPC was then titrated with water. The initial water concentration in the samples was 0.67 mmoles/kg lipid (determined by Karl Fisher analysis) and was increased to 560 mmoles/kg lipid by adding water. The vials were tightly closed with Teflon-lined caps and placed on a stirrer motor at 25 °C for 24 hours before measurements. The final water concentrations were verified by Karl Fisher analysis. The clarity of the samples was measured by light scattering using an Agilent 7010 particle size spectrophotometer. The Agilent 7010 particle size spectrophotometer measures the attenuation of a colloidal dispersion from 350-800 nm. The transparent region was defined as samples with a measured attenuation ≤ 0.2 cm⁻¹ (visibly clear),
while the cloudy region was defined as samples with attenuation > 0.2 cm$^{-1}$.

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

The procedure described by Kanamoto and coworkers (160) was followed to spectrophotometrically determine the CMC of the phospholipids in stripped oil. In short, varying concentrations of DOPC or DCA$_4$PC were dissolved in either SSO or MCT by mixing for 12 h followed by addition of 7,7,8,8-tetracyano-quinodimethane (TCNQ, 5mg) to 5 g of oil/DOPC in a small conical flask and the mixture was agitated using a magnetic stirrer for 5 h at room temperature. After sedimentation of excess TCNQ by centrifugation at 2000 g for 20 min, absorbance measurements at 480 nm were recorded (Shimazu 2014, Tokyo, Japan). SSO oil or MCT without DOPC was used as a control. The CMC was taken as the intersection point of straight lines extrapolated from low and high DOPC concentrations in the curve generated from absorbance and DOPC concentrations on a semi-log plot.

The CMC of DOPC in SSO was also determined by interfacial tension (IFT) measurements using Drop Shape Analysis (DSA100, Krüss GmbH, Hamburg, Germany) equipped with a pendant drop module. A needle with a diameter of $1.830 \times 10^{-3}$ m was used to create a pendent drop (containing the stripped soybean oil and varying concentrations of DOPC). The pendant drop was extruded from the syringe into a quartz cell containing distilled water. Droplet images were taken every 2 s and the drop shape analysis program supplied by the instrument manufacturer (based on the Young-Laplace equation) was used to determine IFT values.
3.2.5 Small angle X-ray scattering (SAXS)

SAXS measurements were performed on the oil samples containing varying concentrations of phospholipids and water using a Rigaku Molecular Metrology SAXS instrument (Rigaku, Inc) at the W.M. Keck Nanostructures Laboratory at the University of Massachusetts-Amherst. The instrument generates X-rays with a wavelength of 1.54 Å and utilizes a 2-D multi-wire detector with a sample-to-detector distance of 1.5 m. Samples were inserted into the 1 mm outer diameter quartz capillary (Hampton Research, Aliso Viejo, CA, USA) which were then sealed by Duco® Cement and then enclosed in an airtight sample holder. This assembly was then put in the X-ray beam path, and data were collected on the samples for 3 hours.

The experimental SAXS intensity curves were corrected for background, sample absorption and detector homogeneity, using MicroCal Origin 5.0™ software. The scattering vector amplitude $q$ is defined by equation (1), where $\theta$ is half of the scattering angle and $\lambda$ is the wavelength:

$$ q = 4\pi \frac{\sin \theta}{\lambda} \quad (1) $$

Fittings for the experimental SAXS curves were obtained using the GNOM program (version 4.5, ATSAS, German) (161). For a set of monodisperse spherical particles randomly distributed, the scattering intensity is given by the following equation (162):

$$ I(q) = \gamma n_p (\Delta \rho)^2 V^2 P(q) S(q) \quad (2) $$

where $\gamma$ is a factor related to the instrumental effects; $n_p$ corresponds to the number of scattering species; $\Delta \rho$ is the electron density contrast between the scattering species.
and the medium; \( V \) is the scattering species volume; \( P \) is the normalized particle form factor \((P(0)=1)\); and \( S \) is the structure factor of the particle system, its value being \( \approx 1 \) for non-correlated systems. In this particular case, the intensity function \( I \) depends solely on the particle form factor and according to theory, the pair-distance distribution function \( p(r) \), can be obtained by Fourier inversion of the intensity function \( I(\mathbf{q}) \): 

\[
p(r) = \frac{1}{2\pi^2} \int_0^\infty I(q) \sin(qr) dq
\]  

(3)

This function provides information about the shape of the scattering particles as well as their maximum dimension, \( D_{\text{max}} \) accounted for by the \( r \) (pair-distance) value where \( p(r) \) goes to zero.

### 3.2.6 Front-Face (FF) Fluorimetric Measurements

Front-face fluorescence with surface active fluorescent probes was used to verify the ability of DOPC and water to form association colloids. The surface active fluorescent probe used was NBD-PE which is a phospholipid analog with a fluorescent functional groups covalently attached to the choline head group. Steady-state emission measurements were recorded with a PTI Spectrofluorimeter (PTI, Ontario, Canada) with the sample holder held at 22 °C. To minimize any reflection of the excitation beam by the cell window and by the underlying liquid surface of the sample into the emission monochromator, samples are stored in triangular suprasil cuvette. The emission was observed at 90° to the incident beam, i.e., 22.5° with respect to the illuminated cell surface. A 2.0 nm spectral bandwidth was used for both excitation and emission slits were employed for the NBD-PE excitation at 468 nm. The integration time was 1 s, and the wavelength increment during emission spectrum scanning was 1 nm. The intensity of the
spectra was determined as the emission signal intensity (counts per second) measured by means of a photomultiplier. In order to avoid self quenching of probes, the concentrations of NBD-PE was 9.5 μM (1/100 of DOPC) (164).

3.2.7 Measurement of oxidation parameters

Lipid hydroperoxides were measured as the primary oxidation product using a method adapted from Shanta and Decker (165). Accurately weighed oil samples were added to a mixture of methanol/butanol (2.8 mL, 2:1, v:v) followed by addition of 15 μL of 3.94 M thiocyanate and 15 μL of 0.072 M Fe²⁺. The solution was vortexed, and after 20 min the absorbance was measured at 510 nm using a Genesys 20 spectrophotometer (ThermoSpectronic, Waltham, MA). The concentration of hydroperoxides was calculated from a cumene hydroperoxide standard curve.

Secondary oxidation products were monitored using a GC-17A Shimadzu gas chromatograph equipped with an AOC-5000 autosampler (Shimadzu, Kyoto, Japan) (147). Sample (1 mL) in 10 mL glass vials capped with aluminum caps with PTFE/silicone septa were pre-heated at 55 °C for 15 min in an autosampler heating block. A 50/30 μmDVB/ Carboxen/PDMS solid-phase microextraction (SPME) fiber needle from Supelco (Bellefonte, PA, USA) was injected into the vial for 2 min to absorb volatiles and then was transferred to the injector port (250 °C) for 3 min. The injection port was operated in split mode, and the split ratio was set at 1:5. Volatiles were separated on a Supleco 30 m × 0.32 mm Equity DB-1 column with a 1 μm film thickness at 65 °C for 10 min. The carrier gas was helium at 15.0 mL/min. A flame ionization detector was used at a temperature of 250 °C. Propanal concentrations were determined from peak
areas using a standard curve prepared from an authentic standard.

3.2.8 Statistical Analysis

Duplicate experiments were performed for all studies. All data shown represents mean values ± standard deviations (n = 3). Statistical analysis of lipid oxidation kinetics was performed using a one-way analysis of variance. A significance level of p<0.05 between groups was accepted as being statistically difference. In all cases, comparisons of the means of the individual groups were performed using Duncan’s multiple range tests. All calculations were performed using SPSS17 (http://www.spss.com; SPSS Inc., Chicago, IL).

3.3 Results and discussion

3.3.1 Attenuation of the water/phospholipid/MCT system

Commercial refined vegetable oils are optically clear and yet potentially contain physical structures such as association colloids. This is possible because many association colloids have dimensions below 100 nm and thus they do not strongly scatter visible light (166). The objective of this initial study was to define the phospholipid/water/lipid concentrations that produce optically transparent oils, corresponding to commercial refined oils. To do this, a partial phase diagram of the three component system water/phospholipid/MCT was constructed to determine the phospholipid and water concentrations that did not form structures that scattered light strongly as determined by light attenuation measurements (Figure 3.2) using a method previously reported for phospholipids and water in olive oil (167). In the presence of
1.25, 2.5, and 5 mg DOPC/g MCT, a large increase in attenuation was observed at a water concentration $\geq 20$ mg/g MCT. At 20 mg/g MCT, the attenuation of the sample containing the highest concentration of DOPC (5 mg DOPC/g MCT) had the lowest attenuation. This is likely due to the formation of a larger number of small non-light scattering association colloids in the presence of high levels of DOPC. In the remainder of the experiments we focused on those phospholipid/water/lipid concentrations that gave optically transparent systems.

![Figure 3.2.](image)

**Figure 3.2.** Attenuation of medium chain triacylglycerols (MCT) samples containing 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and varying concentrations of water.

### 3.3.2 Determination of the CMC of phospholipids in SSO and MCT

Phospholipids can self-assemble into a variety of structures in water or oil phases because of their amphiphilic nature (168). The nature of the structures formed depends on the phospholipid’s chemical structure and solvent type (169). In non-aqueous media, such as oil, phospholipids normally form reverse micelles when their concentration just exceeds the CMC (critical micelle concentration), with the polar head groups pointing
into the hydrophilic core of the reverse micelle and the non-polar tails pointing towards the hydrophobic oil. Therefore, in the region of the phase diagram where the system is clear (Figure 3.2), it is important to determine whether the DOPC concentration is above or below its CMC so as to determine if any association colloid structures form. SSO can rapidly oxidize resulting in the production of additional surface active oxidation products which could alter the CMC. Therefore, the CMC of DOPC was measured in both SSO and MCT to determine if SSO was a suitable solvent for analysis of association colloids.

One of the most important properties of reverse micelles is their ability to increase the solubility of molecules. 7,7,8,8-TCNQ is not lipid soluble and does not absorb light at 480 nm when dispersed in non-polar solvents. 7,7,8,8-TCNQ can undergo charge-transfer (170) interactions with surfactants when the surfactant concentration is above the CMC, which can be observed by absorbance at 480 nm (160). The absorbance values of 7,7,8,8-TCNQ solubilized in MCT were plotted as a logarithmic function of phospholipid concentration (Figure 3.3 A). When the concentration of DOPC was increased, the absorbance of TCNQ initially remained low, but there was a rapid increase in absorbance at DOPC concentrations above 500 \( \mu \text{M} \). Based on the intercept of these two linear regions, the CMC of DOPC was estimated to be \( \sim 550 \mu \text{M} \) in MCT. This experiment was repeated in SSO where the CMC of DOPC was found to be \( \sim 650 \mu \text{M} \) (Figure 3.3 B). Differences of the CMC in MCT and SSO could be due to differences in fatty acid chain length and the presence of other minor surface active components in the oils that were not completely removed by stripping (171). Since the CMC values for MCT and SSO were fairly similar, SSO was used for all subsequent experiments. The CMC of DC\(_4\)PC in the SSO could not be determined by this method because DC\(_4\)PC did not dramatically change
the absorbance of the 7,7,8,8-TCNQ before the DC₄PC reached its solubility limit in the SSO.

Figure 3.3. Determination of critical micelle concentration (CMC) of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) in medium chain triacylglycerols (A) and stripped soybean oil (B) as determined by the absorbance (480 nm) of 7,7,8,8-tetracyanoquinodimethane.

The CMC of DOPC in SSO was also determined by interfacial tension
measurements. At low DOPC concentrations, the interfacial tension decreased with increasing DOPC concentration, but at higher DOPC concentrations the interfacial tension reached a fairly constant value indicating that reverse micelles had been formed. A semi-logarithmic plot between interfacial tension and the concentration of DOPC in SSO is shown in Figure 3.4. Based on the intercept of the two linear regions a CMC of ~950 \(\mu\text{M}\) was calculated. The CMC value obtained by interfacial tension measurements is about 300 \(\mu\text{M}\) higher than that obtained with the spectrophotometric technique, which could be due to some of the DOPC partitioning into the aqueous phase during the interfacial tension measurements. Alternatively, the spectrophotometric and interfacial tension methods may have given different CMC values because they are based on different physical principles. For the purposes of this study, we considered the CMC of DOPC in SSO to be in the range of 650-950 \(\mu\text{M}\). The normal concentration of phospholipids in refined vegetable oil is higher than 950 \(\mu\text{M}\), suggesting that commercial oils could have association colloids formed by phospholipids.
**Figure 3.4.** Determination of critical micelle concentration of 1,2-dioleoyl-sn-glycero-3-phosphocholine in stripped soybean oil using interfacial tension.

### 3.3.3 Detection and characterization of phospholipids physical structures by small angle x-ray scattering

As mentioned above, the specific structures formed by phospholipids in non-aqueous solution depend on both concentration and phospholipid type. In consideration of the complicated compositions of phospholipids in refined bulk oil, we first determined how two types of phospholipids impacted association colloid structure in ternary systems containing SSO, water, and either DOPC or DC₄PC using. **Figure 3.5** shows the SAXS profiles in the SSO systems containing ~60 ppm water (i.e., intrinsic water content after oil stripping) and either 950 μM of DOPC or DC₄PC. These phospholipid concentrations were chosen as these samples did not strongly scatter light (**Figure 3.2**) but were equal to or above the CMC of DOPC (**Figure 3.3 and 3.4**). As shown in **Figure 3.5**, the two different types of phospholipids induced changes of the minor structures in stripped oil in such a way that the scattering profiles are distinct and displaced towards different $q$ values and intensity. The Bragg space (d-spacing) and the intensity of the samples with DOPC was similar to blank stripped oil, being 141 Å and 3 (a.u.) respectively. However, the structures with DC₄PC are quite different to the blank and the one with DOPC. Its Bragg peak shifts towards a higher $q$ value with Bragg space being 68 Å while the intensity rises to 12 (a.u.). In addition, the structures with different types of phospholipids are verified by pair distribution $p(r)$ function analysis (**Figure 3.6**). The $p(r)$ of DC₄PC had a long tail at high $r$, which is typical of a cylindrical structure. The $r$ value at the
inflection (~20 nm) usually represents the diameter of the cylinder (172). The $D(r)$ of DOPC had a symmetrical shape which represents a spherical structure, presumably a reverse micelle. For the bulk stripped soybean oil, there was only a flat line indicating the presence of no structures.

Figure 3.5. Small angle x-ray scattering profiles of stripped soybean oil (SSO) and SSO + 950 μM 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC₄PC).
Figure 3.6. The pair distribution functions, \( p(r) \), calculated from the scattering profiles of stripped soybean oil (SSO) and SSO + 950 \( \mu \)M 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or 1,2-dibutyril-sn-glycero-3-phosphocholine (DC\textsubscript{4}PC) using GNOM analysis of the three small angle x-ray scattering profiles indicated in Figure 3.5.

In the stripped oil, almost all the surface active minor components and the majority of water had been removed by the silicic acid and activated charcoal sandwich chromatography which is consistent to our, Karl Fisher, TLC and previous HPLC results (173). Therefore it is reasonable that the bulk stripped oil did not contain any association colloid structures (174). The structures formed by DC\textsubscript{4}PC are quite different to those formed by DOPC. DOPC and DC\textsubscript{4}PC have the same phosphocholine hydrophilic head group (Figure 3.1), but DOCP possesses \textit{cis}-oleic fatty acids on \textit{sn}-1 and \textit{sn}-2 of glycerol side chains, whereas DC\textsubscript{4}PC has 2 butyl fatty acids. As indicated, amphiphilic molecules, like phospholipids, having a large tail area and a small head group area, can self-assemble into reverse spherical micelles in highly non polar liquids due to a critical packing parameter (CPP) values greater than 1 (175). There are no reported CPP values of DC\textsubscript{4}PC. However, based on the critical packing parameter equation (176):
where \( n \) is the number of carbons in the hydrophobic chain, the CPP of DC_4PC in SSO should be lower than that of DOPC, approximately less than ½ assuming no difference of their effective area of the head group. This is because the CPP is mainly dependent on the hydrocarbon tail length. A low CPP often corresponds to the formation of cylindrical structures.

### 3.3.4 Effect of water/phospholipid molar ratio on the physical properties of DOPC association colloids

Experimental and theoretical approaches have shown that the key structural parameter of reverse micelles is the water/surfactant molar ratio \((W_o)\) \((177, 178)\). On one hand, water will serve to bridge the phosphate head groups between neighboring phospholipids through hydrogen bonds \((179)\). On the other hand, the water content will determine structure size as well as the amount of the water that is strongly associated with the phospholipid head groups \((164)\). Oil-rich phospholipid solutions with micellar morphology can change to lamellar and hexagonal structures as the \(W_o\) is raised in the system \((24)\). In order to characterize the physical structure of stripped soybean oil with DOPC (950 \(\mu\)M) as a function of \(W_o\), a combination of SAXS and Front-Face Fluorimetry were used.
Figure 3.7. Small angle x-ray scattering profiles of stripped soybean oil with different water/1,2-dioleoyl-sn-glycero-3-phosphocholine molar ratios (W_o). Inserted figures are the 2 dimensional small angle x-ray scattering pattern of each sample.

At high $W_o$, water is solubilized into phospholipid reverse micelles and structures such as cylindrical micelles and spherical swollen micelles can form depending upon the amount of water in the system \((176)\). In our system there is a clear Bragg peak on all the SAXS profiles, with $d$-space around 141 nm (Figure 3.7). The SAXS intensity is very
weak (i.e., 3 a.u.) for the system with $W_o = 0$ (no added water), suggesting that DOPC alone forms very little structure. When the $W_o$ increased from 0 to 35 and 47, the scattering intensity increased from 3.0 to 8.0, and 13.0, respectively, presumably due to the formation of association colloids by DOPC. Increasing $W_o$ above 100 results in the 2-D X-ray pattern of the samples becoming aligned, indicating a transformation from an isotropic to an anisotropic system. At the same time, the SAXS intensity increased greatly to 749.6 a.u. In all samples, no other Bragg peaks were observed (Figure 3.7), indicating the absence of cylindrical micelles and spherical swollen micelles. Lack of transformation form reverse micelles to cylindrical micelles and spherical swollen micelles was also reported in a phospholipid in hexane and soybean oil system (180). The fact that the reverse micelles did not transform to these other colloidal structures in our system could be due to: (1) the increase in $W_o$ was too small in our experimental design so that the transformation did not occur; or, (2) the concentration of the DOPC was too low to allow transformation. While the anisotropic transition at $W_o$ values greater than 100 indicates that the structures were changing symmetry. This could be due to a stretching of the symmetrical structure into more of an oblong structure such as shown in Figure 3.8.
Figure 3.8. A postulated structural change of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as phospholipid concentrations are increased to above the critical micelle concentrations (CMC) form a reverse micelle.

Fluorescence intensity of selected probes can also be used to provide information about the micro-environment of association colloids as a function of $W_0$. In this study, we used NBD-PE as a fluorescence probe and Front Face Fluorometry to detect changes in its spectra. This probe is a phospholipid analog grafted with a fluorophore on the phosphate head groups (Figure 3.9 insert) that has previously been used as an indicator of changes in the interfacial properties of reverse micelle systems (164). Theoretically, NBD-PE possesses similar properties as DOPC as both are amphiphilic molecules with the ability to participate in the formation of association colloids and reside at the water-oil interface (164). The probe is useful because its fluorescence decreases when it is exposed to water. Figure 3.9 shows the fluorescence intensity of NBD-PE decreased with increasing water concentrations suggesting that the exposure of the probe to water increased. The exposure of the probe to the water phase can also be confirmed by a change in the wavelength of maximum fluorescence intensity of NBD-PE with a red shift.
occurring when the probe is exposed to a more polar environment \((164)\). A red shift of 4 nm was observed for NBD-PE in the DOPC/stripped soybean system when the \(W_o\) was increased from 11.7 to 46.8.

**Figure 3.9.** The fluorescence intensity of N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE) in stripped soybean oil with different water/1,2-dioleoyl-sn-glycero-3-phosphocholine molar ratios \((W_0)\). Inserted figure is the molecular structure of (NBD-PE).

### 3.3.5 Oxidation of stripped soybean oil containing association colloids

Studies of lipid oxidation in bulk oils have been carried out for several decades, but many of the mechanisms involved in this reaction are still unclear, including whether physical structures in the oil can impact oxidation kinetics. The ultimate purpose of these experiments was to produce soybean oil, water, and phospholipid systems with different physical structures and determine if these physical structures impact lipid oxidation kinetics. Therefore, the lipid oxidation kinetics of stripped soybean oil containing 1000 \(\mu\)M of either DOPC or DC_{4}PC and \(~200\ ppm\) water was studied as determined by lipid hydroperoxides and propanal during storage at 37 °C (**Figure 3.10**). In the presence of
DOPC, the lag phase of lipid hydroperoxides (Figure 3.10A) and headspace propanal (Figure 3.10B) were 8 and 13 days, respectively, compared to 13 and 17 days, respectively, for the no phospholipid control. DC₄PC had the same lag phase times for lipid hydroperoxides and headspace propanal as the control (Figure 3.10A and B).

Figure 3.10. Formation of lipid hydroperoxides (A), propanal (B) in stripped soybean oil containing 1000 μM of 1,2-dioleoyl-sn-glycero-3-phosphocholine(DOPC) or 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC₄PC) and 200 ppm water at 37 °C. Data represent means (n =3) ± standard deviations. Some error bars are within data points.
Phospholipids have previously been reported to have protective effects in bulk oils. The mechanisms for these protective effects have been postulated to be due to the phospholipid’s ability to chelate metals, decompose lipid hydroperoxides, directly scavenge free radicals, and increase the antioxidant activity of tocopherols (181). In this study, DOPC and DC₄PC were chosen since they both have identical choline head groups so any impact of the phospholipids on alteration in lipid oxidation kinetics by chemical pathways would be similar. Therefore, the major differences in the oil systems containing DOPC and DC₄PC would be in how these phospholipids produced different physical structures. As discussed above, 7,7,8,8-TCNQ was an effective tool to monitor the formation of structures by DOPC but this probe did not detect structures formed by DC₄PC. Using small angle x-ray scattering, structures could be seen for both DOPC and DC₄PC but these structures were very different with DOPC forming spherical and DC₄PC forming cylindrical structures (Figure 3.6). Therefore these results suggested that the spherical structures formed by DOPC were prooxidative while the cylindrical structures formed by DC₄PC had no impact on oxidation rates. These results suggest that phospholipids have the ability to form colloidal structures in vegetable oils and that these structures could have an impact on the oxidative stability of food oils.
CHAPTER 4
ROLE OF REVERSE MICELLES ON LIPID OXIDATION IN BULK OILS:
IMPACT OF PHOSPHOLIPIDS ON ANTIOXIDANT ACTIVITY OF
α-TOCOPHEROL AND TROLOX

4.1 Introduction

Lipid oxidation is one of the main factors limiting the shelf life of bulk oils, since it adversely affects flavor and quality, and potentially produces toxic reaction products (182). Preventing or inhibiting the oxidation of bulk oils is therefore of great importance to consumers and the food industry. A variety of mechanisms have been proposed to be responsible for the oxidation of bulk oils during processing and storage, with photosensitized oxidation, metal-promoted and autoxidation being the most well-known. Some factors that impact the oxidative stability bulk oils include: oil extraction and processing conditions; light exposure; temperature; fatty-acid composition; antioxidant composition; oxygen levels; and the presence of minor components (183). Manipulation of these factors can be used to retard lipid oxidation in edible oils.

One of the most effective ways of inhibiting lipid oxidation in bulk oils is to incorporate antioxidants (184). Depending on their mechanism of action, antioxidants can be classified as either “primary” or “secondary” antioxidants (185). Tocopherols are the most common primary antioxidant present in many vegetable oils, which may originate naturally from the extracted oil itself or may be manually added after oil refining (186). However, tocopherols may not be the most effective antioxidants in bulk oil systems.
Indeed, research has shown that hydrophilic antioxidants (Trolox or ascorbic acid) possess better antioxidant activity than their hydrophobic analogues (tocopherol and ascorbyl palmitate) in some bulk oil systems (156). The greater tendency for hydrophilic antioxidants to accumulate at the air-water interface where oxidation may be expected to begin is one of the mechanisms proposed to account for their better antioxidant activity in bulk oils (156). However, other mechanisms have also been proposed to account for the ability of hydrophilic antioxidants to act as better antioxidants than their hydrophobic analogs in bulk oils since air is more hydrophobic than oil and thus there is no driving force to concentrate hydrophilic antioxidants at the oil-air interface (181, 185). Studies have shown that the addition of phospholipids to bulk oils increased the antioxidant activity of tocopherol (181). It was postulated that the phospholipids formed microstructures, known as association colloids, within the bulk oil, which caused the tocopherol molecules to accumulate in the phospholipid microstructures where lipid oxidation primarily occurred. Other researchers have also demonstrated the ability of phospholipids to act as antioxidants in various kinds of bulk oils (90, 187). Several mechanisms were proposed to account for the antioxidant activity of the phospholipids, including their ability to chelate metals, decompose lipid hydroperoxides, and scavenge free radicals. Nevertheless, there is still a poor understanding of the importance and contribution of the combination of phospholipids and tocopherols to the oxidative stability of bulk oils.

In the previous chapter, we characterized the formation of reverse micelles in bulk oils, and their impact on lipid oxidation (188). These reverse micelles were formed by adding phospholipid (1,2-dioleoyl-sn-glycero-3-phosphocholine, DOPC) into stripped
soybean oil (SSO) containing water levels similar to that found in commercial refined oils. Using small angle x-ray scattering, this study showed that the combination of DOPC and water resulted in the formation of reverse micelles in bulk oil. Alternately, when phosphatidylcholine with short chain fatty acyl residues (1,2-dibutyryl-sn-glycero-3-phosphocholine, DC₄PC) was added to the same system, no reverse micelles were formed. The lipid oxidation chemistry of these two systems was different with DOPC reverse micelles demonstrating a prooxidative effect while DC₄PC had no effect on oxidation rates. This study suggests that the combination of phospholipids and water can form physical structures in bulk oils and these structures can impact lipid oxidation chemistry. In the present study, we examined if reverse micelles in bulk oil produced by phosphatidylcholine (i.e., DOPC) were able to impact the activity of free radical scavenging antioxidants. The antioxidants tested included alpha-tocopherol (non-polar) and Trolox (polar), which are chemical analogs. In addition, the system had equal molar concentrations of phospholipids with one system containing reverse micelles (DOPC) and the other containing no measurable reverse micelles (DC₄PC). We also measured how the tocopherol analogs impacted the structure of the reverse micelles with the aim of better understanding the location and properties of antioxidants in bulk oils. By better understanding how physical structure in bulk oils impact the activity of antioxidants such as tocopherols, it might be possible to design systems to improve the activity of these important “natural” antioxidants.

4.2 Materials and methods

4.2.1 Materials
1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC₄PC) were acquired from Avanti Polar Lipids, Inc (Alabaster, AL). N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE, Cat.No. N-360) was acquired from Invitrogen. Soybean oil was purchased from a local store and stored at 4 °C. Silicic acid, activated charcoal, hexane, α-tocopherol, Trolox were purchased from Sigma-Aldrich Co. (St. Louis, MO). All other reagents were of HPLC grade or purer distilled and deionized water was used as needed.

4.2.2 Preparation of stripped soybean oil

Stripped soybean oil with DOPC reverse micelles were prepared according to the method of Chen et al (157). Briefly, formation of reverse micelles was accomplished by pipetting DOPC (1000 μM, final concentration) in chloroform into an empty beaker and then flushing with nitrogen until the chloroform was evaporated. The appropriate amount of medium chain triacylglycerols (MCT) or stripped soybean oil (SSO) was then added followed by double distilled water at a final concentration of 200 ppm. The oil samples were then stirred in a beaker at 1000 rpm in a 20 °C incubator room for a 24 h.

4.2.3 Formation of DOPC reverse micelles in stripped soybean oil

The formation of DOPC reverse micelles was done using our previous procedure (188). Briefly, DOPC (1000 μM) in chloroform was pipetted into an empty beaker and then chloroform was removed by flushing with nitrogen. The appropriate amount of stripped soybean oil was then added followed by double distilled water to a final amount
of ~ 200 ppm. The sample in beaker was magnetically stirred at the speed of 1000 rpm in a 20 °C incubator room for a 24 hrs. Antioxidants were dissolved in ethanol, mixed with stripped soybean oil and stirred for at least 12 hrs to obtain the homogenous samples. In the lipid oxidation studies, a mixture of 75% of medium chain TAG and 25% SSO were used due to the high amount of samples needed. Samples for the oxidation studies were aliquoted into GC vials (1 mL/vial) stored at 55 °C in the dark. For fluorescence, the NBD-PE probe was mixed into stripped soybean oil at the same time as DOPC and water.

### 4.2.4 Measurement of oxidation parameters

Lipid hydroperoxides were measured as the primary oxidation product using a method adapted from Shanta and Decker (165). Accurately weighed oil samples were added to a mixture of methanol/butanol (2.8 mL, 2:1, v:v) followed by addition of 15 μL of 3.94 M thiocyanate and 15 μL of 0.072 M Fe^{2+}. The solution was vortexed, and after 20 min the absorbance was measured at 510 nm using a Genesys 20 spectrophotometer (ThermoSpectronic, Waltham, MA). The concentration of hydroperoxides was calculated from a cumene hydroperoxide standard curve.

Secondary oxidation products were monitored using a GC-17A Shimadzu gas chromatograph equipped with an AOC-5000 autosampler (Shimadzu, Kyoto, Japan) (147). Sample (1 mL) in 10 mL glass vials capped with aluminum caps with PTFE/silicone septa were pre-heated at 55 °C for 15 min in an autosampler heating block. A 50/30 μmDVB/ Carboxen/PDMS solid-phase microextraction (SPME) fiber needle from Supelco (Bellefonte, PA, USA) was injected into the vial for 2 min to absorb volatiles and then was transferred to the injector port (250 °C) for 3 min. The injection
port was operated in split mode, and the split ratio was set at 1:5. Volatiles were separated on a Supleco 30 m × 0.32 mm Equity DB-1 column with a 1 μm film thickness at 65 °C for 10 min. The carrier gas was helium at 15.0 mL/min. A flame ionization detector was used at a temperature of 250 °C. Propanal concentrations were determined from peak areas using a standard curve prepared from an authentic standard.

4.2.5 Small angle x-ray scattering (SAXS) study of bulk oil with DOPC and antioxidants

SAXS measurements were performed on the oil samples containing varying concentrations of phospholipids and water using a Rigaku Molecular Metrology SAXS instrument (Rigaku, Inc) at the W.M. Keck Nanostructures Laboratory at the University of Massachusetts-Amherst. The instrument generates X-rays with a wavelength of 1.54 Å and utilizes a 2-D multi-wire detector with a sample-to-detector distance of 1.5 m. Samples were inserted into the 1 mm outer diameter quartz capillary (Hampton Research, Aliso Viejo, CA, USA) which were then sealed by Duco® Cement and then enclosed in an airtight sample holder. This assembly was then put in the X-ray beam path, and data were collected on the samples for 3 hours.

The experimental SAXS intensity curves were corrected for background, sample absorption and detector homogeneity, using MicroCal Origin 5.0™ software. The scattering vector amplitude \( q \) is defined by equation (1), where \( \theta \) is half of the scattering angle and \( \lambda \) is the wavelength:

\[
q = 4\pi \frac{\sin\theta}{\lambda} \quad (1)
\]
Fittings for the experimental SAXS curves were obtained using the GNOM program (version 4.5, ATSAS, German) \((161)\). For a set of monodisperse spherical particles randomly distributed, the scattering intensity is given by the following equation \((162)\):

\[
I(q) = \gamma n_p (\Delta \rho)^2 V^2 P(q) S(q)
\]  

(2)

where \(\gamma\) is a factor related to the instrumental effects; \(n_p\) corresponds to the number of scattering species; \(\Delta \rho\) is the electron density contrast between the scattering species and the medium; \(V\) is the scattering species volume; \(P\) is the normalized particle form factor \((P(0) = 1)\); and \(S\) is the structure factor of the particle system, its value being \(\approx 1\) for non-correlated systems. In this particular case, the intensity function \(I\) depends solely on the particle form factor and according to theory, the pair-distance distribution function \(p(r)\), can be obtained by Fourier inversion of the intensity function \((163)\):

\[
p(r) = \frac{1}{2\pi^2} \int_0^\infty I(q) q^2 \sin(qr) dq
\]

(3)

This function provides information about the shape of the scattering particles as well as their maximum dimension, \(D_{\text{max}}\) accounted for by the \(r\) (pair-distance) value where \(p(r)\) goes to zero.

4.2.6 Fluorescence measurement of bulk oil with DOPC and antioxidants

Front-face fluorescence with surface active fluorescent probes was used to verify the ability of DOPC and water to form association colloids. The surface active fluorescent probe used was NBD-PE which is a phospholipid analog with a fluorescent functional groups covalently attached to the choline head group. Steady-state emission measurements were recorded with a PTI Spectrofluorimeter (PTI, Ontario, Canada) with
the sample holder held at 22 °C. To minimize any reflection of the excitation beam by the
cell window and by the underlying liquid surface of the sample into the emission
monochromator, samples are stored in triangular suprasil cuvette. The emission was
observed at 90° to the incident beam, i.e., 22.5° with respect to the illuminated cell
surface. A 2.0 nm spectral bandwidth was used for both excitation and emission slits were
employed for the NBD-PE excitation at 468 nm. The integration time was 1 s, and the
wavelength increment during emission spectrum scanning was 1 nm. The intensity of the
spectra was determined as the emission signal intensity (counts per second) measured by
means of a photomultiplier. In order to avoid self quenching of probes, the concentrations
of NBD-PE was 9.5 μM (1/100 of DOPC) (164).

The fluorescence decay of NBD-PE in the systems was carried out at 22 °C using a
PTI Laserstrobe fluorescence lifetime instrument with a PTI GL-3300 nitrogen laser and
a GL-302 tunable dye laser with C-500 laser dye, exciting the oil samples at 500 nm.
Each data point on a lifetime decay curve represents five laser flashes, and each decays
represents 100 of these data points spaced out over the collection time interval.

Data were analyzed using the commercial PTI software. Fluorescence decays were
fitted to sums of two and three exponentials, and the average lifetime was calculated.

4.2.7 Statistical analysis

Duplicate experiments have been performed with freshly prepared samples. All data
shown represents the mean values ± standard deviation of triplicate measurements.
Statistical analysis of lipid oxidation kinetics was performed using a one-way analysis of
variance. A significance level of p<0.05 between groups was accepted as being
statistically difference. In all cases, comparisons of the means of the individual groups were performed using Duncan’s multiple range tests. All calculations were performed using SPSS17 (http://www.spss.com; SPSS Inc., Chicago, IL).

4.3 Results

4.3.1 Impact of phospholipids on oxidative stability of SSO

Commercial soybean oil is a highly complex liquid system containing a number of different components that could potentially alter the rate and extent of lipid oxidation such as tocopherols, chlorophyll, phospholipids, fatty acids, and water (185). Consequently, stripped soybean oil (SSO) was used as a model oil in this study to reduce any effects associated with these minor components. Previous research using small angle x-ray diffraction showed that the combination of DOPC and water resulted in the formation of reverse micelles in bulk oil while DC₄PC did not form structures (10). Initially, we examined the influence of phospholipid type (DOPC and DC₄PC at 1000 μM) on the formation of lipid hydroperoxides and headspace hexanal in SSO during storage at 55 °C (Figure 4.1A and B). In the absence of added phospholipids, the lag phase for both lipid hydroperoxides and headspace hexanal lasted 2 days. Addition of DC₄PC had little impact on the lipid oxidation profile, but the addition of DOPC reduced the lag-period by 1 day, confirming that it acted as a prooxidant.
Figure 4.1. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil containing 1000 μM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or 1,2-dibutryrl-sn-glycero-3-phosphocholine (DC₄PC) during storage at 55 °C. Some of the error bars are within data points.

Previous studies of the oxidative stability of bulk oils containing phospholipids suggest that phosphatidylethanolamine (PE) improves their oxidative stability (189, 190). On the other hand, studies on the impact of phosphatidylcholine (PC) have indicated seemingly contradictory results. Prooxidant, antioxidant, and no effects of PC in bulk oil...
oxidation have been reported in the literature (191, 192). In agreement with our study, a prooxidant activity of DOPC was reported by Takenaka, et al (192) in bonito oil, which was attributed to the unsaturated hydrocarbon chain of the PC used. In our study we used phosphatidylcholine with oleic acid. DOPC was chosen because of its ease of handling and dispersion into bulk oil due to its low melting point and due to its high oxidative stability. In general, the oxidative stability of oleic acid is 10-20 times greater than the linolenic and linolenic acids found in SSO and thus the fatty acyl residues in the added DOPC would not be expected to decrease the oxidative stability of the oil. In addition, Le Grandios, et al (193) showed that oleyl and linolenyl residues in PC are more oxidatively stable than the same residues in triacylglycerols (TAG) again suggesting that the oleyl residues in the DOPC added to the SSO would not be responsible for the observed increase in oxidation rates. These data suggest that the prooxidant activity of DOPC in comparison to DC₄PC is not due to its unsaturated fatty acids.

It is possible that the impact of the phospholipids on the oxidation stability could be due to the polar head groups. However, since both phospholipids tested had the same head group at equal molar concentrations this is not likely. One major difference between DOPC and DC₄PC in SSO is their ability to form physical structure with DOPC forming reverse micelles and DC₄PC forming cylindrical structures (188). In addition, this study showed that DOPC could form physical structures at much lower concentrations than DC₄PC due to its lower critical micelle concentration. Overall, these data suggested that the prooxidant activity of DOPC could be related to its ability to form reverse micelles.

4.3.2 The impact of phospholipids on the oxidative stability of SSO in the presence
Previous studies of the influence of antioxidant polarity on lipid oxidation concluded that polar antioxidants inhibit lipid oxidation in bulk oil systems more effectively than non-polar antioxidants (156, 194, 195). A number of studies have previously examined the effects of combinations of phospholipids and antioxidants on lipid oxidation in bulk oils, such as α-tocopherol, flavonoids, etc (90, 196, 197). Some of these studies used lecithin with a high proportion of PE as the phospholipid source, some of them used commercial oils with unknown intrinsic antioxidant levels, and some used model systems like triolein, methyl linoleate, or methyl laurate which may not accurately reflect commercial bulk oil compositions (197). For this reason, we used a model system that consisted of stripped soybean oil to mimic commercial oil triacylglycerol compositions and well-defined phospholipids and antioxidants to provide a more fundamental understanding of the complex physicochemical phenomenon involved.

The formation of lipid hydroperoxides (LH) and headspace hexanal in SSO containing different levels of phospholipid (0 and 1000 μM DOPC) and the non-polar antioxidant α-tocopherol (0, 10 and 100 μM) was measured during incubation at 55 °C (Figures 4.2). As discussed earlier, in the absence of phospholipid and antioxidants the lag phase of lipid hydroperoxides (LH) and hexanal formation for the control was 3 days for the SSO. The incorporation of 1000 μM DOPC reduced the lag phase of LH and hexanal formation to 2 days, again indicating that this phospholipid was prooxidative. In the absence of DOPC, the duration of the lag phase increased with increasing α-tocopherol concentration compared to the control. The addition of 10 and 100 μM α-tocopherol increased the lag phase for LH to 9 and 27 days, respectively and headspace
hexanal formation to 7 and 27 days, respectively. When DOPC was present, the impact of α-tocopherol on lipid oxidation was more complex (Figures 4.2 A and B). DOPC enhanced the antioxidant activity of 10 μM α-tocopherol by extending the lag phase of LH formation from 9 to 11 days (Figure 4.2 A), and hexanal formation from 7 to 11 days (Figure 4.2 B). The opposite trends were observed when 100 μM α-tocopherol and DOPC were added to the SSO. In this case, the lag time was shorter (less oxidatively stable) in the presence of DOPC than in its absence (Figure 4.2 A and B). For example, DOPC decreased the lag phase of LH formation from 27 to 23 days (Figure 4.2 A) and hexanal formation from 27 to 20 days (Figure 4.2 B) in the presence of 100 μM α-tocopherol.
Figure 4.2. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil containing 1000 μM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) with or with 10 or 100 μM α-tocopherol during storage at 55 °C. Some of the error bars are within data points.

The impact of DC₄PC on the antioxidant activity of α-tocopherol was also determined (Figure 4.3A and B). As observed previously, DC₄PC did not impact with lipid hydroperoxide or hexanal formation rates compared to the control. α-Tocopherol again decreased oxidation rates but in this case DC₄PC decreased the effectiveness or α-
tocopherol at both 10 and 100 µM unlike DOPC which enhances antioxidant activity only at 10 µM.

Figure 4.3. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil containing 1000 µM of 1,2-dibutyl-r-sn-glycero-3-phosphocholine (DC₄PC) with or with 10 or 100 µM α-tocopherol during storage at 55 °C. Some of the error bars are within data points.

4.3.3 The impact of phospholipids on the oxidative stability of SSO in the presence
The impact of different concentrations of Trolox (10 and 100 µM), the polar analog of α-tocopherol, on the oxidative stability of SSO with or without 1000 µM DOPC was also investigated. Again, the formation of LH and hexanal were determined during storage at 55 °C (Figure 4.4 A and B). Trolox at 10 µM increased the lag phase for both lipid hydroperoxides and headspace hexanal formation from 3 (control) to 14 days. When 10 µM Trolox was added to the SSO in combination with 1000 µM DOPC the lag phase for lipid hydroperoxides increased from 14 to 17 days while headspace hexanal formation was similar in the presence and absence of DOPC. Increasing the concentration of Trolox to 100 µM further decreased lipid oxidation rates with no appreciable increase in lipid hydroperoxides or hexanal after 37 days of storage (again more effective than α-tocopherol). However, like α-tocopherol, the presence of 1000 µM of DOPC decreased the antioxidant activity of 100 µM Trolox with the lag phase of LH and hexanal formation decreasing to 27 and 20 days, respectively.
Figure 4.4. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil containing 1000 μM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) with or with 10 or 100 μM Trolox during storage at 55 °C. Some of the error bars are within data points.

In the presence of DC₄PC, the antioxidant activity of Trolox decreased (Figure 4.5A and B). At a concentration of 10 μM Trolox, DC₄PC decreased the lag phase for hydroperoxide formation from 9 to 7 days and hexanal formation from 9 to 5 days. At a concentration of 100 μM Trolox, DC₄PC decreased the lag phase for hydroperoxide and hexanal formation to 25 days (no LH or hexanal formation was observed for Trolox alone).
Figure 4.5. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil containing 1000 \(\mu\)M of 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC4PC) with or with 10 or 100 \(\mu\)M Trolox during storage at 55 °C. Some of the error bars are within data points.

Overall, both \(\alpha\)-tocopherol and Trolox inhibited lipid hydroperoxide and hexanal formation with increasing concentrations from 10 to 100 \(\mu\)M increasing antioxidant activity. The more polar Trolox was more effective than \(\alpha\)-tocopherol which is similar to trends reported by other researchers (156).
4.3.4 The impact of antioxidants on the properties of DOPC reverse micelles in SSO

Aqueous self-assembly of amphphilic surfactants to form association colloids are recognized to be driven primarily by hydrophobic interactions (198). In non aqueous systems, uncertain effects could also drive the self-assembly of amphiphiles into association colloids and this is referred to as reverse self-assembly (199). Our previous research illustrated 1000 µM of amphiphilic DOPC plus 200 ppm water resulted in the formation of reverse micelles, a type of association colloid. Here, the physical structures of the DOPC/water reverse micelles containing different concentrations of antioxidants were measured using small angle x-ray scattering (SAXS) and the fluorescence steady state and lifetime decay of the surface active fluorescent probe, NBD-PE. These studies were not carried out with DC₄PC as this phospholipid does not form association colloids at the concentrations used in this study.

SAXS is a unique technique which has been widely used to distinguish structure transitions in reverse micelles. In our previous study, SAXS was able to determined that DOPC and water formed reverse micelles and that the size and shape of the reverse micelles changed with varying water concentrations. The same technique was employed in this study to determine if α-tocopherol and Trolox had any impact on the structure of DOPC reverse. SAXS patterns revealed that the scattering patterns of DOPC reverse micelles were not altered by either α-tocopherol or Trolox (data not shown) indicating that the antioxidants did not have a major impact on the size and shape of the reverse micelles. The only difference of SAXS between the samples was scattering intensity; however, this difference did not increase with increasing antioxidants concentration. Others reported the phospholipids aliphatic chain is the main factor that impacts the
scattering intensity of phospholipids structures (200). Therefore, the intensity differences caused by the antioxidants could be due to their association with the phospholipid acyl chains thus causing attenuation of the x-ray scattering.

Fluorophores have been reported to provide valuable insights into microenvironmental changes in reverse micelles (164). Previously, we selected NBD-PE, a phospholipid analog grafted with a fluorophore on the phosphate head groups to study the interfacial properties of DOPC reverse micelle systems. NBD-PE emission intensity increases in a polar environment. The lowest recorded emission of NBD-PE was observed in the SSO control. The addition of DOPC and water in SSO increased the emission to 8.5 ×10^5 counts, 30% higher than in SSO alone. Neither α-tocopherol nor Trolox caused a shift in the fluorescence emission peak wavelength (data not shown). The addition of 10 μM α-tocopherol decreased the emission intensity of NBD-PE in the DOPC reverse micelles but further increases in α-tocopherol did not further decrease NBD-PE fluorescence (Figure 4.6). Conversely, a concentration dependent decrease in NBD-PE fluorescence occurred in the presence of Trolox.
Figure 4.6. The neutralized fluorescence intensity of NBD-PE at stripped soybean oil containing 1000 μM of 1,2-dioleoyl-sn-glycero-3-phosphocholine(DOPC) with varying concentrations(0-100 μM) of α-tocopherol and Trolox.

The low NBD-PE emission in the SSO control is likely due to the water concentrations (< 60 ppm in pure SSO). When DOPC and water were added to the SSO, NBD-PE was incorporated into the reverse micelles thus placing it in a more polar environment thus increasing its emission intensity. The ability of increasing concentrations of the water soluble Trolox to decrease fluorescence intensity suggests to that Trolox can interact with water in the reverse micelle thus decreasing NBD-PE-water interactions and thus fluorescence intensity. Additionally, the polar Trolox molecules could directly interact with NBE-PE thus decreasing fluorescence intensity by decreasing the ability of NBD-PE to become excited. Overall, this data suggests that both the NBD-PE probe and Trolox are incorporated into the DOPC reverse micelles.
Figure 4.7. The fluorescence lifetime decay of NBD-PE at stripped soybean oil containing 1000 μM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) with varying concentrations (0-100 μM) of α-tocopherol (A) and Trolox (B) concentrations.

Information about the properties of the antioxidants in the reverse micelles can also be gained by measuring the lifetime decay of NBD-PE. Overall, neither α-tocopherol not Trolox had a major impact on the fluorescence lifetime of NBD-PE (Figure 4.7A and B).
However, there was a slight trend of Trolox decreasing the lifetime of NBE-PE in DOPC reverse micelles. This again suggests that both the NBD-PE probe and Trolox are incorporated into the DOPC reverse micelles.

4.4 Discussion

The main motivation of investigating the impact of DOPC based reverse micelles in stripped soybean oil on the physicochemical properties of antioxidants was to better understand the underlying phenomena of lipid and antioxidant interactions in bulk oil. The parameters evaluated were phospholipids with the same polar head groups and different fatty acyl chains (DC₄PC and DOPC) that would result in formation of different types of physical structures. Our previous study showed that DOPC but not DC₄PC formed reverse micelles in SSO¹⁰. In addition, DOPC reverse micelles accelerated lipid oxidation while DC₄PC, which does not form physical structure had no impact on lipid oxidation rates. This data suggest that lipid oxidation in bulk oils is not only influenced by the traditional chemical factors, such as lipid compositions, transition metals and antioxidants, but is also related to the existence of physical structures of. Since one of the most effective techniques employed for retarding the lipid oxidation is the addition of free radical scavengers, this study was conducted as an extension of the previous study to determine how physical structure impact the activity of antioxidant in bulk oils. In this study, α-tocopherol and Trolox were chosen as hydrophobic and hydrophilic antioxidants that have similar free radical scavenging functional groups (e.g same chromanol ring) with different hydrocarbon tails (201).

DOPC by itself promoted lipid oxidation while DC₄PC had no effect. This would
suggest that the physical structure formed by DOPC increased lipid oxidation rates since both DOPC and DC₃PC have the same polar head groups and thus would impact the chemistry of lipid oxidation in a similar manner. This is supported by the previous observation that DOPC below its critical micelle concentration, where no reverse micelles are formed, does not promote lipid oxidation (188). The prooxidative effect of DOPC suggest that reverse micelles facilitate lipid oxidation.

One would expect the prooxidant activity of DOPC reverse micelles to decrease the activity of the antioxidants. However, at low antioxidant concentrations, DOPC increased the activity of both Trolox and α-tocopherol. Koga and Terao (181) have postulated that physical structures formed by phospholipids can increase the antioxidant activity of tocopherols by allowing them to concentration at the oil-water interface where lipid oxidation is most prevalent. However, increasing α-tocopherol and Trolox concentrations to 100 µM in the presence of a constant DOPC concentrations resulted in a reduction in the effectiveness of the antioxidant. Antioxidants such as tocopherols in bulk oils have been shown to lose effectiveness as their concentrations increase and this could help to explain this observation. However, loss of effectiveness with increasing concentrations is not typically seen at the α-tocopherol and Trolox concentrations used in this study (202). A simple explanation for this observation is not available but it could be due to differences in the number and/or type of association colloids found in this simple model system compared to commercial oils that would contain a much greater variety of endogenous surface active molecules. Another possible explanation is that the DOPC reverse micelles used in this study would produce a negatively charged interface that could attract prooxidative transition metals. Partitioning of high concentrations of α-
tocopherol and Trolox into the same physical location as the transition metals could allow the antioxidants to reduce the metals into their more prooxidative state and thus increase lipid oxidation rates which would be seen as a decrease in the effectiveness of the antioxidants. This pathway could be further increased by the accumulation of lipid hydroperoxides, the substrate for metal promoted lipid oxidation, into these same structures since they have been reported to partition into reverse micelle structures in oils and act as the co-surfactant (145). The presence of DC$_4$PC only decreased the activity of α-tocopherol and Trolox indicating that it was not able to enhance the activity of the antioxidants.

Trolox was a more effective antioxidant in the presence of the DOPC micelles than α-tocopherol which is in agreement with previous research (156). The fluorescence of NBD-PE was affected to a much greater extent by Trolox than α-tocopherol suggesting that Trolox was partitioning into the DOPC reverse micelles differently than α-tocopherol. This could be due to Trolox’s higher water solubility which would allow it to partition into the water phase while α-tocopherol has virtually no water solubility and thus at best would align at the oil-water interface. Differences in the physical location of Trolox and α-tocopherol could alter the overall impact on lipid oxidation by influencing their antioxidant (free radical scavenging) or prooxidant (reduction of transition metals) activity.

This study showed that the antioxidant activity of both Tolox and α-tocopherol were influenced by physical structures in bulk oils. Work such as this can provide important data on how physical structures in bulk oils impact the activity of antioxidants. Since few new antioxidants are available for food applications, this information might provide
information on how to alter the physical structures in bulk oils such that we can utilize the available food antioxidants more effectively.
IRON/ANTIOXIDANTS RELATIONSHIP IN BULK OIL OXIDATION

5.1 Introduction

Lipid oxidation is one of the major factors limiting the shelf life of bulk oils, since it adversely affects flavor and quality by forming a very complex mixture of lipid hydroperoxides, fatty acid chain-cleavage products, and polymeric materials (203,204). In addition, lipid oxidation presents food safety concerns as low concentrations of lipid oxidation products, such as 4-hydroxynonenal (4-HNE) and malonaldehyde (MDA) can promote inflammation, atherosclerosis, neurodegenerative diseases and cancer (205).

The current dietary trends to consume healthier oils, such as omega-3 fatty acids is a major challenge for food scientists since these oils are extremely susceptible to lipid oxidation (206). Therefore, new strategies to prevent or inhibit the oxidation of bulk oils are of major importance to consumers and the food industry. Based on the current knowledge of lipid oxidation mechanisms, some approaches have been developed to fulfill this goal. The application of antioxidants can retard lipid oxidation (8). But in many foods the currently approved antioxidant are not sufficient to prevent rancidity. Since very few new antioxidants are available to the food industry, new technologies are needed to enhance the effectiveness of currently available food-grade antioxidants.

Bulk oil is a deceptively complex food system since it contains numerous minor components, such as free fatty acid, sterols, antioxidants, phospholipids, monoacylglycerol, and water (121,207). Recent studies from our group found that in bulk oils some of these minor components are able to form physical structures known as association colloids. These physical structures can increase lipid oxidation reactions and alter the effectiveness of antioxidants, such as α-tocopherol, Trolox, and chlorogenic acid
esters (188,208,209). For example, the more polar Trolox was able to associate with association colloids more effectively than nonpolar \(\alpha\)-tocopherol. Since association colloids increase lipid oxidation rates, the association of Trolox with these association colloids could help explain by it’s a more effective antioxidant than \(\alpha\)-tocopherol.

The role of transition metals on lipid oxidation in food systems has been intensively studied (210,211). Low valence-state metal ions, such as ferrous and cuprous, can participate in the initiation and propagation steps of lipid oxidation by abstracting hydrogen to directly form free radicals and decomposing lipid hydroperoxides into free radicals such as the alkoxy radical (212). The prooxidative effect of high-valence-state metal ions is less clear although there is some evidence that they can promote oxidation when they are reduced by antioxidants to form the more prooxidative low valence state of the metal ions (213,214). For example, ascorbic acid and gallic acid can promote lipid oxidation by reducing iron to its low valence-state (215,216).

Vegetable seed oils inevitably contain transition metals which originate from the seed and/or manufacturing equipment and ingredients suggesting that they could be important prooxidants in bulk oils. Tocopherols are important antioxidants in bulk oils but they have been reported to have prooxidant activity in vegetable oils at high concentrations (217,218). While association colloids in the bulk oil seem to act as important nano-reactors in bulk oils, it is unclear what promotes this oxidation. In addition, since molecules such as Trolox and tocopherols can also have prooxidative activity, it is possible that association colloids could negatively impact lipid oxidation by enhancing the prooxidant activity of antioxidants. Therefore, the present investigation was undertaken to explore if iron was an important prooxidant in bulk oil containing
association colloids. In addition, the ability of high or low valence state iron to interact with α-tocopherol and Trolox was also determined. Iron-antioxidant interactions were determined in a model system containing non-oxidizable medium chain triacylglycerol (MCT) by measuring the depletion of antioxidants and the formation of free radicals.

5.2 Materials and methods

5.2.1 Materials

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) was acquired from Avanti Polar Lipids, Inc (Alabaster, AL). Soybean oil was purchased from a local store and stored at 4 °C. Medium chain triacylglycerols (MCT, Miglyol®) were purchased from Sasol North America Inc (Houston, TX). Silicic acid, activated charcoal, calcein (CA), deferoxamine (DFO), methanol, hexane, alpha-tocopherol, Trolox, and N-t-butyl-phenylnitrone (PBN) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Anhydrous ferric chloride and anhydrous ferrous chloride beads (99.998 % purity) were purchased from Sigma-Aldrich Co.,(St. Louis, MO). All other reagents were of HPLC grade or purer distilled and deionized water was used as needed.

5.2.2 Formation of DOPC association colloids in stripped soybean oil or MCT

Stripped soybean oil with DOPC reverse micelles were prepared according to the method of Chen et al (188). Briefly, formation of reverse micelles was accomplished by pipetting DOPC (1000 μM, final concentration) in chloroform into an empty beaker and then flushing with nitrogen until the chloroform was evaporated. The appropriate amount
of medium chain triacylglycerols (MCT) or stripped soybean oil (SSO) was then added followed by double distilled water at a final concentration of 200 ppm. The oil samples were then stirred in a beaker at 1000 rpm in a 20 °C incubator room for a 24 h.

5.2.3 Measurement of oxidation parameters

For lipid oxidation kinetics studies antioxidants were incorporated into stripped soybean oil by stirring at 300 rpm for at least 12 h at room temperature to obtain the homogenous samples. Then, samples were aliquoted (1 mL/vial) into GC headspace vials and stored at 55 °C in the dark.

Lipid hydroperoxides were measured as the primary oxidation products using a method adapted from Shanta and Decker (165). Oil samples were weighed and recorded before adding to a mixture of methanol/butanol (2.8 mL, 2:1, v:v) followed by addition of 15 μL of 3.94 M thiocyanate and 15 μL of 0.072 M Fe$^{2+}$. The solution was vortexed, and after 20 min the absorbance was measured at 510 nm using a Genesys 20 spectrophotometer (ThermoSpectronic, Waltham, MA). The concentration of hydroperoxides was calculated from a cumene hydroperoxide standard curve.

The secondary oxidation product, hexanal, was monitored using a GC-17A Shimadzu gas chromatograph equipped with an AOC-5000 autosampler (Shimadzu, Kyoto, Japan). Samples (1 mL) in 10 mL glass vials capped with aluminum caps with PTFE/silicone septa were heated at 55 °C for 15 min in an autosampler heating block before measurement. A 50/30 μm DVB/ Carboxen/PDMS solid-phase microextraction (SPME) fiber needle from Supelco® (Bellefonte, PA, USA) was injected into the vial for 2 min to absorb volatiles and was then transferred to the injector port (250 °C) for 3 min.
The injection port was operated in split mode, and the split ratio was set at 1:5. Volatiles were separated on a Supleco 30 m × 0.32 mm Equity DB-1 column with a 1 μm film thickness at 65°C for 10 min. The carrier gas was helium at 15.0 mL/min. A flame ionization detector was used at a temperature of 250°C. Hexanal concentrations were determined from peak areas using a standard curve prepared from an authentic standard.

5.2.4 Determination of Trolox and α-tocopherol concentrations

For HPLC analysis, 100 μL of each sample was dissolved in 0.9 ml methanol and then was passed through a 0.45 μm filter. A 20 μl aliquot of this sample solution was separated using a Shimadzu HPLC system equipped with a Shimadzu diode array detector (DAD), a Waters 474 Scanning Fluorescent Detector and a 250 mm × 4.6 mm i.d., 5 μm, Inertsil C18 analytical column. The mobile phase consisted of 4 % purified water with 3 mM phosphoric acid at pH 2.6 (solvent A) and 96 % methanol (solvent B) using isocratic gradient at a flow rate of 1 ml/min. Column temperature was set at 38 °C. Trolox was detected using DAD at a wavelength of 280 nm. Detection of α-tocopherol was conducted using both DAD at 295 nm and fluorescence at an excitation wavelength of 290 nm and an emission wavelength of 330 nm. Trolox and α-tocopherol in the samples were identified by comparing their relative retention times and UV spectra with authentic compounds and were quantitated using an external standard method.

Depletion of antioxidants was determined using $\frac{A_t}{A_0} \times 100\%$

Where $A_0$ and $A_t$ were the peak area at time zero and t, respectively.
5.2.5 Determination the concentration of ferric iron in MCT

Ferric iron content was determined by UV-visible spectrophotometric method using calcein (CA) dye method developed by Thomas et al (219). Briefly, calcein solution was prepared by adding 0.75 g of calcein in 400 g MCT and stirred overnight to dissolve the dye. The solution was centrifuged at 10,000 rpm for 10 min and upper clear orange color solution was collected and defined as the dye solution. Equal volume of dye solution was added directly to bulk oil samples containing ferric iron and the absorbance was measured at 352 nm using a Shimadzu UV-2101 PC UV–vis scanning spectrophotometer (Shimadzu, Kyoto, Japan).

Remaining ferric iron in MCT was determined using $\frac{Ab_t}{Ab_0} \times 100\%$

Where $Ab_0$ and $Ab_t$ were the absorbance at time zero and t, respectively.

5.2.6 EPR spectroscopy

EPR measurements were carried out at room temperature using a Bruker EPR Elexsys-500 spectrometer operating at the X-band. The samples were placed in 707-SQ-250 M, thin wall quartz EPR sample tubes (Wilmad Glass Co., Buena, NJ, USA) and inserted into the ER 4122-SHQE high sensitivity TE102 cylindrical mode single cavity (Q ~ 3000) optical window of the EPR system. Instrument settings were: center field, 3470 G; scan range, 100 G; gain, 60; time constant, 128 ms; modulation amplitude, 1 G; phase 0°; microwave power, 20 mW. Data collection was performed using the computerized program Xepr.
5.2.6 Statistical analysis

Duplicate experiments were performed with freshly prepared samples. All data shown represents the mean values ± standard deviation of triplicate measurements. Statistical analysis of lipid oxidation kinetics was performed using a one-way analysis of variance. A significance level of p<0.05 between groups was accepted as being statistically different. In all cases, comparisons of the means of the individual groups were performed using Duncan’s multiple range tests. All calculations were performed using SPSS17 (http://www.spss.com; SPSS Inc., Chicago, IL).

5.3 Results

5.3.1 Effects of the metal chelator, Deferoxamine (DFO) on the oxidative stability of stripped soybean oil

Transition metals are implicated in many lipid oxidation pathways as they are able to generate free radicals that initiate and propagate lipid oxidation in food systems (220,221). The different redox states of iron have different pathways by which they can promote oxidation (222). For example, low-valence transition metals can promote the decomposition of lipid hydroperoxides into free radicals such as the alkoxy radical (reaction 1). The high-valence state of transition metals can also decompose lipid hydroperoxide (reaction 2) but this pathway is very slow and may not be very important in foods. However, compounds such as antioxidants can reduce metals resulting in the formation of the highly prooxidative low-valence state thus promoting oxidation (reaction 3) (207,223).

\[ \text{Fe}^{2+} + \text{LOOH} \rightarrow \text{Fe}^{3+} + \text{LO}^\cdot \cdot \cdot \text{OH} \]  
(reaction 1)
Bulk oil naturally contains transition metals. Therefore, gaining a better understanding of the chemical role of transition metals in bulk oil oxidation is necessary to develop effective prevention strategies to extend the shelf-life of oils. To gain a better understanding of the role of iron on lipid oxidation in bulk oils, we studied the effect of deferoxamine (DFO), a specific Fe(III) chelator, on the oxidative stability of stripped soybean oil (SSO) in the absence and presence of phospholipid reverse micelles.

The formation of lipid hydroperoxides (LH) and headspace hexanal in SSO containing different levels of phospholipids (0 and 1000 μM DOPC) and DFO (0 and 2 mM) was measured during incubation at 55 °C (Figures 5.1 A and B). The lag phase for both lipid hydroperoxides and headspace hexanal for SSO lasted 4 days. The addition of DOPC reduced the lag phase by 2 days, which is in agreement with our previous study indicating that DOPC reverse micelles act as a prooxidant (209). The incorporation of DFO had no significant effect on the lag phase of SSO (p>0.05), but extended the lag phase of SSO containing DOPC from 2 to 7 days (p<0.05).
Figure 5.1. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil containing 2 mM deferoxamine (DFO) and/or 1000 μM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) during storage at 55 °C. Some of the error bars are within data points.

In order to understand if iron played a role in lipid oxidation in the presence of free radical scavenging antioxidants, different combinations of DFO, α-tocopherol and Trolox were added to the SSO in the presence and absence of phospholipids reverse micelles.
during incubation at 55 °C (Figures 5.2 and 5.3). The lag phase of lipid hydroperoxides (LH) and hexanal formation was 25 days for SSO containing 100 μM α-tocopherol (Figures 5.2 A and B). In the presence of DOPC reverse micelles and α-tocopherol, the lag phase decreased to 16 days for both LH and hexanal formation, again showing that α-tocopherol was less effective in the presence of DOPC reverse micelles (188). DFO increased the lag phase of LH and hexanal formation in the presence of α-tocopherol both in the presence and absence of DOPC reverse micelles. In the absence of DOPC reverse micelles, the lag phase was increased by 5 days while in the presence of DOPC reverse micelles the lag phase was increased by 10-11 days. Unlike the previous experiment, DFO was able to inhibit lipid oxidation in the absence of DOPC micelles suggesting that α-tocopherol was somehow increasing the prooxidant activity of iron.
Figure 5.2. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil containing 100 μM α-tocopherol, 2 mM deferoxamine (DFO) and/or 1000 μM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) during storage at 55 °C. Some of the error bars are within data points.

The impact of DFO on the antioxidant activity of Trolox was also tested in the presence and absence of DOPC reverse micelles (Figure 5.3 A and B). The lag phase of lipid hydroperoxides (LH) and hexanal formation was 40 days for the SSO containing 100 μM Trolox showing that the more polar Trolox was a more effective antioxidant than α-tocopherol as predicted by the antioxidant polar paradox (224). However, unlike α-tocopherol, the incorporation of DFO did not increase the lag phase of LH and hexanal formation in the absence of DOPC micelles. As observed previously, DOPC again diminished the effectiveness of Trolox decreasing the lag phase to 21 days for both LH and hexanal formation. Even though DOPC decreased the activity of both α-tocopherol and Trolox, the more hydrophilic Trolox was still the most effective of the two. When DOPC micelles, Trolox and DFO were present in combination, DFO helped partially offset the prooxidative effect of DOPC by increasing the lag phase for LH and hexanal
formation to 29 days.

**Figure 5.3.** Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil containing 100 μM Trolox, 2 mM deferoxamine (DFO) and/or 1000 μM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) during storage at 55 °C. Some of the error bars are within data points.

DFO, a powerful iron chelator, has been successfully used in clinical settings to treat patients with acute and chronic iron overload syndromes (225). DFO has a remarkably high affinity for ferric ion allowing it to inhibit the initiation and propagation of lipid
oxidation (226). Therefore, it was anticipated that oxidative stability of bulk oil would be improved by DFO if iron was an important prooxidant. In this study, DFO was not an effective antioxidant in soybean oil stripped of its minor components including endogenous antioxidants and phospholipids. However, DFO was effective in the presence of DOPC reverse micelle indicating that the iron was an important prooxidant in the micelles. This could occur since phospholipids can bind iron (227) and thus could concentrate iron at the lipid-water interface of reverse micelles. Lipid hydroperoxides are also surface active and could accumulate at the water-oil interface of the reverse micelles (14). Thus, the prooxidant activity of DOPC could be due to their ability to concentrate both endogenous iron and lipid hydroperoxides at the water-lipid interface of reverse micelles thereby increasing the ability of iron to decompose lipid hydroperoxides.

The combination of the free radical scavengers, \( \alpha \)-tocopherol and Trolox, and DOPC reverse micelles also impacted the ability of DFO to inhibit lipid oxidation. In the absence of DOPC reverse micelles, DFO was a weak antioxidant in the presence of \( \alpha \)-tocopherol (increased lag phase approximately 5 days) and did not inhibit oxidation in the presence of Trolox. However, in the presence of DOPC reverse micelles and \( \alpha \)-tocopherol or Trolox, DFO was a strong antioxidant increasing the lag phase of oxidation to 11 and 13 days for \( \alpha \)-tocopherol and Trolox, respectively. This observation could be due to the ability of DFO to inhibit iron-promoted generation of free radical that would deplete the antioxidants in the DOPC reverse micelles system. However, it is also possible that DFO could improve the ability of \( \alpha \)-tocopherol or Trolox by inhibiting direct degradation by iron.
5.3.2 Effects of Fe(III) and Fe(II) on the depletion of α-tocopherol and Trolox in MCT

In order to better understand interactions between high and low-valence iron with α-tocopherol and Trolox, a medium chain triacylglycerides (MCT) model bulk oil system was used. MCT was used in these experiments since it does not contain unsaturated fatty acids and thus there would be no fatty acid oxidation that could alter α-tocopherol or Trolox concentrations. Figure 5.4A shows the depletion of α-tocopherol and Trolox during storage at 55 °C in the presence of high-valence Fe(III). The concentration of both α-tocopherol and Trolox were relatively constant in the absence of Fe(III). Incubation of α-tocopherol or Trolox with 150 μM Fe(III) resulted in a similar trend in antioxidant depletion with 15 and 12 % of α-tocopherol and Trolox consumed, respectively, after one hour storage. α-Tocopherol and Trolox concentrations in the presence of 150 μM Fe(III) reached equilibrium (~50% consumed) after 12 h. When 600 μM of Fe(III) was added, the pattern of antioxidants consumption was different with α-tocopherol being completely consumed after 8 h of storage. Increasing the Fe(III) concentration to 600 μM had much less effect on Trolox consumption with a maximum of approximately 60% depletion of Trolox after 24 h. The observed consumption of α-tocopherol and Trolox in the presence of Fe(III) could be due to the ability of these antioxidants donate an electron to iron thus converting ferric to ferrous ions. Since Fe(III) was more reactive with α-tocopherol than Trolox, this could help explain why DFO was able to improve the antioxidant activity of α-tocopherol but not Trolox in the absence of DOPC reverse micelles (Figure 5.2 and 5.3) as DFO could decrease α-tocopherol’s ability to reduce ferric ions to the more prooxidative ferrous ions and could also decrease Fe(III) promoted α-tocopherol
consumption which would help maintain a higher concentrations of α-tocopherol thus more effectively inhibiting lipid oxidation.

Figure 5.4. Depletion of α-tocopherol and Trolox (100 μM) in medium chain triacylglycerols (MCT) in the presence of (A) Fe(III); and (B)Fe(II)

Since α-tocopherol and Trolox consumption reached an equilibrium and did not continue to decrease in concentrations, this suggests that Fe(II) did not cause antioxidant consumption. To test this hypothesis, Fe(II) was also added to the MCT model system
Initial experiments were performed with reagent grade ferrous chloride powder but rapid \( \alpha \)-tocopherol and Trolox consumption was observed (data not shown). It was thought that this could be due to contaminating Fe(III) so experiments were repeated using a high purity form of anhydrous ferrous supplied under vacuum. In these experiments, 150 \( \mu \)M Fe(II) did not significantly decrease \( \alpha \)-tocopherol and Trolox for the first 12 h of storage compared to a 42 and 50% decrease in \( \alpha \)-tocopherol and Trolox, respectively, during the same time period in the presence of 150 \( \mu \)M Fe(III) (Figure 5.4A). In the presence of 600 \( \mu \)M Fe(II), Trolox consumption was more rapid than \( \alpha \)-tocopherol. In all cases, \( \alpha \)-tocopherol and Trolox consumption increased during prolonged storage but the level of consumption was always significantly lower than Fe(III) (\( p > 0.05 \), Figure 5.4A). The depletion of antioxidants during the later stages of incubation may be due to the oxidation of ferrous to ferric upon reaction with oxygen.

5.3.3 Effects of Fe(III) and Fe(II) on the depletion of \( \alpha \)-tocopherol or Trolox in the presence of DOPC reverse micelles

Previous results showed that DFO more effectively increased the antioxidant activity of \( \alpha \)-tocopherol and Trolox in the presence of DOPC micelles (Figure 5.2 and 5.3). This could be due to DFO’s ability to decrease iron-promoted decomposition of lipid hydroperoxides into free radicals, a factor that would decrease \( \alpha \)-tocopherol and Trolox consumption and thus increase their ability to scavenge free radicals. However, it is also possible that the improved activity of DFO in DOPC reverse micelles could be due to its ability to inhibit the direct consumption of \( \alpha \)-tocopherol and Trolox by iron which again would increase antioxidant concentrations and would also decrease the reduction of ferric...
into the more prooxidative ferrous ions. To test this possibility, the depletion of α-tocopherol and Trolox by Fe(III) or Fe(II) in MCT in the presence of DOPC reverse micelles was determined (Figure 5.5 and 5.6). These experiments were also conducted with DC₄PC, a phosphatidylethanolamine with butyric acid. DC₄PC does not form reverse micelles at the concentrations tested (188), so experiments could be conducted with the same concentration of the choline head group in the absence of association colloids.

**Figure 5.5.** Depletion of α-tocopherol and Trolox (100 μM) in medium chain
triacylglycerols (MCT) containing 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or
1,2-dibutryl-sn-glycero-3-phosphocholine (DC₄PC) in the presence of (A) 40 ppm
Fe(III); and (B) 100 ppm Fe(III)

In the absence of Fe(III), neither DOPC nor DC₄PC promoted the consumption of α-
tocopherol and Trolox and (Figure 5.4A and B) and α-tocopherol and Trolox were
stable in the absence of added iron (data not shown). In the presence of 150 μM Fe(III)
and DOPC reverse micelles, the consumption of both α-tocopherol and Trolox was less
than in the absence of DOPC (Figure 5.5A). For example, after 24 h of incubation, α-
tocopherol concentrations were 10% lower than time 0 in the presence of DOPC reverse
micelles compared to 40% lower in the absence of DOPC reverse micelles. In the
presence of 600 μM Fe(III), Trolox was depleted more rapidly than α-tocopherol in the
presence of DOPC reverse micelles while the opposite was true in the absence of DOPC.
Trolox has been found to more highly associated than α-tocopherol with water in DOPC
micelles (212). This means that it could have greater contact with phosphatidylcholine
bound iron at the water-lipid interface and thus could be more rapidly depleted.

In the presence of DC₄PC which does not form reverse micelles, Fe(III) was rapidly
consumed by both α-tocopherol and Trolox. At low (150 μM) and high (600 μM) Fe(III)
concentrations, DC₄PC slightly increased both α-tocopherol and Trolox consumption
compared to the absence of phospholipid (Figure 5.6A and B). DC₄PC would be
expected to bind iron as would any phosphatidylcholine. The ability of DC₄PC to slightly
promote the consumption of α-tocopherol and Trolox could be due to its ability to
increase the solubility of iron in the oil by acting as a lipid soluble metal chelator and
thus making the iron more reactive if the iron chelated to DC₄PC would still be redox
active. Since DC₄PC does not form reverse micelles at the concentrations tested, this suggests that the ability of DOPC to inhibit both α-tocopherol and Trolox depletion was not due to the phospholipid head group but instead was due to the presence of reverse micelles.

**Figure 5.6.** Depletion of α-tocopherol and Trolox (100 μM) in medium chain triacylglycerols (MCT) containing 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC₄PC) in the presence of (A) 40 ppm Fe(II); and (B) 100 ppm Fe(II)

5.3.4 Measurement of Fe(III) loss by α-tocopherol and Trolox
Many researchers have determined iron concentration in bulk oil (228-230). For example, Mendil and coworkers measured iron content in bulk oil with a flame atomic absorption spectrometer (FAAS) and total iron was found to be 139, 127, 105, and 52 ppm (i.e., 200-800 μM) in olive oil, hazelnut oil, sunflower oil and corn oil, respectively (230). While this experiment is helpful to verify that iron exist in commercially refined oils, it does not provide any information on the redox state of the iron. This is not uncommon since analytic techniques to measure iron redox state in bulk oil are limited. Verifying the existence of iron recycling pathway by measuring the redox form of iron has been extremely helpful in understanding the role of iron redox form in lipid oxidation in both in vivo systems and oil-in-water emulsion (231,232). Therefore, in this study we initially attempted to track the concentration of Fe(III) in MCT with calcein acetoxymethyl ester (calcein-AM), an Fe(III) specific probe used successfully in cells and biological fluids (233). However, calcein-AM solubility in MCT was quite limited so calcein was successfully substituted due to its higher solubility in hydrophobic solvents (219). Preliminary studies showed that calcein had strong absorbance at 365nm in MCT which was quenched by ferric chloride. It also showed a good linear relationship (r>0.99) with Fe(III) in MCT over the range of 0-600 μM Fe(III) (data not shown). Room temperature was chosen in these experiments since the 55 °C used in the previous experiments caused too rapid of a reaction between ferric ions and the antioxidants.

The ability of α-tocopherol and Trolox to reduce Fe(III) was determined at 600 μM Fe(III) and varying concentrations of the antioxidants (Figure 5.7A). After 24 h of incubation, Fe(III) concentrations only decreased slightly (<5%) in the absence of added antioxidants. α-Tocopherol significantly decreased Fe(III) at concentrations as low as 20
μM. Maximal decrease in Fe(III) concentrations was observed at α-tocopherol concentrations of 60 μM. Trolox exhibited a similar trend on Fe(III) concentrations although its maximal decrease in Fe(III) concentrations was 40 μM. At 100 μM, the decreased in Fe(III) concentrations was slightly greater for Trolox than α-tocopherol (p<0.05).

DOPC reverse micelles by themselves caused a greater decrease in Fe(III) (Figure 5.7B) than in the absence of DOPC (Figure 5.7A) suggesting that the DOPC by itself could reduce Fe(III) (Figure 5.7B). DOPC reverse micelles decreased the ability of both α-tocopherol and Trolox to reduce iron as significant decreases in Fe(III) were not seen until α-tocopherol and Trolox concentrations were greater than 40μM. As in the absence of DOPC reverse micelles, at 100 μM, the decreased in Fe(III) concentrations was slightly greater for Trolox than α-tocopherol (p<0.05).

Overall, DC₄PC (Figure 5.7C) changed the concentration of Fe(III) in a similar manner to the absence of phospholipid (Figure 5.7A). For example, DC₄PC had very little impact on Fe(III) concentrations in the absence of antioxidants. In addition, both α-tocopherol and Trolox were able to significantly decreased Fe(III) at concentrations as low as 20 μM as seen in the absence of phospholipid. However, DC₄PC decreased the maximal decrease in Fe(III) concentrations by α-tocopherol from 60 to 40 μM.
A remaining of Fe^{3+} (%)

Antioxidant concentration (µM)

600µM Fe^{3+}  600µM Fe^{3+} + Trolox  600µM Fe^{3+} + Trolox

B remaining of Fe^{3+} (%)

Antioxidant concentration (µM)

600µM Fe^{3+} + DOPC  600µM Fe^{3+} + Trolox + 1000µM DOPC  600µM Fe^{3+} + Trolox + 1000µM DOPC
Figure 5.7. Fe(III) development in medium chain triacylglycerols (MCT) containing (A) α-tocopherol or Trolox; (B) 1000 μM of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC); (C) 1,2-dibutyryl-sn-glycero-3-phosphocholine (DC₄PC) after 24 h storage at room temperature. For each group, different letters on the top of columns represent significant differences (p < 0.05).

5.3.5 Effects of Fe(III) and Fe(II) on the formation of PBN spin adducts in MCT

Since both α-tocopherol and Trolox can scavenge free radicals generated in oils, one may argue that the depletion of antioxidants in this research could be due to the presence of free radicals. In order to clarify this potential pitfall and provide more evidence for the different role of Fe(III) and Fe(II) in bulk oil, an EPR study was employed in an effort to detect the formation of free radicals in our MCT model system under different conditions.

A comparison of the EPR signals of SSO (Figure 5.8A) and MCT (Figure 5.8B) after 24 h storage at 55 °C showed that PBN trapped free radicals were only observed in SSO indicating that there were no detectable free radicals in our MCT model system. When antioxidants and iron were added to MCT, the formation of α-tocopheroxyl (Figure 5.8C) or Trolox (Figure 5.8D) radicals were not detected. This was true in the
presence or absence of DOPC or DC<sub>4</sub>PC (data not shown). To determine if iron was reactive in the MCT model, it was added in combination with cumene hydroperoxide. In this system, Fe(II) was observed to form PBN trapped radicals (Figure 5.8E) but Fe(III) was not (Figure 5.8F). This confirms that Fe(II) could be an active prooxidant in bulk oils and that Fe(III) is much less reactive as has been observed oil-in-water emulsions.

Figure 5.8. EPR detection of radical formations in the reaction consisting of (A) 0.1 M PBN in stripped soybean oil; (B) 0.1 M PBN in MCT; (C) 0.1 M PBN+100 μM α-tocopherol in MCT; (D) 0.1 M PBN+100 μM Trolox in MCT; (E) 0.1 M PBN+10 mM cumene hydroperoxides+40 ppm Fe(II) in MCT; (F) 0.1 M PBN+cumene hydroperoxides+40 ppm Fe(III) in MCT after 24 h storage at 55 °C

5.4 Discussion
DFO was able to inhibit lipid oxidation in SSO in the presence of DOPC reverse micelles indicating that iron was an active prooxidant in the presence of association colloids. Since refined oils contain association colloids due to the presence of surface active minor components (e.g. phospholipids, free fatty acids, lipid hydroperoxides, etc.) and water (121), this could help explain why the metal chelator citric acid is an effective antioxidant in commercial oils. DFO was even more effective at increasing the oxidative stability of oils with DOPC reverse micelles in the presence of \( \alpha \)-tocopherol and Trolox. This could be due to the decreased iron-promoted generation of free radicals or direct iron-promoted decomposition of antioxidants.

Many phenolic antioxidants, such as the chlorogenic, caffeic, sinapic and ferulic acid, can reduce Fe(III) to Fe(II) in aqueous environments resulting in the consumption of the antioxidant. For example, 1 molecule of caffeic acid was reported to reduce 9 atoms of Fe(III) (234,235). Our result showed that \( \alpha \)-tocopherol and Trolox can reduce Fe(III) in bulk oil as seen by both the ability of Fe (III) to decrease antioxidant concentrations (Figure 5.4) and the ability of antioxidants to decrease Fe(III) concentrations (Figure 5.7B). In this study, DOPC reverse micelles decreased interactions between iron and \( \alpha \)-tocopherol or Trolox as seen by both a decrease in iron-promoted \( \alpha \)-tocopherol and Trolox decomposition and a decrease in the ability of \( \alpha \)-tocopherol and Trolox to decrease Fe(III) concentrations. This suggests that DFO did not decrease the iron-promoted decomposition of \( \alpha \)-tocopherol and Trolox. Therefore, it is more likely that the ability of DFO to enhance the activity of \( \alpha \)-tocopherol and Trolox in DOPC reverse micelles was due to its ability to inhibit iron-promoted generation of free radicals through pathways such as hydroperoxides decomposition. This could occur if iron
concentrated at the water-lipid interface of the DOPC reverse micelles and increased its ability to decompose surface active lipid hydroperoxides. Decrease production of free radicals from decomposing lipid hydroperoxides would decrease the consumption of the antioxidants thus increase their effectiveness.
CONCLUSION

This research has shown that 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) can self-assemble in stripped soybean oil (SSO) to form thermodynamically stable association colloid structure, most likely reverse micelles. Interfacial tension and fluorescence spectrometry results showed the critical micelle concentration (CMC) of DOPC in stripped soybean oil was 650 and 950 μM, respectively which are well within the phospholipid concentrations found in refined vegetable oils. Light scattering attenuation results showed water concentrations was a critical factor in DOPC reverse micelle structure with a 500 ppm water threshold above which association colloids altered the optical properties of stripped soybean oil. Small-angle X-ray scattering (SAXS) and fluorescence probes again showed that water had a very strong impact on the properties of the association colloids formed by DOPC.

Measurement of primary and secondary lipid oxidation products revealed that reverse micelles formed by DOPC reverse micelles had a pro-oxidant effect, shortening the lag phase of SSO at 55 °C. This research also showed that 1,2-dibutyl-sn-glycero-3-phosphocholine (DC_{4}PC) did not form association colloid in stripped soybean oil, nor did it change lipid oxidation kinetics of stripped soybean oil indicating that it was not the choline head group that responsible for the observed accelerated lipid oxidation but instead was likely the reverse micelle itself. The addition of ferric ion chelator, deferoxamine (DFO) in stripped soybean oil significantly prevented the lipid oxidation caused by DOPC reverse micelles extending lag phase from 2 to 7 days. This indicated that iron was a strong prooxidant in the DOPC reverse micelles. Lipid hydroperoxides are also surface active and therefore likely concentrate in the DOPC reverse micelles.
Therefore the proposed mechanism of oxidation in DOPC reverse micelles is iron promoted lipid hydroperoxide decomposition into free radicals.

DOPC reverse micelles can also impact the physical location of antioxidants such as non-polar α-tocopherol and its polar analog Trolox. The steady state fluorescence intensity of the probe, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE), revealed that both α-tocopherol and Trolox were associated with DOPC reverse micelle in bulk oil. Trolox primarily concentrated in the water pool of reverse micelle since it quenched the NBD-PE intensity with increasing concentrations. A portion of α-tocopherol was also associated with the aqueous phase as evidence by the fact that it did not impact NBD-PE intensity when its concentration was increased from 50 to 100 μM. The shortened life time of NBD-PE fluorescence by Trolox but not α-tocopherol was provided evidence that Trolox was more highly associated with the water pool of the DOPC reverse micelles. Increasing the concentration of both α-tocopherol and Trolox increased the stability of bulk stripped soybean oil containing DOPC reverse micelles. Hydrophilic Trolox had better antioxidant activity than hydrophobic α-tocopherol. This could be due to Trolox’s ability to more highly associate with the water phase of the reverse micelles where lipid oxidation is prevalent.

Since α-tocopherol and Trolox both associate with the DOPC reverse micelles, it is possible that they could also interact with iron at the oil-water interface as proposed in Figure 6.1. High-valence (FeIII) and low-valence-state (FeII) iron could interact with Trolox and α-tocopherol) causing their depletion or increasing the reactivity of iron if the antioxidant caused iron reduction. However, DOPC reverse micelles minimized iron-
antioxidant interactions as seen by their ability to decrease iron-promoted \( \alpha \)-tocopherol and Trolox degradation as well as decrease the ability of the antioxidants to reduce Fe(III) to Fe(II). It is unclear how the DOPC reverse micelles decrease interactions between iron and \( \alpha \)-tocopherol or Trolox. It does not seem to be due to physical hindrance since the reverse micelles protected both hydrophilic Trolox which would concentrate in the aqueous phase and hydrophobic \( \alpha \)-tocopherol which would concentrate in the lipid phase. Therefore, protection could be due to the phosphate group that would bind iron and decrease its ability to interact with the antioxidants.

**Figure 6.1.** Proposed mechanism of bulk oil oxidation that contains reverse micelles

Overall, this research identified several important new insights into potential lipid
oxidation mechanisms in bulk oil. First, it showed that DOPC can form physical structures such as reverse micelles in vegetable oils at the concentrations of phospholipids and water found in commercially refined oils. These physical structures increase lipid oxidation rates especially oxidation promoted by iron. Antioxidants were also shown to associate with the DOPC reverse micelles with the more hydrophilic Trolox associating with the association colloids than α-tocopherol. The evidence of acceleration of oxidation by DOPC reverse micelles suggests that these physical structures could be a major site for oxidation reactions which could help explain by hydrophilic antioxidants are often more effective than hydrophobic antioxidants in bulk oils.

The results of this research also identified a number of areas where future experiments would be beneficial in developing knowledge of how association colloids impact bulk oil oxidation mechanisms and could present new opportunities for inhibiting lipid oxidation:

(i) Investigation the impact of free fatty acids on bulk oil oxidation containing association colloids;

(ii) Detection dissolved oxygen in oil and its role on iron redox recycling;

(iii) Identification the reaction products between α-tocopherol or Trolox and Fe(III) and their role on bulk oil oxidation;

(iv) Investigation the relationship between lipid hydroperoxides generation, decomposition and the iron recycling in bulk oil system.

(v) Investigation the impact of other phospholipids, such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) on bulk oil oxidation, specifically on the physical
structure formation and chemical stability.

The knowledge gained from this study will improve our understanding of causes and prevention of lipid oxidation in bulk oils. It suggests that DOPC reverse micelles can alter the microenvironment where chemical degradation reactions occur, such as lipid oxidation. In addition, lipid oxidation in bulk oils is not only influenced by the traditional chemical factors, such as fatty acid composition, transition metals and antioxidants, but is also related to the existence of physical structures. Understanding how these physical structures impact lipid oxidation and antioxidant mechanisms could lead to the development of new antioxidant technologies and/or methods to use existing antioxidants more effectively.

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