UNDERSTANDING THE IMPACT OF OXYGEN CONCENTRATION AND ACTIVE PACKAGING ON CONTROLLING LIPID OXIDATION IN OIL-IN-WATER EMULSIONS

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UNDERSTANDING THE IMPACT OF OXYGEN CONCENTRATION AND ACTIVE PACKAGING ON CONTROLLING LIPID OXIDATION IN OIL-IN-WATER EMULSIONS

A Dissertation Presented

By

DAVID RYAN JOHNSON

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 2017

Food Science
UNDERSTANDING THE IMPACT OF OXYGEN CONCENTRATION AND ACTIVE PACKAGING TO CONTROL LIPID OXIDATION IN OIL-IN-WATER EMULSIONS

A Dissertation Presented

By

DAVID R JOHNSON

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Department of Food Science
ACKNOWLEDGMENTS

First and foremost, I would like to thank the University of Massachusetts for maintaining an atmosphere of growth, opportunity, and collaboration that has shaped me into the individual I am today. It truly is a remarkable place. I first stepped foot on this campus nearly 10 years ago, and since then UMass has provided me with amazing opportunities. Leading me in this way, I have been fortunate enough to have great advisers/mentors that took an active role in my education (especially Dr. Eric Decker, Coach Ken O’Brien, & Dr. Julie Goddard).

I cannot forget the support of Dr. McClements, Dr. Xiao, and Dr. Autio who asked me thought provoking questions and gave great advice. A special thanks to Jean, Fran, Debbie, and Stacy who have made my graduate student life at UMass much less complicated. I would also like to thank PepsiCo for their sponsorship and the American Oil Chemists’ Society for their support. As well as, the following companies for providing assistance and materials to support academic research: Ocean Optics Inc., DSM Nutritional Products, and Mitsubishi Gas Chemical.

The best part about the UMass Food Science Department is the collaboration and that extends to the student relationships as well. I am forever grateful for the help and friendship of Leann Barden, Bingcan Chen, Dan Vollmer, Raffaella Inchingolo, Sibel, Maxine Roman, Dana Wong, Kyle Landry, Fang Tian, Rika Homma, Jen K., Sam, Julia Gisder, Said Toro Uribe, and all of the lab members that taught me something along the way. A special thank you to my family who has always encouraged and supported me in my pursuit of higher education. Go UMass!
ABSTRACT

UNDERSTANDING THE IMPACT OF OXYGEN CONCENTRATION AND ACTIVE PACKAGING ON CONTROLLING LIPID OXIDATION IN OIL-IN-WATER EMULSIONS

MAY 2017

DAVID RYAN JOHNSON, B.S., UNIVERSITY OF MASSACHUSETTS AMHERST

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Directed by: Professor Eric A. Decker

Consumer concern over synthetic food antioxidants have led researchers to seek alternative natural, or ‘clean’ label, solutions to prevent lipid oxidation. Unfortunately, natural antioxidants are often not as effective as their synthetic counterparts. As a result, there remains a need to develop active packaging strategies and maximize current antioxidant strategies in food applications.

Active packaging, or packaging that has a function beyond being an inert barrier, is an attractive strategy to limit lipid oxidation in foods. Active packaging performs the work of an antioxidant without appearing on the package label. The work presented here builds upon active packaging development as a means to control prooxidant metals in oil-in-water (O/W) emulsions. A metal-chelating active packaging material was designed and characterized, in which biomimetic poly(hydroxamic acid) (PHA) metal-chelating moieties were grafted from the surface of a common industry used plastic, poly(ethylene terephthalate) or PET. Surface characterization of the plastic film by spectroscopy and scanning electron microscopy (SEM) revealed successful grafting and conversion of the
plastic to contain metal chelating group on the surface. Metal analysis and lipid oxidation studies demonstrated the activity of the PHA grafted PET films to inhibit metal-promoted oxidation in acidified O/W emulsions.

Oxygen removal from food packaging is another practical solution to stabilize foods without additives. Indeed, manufacturers have been using this technique for decades, however there is little evidence to how much oxygen needs to be removed to provide meaningful increases in oxidative stability. Results from this research suggest that the oxidative stability of 1% O/W emulsions can be extended by reducing system oxygen by ~58%, but to have a meaningful antioxidant impact greater than ≥93% oxygen removal is required. Further investigation into simulated commercial oxygen removal strategies (e.g., nitrogen displacement of oxygen and ascorbic acid) demonstrated that current industrial strategies are lacking and need to be optimized in order to enhance stability against lipid oxidation.

Incorporating both strategies, active packaging and oxygen removal, a commercial oxygen scavenging packaging was tested in its ability to reduce packaged oxygen, inhibit lipid oxidation in O/W emulsions, and stabilize oxygen-sensitive vitamins. Dissolved and headspace oxygen measurements determined the material’s ability to scavenge >95% system oxygen across conditions such as pH, sodium chloride, and fat content. Active oxygen scavenging packaging was able to inhibit lipid oxidation in O/W emulsions as well as preserve Vitamin E and Vitamin C in model solutions. This work demonstrates that active packaging and oxygen reduction are promising strategies that warrant more research in their ability to help achieve ‘clean label’ solutions to prevent lipid oxidation in foods.
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LIST OF COMMON ABBREVIATIONS

AA: ascorbic acid

ATR-FTIR: attenuated total reflectance Fourier transform infrared spectroscopy

Brij 35: a nonionic surfactant

EDTA: ethylenediaminetetraacetic acid

ICP-MS: inductively coupled plasma mass spectroscopy

O/W: oil-in-water emulsion

OS: oxygen scavenging

PET: poly(ethylene terephthalate)

PHA: poly(hydroxamic acid)

PHEA: poly(hydroxyethyl acrylate)

PUFAs: Polyunsaturated fatty acids

ROS: Reactive oxygen species

SEM: scanning electron microscopy

TBARS: thiobarbituric acid reactive substances, secondary lipid oxidation product
CHAPTER 1

INTRODUCTION

Lipid oxidation of food products leads to quality loss and can negatively impact human health through the generation of toxic reaction by-products and destruction of essential nutrients. In general, the oxidative susceptibility of food products is increasing because of food industry trends to remove stable hydrogenated oils and use more bioactive unsaturated fatty acids. Addition of antioxidants to foods can provide oxidative stability by controlling prooxidant metals, scavenging oxygen, or quenching free radicals (Decker et al. 2010). However, growing consumer demand for removal of synthetic additives has led researchers to seek alternative natural solutions to prevent lipid oxidation (Pokorný 2007). Unfortunately, natural antioxidants are often not as effective as their synthetic counterparts and expensive government approval for food additives makes it difficult for new antioxidants to emerge (Medina et al. 1999). Thus, there is a need to leverage a combination of current antioxidant strategies, acting via distinct mechanisms, to control oxidative reactions in targeted applications. Specifically, the development of metal chelating packaging materials and the impact of oxygen on lipid oxidation reactions were studied to better understand strategies to prevent lipid oxidation. A literature review on the role of oxygen in lipid oxidation reactions was also performed to guide research capable of defining how to use oxygen reduction as an antioxidant strategy.

The first research study describes the design and synthesis of poly(ethylene terephthalate) (PET) active packaging films covalently grafted with biomimetic iron chelating groups (hydroxamic acid). Active packaging refers to modified plastic’s ability
to have a function besides simple containment of the food. In this case, the non-migratory nature of the common plastic PET active packaging described may offer an opportunity to maintain product quality without the addition of food additives. The covalent grafting of hydroxamic acid groups was accomplished in a two-step UV-initiated process without the use of a photoinitiator. Successful non-migratory grafting was suggested by attenuated total reflectance Fourier transform infrared (ATR-FTIR) and scanning electron microscopy (SEM) imaging. Further, the resulting material demonstrated the ability to bind iron and more than doubled the oxidative stability of soybean oil-in-water (O/W) emulsions under acidified conditions (pH 3.0). The significance of this work is it presents a possible alternative for the effective synthetic metal chelator ethylenediaminetetraacetic acid (EDTA) as a food additive.

Oxygen control of the packaging environment offers another means to control lipid oxidation. The oxygen content of a food system, whether dissolved or in the headspace, is one of the most important factors influencing the rate and extent of lipid oxidation. Food manufacturers typically increase lipid oxidative stability by oxygen removal, such as vacuum packaging or flushing the food package with an inert gas (e.g., nitrogen). Oxygen removal is an attractive strategy for the food industry because it has potential to improve shelf life without the use of food additives. Research has historically looked at the impact of either headspace or dissolved oxygen levels on lipid oxidation rates. In this work, a fish oil-in-water (O/W) emulsion system was used as a model to create different total packaged oxygen concentrations using custom blends of nitrogen and oxygen. The resulting emulsions were characterized over time by non-destructively measuring oxygen concentrations, both in the headspace and dissolved phase, and the
impact of oxygen concentrations on lipid oxidation kinetics was measured. Further, the efficacy of simulated commercial strategies to remove oxygen was investigated. The significance of this work is that there is a global demand to remove synthetic additives from food and beverage formulations, and as described in this work, the level of oxygen reduction is defined that provides meaningful increases in oxidative stability. Results suggest that near complete oxygen removal is necessary, however strategies need to be identified that can reach these low oxygen concentrations in oil-in-water emulsion systems.

Consequently, elucidating the conditions under which antioxidants can be innovatively combined is critical to the successful inhibition of lipid oxidation and removal of synthetic antioxidants. Future research will focus on the impact that reduced oxygen levels used in combination with water-soluble or lipid-soluble antioxidants have on lipid oxidation kinetics. Further investigation will also test the impact of total oxygen concentration on bulk oil and define levels that will have a protective effect against oxidation. If successful, these future studies could help guide design of antioxidant solutions that are multifaceted and cost effective.

1.2 References


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CHAPTER 2

LITERATURE REVIEW: THE ROLE OF OXYGEN IN LIPID OXIDATION REACTIONS

2.1. Abstract

The susceptibility of food oil to quality loss is largely determined by the presence of oxygen. This article reviews the current understanding concerning the effect of oxygen types, location, and concentration on the oxidative stability of foods. It also discusses the major factors that influence the interaction between oxygen and lipids such as antioxidants, prooxidants, reactive oxygen species (ROS), environmental conditions, and oxygen scavengers. Research has shown that the amount of oxygen needed to cause oxidation is generally very small and that by reducing oxygen concentration in containers to less than 2%, oxidative stability can be greatly enhanced. However, very few studies have systematically examined the oxygen levels needed to reduce, or inhibit, lipid oxidation processes. Thus, a more comprehensive understanding of the relationship between oxygen levels and lipid oxidation is necessary for the development of innovative antioxidant solutions and package designs that prolong the quality of foods containing lipids.

Keywords lipid oxidation, dissolved oxygen, headspace oxygen, oxygen scavenging, antioxidant solutions

2.2. Introduction

Lipid oxidation is the principal deteriorative reaction during food processing and storage that limits shelf life of most microbiologically stable foods (Andersson & Lingnert 1997). The oxidation of food products not only sharply curtails shelf life, but
can also pose hazards to human health. The generation of toxic reaction by-products and the destruction of essential vitamins during oxidation are responsible for negatively impacting the safety and wholesomeness of foods (Kubow 1992). In general, the oxidative susceptibility of food products is increasing because of food industry trends to remove hydrogenated oils, use bioactive unsaturated fatty acids, and replace potent synthetic antioxidants with ones of natural origin. Specifically, the incorporation of unsaturated fatty acids presents a difficult challenge in foods because unsaturated fatty acids are prone to deleterious chemical reactions with oxygen. Oxygen, a diatomic gaseous molecule ubiquitously present in the atmosphere, is free to interact with reactive unsaturated fatty acids and is ultimately responsible for quality deterioration of lipids (McClements & Decker 2000). Two forms of oxygen are responsible for the different chemical mechanisms involved in the oxidation of edible oils. The more reactive form, singlet oxygen ($^{1}\text{O}_2$), can quickly promote lipid oxidation in foods containing photosensitizers. The more common form, triplet oxygen ($^{3}\text{O}_2$), is responsible for the free radical chain reaction of lipid oxidation. Both oxidative pathways produce undesirable low molecular weight volatiles that have human sensory threshold values as low as 0.01 ppb, as seen in fish oils (Hsieh & Kinsella 1989). Extremely low sensory thresholds of lipid oxidation products demand the prevention of even minimal lipid degradation. As a result, efforts made to inhibit oxidative reactions in food products should aim to extend the lag time prior to generation of volatile lipid oxidation products.

Antioxidant supplementation is a common strategy for avoiding oxidative reactions in the processing and storage of food. Historically, antioxidants have been used in food systems to prevent or mediate oxidative damage by scavenging free radicals.
before the propagation phase of lipid oxidation (Ahn et al. 1993). However, an industry
trend to replace powerful synthetic antioxidants with natural compounds has recently
emerged, and these natural antioxidants have proven to be a less effective solution to
limit oxidation. Now, innovative solutions are needed to maintain the oxidative stability
of edible oils. Because oxygen is essential for the degradation of lipids, a more complete
understanding of oxygen’s role and reactivity in lipid-containing systems will lead to a
successful design of alternative, effective antioxidative solutions.

2.3. Lipid Oxidation Mechanism

The classical scheme of lipid oxidation involves three stages: initiation, propagation, and termination (Scheme 2.1). Deterioration of fatty acids is initiated when
a hydrogen atom is abstracted from a fatty acid, forming an alkyl radical (L•). Free
radicals on unsaturated fatty acids can form via hydrogen abstraction mechanisms from
initiating free radicals (e.g., hydroxyl radicals) present in foods. Hydrogen abstraction
depends on the bond dissociation energy of the covalent carbon-hydrogen bond, which is
weakened by adjacent electron-rich double bonds in unsaturated fatty acids.

Scheme 2.1

Polyunsaturated fatty acids (PUFAs) are particularly susceptible to hydrogen abstraction
due to the presence of single or multiple methylene-interrupted hydrogens with lower
bond dissociation energies (Table 2.1). After alkyl radical formation, π electrons of the
double bond stabilize the free radical through delocalization over a conjugated diene
structure converting one of the double bonds to the more stable \textit{trans} form. Once a lipid radical has formed, triplet oxygen ($^3\text{O}_2$) reacts quickly with the alkyl radical at a rate of $2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$ under normal oxygen pressure to form a covalent bond with the lipid (Zhu & Sevilla 1990).

Table 2.1. Bond dissociation energy for carbon-hydrogen covalent bond on fatty acids (McClements & Decker 2008)

<table>
<thead>
<tr>
<th>Carbon-hydrogen covalent bond</th>
<th>Bond dissociation energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated (aliphatic chain)</td>
<td>98</td>
</tr>
<tr>
<td>Monounsaturated (adjacent to double bond)</td>
<td>89</td>
</tr>
<tr>
<td>Polyunsaturated (methylene-interrupted carbon)</td>
<td>80</td>
</tr>
</tbody>
</table>

The resulting peroxyl radical ($\text{LOO}^\bullet$) is able to abstract another hydrogen from an unsaturated lipid to form hydroperoxides (LOOH) and propagate the free radical chain reaction by producing another free radical on the second fatty acid. Notably, lipid hydroperoxides do not cause rancidity. Rather, lipid hydroperoxides can be broken down by $\beta$-scission reactions (triggered by light, heat, or transition metals) into aldehydes, ketones, acids, esters, and alcohols that are associated with off-flavors. Eventually, two radicals may react to form a nonradical species that terminates the radical chain reaction.

The damage caused by oxygen incorporation has been studied over the past 60 years (Frankel 2005, Labuza 1971); however, it remains unclear how to limit its reactivity in different foods. Therefore, this review focuses on the role of oxygen in lipid reactions and available strategies to enhance the oxidative stability of foods.

2.4. Factors that Influence Lipid Oxidation

2.4.1 Lipid Compositions

Foods exist as complex chemical systems. There are numerous elements within
and surrounding foods that can often interact in various ways with one another to either promote or inhibit lipid oxidation, a phenomenon that highlights the difficulty in isolating a single cause of deterioration. Lipid composition, minor components of the lipid and aqueous phases, and food environment are some of the major factors determining the oxidative susceptibility of lipids. The chemical structure of the lipid substrate largely determines the oxidative stability of foods. Lipids that possess higher degrees of unsaturated double bonds are more prone to oxidation. For example, the relative oxidation rates for fatty acids containing 18 carbons (18:1, 18:2, 18:3) that differ only in the number of double bonds is 1, 12, 25, respectively (Holman & Elmer 1947). As previously mentioned, the presence of double bonds lowers the energy needed to abstract allylic hydrogens and enhances the rate of lipid radical chain reactions. Until recently, unsaturated lipids were commonly catalytically hydrogenated to remove double bonds and increase oxidative stability. However, insights into the health benefits of PUFAs and the negative effects of trans fatty acids derived from the hydrogenation process have since left food scientists searching for novel ways to incorporate and protect unsaturated fatty acids in food products.

2.4.2 Minor Components

Minor components within the food system interact in a complex balance between antioxidant and prooxidant factors. Minor components that function as antioxidants inhibit or slow oxidative processes. The inhibition of the lipid free radical chain reaction during oxidation is of practical importance because it can limit the extent of degradation of unsaturated fatty acids. Antioxidants inhibit oxidation in foods by inactivating free radicals, quenching oxygen and photosensitizers, and chelating prooxidative metal ions.
(Choe & Min 2009). Tocopherols, carotenoids, ascorbic acid, and phenolics are major antioxidant components commonly found in foods (Frankel 2005). Yet, antioxidant activity is highly dependent on the concentration and location of the antioxidant within foods. In some cases, the same antioxidant that inhibits oxidation in one food system may impart a prooxidative effect in another. For instance, ascorbic acid (1,161 μM) added to a bulk stripped corn oil was shown to exhibit antioxidant activity by inhibiting the formation of the secondary lipid oxidation product, hexanal (Frankel et al. 1994). However, ascorbic acid added at a low concentration (50 μM) to an oil-in-water emulsion system resulted in a prooxidant effect (Alamed et al. 2009). One explanation for this phenomenon is that in the bulk oil system high levels of ascorbic acid concentrate at the oil-water interfaces and limit oxidation by free radical scavenging activity. On the contrary, at lower concentrations, in oil-in-water emulsions, ascorbic acid is dispersed throughout the matrix, and its free radical scavenging activity is outweighed by its strong reduction potential, which redox cycles transition metals to their more active prooxidative state (Alamed et al. 2009, Decker & Hultin 1992). Alternatively, ascorbic acid might not be prooxidative in bulk oils because iron is not a major prooxidant. Clearly, the overall antioxidant-prooxidant balance regarding location and concentration is important to consider when minor components are supplemented in foods.

Although antioxidant components contribute to inherent oxidative stability of edible oils, foods are constantly exposed to prooxidants that threaten to destabilize oxidative stability. Transition metals, such as iron and copper, are minor components found in foods that can serve to both initiate and accelerate lipid oxidation. Oxidation can be initiated by transition metals through electron transfer reactions with $^{3}$O$_{2}$ and lipids
that produce reactive oxygen species (ROS) or alkyl radicals, respectively (Scheme 2.2).

Iron and copper can accelerate oxidation when they undergo redox cycling to decompose hydroperoxides into reactive lipid radicals, as shown in Scheme 2.2.

Scheme 2.2.

Although copper is more reactive at promoting lipid breakdown, iron is much more abundant in both aqueous and oil environments. Iron was found in tap water at 200 μg * L\(^{-1}\) \(^{-1}\), whereas copper was at 20 μg * L\(^{-1}\) \(^{-1}\), and iron concentrations were at levels 100–1,000 times higher than copper in bulk olive and soybean oils (Choe & Min 2006, Hu et al. 2002). Importantly, iron is still a contaminant in bulk oils after refining at levels high enough (0.20 ppm iron in soybean oil) to cause significant oxidative instability (Sleeter 1981). Additionally, many foods are fortified with iron to supplement health; thus, its inclusion in foods contributes to potential prooxidative effects. Mancuso et al. (1999) clearly demonstrated the prooxidant effects of iron in oil-in-water emulsions when addition of apo-transferrin, a highly iron-specific binding transport protein, nearly resulted in complete inhibition of lipid oxidation in Tween 20–stabilized salmon oil-in-water emulsions. As with other transition metals, iron can exist in multiple chemical oxidation states. The ferrous (Fe\(^{+2}\)) state is an order of magnitude more reactive at catalyzing LOOH breakdown than the ferric state (Fe\(^{+3}\)) (Choe & Min 2006). Thus, reducing compounds found in foods can promote lipid oxidation by redox cycling.
transition metals to their more reactive state. The effect of iron’s oxidation state can be observed from oxygen consumption data in a liposome system following addition of ferrous iron. Consumption of oxygen, following addition of Fe$^{+2}$, increased exponentially as ferrous iron promotes the generation of fatty acid radicals that allow oxygen to form peroxyl radicals (Mozuraityte et al. 2008). Kristinova et al. (2014) showed that one ferrous molecule could promote the consumption of five oxygen molecules via lipid oxidation reactions in a herring oil emulsion system.

2.4.3 Temperature

Environmental factors, such as temperature, can either increase or decrease lipid oxidation rates depending on the system. For instance, in most foods a 10°C increase in temperature corresponds to approximately a doubling in the rate of oxidation (Matthäus 2010). Elevated temperatures can increase lipid oxidation rates by promoting hydroperoxide breakdown in a process that generates free radicals. Accordingly, an effective way to control lipid oxidation is to decrease the storage temperature of the oil. Lowering the storage temperature of fish oil from +4°C to −18°C showed a decrease in both primary and secondary lipid oxidation products, nearly doubling the shelf life of the fish oil (Boran et al. 2006). Unfortunately, low-temperature storage is not suitable for all foods. For example, low temperatures can cause solidification of the oil resulting in changes in appearance (cloudiness), inability to pour, and disruption of oil-in-water emulsions (McClements & Decker 2008). In general, high temperatures will increase free radical formation, but this may not result in immediate alterations in food quality because most foods contain inherent antioxidants that provide a natural resistance to oxidation. Therefore, the impact of high-temperature processing is not affected by temperature
alone, but also the duration of time that temperature is applied. Broncano et al. (2009) demonstrated during meat roasting that heating for a longer time at a lower temperature caused a higher increase in secondary lipid oxidation products compared to heating at a higher temperature for a shorter time. Other studies (Rastogi et al. 2006, Santos et al. 2013) support the finding that lipid oxidation during cooking processes may be more affected by longer cooking times and lower temperatures than higher temperatures and shorter cooking times. There exist instances when an extreme increase in temperature actually decreases the development of oxidative rancidity, such as in heated frying oils that are not being actively used to cook foods. In this situation, elevated temperatures decrease oxygen levels so that oxygen is not available for the formation of hydroperoxides, and the oxidation process results in termination reactions between alkyl radicals (Matthäus 2010). This reaction yields fatty acid polymers that do not impact flavor, unlike volatiles derived from hydroperoxide decomposition (Choe & Min 2007). The oxidative stability resulting from decreased amounts of oxygen in unused, heated frying oil shows that molecular oxygen is ultimately responsible for the deterioration of edible oils. The effects of oxygen in lipid oxidation reactions are discussed further in subsequent sections.

2.5 Oxygen Species

2.5.1 Triplet Oxygen

An important step in lipid oxidation occurs when atmospheric triplet oxygen ($^3$O$_2$) reacts with a fatty acid radical. The chemical reactivity of triplet oxygen with fatty acid radicals is more easily understood when the state of oxygen’s electron molecular orbitals
is considered (Figure 2.1). Abiding by Hund’s Rule, oxygen most commonly exists as a biradical in its ground state ($^3\text{O}_2$) that results in two unpaired electrons with parallel spins (Halliwell & Gutteridge 1990). The nomenclature for the so-called triplet and singlet electronic states is derived from the molecule’s spin multiplicity, defined as $2S+1$, where $S$ is the total spin number. Depending on the angular direction of the electronic spin, the $S$ value can be either $+1/2$ or $–1/2$. If oxygen’s two unpaired electrons have parallel spins, then the spin multiplicity will be $2(1/2 + 1/2) + 1 = 3$, or simply, in the triplet state. Electrons that have antiparallel spin values within the same orbital will cancel each other out, which results in the singlet state. Because the electronic configuration of unsaturated fatty acids exists in the singlet state, its reaction with triplet oxygen would result in an unlikely highly endothermic reaction and is thus an improbable occurrence (Korycka-Dahl & Richardson 1978). Therefore, oxidation of edible oils first requires the formation of a free radical to overcome electronic spin restrictions and make the reaction between oxygen and the fatty acid thermodynamically favorable.

Figure 2.1. Molecular orbitals of singlet and triplet oxygen. Singlet oxygen addition to linoleic acid’s electron-rich double bonds in photosensitized oxidation (redrawn from McClements & Decker 2008).
2.5.2 Reactive Oxygen Species

Reactivity of triplet oxygen with unsaturated fats is forbidden because of spin restrictions, but spin restrictions can be overcome by the generation of ROS (Hsieh & Kinsella 1989). ROS are formed when oxygen is reduced during oxidation of other molecules. Hydroxyl (HO•), peroxyl (ROO•), alkoxyl (RO•), and hydroperoxyl (HOO•) radicals, as well as the superoxide anion (O2−), are ROS that can accelerate lipid oxidation in foods (Choe & Min 2005). Hydroxyl radicals are ROS primarily formed from degradation of H2O2 by UV light and metals (Gutteridge 1995). Hydroxyl radicals are important initiators in lipid oxidation, abstracting hydrogen from a lipid and creating a carbon-centered radical on the alkyl chain. As previously discussed, peroxyl and alkoxyl radicals function as intermediates in the propagation phase of lipid oxidation. The high-energy peroxyl and alkoxyl radicals can abstract hydrogens from neighboring lipids, or in the case of alkoxyl radicals can break the aliphatic chain of a fatty acid.

Other ROS in foods indirectly influence lipid oxidation through enhancement of prooxidant activity or as precursors to free radicals. The superoxide anion is the result of a one-electron reduction of triplet oxygen forming a radical anion (O2−). The superoxide anion can be produced in foods enzymatically (xanthine oxidase and nicotinamide adenine dinucleotide phosphate oxidase), by photoactivation of tetrapyrroles (e.g., hemoglobin and chlorophyll), or by irradiation that allows hydrated electrons to reduce triplet oxygen (Choe & Min 2005). The superoxide anion itself is not an initiator of free radical lipid oxidation because its reduction potential is too low to abstract hydrogen from unsaturated fatty acids (Bielski et al. 1983). However, the superoxide anion can reduce transition metals to a more active state, thus promoting lipid oxidation. The
superoxide anion can also become protonated at low pH to form hydroperoxy radicals that carry a reduction potential capable of abstracting hydrogen from unsaturated fatty acids. To limit the initiating and propagating effect of the superoxide anion, almost all biological tissues contain superoxide dismutase, which converts the superoxide anion into triplet oxygen and hydrogen peroxide (McClements & Decker 2008).

### 2.5.3 Singlet Oxygen

Photosensitized oxidation differs from oxidation by triplet oxygen because it can produce high-energy oxygen species that abstract hydrogen atoms from unsaturated fatty acids, or ROS, without formation of a carbon-based free radical (Figure 2.1). Photosensitizers such as chlorophyll, flavins (e.g., riboflavin), and myoglobin absorb light in the visible or near-UV region and become electronically excited (Frankel 2005). Depending on the concentration of oxygen, the electronically excited sensitizer can cause photosensitized oxidation by two distinct mechanisms. Under lower oxygen concentrations, type I mechanisms proceed with the photosensitizer possessing enough energy to independently abstract a hydrogen atom from a fatty acid generating a free radical. Reaction products of type I mechanisms are consistent with those of nonsinglet oxygen–promoted oxidation, and the photosensitizer acts as a free radical generator (Min & Boff 2002). Type II reactions occur when oxygen is readily available and involve the transfer of energy from the photosensitizer to oxygen. Energy transfer to oxygen causes the spin of 2p orbital electrons to shift into opposing directions, thereby generating singlet oxygen ($^1\text{O}_2$).

Singlet oxygen may exist in one of five configurations with the $^1\Delta$ configuration, indicating two antiparallel electrons existing in one orbital, which is the most common in
food (Choe & Min 2006). The higher electronic state of singlet oxygen creates an energy difference of 22.5 kcal above the triplet state of oxygen (Korycka-Dahl & Richardson 1978). Singlet oxygen’s electron configuration allows it to directly attack the electron-rich double bonds of unsaturated lipids, at a rate 1,500 times greater than triplet oxygen (García-Torres et al. 2009). Andersson & Lingnert (1998) demonstrated the influence of photosensitized oxidation when hexanal, a secondary lipid oxidation product, was produced at concentrations 330 times higher in spray-dried cream stored in fluorescent light compared to a control stored in the dark. In this case, the photosensitizer, riboflavin, present in the cream powder was capable of generating singlet oxygen that enhanced the rate of oxidation. Researchers can detect the presence of singlet oxygen because of its ability to react with the carbons at either end of the unsaturated fatty acid double bond, forming unique lipid oxidation hydroperoxides. For example, in a linoleate system singlet oxygen has the potential to generate four lipid hydroperoxides (conjugated and non-conjugated) compared to the typical two (conjugated double bond configuration) formed in traditional oxidation (Table 2) (Frankel 2005, Rawls & Van Santen 1970).

Table 2.2. Hydroperoxides formed from oxidation of linoleate by singlet and triplet oxygen (modified from Min & Boff 2002).

<table>
<thead>
<tr>
<th>Oxygen type</th>
<th>Lipid hydroperoxides from linoleate</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conjugated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Singlet oxygen</td>
<td>9-OOH</td>
<td>10-OOH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13-OOH</td>
<td>12-OOH</td>
<td></td>
</tr>
<tr>
<td>Triplet oxygen</td>
<td>9-OOH</td>
<td>Minimal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13-OOH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Rawls & Van Santen (1970) reported that photosensitized oxidation is temperature independent as a result of the low activation energy (0 to 6 kcal/mol) required for singlet oxidation of edible oils (Choe & Min 2006). Thus, at lower temperatures singlet oxygen
threatens oxidative stability because of its potential to initiate lipid oxidation reactions.

Given singlet oxygen’s potential to initiate lipid oxidation, control measures are needed to protect foods. Besides packaging that excludes light, singlet oxygen quenchers are the most effective means of preventing photosensitized oxidation. Quenchers function by either physical or chemical mechanisms to inactivate singlet oxygen. Physical quenching returns singlet oxygen, or an excited photosensitizer, to its ground state without generating oxidized products or consuming oxygen (Min & Boff 2002).

Carotenoids are a diverse group of yellow to red colored singlet oxygen quenchers (e.g., β-carotene, lycopene, and lutein) that physically inactivate $^1$O$_2$ by absorbing energy over nine or more conjugated double bonds. The absorption produces an excited-state carotenoid that vibrates to transfer the absorbed energy to the surrounding system giving off heat. Lee & Min (1990) found that carotenoids could protect against photosensitized oxidation in soybean oil and that antioxidant effectiveness increased with the number of conjugated double bonds. β-Carotene is the most common carotenoid in foods and one of the most potent singlet oxygen quenchers, with 1 mol inactivating between 250 and 1,000 molecules of singlet oxygen (Foote et al. 1970). Tocopherols are another class of singlet oxygen quenchers that physically convert singlet oxygen to triplet oxygen by charge transfer and electron donation (Kamal-Eldin & Appelqvist 1996). Carotenoids and tocopherols, as well as ascorbic acid, can also chemically quench singlet oxygen, which involves singlet oxygen attacking the double bond of the antioxidant. However, chemical quenching consumes both oxygen and the quencher to generate an oxidized antioxidant. For carotenoids, protection by physical quenching is preferred because chemical inactivation of singlet oxygen rapidly degrades color (Min & Boff 2002).
2.6 Oxygen in Foods Containing Lipids

2.6.1 Oxygen Location

Oxygen is the primary reactant in lipid oxidation reactions that cause rancidity in foods. The most abundant and stable form of oxygen, triplet oxygen, can be present as a dissolved gas within the food matrix, as nondissolved gas in the headspace above the food, or as a nondissolved gas trapped in solid or semisolid matrices (Figure 2.2) (e.g., whipped cream, mayonnaise, processed meats). As a dissolved gas, the proximity and accessibility of dissolved oxygen to lipids reduce oxidative stability.

Figure 2.2. Location of oxygen in an enclosed food package.

Specifically, dissolved oxygen content has been correlated with oxygen disappearance, generation of free radicals, and formation of volatile lipid oxidation products in food (Min & Wen 1983, Parenti et al. 2007). Dissolved oxygen concentration follows Henry’s Law, meaning that at equilibrium the solubility of oxygen is directly proportional to the partial pressure of the oxygen above the food (Ke & Ackman 1973). In addition to pressure effects, the food’s composition influences the level of dissolved oxygen. Oxygen is approximately 5–10 times more soluble in bulk oil than pure water at 20°C, with
saturation occurring at 55 ppm and 5–10 ppm oxygen, respectively (Aho & Wahlroos 1967, Montgomery et al. 1964). In comparison to the same volume of water, atmospheric air contains approximately 30 times more oxygen (Andersson 1998). The solubility of oxygen in food is temperature dependent, with an increase in temperature driving oxygen from the dissolved state into the headspace. However, at temperatures greater than 60°C, dissolved oxygen concentrations decrease rapidly by both heat-accelerated oxidation reactions and reduced oxygen solubility (Chen et al. 2011).

Headspace oxygen is the nondissolved gas present above the foodstuff. Although headspace oxygen has less surface area contact with lipids than dissolved oxygen, it still poses a threat to lipid oxidation as a reservoir of oxygen (Quast & Karel 1971). Oxygen can be transported from the headspace and dissolved in the food by either diffusion or mechanical agitation (Andersson 1998). Oxygen diffusion into food is driven by a concentration differential between headspace and dissolved oxygen. Assuming that a steady state has been reached between diffusion and oxygen consumption (e.g., lipid oxidation reactions), the oxygen concentration in the oil can be expressed as

\[
\frac{dc}{dt} = \frac{DS}{VZ} (c_0 - c) - kc = 0,
\]

where \(c\) is the oxygen concentration, \(D\) is the diffusion coefficient, \(S\) is the oil-oxygen interfacial area, \(V\) is the volume of the oil, \(Z\) is the thickness of the diffusion film in the oil, and \(k\) represents the rate constants of oxidative processes occurring in the induction phase of lipid oxidation (Šimon et al. 2000).

Moreover, the rate that oxygen can travel through a medium, referred to as diffusivity, is quantified by diffusion coefficients. Davidson et al. (1952) found that olive oil had an oxygen diffusion coefficient of \(0.75 \times 10^{-9} \text{ m}^2 \text{s}^{-1}\), whereas lard’s rate of oxygen transfer was found to be \(0.40 \times 10^{-9} \text{ m}^2 \text{s}^{-1}\). Whether this difference was due to the
medium’s viscosity or is the result of oxidative reactions interfering with the measurement is difficult to determine. What is certain is that the food composition and environmental conditions affect the location of oxygen and its ability to diffuse into a food.

2.7. The Effect of Oxygen Concentration

Reducing the amount of oxygen within foods has been widely regarded as an effective solution to retard lipid oxidation (Chen et al. 2011, Choe & Min 2006, McClements & Decker 2000). The reaction between triplet oxygen and an alkyl radical proceeds at a very fast, diffusion-limited rate with a rate constant of \( \sim 10^9 \text{ M}^{-1} \text{s}^{-1} \) at normal oxygen pressure. This reaction is fast because no quantum mechanical, thermodynamic, or kinetic barriers exist between the unpaired electrons of oxygen and the alkyl radical (Dunford 1987). Researchers hypothesize that at higher concentrations of oxygen the oxidation rate is not limited by oxygen diffusion, but rather is dependent on the lipid substrate and/or prooxidant concentrations (Labuza 1971, McClements & Decker 2008). Evidence suggests that if the oxygen concentration is reduced significantly, then the rate of lipid oxidation could be limited. Unfortunately, there is a lack of comprehensive studies detailing the effect of concentrations on the rate and extent of lipid oxidation.

Many different units have been used to describe the concentration of oxygen, but because most authors express oxygen as a percentage of the total atmosphere this review uses the term percentage (\%) to refer to oxygen concentration in the headspace, instead of oxygen partial pressure. Headspace oxygen concentrations as a percentage describe the oxygen volume/volume concentration at 20°C and standard pressure (101.3 kPa),
whereas dissolved oxygen is reported as ppm. Marcuse & Fredriksson (1968) demonstrated that lowering the oxygen in the headspace above linoleate from 2.0% to 1.0% decreased oxidation to a greater extent than a reduction from 21% to 2.0%. Their study highlights that lower oxygen concentrations have a higher relative effect on oxidation rates, likely because diffusion-limited reaction rates can only be decreased when oxygen levels are very low. However, as discussed above, the composition and structure of foods determine the likelihood of oxygen-lipid radical interactions. To highlight this, we discuss the relationship between oxygen concentrations and lipid oxidation in different foods below.

### 2.7.1 Bulk Oil

Oxygen in bulk oil systems, either present in the headspace or dissolved in the oil, is a primary determinant of the extent of lipid oxidation. In frying oils, decreased headspace oxygen concentrations have also been shown to enhance stability. Decreasing the concentration of headspace oxygen from 20% to 2% above high-oleic safflower frying oil completely inhibited the formation of carbonyls, a product of lipid oxidation, whereas 20% oxygen increased the carbonyls to greater than 150 meq/kg oil after 30 h (Fujisaki et al. 2000). Additionally, the endogenous tocopherols in the frying oil were completely lost at 20% headspace oxygen concentration, but no detectable change in tocopherol concentration could be seen at 2.0% oxygen. Andersson & Lingnert (1999) showed that in lipid oxidation of rapeseed oil, as measured by oxygen consumption and peroxide value, headspace oxygen concentrations below 0.5% strongly influenced the oxidation rate. The increasing influence of oxygen at low levels is also supported in studies of dissolved oxygen as well. A study conducted by Min & Wen (1983) found that
the rate of dissolved oxygen disappearance in soybean oil was three times as fast at 8.5 ppm oxygen compared to that of soybean oil containing 2.5 ppm oxygen. Limiting the dissolved oxygen content is also important because it appears that dissolved oxygen serves as a starter for oxidation in bulk extra virgin olive oil (Masella et al. 2010). Levels of dissolved oxygen contents commonly found in bulk commercial oils are capable of generating hydroperoxides at a value of approximately 10 meq/kg oil (Przybylski & Eskin 1988). At this peroxide level, the eventual breakdown into secondary reaction products would yield volatiles that would render the food unacceptable to consumers.

2.7.2 Heterogeneous Lipid Systems

Heterogeneous food systems (e.g., muscle foods, emulsions, powders) are inherently more complex than bulk oil; however, the oxidative stability of these foods remains just as dependent on oxygen levels. O’Grady et al. (2000) determined the effect of high-oxygen concentrations on lipid oxidation in packaged minced beef by thiobarbituric acid reactive substances (TBARS). The researchers found that increasing oxygen concentration to 40%, 60%, and 80% total headspace did not show a statistical difference in level of oxidation, whereas 20% oxygen showed lipid oxidation to a lesser extent. The results from this study emphasize that at levels of oxygen greater than atmospheric conditions, lipid oxidation is not limited by oxygen availability. In the case of muscle foods, oxygen accessibility is also limited by diffusivity given that oxygen only penetrates 2–10 mm into the food within 24 h and the rate slows as time progresses (Kilic & Cassens 1998). The oxygen diffusivity limitation explains, in part, why the non-minced packaged meat in O’Grady et al.’s (2000) study experienced less oxidation across all levels of oxygen compared to the minced variety. The processing of muscle foods
produces an increase in surface area and disrupts cellular membranes, which permits an increase in the rate of oxygen diffusion to unsaturated lipids (Andreo et al. 2003). In a similar manner, changes to the molecular environment of emulsified foods also dictate oxidative stability. For instance, at low oxygen concentrations (i.e., below 2.0% oxygen), oxidation reactions in a Tween 20–stabilized oil-in-water emulsion were limited by oxygen diffusion through the aqueous phase (Marcuse & Fredriksson 1968, 1969). Not surprisingly, the shelf life of powdered foods is likewise limited by oxygen concentrations. Andersson & Lingnert (1998) showed that lowering the headspace oxygen level down to 0.03% above powdered cream greatly reduced the formation of secondary lipid oxidation products compared to a control at atmospheric conditions. However, the 0.03% oxygen level in the study was still not low enough to protect the sample from the production of rancid off-flavors as determined by sensory studies after 35 weeks.

Examinations that evaluated the effect of oxygen concentration on lipid oxidation definitively show that the influence of oxygen on lipid oxidation decreases with increasing oxygen concentration. That is, across a myriad of food systems oxygen concentrations below 1–2% strongly dictate the rate of lipid oxidation because of oxidation’s dependency on the diffusion of oxygen. Nevertheless, research suggests that any decrease in oxygen concentration under atmospheric levels (~21%) will result in improved oxidative stability. Additional work is needed to better understand the effect that oxygen concentrations ranging from 21% to 2% have on food systems. A pragmatic strategy is to analyze the effect of very low oxygen concentrations because processes to eliminate oxygen can lower the oxygen level of foods to <1.0–2.0%. Unfortunately, even
this level of oxygen may still be able to trigger oxidative rancidity (Andersson & Lingnert 1997). Considering this, measures must be taken to reduce the content of oxygen within a system to the lowest level obtainable during food processing and packaging.

2.8 Methods to Enhance Oxidative Stability

Currently, most strategies to enhance oxidative stability rely on antioxidants to combat free radicals or prooxidative metals present in food systems. Recent trends to remove highly effective synthetic antioxidants and utilize more natural antioxidants have made this strategy less practical. Therefore, new, or additional, methods of preventing oxidation of lipid components are needed. Oxygen concentration is one of the most important extrinsic factors affecting lipid oxidation, and decreasing it can lead to increased lipid stability. Physical methods for removing oxygen from packaging are routinely employed in the food industry and focus on alteration of the gas contents of packaging.

2.8.1 Processing: Controlling Oxygen Level

Decreasing, and maintaining, low oxygen concentrations can be extremely effective in increasing the shelf life of lipid-containing products. However, one must remember that these techniques are only effective prior to the consumer opening the product, with the exception of single-serve food containers.

2.8.1.1 Nitrogen Flushing/Purging

One strategy to reduce the oxygen concentration of foods containing oil is to flush inert gases, typically nitrogen, into the package to displace oxygen. Nitrogen flushing is
particularly useful for extending oxidative stability in fragile foods (e.g., potato chips) or
displacing the air in the headspace above foods. Lloyd et al. (2009) showed that nitrogen
flushing of packaged whole milk powder could reduce the peroxide and volatile
formation when stored in the dark compared to a nonflushed control. Findings by
Pristouri et al. (2010) show that large headspace oxygen volumes contribute to substantial
quality deterioration in bottled extra virgin olive oil. Sionek et al. (2013) showed that
removal of the oxygen reservoir, via nitrogen headspace flushing, could reduce the
formation of primary lipid reaction products (hydroperoxides) in refined rapeseed oil by
22%. Nitrogen purging, bubbling the inert gas through the liquid phase, can also improve
oxidative stability, as Masella et al. (2010) demonstrated when nitrogen purging of olive
oil was able to remove 50% of dissolved oxygen, significantly reducing peroxide
formation.

2.8.1.2 Modified Atmosphere Packaging

Shelf-life extension of foods can also be achieved by modified atmosphere
packaging (MAP), which changes the internal gas contents in packaged foods to limit the
amount of oxygen. MAP is routinely accomplished by pumping a food system with an
inert gas, such as nitrogen or carbon dioxide, to displace oxygen. MAP is similar to
nitrogen flushing/purging, but the goal is to achieve predetermined gas concentrations to
maximize stability and food quality. For instance, Lund et al. (2007) found that
modifying a package of beef patties in chilled storage to 100% nitrogen decreased lipid
oxidation, as measured by TBARS, by more than 70% compared to patties stored in MAP
with 80% oxygen.
2.8.1.3 Vacuum Packaging

The most complete removal of oxygen, vacuum packaging, involves air removal and a hermetic seal so that a near perfect vacuum is achieved inside the food container. Nam & Ahn (2003) demonstrated that vacuum packaging of meats significantly reduced lipid oxidation products compared to atmospherically stored samples.

Removal of oxygen from the enclosed food system is necessary for improved stability, but the packaging materials that determine gas transfer with the outside environment are equally important. Materials used in edible oil packaging are chosen on the basis of their weight, ease of handling, flavor absorption, and oxygen transmission rate (OTR) (Table 3).

Table 2.3. Packaging material OTRs (Lange & Wyser 2003; Duncan 2011)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Packaging material</th>
<th>Oxygen permeability (cm\textsuperscript{3} \cdot mm/m\textsuperscript{2} \cdot day \cdot atm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>Negligible</td>
</tr>
<tr>
<td>Metal (aluminum sheet)</td>
<td>Negligible</td>
</tr>
<tr>
<td>PET</td>
<td>1–5</td>
</tr>
<tr>
<td>PVC</td>
<td>2–8</td>
</tr>
<tr>
<td>PE</td>
<td>50–200</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Abbreviations: OTRs, oxygen transmission rates; PE, poly(ethylene); PET, poly(ethylene terephthalate); PVC, poly(vinyl chloride).

Recently, the use of plastic and paperboard packaging has increased over glass and metals due to lower costs, low weight, and ease of handling. However, because plastic and paperboard packaging allow oxygen transmission, they are sometimes modified (e.g., aluminum foil lamination) to limit the amount of oxygen that can permeate into the product (Piergiovanni & Limbo 2009). Additionally, oxygen ingress can occur through package linings (e.g., seals and caps), allowing oxygen to enter the product. Another hurdle in edible oil stability is that regardless of the oxygen removal
method and type of package, residual oxygen may still remain. Typically, flushing with an inert gas, MAP, and vacuum packaging leaves behind 2.0–5.0%, 0.9–1.1%, and 0.11–0.15% residual oxygen, respectively (Smiddy et al. 2002, Warmbier & Wolf 1976). Additionally, it is imperative that a packaging material with a negligible OTR (e.g., glass or a metal laminate layer) be used when oxygen removal techniques are employed. To illustrate this point, imagine a low-moisture food (e.g., crackers or ready-to-eat cereal) is packaged in plastic (from Table 3, surface area ~1,600 cm²) and then subjected to a process that removes all oxygen. A conservative estimate would be that within approximately 24–32 days the headspace oxygen concentration would return to full atmospheric concentration (21.0%) in the absence of a metal laminate layer. Using this knowledge, industry should consider the efficacy of oxygen removal techniques when utilizing packaging with a high OTR.

### 2.8.2 Oxygen Scavengers

Oxygen scavengers can be employed to completely remove residual oxygen from the system. Either edible oxygen scavengers or oxygen-absorbing sachets can be incorporated to reduce the oxygen content to less than 0.01% (Cruz et al. 2012). Oxygen scavengers can be used in addition to processing techniques, or as stand-alone scavengers, to reduce the costs associated with the oxygen removal process. Scavengers can also be incorporated into packaging by dissolving them in plastics used for bottle manufacture. Cecchi et al. (2010) demonstrated the efficacy of an oxygen scavenger catalyst, Amosorb®, dispersed within the plastic poly(ethyleneterephthalate) (PET) that decreased peroxide formation by 18% in olive oil stored in light and evaluated after 13 months. Importantly, the oxygen diffusivity of the packaging material, and the amount of
oxygen present in the sealed system upon manufacture, must be accounted for when calculating the amount of scavengers needed to achieve, and maintain, an oxygen-free package.

2.8.2.1 Iron-Based Oxygen Scavenging

Metallic-reducing agents such as iron oxide, ferrous carbonate, and metallic platinum are commonly used in the food industry to scavenge oxygen. Iron-based scavengers work by the preferred oxidation of iron to consume oxygen present in the enclosed package (Scheme 2.3). Generally, in commercial scavengers 1 g of iron can scavenge approximately 300 mL of oxygen (Cruz et al. 2012). The scavengers are packaged in small sachets to partition the reactive metals away from sensitive food ingredients and prevent ingestion of toxic components. In a cracker system, an iron oxide scavenger was able to reduce the level of oxygen from 21% to 3.0% within one day and was able to extend sensory acceptance by more than 20 weeks (Berenzon & Saguy 1998). Mexis & Kontominas (2010) showed that iron-based oxygen absorbers could extend the oxidative stability of almonds regardless of storage temperature and initial oxygen concentrations. However, iron-based scavengers have drawbacks due to their toxicity, sachet storage, and also their ability to set off metal detectors.

Scheme 2.3. Iron-based oxygen scavenging
2.8.2.2 Iron-Based Oxygen Scavenging

Other oxygen scavengers function enzymatically, such as the glucose oxidase-catalase system, to remove oxygen from the environment. Glucose oxidase donates hydrogen atoms from the −CHOH group of glucose to oxygen, forming a glucono-delta-lactone and hydrogen peroxide: \[2 \text{glucose} + 2 \text{O}_2 + 2 \text{H}_2\text{O} \rightarrow 2 \text{gluconic acid} + 2 \text{H}_2\text{O}_2\] (Labuza & Breene 1989). However, hydrogen peroxide must be reduced to water and oxygen to prevent decomposition into free radicals that promote lipid oxidation: \[2 \text{H}_2\text{O}_2 + \text{catalase} \rightarrow 2 \text{H}_2\text{O} + \text{O}_2.\] Thus, the system becomes inefficient in reducing oxygen.

Another disadvantage to this system is that it is very sensitive to changes in pH, water activity, temperature, and salinity. Moreover, Min et al. (2003) found that glucose oxidase-catalase acted as an antioxidant at lower concentrations in salad dressing, but at higher concentrations above 0.3 unit/g, there was a prooxidative effect. The prooxidant effect results potentially from the increased concentration of prooxidative heme groups from catalase or by free radicals produced from hydrogen peroxide decomposition. Due to the drawbacks of glucose oxidase-catalase, it is often only used under a narrow range of food products such as beer and wine bottle caps.

2.8.2.3 Ascorbate Oxygen Scavenging

An alternative edible oxygen scavenger that has been gaining attention due to its safety, “natural” label, and efficacy is ascorbic acid. Ascorbic acid is able to scavenge oxygen by an oxidation reaction that produces dehydroascorbic acid (DHAA) (Scheme 2.4). The complete reduction of 1 mol of atmospheric oxygen requires 2 mol of ascorbic acid and results in the formation of DHAA and water. This reaction reduces the level of oxygen over the course of days, but can be accelerated by transition metals and light
(Cruz et al. 2012). In model aqueous solutions, 3.4–3.6 mg ascorbic acid can scavenge the oxygen in 1 cm$^3$ of headspace air, which is close to the theoretical value of 3.3 mg cm$^{-3}$ (Cort 1982). Ascorbic acid can also be conjugated to a fatty acid, such as palmitic acid (ascorbyl palmitate), to increase its solubility in lipid systems. Researchers debate the efficacy of ascorbyl palmitate because it may be difficult to reach high enough concentrations to scavenge substantial amounts of oxygen because of solubility constraints in some foods (Cort 1982, Frankel 2005). A drawback to using ascorbate in oxygen scavenging applications is its ability to reduce ferric iron to the more prooxidative ferrous state (Alamed et al. 2009). Thus, the relationship between ascorbic acid and oxygen scavenging is contradictory. Trace metals must be present within the system to help catalyze the scavenging reaction, but the transition metals (iron and copper) need to be chelated or ascorbate could increase oxidation rates (Figure 2.3).

Scheme 2.4. Ascorbate Oxygen Scavenging

![Scheme 2.4. Ascorbate Oxygen Scavenging](image)

Figure 2.3. Ascorbic acid interacting in a complex system with the capacity to scavenge environmental oxygen (left). Ascorbic acid also possesses potential prooxidative properties by recycling metals to their more active form, causing free radical generation (middle). Metal chelators and FRS can be employed to decrease potential lipid oxidation initiating events (right). Abbreviations: AA, ascorbic acid; DHAA, dehydroascorbic acid; FRS, free radical scavengers.
2.8.2.4 Sulfite Oxygen Scavenging

Other edible scavengers include sulfites that have been incorporated into packaging material and possess enough thermal stability to maintain their efficacy after thermoplastic processing (Cruz et al. 2012). The drawback to sulfite usage is that oxygen scavenging can produce sulfur dioxide as a by-product, which decreases the sensory acceptability of the product and may pose allergenic concerns (Brody et al. 2001).

2.9. Measuring Oxygen Concentration

Measuring oxygen concentration and diffusivity helps to identify a critical substrate that can impact all oxidative reactions in foods. However, data on oxygen within food systems are difficult to obtain. Interference of atmospheric oxygen during sampling and oxygen consumption in chemical reactions are two obstacles facing oxygen measurements in foods. Most of the techniques used today have been available since the 1950s, each with its own utility. The Winkler test, commonly used to calibrate other methods, chemically reacts with oxygen to quantify oxygen in water via colorimetric titration of iodine. The Winkler test is not suitable for complex foods containing components that can oxidize or reduce iodine, such as hydroperoxides. Gravimetry is
used to measure the global system oxygen gain by monitoring weight changes, but mass loss due to volatiles cannot be differentiated from oxygen changes. Manometric and volumetric oxygen analysis methods are the oldest means of quantifying oxygen and measuring the difference in total pressure or total volume, respectively. Many early studies used manometry as the basis of determining oxygen solubility in oils (Pénicaud et al. 2012). However, problems arise because significant amounts of pressure or volume changes are needed and contamination from atmospheric gases is common. Oxygen and its stable radicals can be directly measured by the interaction between the biradical of triplet oxygen and a magnetic field via electron paramagnetic resonance (EPR). Wagner et al. (1994) used EPR in combination with an oxygen monitor to follow the generation of lipid-derived free radicals during lipid peroxidation in cells. Unfortunately, technical challenges and the cost of instrumentation have limited EPR use for quantifying oxygen. Electrochemical methods, such as the Clark electrode, have also been used to quantify oxygen in foods (Ohashi et al. 1994). Oxygen determination is achieved when the sample is submitted to an electrochemical potential difference causing oxygen to be reduced to hydroxide ions. The hydroxide ions generate an electric current proportional to the oxygen chemical potential, but the procedure requires calibration to correlate to oxygen concentration. The major disadvantage of electrochemical measurements is that oxygen consumption during determination prevents the ability to measure continuously. Gas chromatography is a widely used method to monitor oxygen levels, with the major limitation being its ability to perform only headspace analysis (Andersson & Lingnert 1997).

The most recent advance in oxygen quantification is the use of luminescent dyes
that are specifically sensitive to oxygen. Luminescent dyes can be used to quantify oxygen in foods based on energy transfer from the ruthenium-containing fluorophore to oxygen (Tikekar et al. 2011), which decreases the intensity of fluorescence with increasing oxygen content (Pénicaud et al. 2010). The system is calibrated against atmospheric oxygen and expresses oxygen as a partial pressure. A fiber optic cable probe can be added to this system to simultaneously detect dissolved or headspace oxygen concentration (Figure 2.4).

Figure 2.4. Example of a noninvasive experimental setup for simultaneous analysis of dissolved and headspace oxygen using a ruthenium-containing dye and a fiber optic cable probe (modified with permission from Chaix et al. 2014).

Optical probes appear to be the most versatile given that they do not consume oxygen, are sensitive to low levels of oxygen, provide continuous analysis, and have fast response times (García-Torres et al. 2009). The drawbacks associated with this method are derived from its sensitivity to photobleaching of the ruthenium-containing fluorophore and position dependence of the fiber optic cable. Smiddy et al. (2002) used optical oxygen probes to continuously monitor oxygen levels in packaged meats to demonstrate that the shelf life of MAP chicken patties was less oxidatively stable than vacuum packed samples. More in-depth discussions of analytical techniques measuring
oxygen solubility and diffusivity in foods are provided in recently completed reviews by Pénicaud et al. (2012) and Chaix et al. (2014).

2.10 Conclusions

This review has highlighted the importance of oxygen content and location in determining the oxidative stability of lipid-containing foods. Our understanding is that any decrease in oxygen content is advantageous in protecting oxidatively susceptible foods. However, the relative influence of oxygen becomes greater as lower and lower oxygen concentrations are reached. The importance of very low levels of oxygen (i.e., <1–2% oxygen) for extending shelf life has been shown; however, any residual oxygen will still eventually allow lipid oxidation to proceed. Practically speaking, a complete removal of oxygen in some food systems may not be feasible. Thus more knowledge regarding lipid oxidation in the 1–2% oxygen range is needed to determine resources necessary to process and package oxygen-sensitive foods. Further studies are also needed to detail the effects of varying oxygen contents and their relationship to various pro/antioxidants in foods. For example, would oxygen reduction be more effective in foods susceptible to singlet oxygen degradation or in food systems whose stability is dependent on metal-promoted oxidation?

Reaching oxygen concentrations below 2% could be difficult and/or expensive for many foods. It might be more feasible to use combinations of technologies such as nitrogen flushing with edible oxygen scavenging such as ascorbic acid. However, before these combinations can be used, more research is needed to determine conditions where residual antioxidants (e.g., ascorbic acid) would not be prooxidative. Controlling oxygen concentrations could also help enhance the activity of natural antioxidants such that their
effectiveness could be similar to that of synthetic antioxidants. Antioxidant applications incorporating knowledge explained in this review are needed, especially as the food industry faces the major challenge of increasing incorporation of healthy, but also unstable, polyunsaturated fatty acids.

2.11 References


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CHAPTER 3

DEVELOPMENT OF IRON CHELATING POLY(ETHYLENE TEREPHTHALATE) PACKAGING FOR INHIBITING LIPID OXIDATION IN OIL-IN-WATER EMULSIONS

3.1 Abstract

Foods such as bulk oils, salad dressings, and nutritionally fortified beverages that are susceptible to oxidative degradation are often packaged in poly(ethylene terephthalate) (PET) bottles. In the present work, the ability to graft poly(hydroxamic acid) (PHA) metal chelating moieties from the surface of PET was investigated. Biomimetic PHA groups were grafted in a two-step UV-induced process without the use of a photoinitiator. Surface characterization of the films by attenuated total reflective Fourier transform infrared spectroscopy (ATR-FTIR) and scanning electron microscopy (SEM) suggested successful grafting and conversion of poly(hydroxyethyl acrylate)(PHEA) to PHA chelating moieties from the surface of PET. Colorimetric (ferrozine) and inductively coupled plasma mass spectroscopy (ICP-MS) assays demonstrated the ability of PET-g-PHA to chelate iron in a low pH (3.0) environment containing a competitive metal chelator (citric acid). Lipid oxidation studies showed the antioxidant activity of PET-g-PHA films in inhibiting iron-promoted oxidation in an acidified O/W emulsion model system (pH 3.0). Particle size and ζ-potential analysis indicated that the addition of PET-g-PHA films did not impact the physical stability of the emulsion system. This work suggests that biomimetic chelating moieties can be grafted from PET and effectively inhibit iron-promoted degradation reactions.
3.2 Introduction

The presence of transition metals (e.g. iron) enhances lipid oxidation in foods containing lipids, leading to nutrient loss and the generation of off-flavors. This is due to the ability of iron to promote the breakdown of lipid hydroperoxides to generate reactive radicals that degrade bioactive molecules such as fatty acids, antioxidants, and carotenoids (Gutteridge 1995). Synthetic metal chelators (e.g. ethylenediaminetetraacetic acid, EDTA) are commonly used in food and beverage products and have been shown to be potent inhibitors of lipid oxidation in emulsion-based systems (Mancuso et al. 1999). However, growing concern over the use of synthetic additives has led researchers to seek alternative solutions to prevent lipid oxidation, such as natural additives and active packaging (Frankel & Finley 2008, López-de-Dicastillo et al. 2012, Tian et al. 2013).

Unfortunately, natural additives are often not as effective as their synthetic counterparts and their safety limits are largely unknown (Medina et al 1999, Pokorný 2007). Active packaging represents an attractive strategy for protecting labile food ingredients, but in some cases may involve migration of active compounds or alteration of the bulk properties (mechanical strength, appearance) of the packaging material (Goddard et al. 2012, Pereira de Abreu et al. 2011, Vermeiren et al. 1999). Therefore, non-migratory covalent modifications to packaging materials capable of chelating prooxidative transition metals have been investigated (Tian et al. 2013b).

Our group previously reported grafting of non-migratory biomimetic hydroxamic acid (PHA) chelating groups from a polypropylene (PP) surface (Tian et al. 2015). The resulting materials chelated iron across a pH range relevant to foods and performed better than EDTA in inhibiting volatile lipid oxidation product formation in oil-in-water (O/W)
emulsions (Tian et al. 2013b). However, PP-g-PHA synthesis requires the use of an added chemical photoinitiator (e.g. benzophenone) to enable the grafting from the PP surface. To expand the industrial potential of non-migratory chelating packaging, the overall goal of this work was to graft PHA from an alternative food packaging material and determine its efficacy in an acidified (pH 3.0) model food emulsion system. Poly(ethylene terephthalate) (PET) was chosen because of its lower oxygen transmission rate, clarity, moisture barrier, temperature tolerance, lightweight nature, and widespread use in the food and beverage industry (Lange & Wyser 2003, Uchida et al. 1994). In addition, an advantage of PET is that its chemical structure can be leveraged to graft polymerize a monomer without the need for a photoinitiator. The challenge in hydroxamic acid grafted PET (PET-g-PHA) is that it requires an alternative chemical synthesis to polymerize a monomer PHA precursor from the PET surface.

Herein, we describe a two-step grafting procedure in which hydroxyethyl acrylate (HEA) was grafted from the surface of PET films via UV-induced graft polymerization. PHEA groups were then converted to PHA groups by exposure to hydroxylamine. The resulting PET-g-PHA was capable of chelating iron at pH 3.0 in the presence of a competitive chelator (citric acid). Materials were characterized for changes in surface chemistry, and the ability to inhibit lipid oxidation was demonstrated in an acidified (pH 3.0) O/W emulsion. This work demonstrates that biomimetic chelating moieties can be grafted from PET and effectively inhibit iron promoted degradation reactions.
3.3 Materials and Methods

3.3.1 Materials

Commercial soybean oil (Wesson 100% natural vegetable oil) was purchased from a local grocer. Poly(ethylene terephthalate) (PET, oriented) sheet with the thickness of 178 ± 18 μm was purchased from McMaster-Carr (Elmhurst, IL). Acetone, ferric chloride anhydrous, hydrochloric acid, trichloroacetic acid (TCA), 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), water (HPLC), 2-propanol, isoctance, 1-butanol, methanol, sodium citrate dihydrate, sodium acetate trihydrate, sodium hydroxide, disodium ethylenediaminetetraacetic acid (EDTA), and Brij 35 (a nonionic surfactant) were purchased from Fisher Scientific (Fair Lawn, NJ). Citric acid monohydrate and hydroxyethyl acrylate (HEA, 97%, stabilized) were purchased from Acros Organics (Morris Plains, NJ). 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p’-disulfonic acid monosodium salt hydrate (ferrozine, 97%), imidazole, hydroxylamine solution (50 wt% in water), barium chloride dihydrate, ammonium thiocyanate, cumene hydroperoxide (80%), and hexanal (98%) were purchased from Sigma-Aldrich (St. Louis, MO). All chemicals and solvents were used without further purification.

3.3.2 Grafting of Poly(hydroxamic acid) from the PET film surface

PET film was cut into 1 cm × 2 cm coupons, which were cleaned sequentially in isopropanol, acetone, and deionized water (2x10 min cleanse per solvent) by sonication. Cleaned PET films were dried in a desiccator overnight (25 °C, 15% RH) for future use. Poly(hydroxamic acid) (PHA) was grafted from the surface of PET films using a two-step process (Figure 3.1). In the first step, poly(hydroxyethyl acrylate) (PHEA) was grafted from the PET film surface using UV-induced graft polymerization. Briefly, PET
coupons were submerged in 6 mL 30 wt% HEA in water using individual septum-screw cap vials. The monomer solution was purged with nitrogen for 15 min to remove oxygen, followed by sonication to disrupt air bubble formation on the films. Films were then subjected to ultraviolet (UV) irradiation for 2.5 min in a Dymax light-curing system (Model 5000 flood, 320-395 nm, 200 mW/cm², Dymax Corporation, Torrington, CT) to graft PHEA from the surface of PET. The resulting PET-g-PHEA films were then washed in deionized water for 30 min at room temperature followed by washing 1 h at 60°C followed by the same washing overnight at room temperature, to remove adsorbed HEA monomers and PHEA homopolymers.

Figure 3.1 Schematic of two step UV-initiated graft polymerization and conversion of (poly)hydroxamic acid from the surface of poly(ethylene terephthalate) PET-g-PHA.

PHEA grafts were then converted to PHA grafts by a modification of the method reported by Polomoscanik et al. (2005) PET-g-PHEA films were submerged in an aqueous solution of hydroxylamine (16.67 wt%) at a surface area to volume ratio of 1 cm² to 2.5 mL and stirred at 55°C for 1 h (Figure 3.1). The resulting PET-g-PHA films were then rinsed three times in deionized water followed by 30 min exposure to acidic water (adjusted by HCl, pH 3.0) to protonate hydroxamic acid groups and finally rinsed again in copious deionized water. The obtained PET-g-PHA films were dried and stored over anhydrous calcium sulfate (25°C, 15% RH) until further use.
3.3.3 Surface Characterization

The surface chemistry of films, before and after PHA grafting, was characterized using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) by an IRPrestige-21 FTIR spectrometer (Shimadzu Scientific Instruments, Inc., Kyoto, Japan) equipped with a diamond ATR crystal. Using clean, native PET as background spectra each spectrum was generated from 32 scans at a 4 cm\(^{-1}\) resolution and collected from three independent films. Representative spectra were replotted with SigmaPlot 12.2 (Systat Software, Inc., Chicago, IL). The surface morphology of films was analyzed by field emission scanning electron microscopy (SEM) (JCM-6000 NeoScope, JEOL, Japan) at 10kV. Prior to imaging, samples were sputter-coated with gold to prevent charging.

3.3.4 Iron Chelating by the Ferrozine Assay

The ferric iron (Fe\(^{3+}\)) chelating activity of PET-g-PHA films was determined at pH 3.0 in citric acid/citrate buffer (10 mM). Citrate was used to both mimic the acid used in many foods and also to help maintain iron solubility. Films (native PET, PET-g-PHEA, and PET-g-PHA) were submerged in 0.06 mM Fe\(^{3+}\) (from ferric chloride anhydrous) in citric acid/citrate buffer, with Fe\(^{3+}\) solution without films serving as a control. Films were allowed to chelate in the dark for 24 h at room temperature while shaking. The Fe\(^{2+}\) chelating activity of films was calculated by the difference of the Fe\(^{3+}\) concentration (determined by ferrozine assay, described below) in Fe\(^{3+}\) solution with films against the control group (Fe\(^{3+}\) solution with no films) (Tian et al. 2013b).

A modification of the ferrozine assay was performed to quantify the Fe\(^{3+}\) concentration of chelating solutions, in which a colorimetric complex is formed between Fe\(^{2+}\) and ferrozine reagent (Bou et al. 2008, Tian et al. 2012). First, Fe\(^{3+}\) was reduced to
Fe$^{2+}$ by exposure to 5 wt% hydroxylamine hydrochloride in 10 wt% trichloroacetic acid followed by addition of ferrozine reagent (18 mM in 0.05 M HEPES buffer, pH 7.0) and mixing for 1 h at room temperature to enable color formation. Absorbance was measured at 562 nm. Fe$^{3+}$ concentration was quantified by comparison to a standard curve made from ferric chloride anhydrous. Results are representative of two experiments performed on independent days.

3.3.5 Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS)

As a direct quantification of iron chelation to support results from ferrozine assay, native and modified PET films (performed in duplicates) were subjected to ICP-MS analysis after films were exposed to iron in the ferrozine assay (see above). Films were prepared for ICP-MS using a standard method (Talbot & Weiss 1994). Approximately 100 mg of the PET film were weighed directly into microwave digestion vessels, followed by addition of nitric acid (5.0 mL). Microwave digestion by a Mars Xpress (CEM, Matthews, NC) was carried out at 210°C for 10 min. Digested samples were then diluted with deionized water and held at 4°C until analysis. ICP-MS analysis was conducted on a Perkin Elmer Elan 9000 equipped with an autosampler (Waltham, MA). Iron concentration was determined by a standard curve prepared using an iron ICP-MS standard (Ricca Chemical Company, Arlington, TX).

3.3.6 Effect of PET-g-PHA films of the Oxidative Stability of Oil-in-Water (O/W) Emulsions

To demonstrate the effectiveness of chelating PET-g-PHA films in preventing iron promoted degradation reactions, an accelerated lipid oxidation study was performed using an acidified O/W emulsion as a model food system. Emulsions were prepared by
dissolving Brij 35 (0.10% w/w) and commercial soybean oil (1.0% w/w) in a 0.05 M sodium acetate/imidazole buffer (pH 3.0), followed by homogenization with a hand-held homogenizer (Biospec Products, Inc., Bartlesville, OK) at 7000 rpm for 2 min. The coarse emulsion then underwent three passes through a microfluidizer (Microfluidics, Newton, MA) at 9 kPSI. Films (1 cm²) were submerged in 1 mL emulsion and stored in the dark at 55°C for 29 days. Emulsions without films served as a negative control; emulsions containing the synthetic chelator EDTA at two concentrations (0.01 mM, equivalent to the chelating capacity of the PET-g-PHA films, and 0.08 mM, the maximum legal limit) served as positive controls (FDA Food Additive Regulation 21 CFR 172.120).

The formation of primary and secondary lipid oxidation products (lipid hydroperoxides and hexanal, respectively) were quantified throughout the study to determine the oxidative stability of the O/ W emulsions. Lipid hydroperoxides were quantified according to the method reported by Waraho et al. (2009). Briefly, 0.30 mL of emulsion was mixed with 1.5 mL of iso-octane/isopropanol (3:1 v/v) by vortex for 3 × 10 s and then centrifuged at 1785g for 2 min. The upper layer, containing the lipid hydroperoxides, was collected and added to 2.8 mL of methanol/1-butanol (2:1 v/v), followed by the addition of 30 μL of thiocyanate/Fe²⁺ solution (obtained from mixing an equal volume of 3.94 M thiocyanate solution with 0.072 M Fe²⁺). The reaction samples were held for 20 min at room temperature to enable color formation. Absorbances were read at 510 nm, and lipid hydroperoxide concentrations were determined by comparison to a standard curve prepared using six concentrations of cumene hydroperoxide between
0 and 600 mmol/kg oil \((r^2 = 0.99, \text{linear regression})\). Samples were diluted with methanol/1-butanol to maintain their absorbance within the prepared concentration range. The secondary lipid oxidation product hexanal was quantified by gas chromatography (GC, model GC-2014, Shimadzu, Tokyo, Japan) equipped with an autosampler and flame ionization detector (FID) as in Tian et al. (2013b). Samples (1 mL) were incubated at 55 °C for 10 min to allow volatile compounds from the emulsion to enter the headspace of the 10 mL headspace gas chromatography vial. A divinylbenzene/carboxen/polydimethylsiloxane (DVB/carboxen/PDMS) solid-phase microextraction (SPME) fiber (50/30 μm, Supelco, Bellefonte, PA, USA) was exposed to the headspace above the sample for 2 min to adsorb volatile components, followed by desorption for 3 min at 250 °C in the injector at a split ratio of 1:7. Volatile compounds were separated on a fused-silica capillary column (30 m × 0.32 mm inner diameter × 1 μm) coated with 100% poly(dimethylsiloxane) (Equity-1, Supelco). The run time was 10 min per sample with temperatures of 250, 65, and 250 °C for the injector, oven, and detector, respectively. Hexanal concentration was calculated from the peak areas using a standard curve prepared using authentic hexanal (concentrations ranging from 0 to 43.6 mmol/kg oil, \(r^2 = 0.91, \text{linear regression}\)).

Time to lipid oxidation was quantified by determining the lag phase, defined as the day before a statistically significant increase in concentration of primary or secondary oxidation products as compared to initial concentrations.

To demonstrate that the PET-g-PHA films did not affect emulsion stability, emulsion particle size distribution and electrical charge were characterized using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Particle size was
determined by dynamic light scattering (DLS) after 10-fold dilution of the emulsion with sodium acetate/imidazole buffer (0.05 M, pH 3.0). The diluted emulsion was equilibrated for 60 s before particle size measurements were recorded with an average of 11 readings, reported as the Z-average in mean diameter (nm). Size measurements were followed immediately by electrical charge measurements (10 readings for each measurement) with an equilibration time of 60 s, and the zeta (ζ) potential (mV) of the emulsion droplets was collected. Stability tests were both performed for two measurements per sample and performed in duplicates.

### 3.3.7 Statistical Analysis

Results are presented as means and standard deviations of analyses conducted in triplicate, unless otherwise stated. Oxidation lag phases were defined as the first data point statistically greater than the 0 time within each treatment tested using one-way analysis of variance (ANOVA) with comparison of the oxidation measurement means performed using Tukey’s HSD post hoc test (p = 0.05). Calculations were performed using IBM SPSS Statistics version 22 (IBM SPSS, Chicago, IL, USA).

### 3.4. Results and Discussion

#### 3.4.1 Characterization of Biomimetic PET-g-PHA Active Packaging Films

Metal chelating active packaging films were produced in a two-step process in which PHEA was grafted from the surface of PET by UV-initiated graft polymerization followed by conversion to PHA by exposure to hydroxylamine (Figure 3.1). The surface chemistry at each step in the modification was analyzed by ATR-FTIR (Figure 3.2). Using clean, native PET as the background, the spectrum of PET-g-PHEA showed strong absorption band of 3500-3300 cm⁻¹ characteristic of an –OH stretch and two smaller
peaks 2900-2800 cm\(^{-1}\) corresponding to –CH stretch, suggesting successful covalent grafting of poly(hydroxyethyl acrylate) PHEA from PET. Conversion of PHEA to chelating hydroxamate groups (PHA) was confirmed by the introduction of an –OH stretch at 3300-3100 cm\(^{-1}\) typical of a hydroxyl group adjacent to an amide as present in the hydroxamic acid chelating moiety (Figure 3.2). The absorption in the 3000-2900 cm\(^{-1}\) range, attributed to the –CH stretching for the long alkyl backbone of PHEA, did not change suggesting specific conversion of the ester to a hydroxamic acid moiety. ATR-FTIR spectroscopy on both sides of the film confirmed that grafting did not occur on the backside of PET films.

Figure 3.2. ATR-FTIR spectra of PET-g-PHEA and PET-g-PHA films using native PET as the background. Each spectrum is representative of measurements collected from three independent films.

Figure 3.3. SEM surface analysis (9000x) of (a) PET (b) PET-g-PHEA (c) PET-g-PHA films.
The effect of PHA grafting on the film surface morphology was determined by electron microscopy (Figure 3.3). Native PET (Figure 3.3A) exhibited small irregularities, similar to that observed by others in commercial PET films (Fasce et al. 2008). PET-g-PHEA films had uniform surface morphology across and between samples (Figure 3.3B), exhibiting ridge-like structures typical of hydrophilic polymer/hydrogels that shrink upon dehydration (Tian et al. 2013b, Curti et al. 2005). As expected, the films lost the ridge-like structure after conversion to PET-g-PHA (Figure 3.3C) due to the change in surface hydrophilicity by conversion to the relatively hydrophobic hydroxamate groups.

### 3.4.2 Iron Chelation by Biomimetic PET-g-PHA Active Packaging Films

The ability of the hydroxamic acid grafted PET (PET-g-PHA) films to chelate iron was quantified by the colorimetric ferrozine iron-binding assay (Figure 3.4).

Figure 3.4. Ferric iron chelation by PET, PET-g-PHEA, and PET-g-PHA films (24 hr incubation, pH 3.0 citric acid/citrate buffer). Values represent means plus standard deviation (n=4). Results are representative of two experiments performed on independent days.

Iron chelating of native PET was negligible (PET 0.60 ± 3.80 nmol Fe$^{3+}$/cm$^2$) and grafted PHEA also exhibited negligible activity (PET-g-PHEA 2.22 ± 1.10 nmol Fe$^{3+}$/cm$^2$). PET-g-PHA films demonstrated significant iron chelating activity (13.39 ± 2.30 nmol Fe$^{3+}$/cm$^2$) in competitively chelated citrate-Fe$^{3+}$ solutions. Given these data,
the density of the chelating groups on the PET-g-PHA film can be estimated between 13 to 39 nmol HA groups, assuming a HA/Fe$^{3+}$ ratio of between 1:1 and 3:1. The lack of chelating by native PET and PET-g-PHEA suggests that chelating shown by PET-g-PHA groups is due to specific iron-HA interactions. The minimal chelation of PET-g-PHEA films was likely the result of non-specific absorption or swelling into the grafted layer. Previously, our group demonstrated the effectiveness of grafted PHA from polypropylene (PP-g-PHA) (Tian et al. 2015). The chelating activity of the PET-g-PHA materials reported herein are lower than our previous reports on PP-g-PHA materials, however, it is important to note that those studies were performed at a higher pH value (pH 5.0), at which the film chelated 21.39 ± 1.28 nmol Fe$^{3+}$/cm$^2$. In this work, we have demonstrated the ability of the synthesized PET-g-PHA films to maintain chelating abilities in an environment representative of an acidified liquid food system (pH 3.0), in which citric acid was used as an acidulant (10mM) and competitive chelator.

Direct quantification of iron chelation by ICP-MS was performed to complement ferrozine assay results. After exposure to ferric iron solution, native PET films presented insignificant iron (-1.00 ± 1.70 nmol Fe$^{3+}$/cm$^2$) while PET-g-PHA films presented 7.20 ± 1.10 nmol Fe$^{3+}$/cm$^2$. These values are on the order of, but lower than, those obtained using the ferrozine assay. This discrepancy may be a result of differences in assay sensitivities or incomplete microwave digestion of the PET-g-PHA films during the preparation step for ICP-MS analysis.

3.4.3 Controlling Lipid Oxidation by Iron Chelating PET-g-PHA Films
The ability of PET-g-PHA films to delay lipid oxidation in acidified (pH 3.0) soybean oil-in-water emulsions was determined by quantifying formation of lipid hydroperoxides
and hexanal during storage at 55°C in the dark (Figure 3.5, 3.6). As expected, emulsions in the absence of metal chelators (control and native PET samples) oxidized rapidly with a lag phase of 3 days for both lipid hydroperoxide and hexanal production. The addition of PET-g-PHA films to the emulsions extended the lag phase to 5 days for lipid hydroperoxide production and to 7 days for hexanal production. The PET-g-PHA films were less effective than emulsions containing the positive controls (EDTA and no film), which at both concentrations extended lag phase to 20 days and 26 days for lipid hydroperoxides and hexanal, respectively. The effective inhibition of lipid oxidation by EDTA suggests that transition metal-promoted degradation was a major oxidative instability mechanism in the O/W emulsions. The iron content of the commercial soybean oil was determined to be 330 ppb by ICP-MS analysis, which is in agreement with prior reports on iron content of refined soybean oil (200 ppb) (Choe & Min 2006). The results indicated that both EDTA concentrations were in excess of the transition metals (e.g. iron or copper) present in the emulsion system because an increase in EDTA concentration did not extend the lag phase of lipid hydroperoxide or hexanal production. It is interesting to note that although the PET-g-PHA film had theoretical equivalent chelating capacity to the emulsion containing 0.01 mM EDTA, it was significantly less effective at inhibiting lipid oxidation. A possible explanation for the higher efficacy of EDTA, as compared to PET-g-PHA may be related to physical structure and chelation geometry (Winston & McLaughlin 1976). PET-g-PHA chelating groups are tethered to the PET polymer and chelating may be dependent upon iron diffusion to the HA groups, whereas EDTA molecules are uniformly dispersed in the solution. In addition, the enhanced oxidative stability of emulsions containing EDTA could be due to EDTA’s low pK$_{1,2}$ (1.99, 2.67)
that allows greater chelating capacity under acidic conditions (pH 3.0).

Figure 3.5. Formation of lipid hydroperoxides in 0.05 M sodium acetate-imidazole buffered (pH 3.0) soybean oil-in-water emulsions stored at 55°C: in the absence of a metal chelator (control and native PET), a metal chelator (EDTA 0.01mM and 0.08mM), or PET-g-PHA film. Values represent means ± standard deviation (n=3).

Figure 3.6. Formation of hexanal in 0.05 M sodium acetate-imidazole buffered (pH 3.0) soybean O/W emulsions stored at 55°C: in the absence of a metal chelator (control and native PET), a metal chelator (EDTA 0.01mM and 0.08mM), or PET-g-PHA film. Values represent means ± standard deviation (n=3).

Previously, our group demonstrated the effectiveness of grafted PHA from polypropylene (PP-g-PHA) in controlling lipid oxidation in an oil-in-water emulsion system (pH 3.0) composed of stripped soybean oil (Tian et al. 2013b). Although PP-g-PHA films were unable to effectively inhibit lipid hydroperoxide development they
demonstrated better performance than EDTA (0.01 mM) in preventing the formation of hexanal. Herein, the developed PET-g-PHA film demonstrated ability to delay development of both lipid hydroperoxide and hexanal, albeit to a lesser extent than EDTA. In this study, an acidified food or beverage system using commercial oil was studied to demonstrate the potential application of PET-g-PHA modified packaging in extending the shelf life of an O/W emulsion system.

Physical stability of the emulsions at the Day 0 and Day 19 were evaluated to confirm that the physicochemical properties of the emulsion did not change during the course of lipid oxidation. Throughout the study, there was no significant change in the size and charge of the Brij 35-stabilized emulsion, suggesting that the reported PET-g-PHA films do not influence the physical stability of emulsions (Table 3.1).

Table 3.1. Physical stability of 0.05 M sodium acetate-imidazole buffered (pH 3.0) soybean O/W emulsions stored at 55°C at day 0 and day 19 for particle size diameter (nm) and ζ potential (mV). Values are means ± standard deviation of two measurements per sample, performed in duplicate (n=4).

<table>
<thead>
<tr>
<th>Sample emulsion</th>
<th>Particle size diameter (nm)</th>
<th>ζ potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>original emulsion (Day 0)</td>
<td>290 ± 61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-1.13 ± 0.62</td>
</tr>
<tr>
<td>no film</td>
<td>221 ± 18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.24 ± 0.10</td>
</tr>
<tr>
<td>PET</td>
<td>224 ± 17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.08 ± 0.04</td>
</tr>
<tr>
<td>PET-g-PHA</td>
<td>198 ± 14&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.57 ± 0.45</td>
</tr>
<tr>
<td>EDTA (0.01 mM)</td>
<td>182 ± 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-0.99 ± 0.30</td>
</tr>
<tr>
<td>EDTA (0.08 mM)</td>
<td>180 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-0.58 ± 0.12</td>
</tr>
</tbody>
</table>

In conclusion, the potential applicability of a biomimetic iron-chelating moiety (PHA), grafted from a commercially applicable food-packaging polymer (PET), was
demonstrated in controlling lipid oxidation of commercial soybean oil-in-water emulsion system. Herein, we developed a synthesis method to graft iron chelating group precursors and convert them into active iron chelating HA groups. PET-g-PHA films demonstrated strong iron-chelating activity in buffered systems relevant for chelating iron levels found in acidified food and beverages (pH 3.0) with competitive chelators. Results from this study demonstrate the applicability of biomimetic active packaging technology that can be utilized to protect food and beverage from iron-promoted degradation.

3.5 Acknowledgement

The authors gratefully acknowledge D. Julian McClements for the use of his emulsion preparation equipment and Zetasizer Nano ZS. ICP-MS sample preparation and analysis was conducted in the laboratories of Julian Tyson and David Reckhow with technical assistance from Nan Wang, Joe Goodwill, and Rassil Sayess.

3.6 References


18) Talbot, J.; Weiss, A. Laboratory methods for ICP-MS analysis of trace metals in precipitation. *Hazardous Waste Research and Information Center* **1994**.


CHAPTER 4

IS OXYGEN REDUCTION A Viable Antioxidant Strategy for Oil-In-Water Emulsions?

4.1 Abstract

The impact of 0, 40, 58, 79, 93, and 98% total oxygen reduction on lipid oxidation kinetics in a 1.0% fish oil-in-water emulsion (pH 3.0; 32°C) was determined. Atmospheres were modified using nitrogen/oxygen gas blends or high purity nitrogen. Headspace and dissolved oxygen were monitored throughout the study using a non-destructive technique in which fluorescent sensors were fixed in sealed vials. Lipid oxidation, as measured by lipid hydroperoxides and thiobarbituric acid reactive substances (TBARS), was inhibited at oxygen reductions ≥58%. However, meaningful protection against lipid oxidation was only achieved when oxygen reductions ≥93%. Potential commercial strategies, nitrogen flushing/sparging and ascorbic acid, were unable to effectively reduce oxygen. Results suggest that near complete oxygen removal is necessary to protect oxygen-sensitive ingredients, but a need still exists to identify new strategies that sufficiently reduce the oxygen content of emulsions.

4.2 Introduction

The presence of oxygen in lipid-containing foods leads to oxidation reactions that generate off-flavors and destroy nutrients. In general, the oxidative susceptibility of food products is increasing because of food industry trends to remove hydrogenated oils and use more bioactive, oxidatively liable unsaturated fatty acids. Antioxidant compounds are often added to foods as a way to limit lipid oxidation through control of prooxidant metals, scavenging oxygen, or quenching free radicals (Decker et al. 2010). However, the
growing health concerns over synthetic additives have led researchers to seek alternative natural solutions to prevent lipid oxidation (Pokorný 2007). Unfortunately, natural antioxidants are often not as effective as their synthetic counterparts and expensive government approval for additives makes it difficult for novel antioxidants to emerge. As a result, there remains a need to maximize current antioxidant strategies in food applications.

Removal of oxygen is a potential antioxidant strategy because atmospheric triplet oxygen exists as a biracial molecule (•OO•), which can react at near diffusion-limited rates with other radicals (Zhu & Sevilla, 1990). In the case of lipid oxidation, oxygen biracial reacts with alkyl radicals on fatty acids (L•) to form higher energy peroxyl radicals (LOO•). Peroxyl radicals can then abstract a hydrogen from other molecules (e.g. unsaturated fatty acids and free radical scavenging antioxidants) to form a fatty acid hydroperoxide (LOOH), and thereby transferring the radical to the other molecule (McClements & Decker, 2000). Because the oxygen biradical reacts so quickly with fatty acid radicals, and many lipid oxidation products have very low sensory thresholds (e.g., ppb) (for a compilation of sensory thresholds see Frankel 2005), this suggests that only small amounts of oxygen are needed to cause product quality loss by oxidation.

Oxygen reduction in packaged foods is an attractive antioxidant strategy as shelf life can be prolonged without the use of food additives. Indeed, oxygen reduction in packaged foods has been a recommended strategy to prevent lipid oxidation for decades. Strategies to remove oxygen from foods have included vacuum packaging, addition of edible oxygen scavengers, or displacement of oxygen by nitrogen flushing (for review see Johnson & Decker, 2015). Surprisingly, there are relatively few studies where
oxygen concentrations are varied to study the impact on lipid oxidation rates (Table 4.1). Of the nine papers identified, four studied powdered foods and four other papers studied bulk oils (two actually studied bulk oil placed on cotton (Andersson & Lingnert 1998, Andersson & Lingnert 1999), so the studies might not truly represent bulk oil due to the large surface area). The remaining paper studied the effect of oxygen concentrations in fatty acid and fatty acid ethyl ester-in-water emulsions over a short time period of 18 h with shaking. While these studies have provided significant guidance that extremely low oxygen levels are typically required to give extensive oxidative stability, there still remains a need to identify a minimum amount of oxygen reduction to provide an antioxidant effect.

Table 4.1. Studies identified that researched the influence of oxygen concentrations on lipid oxidation rates.

<table>
<thead>
<tr>
<th>First author</th>
<th>Year</th>
<th>Food matrix</th>
<th>Oxygen measurement method</th>
<th>Oxygen concentration tested</th>
<th>Oxygen conc.: initial or constant (headspace, ppm dissolved oxygen)</th>
<th>Sample amount and container size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marchese</td>
<td>1968</td>
<td>Fatty acid ester emulsion</td>
<td>Beckman/Worthing-type apparatus</td>
<td>0.5, 1, 2, 5, 21%</td>
<td>Constant</td>
<td>Not available</td>
</tr>
<tr>
<td>[10] Kacyn</td>
<td>1983</td>
<td>Methionine in cellulose and melon powder</td>
<td>Beckman oxygen electrode</td>
<td>1.05, 2.17, 5.49, 10.69%</td>
<td>Constant</td>
<td>Not available</td>
</tr>
<tr>
<td>Min</td>
<td>1983</td>
<td>Bulk refined soybean oil</td>
<td>Beckman model 200/0 oxygen analyzer</td>
<td>2.5, 4.5, 6.5, 8.5 ppm dissolved oxygen</td>
<td>Initial</td>
<td>Filled 50 mL glass bottles</td>
</tr>
<tr>
<td>Koelich</td>
<td>1991</td>
<td>Powdered cellulose soybean oil mixture</td>
<td>Sensors (no more details given)</td>
<td>1.2, 4.5, 10, 15.4%</td>
<td>Constant</td>
<td>Not available</td>
</tr>
<tr>
<td>Anderson</td>
<td>1997</td>
<td>Cream powder</td>
<td>GC thermal conductivity detector</td>
<td>0.06, 0.07, 0.15, 0.34, 0.83, 1.7, 20.9%</td>
<td>Initial with significant ingress</td>
<td>50 g sample in 50 mL glass bottle</td>
</tr>
<tr>
<td>Anderson</td>
<td>1998</td>
<td>Bulk rapeseed oil</td>
<td>GC thermal conductivity detector</td>
<td>0.04, 0.17, 1.1, 20.9% headspace</td>
<td>Initial with minimal ingress</td>
<td>2.5 mL sample on cotton in 500 mL glass bottle</td>
</tr>
<tr>
<td>Anderson</td>
<td>1998</td>
<td>Cream powder</td>
<td>GC thermal conductivity detector</td>
<td>0.03, 0.04, 0.35, 1.3, 20.9% headspace</td>
<td>Initial with minimal ingress</td>
<td>50 g sample in 560 mL glass bottles</td>
</tr>
<tr>
<td>Anderson</td>
<td>1999</td>
<td>Bulk rapeseed oil</td>
<td>GC thermal conductivity detector</td>
<td>0.03, 0.3, 1.0, 1.8% headspace</td>
<td>Constant</td>
<td>2.5 mL sample on cotton in 500 mL glass bottle</td>
</tr>
<tr>
<td>Fujisaki</td>
<td>2000</td>
<td>Bulk refined safflower oil (180°C frying)</td>
<td>GC thermal conductivity detector</td>
<td>2, 4, 10, 20% headspace</td>
<td>Constant</td>
<td>200 g sample in 500 mL glass flask</td>
</tr>
</tbody>
</table>

Studies that maintained or resupplied the oxygen concentrations over time are identified as constant.

One previous challenge to establishing a minimum oxygen reduction was to account for the total amount of oxygen within a food system. In sealed liquid systems,
oxygen exists dissolved within the food matrix as well as present above the food in the headspace. Most previous studies regarding oxygen concentration typically only used headspace oxygen analysis to create and monitor oxygen conditions. In some cases, such as powdered systems, this headspace oxygen monitoring might be sufficient since there is very little dissolved oxygen and it would be very difficult to directly measure oxygen changes in the encapsulated lipid. However, in bulk oils and oil-in-water emulsions, dissolved oxygen is likely to impact oxidation reactions since the oxygen is in direct contact with the lipid. During the course of oxidation, dissolved oxygen is proposed to be consumed first and thus should be more closely related to shelf life. Unfortunately, as seen in Table 1, of the five papers on bulk oils and oil-in-water emulsions, only one measured dissolved oxygen (Min & Wen 1983), and no paper monitored both headspace and dissolved oxygen. Therefore, many previous accounts have not accounted for these oxygen phases in the system. Monitoring oxygen in both the headspace and dissolved phase simultaneously, along with fatty acid oxidation products, would help explain the relationship between these two phases with lipid oxidation.

In this paper, the role of oxygen concentration on the rate of lipid oxidation in a fish oil-in-water emulsion was studied. Emulsions were chosen because their very high lipid surface area makes them extremely susceptible to oxidation (for review see Waraho et al. 2011), which in turn makes them difficult to stabilize with natural antioxidants. In the only other published paper on varying oxygen concentrations in emulsions (Marcuse & Fredriksson 1968), the authors monitored oxygen consumed by fatty acid and fatty acid ester-in-water emulsions, but did not measure the formation of fatty acid oxidation products. Without measuring fatty acid oxidation products, it is difficult to relate changes
in headspace oxygen to fatty acid oxidation as well as determine the impact on shelf-life
due to off-flavor from fatty acid decomposition. Further, an area to expand on the
previous authors’ work would be to analyze oxygen reductions across a broader range
since they did not measure >0 and <75% oxygen removal. Studying lower levels of
oxygen reduction is important because it is not always realistic for food manufacturers,
particularly in emulsified systems, to reach such low oxygen levels. Thus, knowing if less
reduction of oxygen decreases lipid oxidation rates might make this antioxidant strategy
more practical. In the past, a hurdle to utilizing dissolved oxygen analysis to study lipid
oxidation was the difficulty in collecting data from large sample sets since dissolved
oxygen probes and Warburg apparatuses are expensive, cumbersome, and consume
oxygen. However, fluorescent techniques are now available to measure both dissolved
and headspace oxygen to overcome this obstacle (Pénicaud et al. 2012). One such
technology utilizes inert sol–gel (organically modified silica) patches to encase an
oxygen sensitive fluorescence-based transducer material (platinum-porphyrin) from
Ocean Optics Inc., 2015 (Dunedin, FL, USA). The fluorescent patches can be placed
inside sample vials at locations to measure both the headspace and dissolved oxygen non-
destructively. Thus, the impact of oxygen concentration on lipid oxidation kinetics can be
measured without altering oxidation rates by the instrument consuming oxygen.

4.3 Materials and Methods

4.3.1 Materials

Commercial fish oil (product name: Omega 30 TG food grade fish oil), with
omega-3 fatty acids present as 326 mg/g of triglycerides, was obtained from DSM Food
Specialties B.V. (The Netherlands) and stored in 15 mL glass vials at -80°C in the dark
until use. The initial quality of the oil was found to contain _1.6mmol lipid hydroperoxide/kg oil and 0.5mmol TBARS/kg oil. 2-Propanol, isoctance, 1-butanol, methanol, sodium acetate trihydrate, sodium hydroxide, hydrochloric acid, oxalic acid dihydrate, L-ascorbic acid, 2,6-dichloroindophenol, disodium EDTA, ammonium thiocyanate, and Brij 35 (a nonionic surfactant) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Trichloroacetic acid was purchased from Acros Organics (Morris Plains, NJ, USA). Thiobarbituric acid, imidazole, 4-hydroxymethyl-2,6-ditertiarybutylphenol (BHT), ferrous sulfate heptahydrate, barium chloride dihydrate, and cumene hydroperoxide (80% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). High purity custom nitrogen and oxygen gas blends (5.0 and 2.5 mole % oxygen balanced with pure nitrogen) were obtained from AirGas Specialty Gases (Radnor, PA). All chemicals and solvents were used without further purification.

4.3.2 Emulsion Preparation & Characterization

To determine the effectiveness of oxygen reduction in preventing oxidative degradation reactions, a lipid oxidation study was performed using a fish O/W emulsion as a model food system. Fish O/W emulsions were used in this study because fish oils are highly susceptible to oxidation and thus need more protection than most other emulsions. Emulsions were prepared by dissolving Brij 35 (0.10% w/w) in a 10 mM sodium acetate/imidazole buffer (pH 3.0), adding the fish oil (1.0% w/w), and then dispersing with a hand-held homogenizer (Biospec Products, Inc., Bartlesville, OK) at 7000 rpm for 2 min. The coarse emulsion then underwent three passes through a microfluidizer (Microfluidics, Newton, MA) at 10 kPSI. The emulsion was collected and 5.0 mL was dispensed into glass vials (total volume 12 mL) with a headspace of 7.0 mL. The oxygen
concentration in the closed system was modified with nitrogen or nitrogen/oxygen blends as described below. The O/W emulsion was stored in the dark at 32°C for up until 27 days. When ascorbic acid (500 μM) was evaluated, it was added following emulsion preparation and stirred in the dark for 15 min. An emulsion made with medium-chain triglycerides (MCT) as the oil phase was also produced in order used to create an oxidatively stable 1.0% O/W emulsion, prepared in the same manner as described above. This emulsion was used to determine the impact of nitrogen flushing and sparging on the O/W emulsion systems so that the oil phase would not consume oxygen during the test.

Stability of the emulsions was characterized by monitoring emulsion particle size distribution and electrical charge throughout the storage studies using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Particle size was determined by dynamic light scattering (DLS) after diluting the emulsion 10-fold with sodium acetate/imidazole buffer (0.01 M, pH 3.0). The diluted emulsion was equilibrated for 60 s before particle size measurements were recorded with an average of 11 readings. Size measurements were followed immediately by electrical charge measurements (10 readings for each measurement) with an equilibration time of 60 s and the zeta (ζ) potential (mV) of the emulsion droplets was collected. Stability tests were performed in duplicates. In all experiments, the mean particle diameters and zeta potential remained consistent from Day 0 until the samples were oxidized (data not shown).

4.3.3 Modifying the Oxygen Concentration of Oil-in-Water (O/W) Emulsions by Nitrogen Gas

Emulsion samples with 0±6%, 40±7%, 58±2%, 79±1%, 93±3%, 98±1% (n=3) oxygen reduction were obtained by flushing, flow of gas without disturbing the solution,
custom blends of nitrogen/oxygen or pure nitrogen into the sample headspace. Total oxygen reduction percentage was determined by the relative decrease in oxygen content (on a molar basis) compared to saturated oxygen conditions. Moles of oxygen were calculated from volumes and known oxygen concentrations in the headspace and dissolved states. A saturated oxygen sample (0% reduction) was achieved by exposure of the emulsion to atmospheric conditions (20°C) followed by sealing with hermetically sealed crimped caps. A 40% reduction was reached by flowing nitrogen over the vial for 30 s followed by sealing with a crimp cap. Oxygen reduction of ~58% and ~79% were achieved by flushing custom nitrogen/oxygen blends (5.0 and 2.5% moles oxygen, respectively) for 1 min into the headspace of vials that were already sealed with an airtight crimp cap vials and polytetrafluoroethylene/rubber septa (20 mm) (Figure 4.1). The gas blends were introduced into the vial via a 0.6 mm x 38 mm needle (Becton, Dickinson and Company, Franklin Lakes, NJ) protruding 5 mm into the headspace.

Figure 4.1 Oxygen Modification Setup. Schematic drawing of oxygen modifications performed on a 1.0% oil-in-water emulsion within sealed glass crimped cap vials containing dissolved and headspace oxygen sensor patches (pink circles).

Gases were allowed to exit the vial through another needle inserted through the septa. A ~98% reduction of oxygen was obtained by sparging, or bubbling a chemically inert gas through a liquid, high purity nitrogen into the emulsion solution for 1 min
through a sealed septa. After reaching desired oxygen concentrations by introducing the
gas through the septa, the needles were immediately removed and hot adhesive was then
placed over the septa holes. The function of the adhesive was to fill the holes in the septa
and allowed the subsequent attachment of aluminum foil to prevent oxygen ingress.

An emulsion made with medium-chain triglycerides (MCT) as the oil phase was
also produced in order to create an oxidatively stable 1.0% O/W emulsion, prepared in
the same manner as described above. The ability of nitrogen flushing and sparging,
bubbling nitrogen through the emulsion, to reduce dissolved oxygen levels was
determined using the MCT O/W emulsion (475 mL) in an opened commercial plastic
poly(ethylene terephthalate) (PET) salad dressing bottle (courtesy Kraft-Heinz Co.,
Chicago, IL, USA) at 20°C. The headspace of the salad dressing bottle was nitrogen
flushed through silicone tubing (5mm diameter, MasterFlex) at 20 L/min from a distance
of 5mm above the bottle’s lid to simulate conditions on a food manufacture’s bottling
line. Nitrogen sparging was performed by bubbling nitrogen (<2 L/min) through silicone
tubing placed at the bottom of the packaged O/W emulsion. In both flushing and sparging
experiments, nitrogen flow rates were chosen by the maximum flow rate without losing
product through surface splashing. Oxygen sensing patches were placed at the midpoint
(dissolved) and in the headspace of the bottle.

4.3.4 Oxygen Analysis

A NEOFOX oxygen phase fluorometer (Ocean Optics, Inc., Dunedin, FL) with
fluorescent optical sensors, encased in a chemically inert polymeric (acrylate with a
silicone overcoat) coating that allows the diffusion of oxygen, was used to determine the
dissolved and headspace oxygen concentration in, and above, a fish O/W emulsion.
Sensor patches with adhesive (5.0 mm diameter) were placed at 5 mm (dissolved oxygen patch) and at 25 mm (headspace oxygen patch) from the bottom of the vials, respectively. Oxygen analysis was conducted at 32°C (the temperature of the storage study) on triplicate samples, which were monitored over 19 days. Analysis occurs through a bifurcated cable that excites the sensor (475/600 nm) with LED light and the emitted energy is carried back through the fiber back to the phase fluorometer. The fluorescence emission is then used to calculate excited state lifetime, or \( \tau \) (Tau), \( \tau \) can then be used to calculate the partial pressure of oxygen using the Stern-Volmer equation (Pénicaud et al. 2010). Ocean Optics, Inc. calibrates the sensor patches against known oxygen concentrations and temperatures. Control patches were tested under atmospheric conditions to confirm accuracy to ~20.9% oxygen. Dissolved oxygen is reported as ppm oxygen and was calculated from the partial pressure reading and the maximum oxygen solubility of water at 32°C based on an equation by Weiss (1970). The contribution of the fish oil would slightly increase the amount of dissolved oxygen since oxygen is 3-10 times more soluble in bulk oil compared to water (for review see Chaix et al. 2014). However, due to a lack of references for the solubility of oxygen in fish oil at 32°C, and that the oil phase made up only 1.0% of the system, the contribution of DO in the oil phase to the DO content of the total emulsion was taken to be minor and was not included in calculations.

Oxygen concentration in dissolved and headspace locations varies across systems because of sample composition, sample volume, and headspace volume. Representing the data treatments as total % oxygen reduction is therefore more applicable across O/W
emulsion systems. The total % oxygen reduction was calculated from the sum of initial oxygen contents present in the headspace and dissolved phases.

4.3.5 Quantification of Lipid Oxidation Products in Fish O/W Emulsions

The formation of primary and secondary lipid oxidation products (lipid hydroperoxides and thiobarbituric acid reactive substances (TBARS), respectively) were quantified throughout the study to determine the oxidative stability of the O/W emulsions. Lipid hydroperoxides were quantified by the method reported by Hu et al. (2003). Briefly, 0.3 mL emulsion was mixed with 1.5 mL iso-octane/2-propanol (3:1 v/v) by vortex for 3 x 10 s and then centrifuged at 1785g for 2 min. The upper layer, containing the lipid hydroperoxides, was collected and then added to 2.8 mL of methanol/1-butanol (2:1 v/v), followed by addition of 30 μL thiocyanate/Fe²⁺ solution, which was obtained by mixing an equal volume of 3.94 M thiocyanate solution with 0.072 M Fe²⁺. The reaction samples were held for 20 min at room temperature to enable color formation. Absorbance of the samples was read at 510 nm and lipid hydroperoxide concentrations were calculated from a calibration curve prepared using six concentrations of cumene hydroperoxide between 0 and 60 mmol/kg oil (r² = 0.99, linear regression). Samples were diluted with methanol/1-butanol to maintain their absorbance within the prepared calibration concentration range.

The thiobarbituric acid reactive substances (TBARS) assay was used to measure secondary lipid oxidation products as described by Alamed et al. (2005) with slight modification. Emulsions (1.0 mL) were combined with 2.0 mL TBA reagent (15% w/v TCA, 0.375% w/v TBA in 0.25 M HCl) mixed with 2% w/v BHT in ethanol for a final volume ratio of 467 to 13.6, respectively. Samples were then vortexed (5 s) before being placed in a 75°C water bath for 15 min. The test tubes were immediately cooled to room
temperature and were centrifuged at 595g for 15 min. The absorbance was measured at 532 nm and TBARS concentrations were calculated from a calibration curve prepared using seven concentrations of 1,1,3,3-tetraethoxypropane between 0 and 4 mmol/kg oil ($r^2 = 0.99$, linear regression). Samples were diluted with double distilled water to maintain their absorbance within the prepared calibration concentration range.

Wavelength scans were conducted from 300-700 nm for studies utilizing ascorbic acid since ascorbic acid can react with TBA to produce TBARS (Guillén-Sans & Guzmán-Chozas, 1998). Wavelength scans did not show the appearance of ascorbic acid-TBA adducts (Gutteridge & Wilkens, 1982), which could cause interference under the conditions tested (data not shown).

Our laboratory typically measures headspace aldehydes to monitor the formation of secondary lipid oxidation products. However, the need to seal the headspace vial septa with adhesive after gas flushing made headspace analysis impossible. TBARS is a viable alternative since the high degree of fatty acid unsaturation in fish oil produces significant yields of TBARS (McClements & Decker 2008). In addition, it has been previously reported that different oxygen partial pressures can influence the pathways of lipid oxidation to alter the profile of volatile aldehyde oxidation products (Andersson & Lingnert 1998b, Schieberle & Grosch 1981). Thus, the TBARS assay provides an additional advantage in this respect by avoiding this potential complication.

Lag phase calculations for lipid oxidation studies were determined by measuring the intersection of the tangent lines of the lag and exponential phases of lipid oxidation as described in (O’Keefe & Pike, 2010). Lag phases were used to determine the effectiveness of treatments since after the lag phase the sample is rancid.
4.3.6 Influence of Oxygen Concentration on the Oxidative Stability of Ascorbic Acid

In select studies the ability of L-ascorbic acid (AA) to decrease oxygen concentrations and alter lipid oxidation kinetics was determined. AA degradation was determined by a spectrophotometric method described by Uluata et al. (2015) with slight modification. Briefly, samples (0.50 mL) were mixed with sodium oxalate (0.08% w/v) (1.0 mL) by vortex for 10 s to stabilize AA. A portion of the stabilized ascorbic acid mixture (0.70 mL) was then mixed by vortex for 2 x 5 s with 0.20 mM 2,6-dichloroindophenol (4.30 mL). The reduction of the indicator dye to a colorless compound in the presence of ascorbic acid was measured at 520 nm using an Ultrospec 3000 pro UV-vis spectrophotometer (Cambridge, England). AA concentrations were calculated from a calibration curve prepared using seven concentrations of reagent grade ascorbic acid between 0.0 and 3.0 mM ($r^2 = 0.99$, linear regression). The oxidative stability of AA, and thus its ability to scavenge dissolved and/or headspace oxygen, was measured across saturated and reduced oxygen environments (manufactured as described above).

4.3.7 Statistical Analysis

Results are presented as means and standard deviation of experiments conducted in triplicate, unless otherwise stated. Statistical difference in means for oxygen and ascorbic acid data, compared to time 0, was defined using one-way analysis of variance (ANOVA) with multiple comparisons of means performed using a one-tailed Dunnett’s post hoc test ($p = 0.05$). Calculations were performed using IBM SPSS Statistics version 22 (IBM SPSS, Chicago, IL, USA).
4.4 Results and Discussion

4.4.1 Modification of Oxygen Concentrations of O/W Emulsions

The level of dissolved oxygen in solutions could be reduced in emulsions by techniques such as boiling, reducing the pressure, sonication, and flushing with an inert gas (e.g., nitrogen) (Butler et al. 1994). Preliminary experiments using these methods revealed that preparation of a large amount of emulsion and sequential transfer to individual vials for storage resulted in significant contamination with oxygen. Thus, flushing individual headspace vials with nitrogen/oxygen gas blends (5.0 and 2.5 mole % oxygen basis balanced with pure nitrogen), or high purity nitrogen, produced emulsions with reduced oxygen concentrations. Preliminary studies showed that while atmospheric oxygen could leak into the vials with screw caps, sealed crimp cap vials were effective in preventing oxygen from permeating into the vials for the duration of the study. Samples with ≥ 58% oxygen reduction required that the vials first be sealed followed by flushing gas blends through a needle that penetrated the septa. Hot glue adhesive (FPC Corporation, Wauconda, IL) was then placed over the septa holes to both seal the holes and allow attachment of aluminum foil, which further prevented oxygen ingress. These methods seemed to overcome a previous challenge where significant oxygen ingress occurred over the time course of studies, which makes it difficult to determine the oxygen concentration that actually decreases lipid oxidation rates. The oxygen reduction methods described above was able to produce a range of oxygen concentrations represented as total percent oxygen reduction (~0, ~40%, ~58%, ~79%, ~93%, and ~98%), which was calculated from the oxygen content present in each phase.
The oxygen detection technology described herein provides significant advantages over previous assays in its ability to simultaneously monitor headspace and dissolved oxygen in a non-destructive and continuous manner. Fitzgerald et al. (2001) showed that the applicability of this type of technology compared well to traditional headspace gas analysis methods. Nevares & Del Alamo (2008) used this technology in studies for understanding the influence of oxygen in accelerated aging of wine.

4.4.2 Impact of Oxygen Concentrations on the Oxidative Stability of O/W Emulsions

The effect of oxygen reduction on the oxidative stability of 1.0% fish O/W emulsions was determined by quantification of primary (lipid hydroperoxides) and secondary (TBARS) (Figure 4.2) lipid oxidation products during storage at 32°C in the dark. Once samples exited lag phase, as evidenced by measurable primary and secondary oxidation products, they were removed from the study and not further characterized. Both emulsions saturated with oxygen (0% oxygen reduction) and emulsions with ~40% oxygen reduction exhibited lag phases of 6 days for both lipid hydroperoxides and TBARS production. Further decreasing oxygen, ~58% and ~79% reductions, extended the lag phase to 8 days for both lipid hydroperoxides and TBARS production. The extension of lag phase only became pronounced when oxygen was reduced by ~93%, where the lag phase was 19 days for both lipid hydroperoxides and TBARS production. A further reduction in oxygen, removing ~98% of the initial oxygen, resulted in enhanced stability with the sample not exiting lag phase over the course of the 27 day study. Marcuse & Fredriksson (1968) also showed that by decreasing headspace oxygen above a linoleic acid emulsion by 90–98% slowed the rate of oxygen consumption more than emulsions with 0–76% of headspace oxygen reduction. However, their study did not
compare reduction of headspace oxygen to formation of lipid oxidation products. The
current study provides a correlation between oxygen concentrations and fatty acid
oxidation product lag phases and suggest that only extremely low levels of oxygen are
able to meaningfully stabilize the emulsion against lipid oxidation.

Figure 4.2. Lipid Oxidation Across Oxygen Concentrations. Formation of lipid
hydroperoxides (top) and thiobarbituric acid reactive substances (TBARS; bottom) in 10
mM sodium acetate-imidazole buffered (pH 3.0) fish oil-in-water emulsions stored at
32°C across a range of total oxygen reductions. Values represent averages ± standard
deviation of n=3 determinations.

The influence of oxygen content in the emulsion (dissolved, as ppm) and in the
headspace (as % atm) on lipid oxidation was characterized by in situ oxygen
measurement of both phases throughout lipid oxidation study (Figure 4.3a and 4.3b,
respectively). Preliminary analysis of buffer in the sealed vials showed that oxygen neither decreased nor increased over the course of 13 days at 37°C (data not shown), thus indicating that the buffer components were not consuming oxygen and that the sealed vials were stable. The overall trend for samples showed that dissolved oxygen was consumed first, followed by a decrease in headspace concentration. For example, in O/W emulsions saturated with oxygen (0% reduction), a statistically significant decrease of 12% dissolved oxygen was observed at Day 4 whereas no statistically significant decrease in headspace oxygen appeared until Day 8 (p≤0.05). The lag phase of both lipid hydroperoxides and TBARS was six days indicating that dissolved oxygen was consumed before lipid oxidation exited the lag phase. Once the exponential phase of oxidation began, dissolved oxygen was consumed rapidly and headspace oxygen began to decrease, presumably from diffusing into the oxygen depleted emulsion. Similar trends were observed for the 40-79% reduced oxygen samples. When more than 93% of oxygen was removed, no statistical significant difference between the means of headspace oxygen across the 19 days of analysis were detected.

Figure 4.3. Dissolved and Headspace Oxygen Consumption. Consumption of dissolved (left;a) and headspace (right;b) oxygen in 10 mM sodium acetate-imidazole buffered (pH 3.0) fish oil-in-water emulsions stored at 32°C across a range of total oxygen reductions. Values represent averages ± standard deviation of n=3 determinations.
Lipid oxidation starts with the formation of alkyl radicals, which then react with biradical molecular oxygen at near oxygen diffusion limited rates \((2–8 \times 10^9 \text{ M}^{-1} \text{ s}^{-1})\); Zhu & Sevilla, 1990). This reaction forms peroxyl radicals that react more quickly with free radical scavenging antioxidants such as tocopherol than unsaturated fatty acids (McClements & Decker, 2008). Preferential oxidation of tocopherols can be seen by the depletion of tocopherols in emulsions prior to formation of lipid oxidation products (Panya et al., 2012). In O/W emulsions, transition metals decompose hydroperoxides into free radicals, which can oxidize unsaturated fatty acids once the majority of tocopherols are depleted. The unprotected oil then rapidly oxidizes and dissolved oxygen is rapidly consumed. It is possible that once enough oxygen is removed from the emulsion, there is not enough free radical production to deplete the tocopherol and they are more effective at delaying oxidation. Headspace oxygen does not decrease until the lag phase of oxidation is completed as it diffuses into the emulsion to replace the lost dissolved oxygen. This suggests that headspace oxygen is not an accurate measurement of the shelf life of emulsions as its delayed consumption is after the lag phase and thus would over-estimate the stability of foods to oxidation. While prior reports have done an excellent job of establishing that headspace oxygen consumption can approximate oxidative stability, to the best of the authors’ knowledge this is the first demonstration that monitoring DO content in emulsions can more precisely predict oxidative stability.

4.4.3 Influence of Nitrogen Flushing and Sparging on Dissolved Oxygen Concentrations in O/W Emulsions

Commercial food manufacturers modify the oxygen content of packaged foods by flushing with an inert gas (e.g., nitrogen) in an attempt to reduce the sensitivity to
oxidation (Johnson & Decker 2015). While this may be applicable for solid foods, where rapid removal of headspace oxygen is possible, emulsions retain oxygen in the dissolved phase. Oxygen removal in emulsions by nitrogen flushing of the headspace is likely to be slow, as dissolved oxygen needs time to equilibrate with the partial pressure above the solution. Headspace oxygen concentration dropped to ~2% immediately during nitrogen flushing, but did not decrease any further presumably due to a small amount of atmospheric contamination from the bottle not being capped or oxygen diffusing from the emulsion to the headspace. As previously demonstrated, the limiting factor regarding nitrogen flushing is believed to be the difficulty in reducing dissolved oxygen. Thus, dissolved oxygen measurements were taken continuously while flushing the sample to determine the time needed to reach oxygen levels necessary to extend the lag phase of lipid oxidation.

Figure 4.4. Dissolved Oxygen During Commercial Nitrogen Modification Strategies. Percent dissolved oxygen reduction by nitrogen flushing and sparging in an oxidatively stable 1% medium chain triglyceride (MCT) oil-in-water emulsion in salad dressing bottle (values represent averages ± standard deviation of duplicates).

Dissolved oxygen of the oxidatively stable MCT O/W emulsion was measured over 60 min of either flushing or sparging. Results demonstrate that 60 min of nitrogen flushing
was required to reach the minimum ~58% dissolved oxygen reduction needed for enhanced oxidative stability. Nitrogen sparging was both more effective and more efficient than headspace flushing. At a lower nitrogen flow rate, sparging reduced oxygen concentrations by >58% within 15 minutes and about 45 min to reach ~90% oxygen reduction. Commercial bottling operations are too fast to allow the 15-60 minutes needed to reduce dissolved oxygen levels to the levels that would inhibit lipid oxidation. As lipid contents increase in higher fat emulsions, and thus more dissolved oxygen and increased viscosity, even more time is expected to reach sufficient reductions of oxygen. Higher fat emulsions would thus make it even more difficult for oxygen to be reduced to levels that would inhibit lipid oxidation during rapid food manufacturing operations. The model simulation tested suggests that neither flushing nor sparging is an effective antioxidant strategy in bottled O/W emulsions.

4.4.4 Impact of Ascorbic Acid on Oxygen Concentrations and Oxidative Stability of O/W Emulsions

The inability of nitrogen flushing and sparging to obtain oxygen levels able to inhibit oxidation in a commercially relevant time frame suggests that nitrogen flushing might need to be used in combination with other oxygen reduction strategies. The antioxidant mechanisms of ascorbic acid are commonly referred to as a combination of free radical and oxygen scavenging (Cort 1982). An objective of this investigation was to determine if the reported antioxidant function of ascorbic acid as an oxygen scavenger could be used in combination with nitrogen flushing to reduce oxygen to levels that would impact lipid oxidation rates.
The oxygen reduction and degradation of L-ascorbic acid (2.9 mM) in O/W emulsions with 0±6%, 60±1%, and 75±1% (n=3) of oxygen removed at 32°C for 19 days in the dark is shown in Figure 4.5a and 4.5b, respectively.

Figure 4.5. (a) Ascorbic Acid and Oxygen Reduction: Reduction of total oxygen by ascorbic acid (2.9 mM) under different percentages of initial oxygen reductions in 10 mM sodium acetate-imidazole buffered (pH 3.0) stored at 32°C. Values represent averages ± standard deviation of n=3 determinations. (b) Degradation of ascorbic acid (2.9 mM) in 10 mM sodium acetate-imidazole buffered (pH 3.0) stored at 32°C across a range of oxygen reductions. Values represent averages ± standard deviation of n=3 determinations.

Higher concentrations of ascorbic acid were tested, but these resulted in browning reactions making them impractical in food applications (data not shown). Oxygen reduction occurred under saturated oxygen conditions (0% oxygen reduction), which saw a ~29% oxygen reduction over the course of the study (Figure 4.5a). In buffered solutions
where initial oxygen was reduced by ~60%, AA’s ability to scavenge oxygen was much less (~11%) and when ~75% of the oxygen was removed, there was no statistically significant difference in oxygen concentrations at time 0 and 19 days. The data indicate that in all three buffered solutions, AA alone was not able to reduce oxygen enough to get to levels ≥ 58% reduction that would extend the lag phase of lipid oxidation.

An argument could be made that the ratio of headspace to sample volume is large within this study (7 to 5 cm³, respectively) and that with a smaller headspace to volume ratio ascorbic acid could protect against lipid oxidation by oxygen scavenging. However, this is not likely as the data suggests that as the partial pressure of oxygen decreases, the ability of ascorbic acid to scavenge oxygen also decreases. Although AA only had minor effects on oxygen concentrations, it was observed to degrade in all samples including the 75% reduced oxygen treatment where AA was not able to decrease oxygen (Figure 4.5b). AA degradation can occur by acid and metal driven anaerobic mechanisms that do not result in the consumption of oxygen (Bradshaw et al. 2011). Loss of AA as a function of oxygen concentrations is in agreement with previous research that showed AA degradation was strongly influenced by initial DO contents in fruit juice (Kennedy et al. 1992, Trammel et al. 1986).

The previous results suggested that it is highly unlikely that ascorbic acid functions as an antioxidant in O/W emulsion system by its oxygen scavenging abilities. However, ascorbic acid’s free radical scavenging capacity could be enhanced under reduced oxygen if reduced oxygen levels resulted in decreased peroxyl radical formation. To test this possibility, ascorbic acid (500 μM) was added after emulsion formation
followed by removal of 79% of the oxygen by nitrogen flushing as described above and then formation of lipid oxidation products was monitored (Figure 4.6).

Figure 4.6. Ascorbic Acid and Oxygen Reduction Impact on Lipid Oxidation: Formation of lipid hydroperoxides (left) and thiobarbituric acid reactive substances (TBARS; right) in 10 mM sodium acetate-imidazole buffered (pH 3.0) fish oil-in-water emulsions where 79% of oxygen had been removed from the emulsion. Dotted lines represent when separate samples were opened and exposed to oxygen to saturate the emulsion. Values represent averages ± standard deviation of n=3 determinations.

Reduction of oxygen by 79% was chosen because ascorbic acid concentrations did not decrease at this oxygen level and thus concentrations would be highest and most likely to inhibit oxidation. Lipid oxidation, as measured by both lipid hydroperoxides and TBARS was increased by the addition of ascorbic acid under reduced oxygen conditions (~79% reduced oxygen) compared to the no AA control. The addition of ascorbic acid caused lipid hydroperoxides and TBARS values to rise after 2 days followed by a plateau of the oxidation markers, presumably due to oxygen limiting further oxidation. Lipid hydroperoxides and TBARS did not increase until 6 days of storage for sample without AA. To confirm the hypothesis that plateauing of oxidation markers were due to limited oxygen concentrations, reduced oxygen samples containing oxygen sensors were opened and allowed to equilibrate with the atmosphere until they reached headspace saturation (2 min). The samples were then resealed with a crimp cap, inverted, and placed back in storage. Compared to samples where oxygen was not reintroduced, oxygen renewal
allowed lipid hydroperoxide and TBARS formation to resume, especially in the presence of ascorbic acid. An explanation for this phenomenon could be that ascorbic acid recycles iron to its more reactive state that can generate free radicals by promoting hydroperoxide breakdown. This experiment also suggests that even if AA could stabilize emulsions in a closed low oxygen environment, that once the consumer opens the container, it would be a strong prooxidant causing product failure. Taken together, under the conditions tested the data suggests that not only does ascorbic acid not function as an antioxidant by oxygen scavenging, it is likely to be a prooxidant by recycling transition metals even when oxygen concentrations are greatly reduced.

4.5 Conclusion

This work follows oxygen concentration and reports that reducing oxygen levels had a non-linear impact on inhibition of lipid oxidation rates in a 1%O/W emulsion. A minimum of ≥58% total oxygen needed to be removed in O/W emulsions to provide any antioxidant effect. Further, more than doubling of the lag phase of lipid oxidation was observed when oxygen reduction was ≥93%. Current methods, including oxygen reduction by nitrogen flushing or sparging and addition of edible oxygen scavengers such as AA, were unable to reduce oxygen concentrations enough to inhibit lipid oxidation in a reasonable time frame. To the best of the authors’ knowledge, this is the first demonstration of in situ dissolved and headspace oxygen analysis as a practical, nondestructive method for characterizing influence of oxygen content on lipid oxidation.

4.6 Acknowledgements

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4.7 Conflict of Interest

The authors do not have any conflicts of interest to declare.

4.8 References


CHAPTER 5

THE INFLUENCE OF ANTIOXIDANTS UNDER REDUCED OXYGEN ENVIRONMENTS IN STRIPPED FISH OIL-IN-WATER EMULSIONS

5.1 Abstract

The work presented in this chapter strove to understand if antioxidant functionality of naturally derived antioxidants could be enhanced under reduced oxygen atmospheres to synergistically prevent lipid oxidation. Atmospheres above 1% fish O/W emulsions were modified using nitrogen/oxygen gas blends to create intermediate ~60-75% reduced oxygen atmospheres. Lipid oxidation, as measured by lipid hydroperoxides and thiobarbituric acid reactive substances (TBARS), was inhibited by lipophilic antioxidants in O/W emulsions. Aqueous phase antioxidants promoted lipid oxidation. Notably, the intermediate oxygen removal did not appear to enhance functionality of the antioxidants at the concentrations tested. Results suggest that intermediate oxygen reduction in addition to antioxidants is not a viable commercial antioxidant strategy.

5.2 Introduction

The impact of how naturally derived polar and nonpolar antioxidants function under saturated and reduced oxygen atmospheres in 1% stripped fish O/W emulsions was determined. In previous work, it was shown that 1% fish O/W emulsions that were packaged with total oxygen reduction concentrations of ≥93% exhibited a three-fold increase in oxidative stability compared to a control that was stored under saturated oxygen conditions (Johnson et al. 2016, Chapter 4). Unfortunately, reaching total package oxygen reduction concentrations of ≥93% may not be cost effective or even a feasible strategy for emulsified foods and beverages (Johnson & Decker 2015). Total
package oxygen reduction concentrations of 60-80% may be more practical, yet when tested they provided only a slight increase of 33% in oxidative stability (Johnson et al. 2016). A more practical approach would be to use reduced oxygen atmospheres in combination with other antioxidant strategies, such as the addition of naturally derived antioxidants. A combination strategy may help to achieve maximum shelf life of emulsion systems without the use of synthetic additives, such as the metal chelator EDTA. Natural antioxidants, including tocopherols and plant extracts containing phenolic acids like protocatechuic acid from a wide range of plants including açaí, are attractive strategies for food manufacturers as they are more in line with the ‘clean label’ food movement. Therefore, an understanding of how these natural antioxidants function under reduced intermediate oxygen levels will help guide better antioxidant implementation in emulsified systems.

5.3 Materials and Methods

5.3.1 Materials

Commercial fish oil, with omega-3 fatty acids present as 326 mg/g of triglycerides, was obtained from DSM Food Specialties B.V. (The Netherlands) and stored in 15 mL glass vials at -80°C in the dark until use. 2-Propanol, isoctance, 1-butanol, methanol, sodium acetate trihydrate, sodium hydroxide, hydrochloric acid, ammonium thiocyanate, and Brij 35 (a nonionic surfactant) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Trichloroacetic acid was purchased from Acros Organics (Morris Plains, NJ, USA). Thiobarbituric acid, imidazole, 4-hydroxymethyl-2,6-diteriarybutylphenol (BHT), alpha-tocopherol (96% purity), ferrous sulfate heptahydrate, barium chloride dihydrate, cumene hydroperoxide (80% purity), activated
charcoal, and sand were purchased from Sigma-Aldrich (St. Louis, MO, USA). Silicic acid (100-200 mesh size) was purchased from Clarkson Chromatography (South Williamsport, PA, USA). Protocatechuic acid (CO) and protocatechuic acid alkyl ester (C8) were obtained courtesy of collaborators at CIRAD UMR IATE (Montpellier, France) and were manufactured in similar manner to that performed by Lecomte et al. (2010). High purity custom nitrogen and oxygen gas blends (5.0 and 2.5 mole % oxygen balanced with pure nitrogen) were obtained from AirGas Specialty Gases (Radnor, PA). All chemicals and solvents were used without further purification.

5.3.2 Emulsion Preparation

To determine the effectiveness of oxygen reduction in preventing oxidative degradation reactions, a lipid oxidation study was conducted using a stripped fish O/W emulsion as a model system. Oil stripping, as described in Homma et al. (2016), was performed to ensure that endogenous minor components (e.g., tocopherols and prooxidant metals) were removed before emulsion manufacture and that antioxidant activity was dependent on oxygen concentration and exogenous antioxidants. Stripped fish oil was then used to create 1% O/W emulsions by dissolving Brij 35 (0.10% w/w) in a 0.01 M sodium acetate/imidazole buffer (pH 3.0) followed by the addition of stripped fish oil (1.0% w/w). The mixture was then dispersed with a hand-held homogenizer (Biospec Products, Inc., Bartlesville, OK) at 7000 rpm for 2 min, followed by three passes through a microfluidizer (Microfluidics, Newton, MA) at 10 kPSI.

5.3.3 Antioxidant Addition and Oxygen Reduction

Naturally derived antioxidants, both water-soluble (protocatechuic acid) and liposoluble (alpha-tocopherol and protocatechuic acid esters) were added at different
manufacturing steps in stripped fish O/W emulsions. α-Tocopherol (500 μM of the oil phase) was added to the stripped oil phase prior to emulsion manufacture, whereas protocatechuic acid and its ester (30 μM of the total emulsion as shown to be an effective concentration for similar compounds in Panya et al. (2012) were dissolved in methanol and stirred into the emulsion post manufacture (1 h at 300 rpm in the dark). The emulsion was collected and 5.0 mL was dispensed into glass vials (total volume 12 mL) with a headspace of 7.0 mL. Oxygen removal in the finished, sealed emulsion system was carried out as described in Johnson et al. (2016) and the final O/W emulsion sample was stored in the dark at 20°C.

5.3.4 Lipid Oxidation and Oxygen Analysis

Lipid oxidation primary (lipid hydroperoxides) and secondary (TBARS) products were quantified as described in Johnson et al. 2016 (Chapter 4) throughout the study to determine the oxidative stability of each emulsion. Lag phase time for lipid oxidation studies were determined by the intersection of tangent lines of the lag and exponential phases of lipid oxidation as described in O’Keefe et al. (2010).

A NEOFOX oxygen phase fluorometer (Ocean Optics, Inc., Dunedin, FL) with fluorescent optical sensors was used to determine the oxygen reduction and in some cases to monitor headspace and dissolved oxygen concentration in a fish O/W emulsion as described previously in Johnson et al. (2016).

5.3.5 Statistical Analysis

Results are presented as means including standard deviations for experiments conducted in triplicate, unless otherwise stated.
5.4 Results and Discussion

5.4.1 Antioxidant Effect of α-tocopherol at Saturated and Reduced Oxygen Concentration in Emulsions

α-Tocopherol (500 μM of the oil phase) was tested under saturated (i.e., atmospheric) oxygen conditions and a system where oxygen was reduced by 75%. Dissolved oxygen measurements demonstrated that distinct levels of oxygen were created by flushing with different oxygen/nitrogen blends and oxygen also served as an initial marker to monitor lipid oxidation (Johnson et al. 2016) (Figure 5.1). Sharp decreases in dissolved oxygen content, for samples without antioxidant, suggested instability and that lipid oxidation was consuming oxygen. At both oxygen levels, tocopherol presence resulted in a delay in oxygen consumption.

As seen in Figure 5.2, the control emulsion under saturated oxygen conditions began to oxidize almost immediately as determined by both lipid hydroperoxides and TBARS formation. The ~75% reduced oxygen system only had slightly more protection against oxidation. Previous results from our laboratory (Johnson et al. 2016) demonstrated that this level of oxygen reduction provides a minimal protection against oxidation in 1% O/W emulsions. A clear extension of the lag phase of lipid oxidation was only achieved by the addition of α-tocopherol, which saw an extension to 14 days of oxidative stability for both lipid hydroperoxides and TBARS. Reducing the amount of system oxygen in the presence of α-tocopherol reduced overall lipid oxidation products after 20 days storage compared to either treatment alone. However, the combination of reduced oxygen and α-tocopherol still allowed production of lipid oxidation products slowly over time.
Figure 5.1. Dissolved (A) and headspace (B) oxygen of 0.01 M sodium acetate-imidazole buffered (pH 3.0) stripped fish oil-in-water emulsions stored at 20°C in saturated and reduced oxygen environments and tested with and without the presence of α-tocopherol (500 μM of the oil phase). Values represent means ± standard deviation (n=3).
Figure 5.2. Formation of lipid hydroperoxides and TBARS in 0.01 M sodium acetate-imidazole buffered (pH 3.0) stripped fish oil-in-water emulsions stored at 20°C in saturated and reduced oxygen environments and tested with and without the presence of α-tocopherol (500 μM of the oil phase). Values represent means ± standard deviation (n=3).

These results suggest that α-tocopherol under intermediate oxygen (~75%) reductions does not see an increase its antioxidant functionality since lipid
hydroperoxides and TBARS continue form at a slow rate after 14 days. The reaction rate is likely limited as dissolved oxygen values slowly decrease to greater than ~90% oxygen reduction (Figure 5.1). A previous report by Marcuse and Fredriksson (1969) shows general agreement that a reduction of initial oxygen concentrations above linoleic acid emulsions containing 0.005% (w/w) tocopherol did not enhance antioxidant functionality. It is possible that tocopherol degradation is independent of oxygen concentration down to these oxygen levels tested, and that to see enhanced functionality lower oxygen values must be achieved. However, as mentioned previously, reaching really low oxygen levels can be quite difficult with current technology.

5.4.2 Antioxidant Effect of Protocatechuic Acid and its Alkyl Ester at Saturated and Reduced Oxygen Concentrations in Emulsions

To understand more broadly if natural antioxidants of different polarities and origins could function more effectively under reduced oxygen (~60% oxygen reduction), protocatechuic acid (PCA) and its C8 alkyl ester were tested in the same model emulsion system. Whereas α-tocopherol is a non-polar antioxidant, PCA and its C8 alkyl ester are likely to be located in the aqueous phase or interfacial region, respectively (Panya et al., 2012). Lipid oxidation markers suggest that PCA, the aqueous antioxidant, promoted lipid oxidation in 1% stripped fish O/W emulsions and the prooxidant effect was mitigated by the reduced oxygen, albeit mildly. On the other hand, the PCA-alkyl ester had a modest antioxidant effect under saturated oxygen conditions extending the lag phase from 4 to 6 days, and was slightly enhanced under reduced oxygen atmospheres with a lag phase of 8 days.
PCA exhibited a prooxidant effect as similar to effects caused by the polar gallic acid in emulsions, presumably by activating or recycling prooxidant transition metals in the aqueous phase (Gonzalez et al. 2010). The PCA alkyl ester (C8) location at the interface could help as seen in previous experiments to concentrate its free radical scavenging at the interface where lipid oxidation occurs (Laguerre et al. 2009).

Intermediate oxygen reduction can slow the oxidation rate down enough to alter the rate that which lipids oxidize (Johnson et al. 2016), but results presented herein suggest that this slowing of rate is not enough to make important alterations in the efficacy of the C8 ester or decrease the prooxidant activity of the PCA.

Figure 5.3. Formation of lipid hydroperoxides and TBARS in 0.01 M sodium acetate-imidazole buffered (pH 3.0) stripped fish oil-in-water emulsions stored at 20°C in saturated and reduced oxygen environments and tested with and without the presence of protocatechuic acid (PCA) or PCA-alkyl ester (30 μM of the emulsion). Values represent means ± standard deviation (n=3).
Figure 5.3. (continued) Formation of lipid hydroperoxides and TBARS in 0.01 M sodium acetate-imidazole buffered (pH 3.0) stripped fish oil-in-water emulsions stored at 20°C in saturated and reduced oxygen environments and tested with and without the presence of protocatechuic acid (PCA) or PCA-alkyl ester (30 μM of the emulsion). Values represent means ± standard deviation (n=3).

In conclusion, the intermediate oxygen reductions that provide some protection against oxidation did not enhance antioxidant functionality (regardless of polarity) in stripped fish O/W emulsions. In this model system, the removal of oxygen merely limited the final extent of lipid oxidation in the system, and did not function synergistically to enhance antioxidants. However, the data presented herein did indicate that regardless of oxygen concentration, α-tocopherol and phenolic alkyl esters can be effective natural inhibitors of lipid oxidation in emulsified systems. Therefore, it might not be worth the investment for food companies to attempt intermediate oxygen removal if antioxidants are already contributing to the majority of the oxidative stability. Instead, focusing resources on the quality and location of antioxidants might be more productive. Another
potential option is to explore new technology that effectively removes more oxygen (≥90%) from emulsions in order to provide ‘clean label’ oxidative stability.

5.5 References


CHAPTER 6

OXYGEN SCAVENGING PACKAGING INHIBITION OF VITAMIN DEGRADATION AND LIPID OXIDATION IN FISH OIL-IN-WATER EMULSIONS

6.1 Abstract

The impact of commercial oxygen scavenging packaging on inhibition lipid oxidation in fish oil-in-water emulsions and vitamin degradation was determined. Oxygen scavenging packaging effectively reduced dissolved oxygen concentration in buffer (pH 3 to pH 7) by greater than 95% as measured by an oxygen phase fluorometer over 3 days at 32°C. Lipid oxidation in emulsions, as measured by lipid hydroperoxides and thiobarbituric acid reactive substances (TBARS), was inhibited by oxygen scavenging packaging across pH, fat concentration, and in the presence of sodium chloride. Analysis of ascorbic acid and α-tocopherol demonstrated the antioxidant activity of oxygen scavenging packaging to preserve both water and fat-soluble vitamins. This work suggests that active packaging can sufficiently reduce oxygen levels in liquid foods to greatly extend the oxidative stability without the addition of exogenous antioxidants.

6.2 Introduction

New strategies that sufficiently protect against the oxidation of bioactive polyunsaturated fatty acids and vitamins are needed to increase the nutritional value of food. Both synthetic and natural antioxidants have been previously employed to preserve these nutrients and extend product shelf life. However, many consumers are demanding ‘cleaner labels’ that have fewer or no added antioxidant ingredients. One potential
strategy to inhibit oxidation without exogenous antioxidants is the use of antioxidant active packaging. Active antioxidant packaging is packaging that goes beyond just providing an inert barrier to the external environment by adding functional attributes to the packaging, such as metal chelation or oxygen scavenging, in order to inhibit oxidation (for reviews see Cruz et al. 2012; Tian et al. 2013). The appeal of this type of packaging is that it performs an antioxidant function, yet does not appear on the product ingredient label. However, concerns over the maintenance of antioxidant activity after being incorporated into the packaging, increased cost of active packaging, ability to add antioxidant function to typical food packaging (e.g., poly(ethylene terephthalate), PET and lack of consumer understanding has limited their commercial success.

An alternative “clean label” strategy to inhibit lipid oxidation is oxygen reduction. Unfortunately, significant oxygen reduction in liquid systems, such as emulsions, by traditional methods (e.g. nitrogen flushing or vacuum packing) has proven difficult since >90% of the oxygen needs to be reduced to substantially extend oxidative stability (Johnson & Decker 2015, Johnson et al. 2016). If active packaging could be used to remove the majority of oxygen in a food, this could greatly protect unsaturated fatty acids and vitamins. Oxygen scavenging systems have long been used in foods such as meats, nuts, and seafood (for review see Cichello 2015). These scavenging systems can remove oxygen in foods by utilizing either enzymatic (e.g., glucose oxidase) or chemical (e.g., metal and ascorbic acid) mechanisms. However, enzymatic methods are not always practical in foods because of the cost of enzymes like glucose oxidase and while active packaging containing glucose oxidase can reduce oxygen, it may not be sufficient to prevent lipid oxidation (Wong et al. 2017).
The Mitsubishi Gas Chemical Co. first commercialized the widely used Ageless® technology in the 1970s that relies on oxygen scavenging by oxidation of iron to iron oxide (mechanism shown in Figure 6.1). This technology has long been a cost effective and convenient solution in reducing package oxygen levels to <0.01% headspace oxygen (de Kruijf et al. 2002). However, the iron powder is typically delivered in small sachets within the package. This format presents problems such as accidental consumption, consumer confusion, and incompatibility with semi-solid and liquid food matrices (Ozdemir & Floros 2010). Recently, development of packaging material that is embedded with iron-based oxygen absorption capability could overcome these obstacles (Hatakeyama et al. 2000, Cruz et al. 2012). Mitsubishi Gas Chemical Company produces Ageless OMAC® packaging, referred to hereafter as oxygen scavenging (OS) packaging, which has a multilayered packaging material (Figure 1) with four basic layers that allow for iron-based oxygen consumption within the package without allowing external oxygen to enter. The advantage of this packaging is that its compatible with liquid food systems and it is part of the packaging film these removing the need for a sachet. However, Biji et al. (2015) suggested that such packaging systems have lower oxygen scavenging capacity than sachets. In addition, very little has been published on the conditions (e.g. pH, ionic strength, storage under air) under which the packaging will be effective. To the authors’ best knowledge, no research has been published to determine if this type of technology can inhibit oxidation of lipids in O/W emulsions and oxygen-based degradation of vitamins.

Therefore, the objective of this work was to investigate the potential of a commercially available oxygen scavenging packaging to reduce oxygen in buffered
solutions under different conditions and for its ability to inhibit oxidation of O/W
emulsions and the degradation of oxygen-sensitive vitamins. Oxygen absorption capacity
was determined in model buffered solutions and emulsions by measuring dissolved and
headspace oxygen using an oxygen phase fluorometer. Successful antioxidant application
of the active oxygen scavenging packaging was demonstrated in O/W emulsions and
inhibition of vitamin degradation was determined with ascorbic acid and α-tocopherol.

Figure 6.1. Schematic diagram and mechanism of oxygen scavenging (OS) packaging
(OMAC®).

6.3 Materials and Methods

6.3.1 Materials

Commercial anchovy oil, with omega-3 fatty acids present as 326 mg/g of
triglycerides, was obtained from DSM Food Specialties B.V. (The Netherlands) and
stored in 15 mL glass vials at -80°C in the dark until use. 2-Propanol, iso-octance, 1-
butanol, methanol, glacial acetic acid, sodium acetate trihydrate, sodium hydroxide,
sodium chloride, hydrochloric acid (HCl), oxalic acid dihydrate, L-ascorbic acid, 2,6-
dichloroindophenol, ammonium thiocyanate, n-hexanes (HPLC grade), diooxane (HPLC
grade), and sodium phosphate dibasic anhydrous were purchased from Thermo Fisher
Scientific (Waltham, MA, USA). Trichloroacetic acid (TCA) was purchased from Acros Organics (Morris Plains, NJ, USA). Thiobarbituric acid (TBA), 4-hydroxymethyl-2,6-ditertiarybutylphenol (BHT), Tween80®, ferrous sulfate heptahydrate, barium chloride dihydrate, sodium phosphate monobasic, sodium bromide, citric acid, and cumene hydroperoxide (80% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and solvents were used without further purification.

Ageless OMAC® (thickness: 100-130 μm) active oxygen packaging was obtained from Mitsubishi Gas Chemical (Tokyo, Japan). In this particular instance, one side contained the oxygen scavenging material, an oxygen permeable polyethylene to separate the metal from the food, and an aluminum layer to produce an oxygen transmission rate (OTR) of practically zero. The other side is a transparent poly(ethylene terephthalate), oriented nylon, and polyethylene (thickness: 70 μm) that has a high oxygen barrier (OTR 0.2 cc/cm² • day • atm) and allows visualization of the food product.

Transparent control plastic packaging (thickness: 225 μm) was purchased from WinPak (Winnipeg, MB, Canada). The control packaging consisted of multiple layers including: nylon, ethylene vinyl alcohol, and enhanced linear low-density polyethylene that together provide a high oxygen barrier (OTR of 0.2 cc/m² • day • atm). For ascorbic acid oxidation studies, a substitute high barrier multilayered transparent control plastic packaging (Technopack Corporation, FL, USA) was used that consisted of polyethylene, ethylene vinyl alcohol, and polyamide, which have an OTR on a similar scale as the other control (Lange & Wyser 2003).
6.3.2 Sample Preparation

To measure the effectiveness of the oxygen scavenging packaging, pH 3 (10 mM acetate), pH 5 (10 mM acetate), and pH 7 (10 mM phosphate) buffered solutions (25 ± 1g) were placed into packaging with no headspace and the dissolved oxygen content was quantified (described below). The manufacturer recommends that the oxygen reducing packaging material is stored in airtight secondary containers to limit the packaging from absorbing oxygen from the air and using up its scavenging capacity before the food is added and the package is sealed. However, this might not be practical in all applications so we determined if the OS packaging would lose its oxygen scavenging capacity over storage time. This was tested by storing the OS packaging material in a constant relative humidity of 60% RH, created using a saturated salt solution of sodium bromide, at 20°C. During storage for up to 35 days, stored packaging material samples were periodically removed from storage, filled with buffered solutions, sealed, and dissolved oxygen was determined daily for 3 days at 32°C.

Emulsions were prepared by dissolving Tween80® (at a concentration equal to a surfactant-to-oil ratio of 1:10) in pH 3 (10 mM acetate) and pH 7 (10 mM phosphate) buffer. Fish oil (1 or 20%, w/w) was then added followed by dispersing with a hand-held homogenizer (Biospec Products, Inc., Bartlesville, OK) at 7000 rpm for 2 min. This coarse emulsion then underwent three passes through a microfluidizer (Microfluidics, Newton, MA) at 10 kPSI. The emulsion (25 ± 1g) was dispensed into the OS packaging or control packaging and heat sealed using a Vacmaster SVP20 (Overland Park, KS, USA) without pulling a vacuum on the sample. The headspace to emulsion volume ratio was ~1:1 in order to have sufficient oxygen present to produce lipid oxidation markers.
that could be accurately measured. The O/W emulsions were stored in the dark at 32°C for up to 14 days.

Ability of the OS packaging to inhibit the formation of primary and secondary lipid oxidation products (lipid hydroperoxides and thiobarbituric acid reactive substances (TBARS), respectively) in O/W emulsions was quantified throughout these studies. Lipid hydroperoxides were quantified in emulsions by the method reported by Shantha & Decker, (1994) as modified by Hu, McClements and Decker (2003). Briefly, 0.3 mL emulsion was mixed with 1.5 mL iso-octane/2-propanol (3:1, v/v) by vortex for 3 x 10 s and then centrifuged at 1785g for 2 min. The upper layer (0.2 mL), containing the lipid hydroperoxides, was collected and then added to 2.8 mL of methanol/1-butanol (2:1, v/v). Next, 30 μL of thiocyanate/Fe²⁺ solution, which was obtained by mixing an equal volume of 3.94 M thiocyanate solution with 0.072 M Fe²⁺, was added and methanol/1-butanol (2:1, v/v) was added to bring the sample to a final volume of 3.0 mL. Reaction samples were held for 20 min at room temperature to enable color formation. Absorbances of the samples were read at 510 nm on a Genesys20 spectrophotometer Thermo Fisher Scientific (Waltham, MA, USA) and lipid hydroperoxide concentrations were calculated from a standard curve prepared using 0 to 0.6 mM cumene hydroperoxide (r² = 0.99, linear regression).

The thiobarbituric acid reactive substances (TBARS) assay was used to measure secondary lipid oxidation products as described by Alamed, McClements and Decker (2005) with slight modification. TBARS were used instead of headspace aldehydes because the headspace of the package could not be accurately sampled. Emulsions (1.0 mL) were combined with 2.0 mL of TBA reagent (14.6%, w/v TCA, 0.36%, w/v TBA,
0.06% w/v BHT in 0.25 M HCl). In some cases, emulsion samples were diluted with double distilled water prior to reagent addition to maintain their absorbance within the prepared standard curve concentration range. Samples were then vortexed (3 x 10 s) before being placed in a 75°C water bath for 15 min. The test tubes were immediately cooled to room temperature and were centrifuged at 1190g for 5 min. The absorbance of the aqueous phase was measured at 532 nm on a Genesys20 spectrophotometer Thermo Fisher Scientific (Waltham, MA, USA) and TBARS concentrations were calculated from a standard curve prepared using 0 to 40 μM 1,1,3,3-tetraethoxypropane ($r^2 = 0.99$, linear regression).

### 6.3.3 α-Tocopherol Stability in Fish O/W Emulsions

The ability of OS packaging to protect α-tocopherol (190 μM ± 25 of the oil phase), in a 1% fish O/W emulsion (pH 3.0, 10 mM acetate buffer) was determined over 14 days of storage in the dark at 32°C. α-Tocopherol was extracted from 1 mL of emulsion by vortexing for 3 x 10 s with 2.6 mL of iso-octane:2-propanol solution (3:1, v:v). The mixed solution was centrifuged at 3570g for 5 min (CL10 centrifuge, Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1 mL of upper solvent phase was evaporated under nitrogen. α-Tocopherol was re-dissolved in 200 μL of n-hexane and injected into a Shimadzu 10A VP HPLC (Shimadzu, USA), which was coupled to a SUPELCOSIL LC-DIOL column (25 cm x 4.0 mm, particle size 5μm, Bellefonte, PA), a Supelguard LC-Diol security guard column (2 cm x 4.0 mm, particle size 5 μm) and a fluorescence detector (RF-20 A xs, Shimadzu). α-Tocopherol was separated by using a normal-phase method outlined by Xu et al. (2015) with a isocratic mobile phase (95% hexane:5% dioxane) at a flow rate of 1 mL/min. α-Tocopherol detection was performed
using an excitation wavelength of 296 nm and emission wavelength of 340 nm. The \( \alpha \)-tocopherol peak was identified by comparing its retention time to a reference standard. Concentration of \( \alpha \)-tocopherol was performed using an external standard curve (linear concentration range = 0.04\( \mu \)M - 1.4 \( \mu \)M, \( r^2 = 0.99 \)).

### 6.3.4 Ascorbic Acid Stability

The ability of OS packaging to protect L-ascorbic acid (1 mM), from degradation was determined in 10 mM acetate (pH 3) or 10 mM phosphate buffer (pH 7) during storage in the dark at 32°C. Samples (25 ± 1 g) were heat sealed using a Vacmaster SVP20 (without pulling a vacuum) in either the OS or control packaging with a headspace of approximately 20%. Ascorbic acid concentrations were determined by a spectrophotometric method as described in Johnson et al. (2016) with slight modification. Briefly, samples (0.50 mL) were mixed with sodium oxalate (0.08%, w/v; 1.0 mL) by vortexing for 10 s to stabilize ascorbic acid. A portion of the stabilized ascorbic acid mixture (0.70 mL) was then mixed by vortex for 3 x 10 s with 0.20 mM 2,6-dichloroindophenol (1.80 mL) indicator dye. The reduction of the indicator dye to a colorless compound in the presence of ascorbic acid was measured at 520 nm using a Thermo Fisher Scientific spectrophotometer (Waltham, MA, USA). Ascorbic acid concentrations were calculated from a standard curve ranging from 0.0 to 1.2 mM ascorbic acid (\( r^2 = 0.99 \), linear regression).

### 6.3.5 Oxygen Analysis in Packaging

A NEOFOX oxygen phase fluorometer from Ocean Optics, Inc. (Dunedin, FL, USA) with fluorescent optical sensors was used to determine the dissolved and headspace oxygen concentration in the fish O/W emulsion or buffered solutions as described in
Johnson et al. (2016). Adhesive FOSPOR sensor patches (8.0 mm diameter) were attached to the clear packaging film in contact with (dissolved) or above (headspace) the emulsion or buffered system. Oxygen concentrations were measured non-destructively by holding the NEOFOX oxygen phase fluorometer probe up to the FOSPOR sensor patches. Calibration was performed with a solution of <0.01% oxygen obtained by purging pure nitrogen, 99.9% purity continuously at 25°C) and atmospheric air to standardize the sensor patches against known oxygen concentrations and temperatures as instructed by Ocean Optic, Inc.. Representative triplicate samples were monitored over time in samples stored at 32°C.

6.3.6 Statistical Analysis

Results from all studies are presented as means plus standard deviations of experiments conducted in triplicate. Statistical difference in means for ascorbic acid and tocopherol during storage, compared to time 0, was defined using one-way analysis of variance (ANOVA) with multiple comparisons of means performed using a one-tailed Dunnett’s post hoc test (p=0.05). Calculations were performed using IBM SPSS Statistics version 22 (IBM SPSS, Chicago, IL, USA).

Lag phase calculations for lipid oxidation studies were determined using the intersection of the tangent lines of lag and exponential phases of lipid oxidation to evaluate efficacy of active oxygen packaging (O’Keefe & Pike 2010).

6.4 Results and Discussion

6.4.1 Functionality of OMAC Oxygen Scavenging Packaging in Buffered Solutions

6.4.1.1 Buffer without Headspace

The ability of OS packaging to remove dissolved oxygen was demonstrated
across a pH range seen in foods (Figure 6.2). Results suggest that pH did not seem to affect the rate of oxygen scavenging. The dissolved oxygen content of buffers packaged in the OS package reduced steadily by approximately 13%/h of the initial value after a 2-3 h delay. According to the packaging patent (Hatakeyama et al. 2000), the iron oxygen absorbance system is activated by moisture from the sample. The delay in oxygen reduction is therefore likely the result of the time required for the water in the sample to migrate through the packaging material until it reaches the iron layer. After 48 h, >90% DO was removed across all samples and after 72 h >95% DO had been removed across all samples (Figure 6.2). Control packaging of buffered solutions showed no oxygen consumption in pH 3.0 acetate buffer over 3 days (Figure 6.2), and remained stable for at least 10 days (data not shown).

Figure 6.2. Scavenging of dissolved oxygen by oxygen scavenging (OS) packaging as a function of pH over 3 days at 32°C. Values represent averages and standard deviation of n=3.

The above experiments were performed with no headspace air, but in reality, most foods would have some headspace. The impact of approximately 50% headspace air on OS packaging was determined in acetate buffer (pH 3; Figure 6.3). Dissolved oxygen
decreased faster than headspace oxygen. The slower oxygen consumption rate in the headspace is due to dissolved oxygen being scavenged first and as it decreases, headspace oxygen migrates into the sample. Thus, the headspace serves as an oxygen reservoir which contains ~100x more moles of oxygen than the oxygen in a 1% O/W emulsion for the same volume. Thus, more time is required for removal of dissolved oxygen than in the samples without headspace (Figure 2) as headspace oxygen must migrate into the sample where it is then scavenged by the water activated packaging material. The time needed to reduce DO by ~90% was greater at 5 days, compared to 2 days in packaging without headspace.

Figure 6.3. Scavenging of headspace and dissolved oxygen by oxygen scavenging (OS) packaging over 8 days at 32°C in 10 mM acetate buffer (pH 3) at 32°C with headspace to sample volume ratio of ~1:1. Values represent averages and standard deviation of n=3.

The manufacturer recommends storing the OS packaging material in airtight containers since it is possible that the packaging material could be activated by atmospheric moisture. To test the extent of this problem the OS packaging was stored at 60% RH at 20°C for up to 35 days, and oxygen scavenging capacity was determined. No
statistically significant ($p \leq 0.05$) loss in oxygen scavenging capacity was observed (Figure 6.4).

Figure 6.4. Scavenging of dissolved oxygen by oxygen scavenging (OS) packaging over 3 days at 32°C after the packages had been incubated for up to 35 days storage under 60% RH at 20°C. Values represent averages and standard deviation of $n=3$.

6.4.2 Impact of Oxygen Scavenging Packaging on O/W Emulsions

Figure 6.5 shows the effect of the OS packaging on lipid oxidation in fish oil-in-water emulsions at pH 3 and 7. The lag phase of both lipid hydroperoxides and TBARS was 2 days at both pH 3 and 7. The OS packaging was very effective at decreasing lipid hydroperoxide and TBARS formation at pH 3 with neither lipid oxidation product exiting the lag phase during the entire study. At pH 7, OS had not effect on the lag phase of lipid hydroperoxide formation but extended the lag phase of TBARS formation to 6 days. The reduced effectiveness at pH 7 could occur if lipid oxidation began to occur before the OS packaging was able to totally remove oxygen from the package. It should be noted that both lipid hydroperoxides and TBARS were lower in the OS package than that of control after the lag phase. This would occur if oxygen was removed after the lag phase stopping
further oxidation.

The OS packaging was very effective in removing almost all the oxygen from buffered solutions. While beneficial to potentially stabilizing vitamins and preventing lipid oxidation, it could present a risk if anaerobic pathogens, such as *C. botulinum*, could grow under these reduced oxygen conditions. Thus, all further studies were conducted in acidified O/W emulsion (pH 3) because *C. botulinum* does not grow below pH 4.6 (FSIS, USDA, Internet Access). In addition, acid pH values are also more common to food emulsions (*e.g.*, salad dressings and mayonnaise).
Figure 6.5. Formation of (A) lipid hydroperoxides and (B) thiobarbituric acid reactive substances (TBARS) in 1% fish oil-in-water emulsions (buffered at pH 3 and 7) stored in control and oxygen scavenging (OS) packaging at 32°C in the dark. Values represent averages and standard deviation of n=3.
Figure 6.6. Dissolved oxygen analysis of 1 and 20% fish oil-in-water emulsions (buffered at pH 3) stored in control and oxygen scavenging (OS) packaging over 2 days at 32°C with a headspace to sample volume ratio of ~1:1. Values represent averages and standard deviation of n=3.
Figure 6.7. Formation of (A) lipid hydroperoxides and (B) thiobarbituric acid reactive substances (TBARS) in 1 and 20% fish oil-in-water emulsions (buffered at pH 3) stored in control and oxygen scavenging (OS) packaging at 32°C in the dark. Values represent averages and standard deviation of n=3.

Lipid concentration in O/W emulsions could alter active packaging performance since oxygen solubility is higher in oils than water. Results of oxygen analysis (Figure
6.6) demonstrated similar oxygen scavenging performance for both 1% and 20% emulsions with 73±5.5% and 69±12% % reduction in DO, respectively, after 48 h. Results of lipid oxidation studies (Figure 6.7) showed that the lag phase of lipid hydroperoxides formation was similar in both the 1 and 20% O/W emulsions. However, TBARS had lag phases of 5 days in the 1% lipid control and 8 days in the 20% lipid control. It is unclear why formation of secondary lipid oxidation products would be faster in emulsions with lower fat. Another possibility is that high lipid levels require more surfactant to form physically stable emulsions. At these higher emulsifier levels, the amount of surfactant in the aqueous phase would be higher and thus the concentration of surfactant micelles would be larger. Surfactant micelles have been shown to inhibit lipid oxidation by removing prooxidants (e.g. iron and hydroperoxides; Cho et al. (2002) and Nuchi et al. (2002) from the emulsion droplet and increasing antioxidants at the interface of emulsion droplets (Panya et al. 2012). If the surfactant micelles were able to reduce the ability of iron to decompose lipid hydroperoxides this would cause a decrease in secondary lipid oxidation products such as TBARS. In addition, since the fish oil contains tocopherols, it’s also possible that the increase surfactant micelles could make them more effective thus slowing down oxidation in the high fat emulsions.

The OS packaging was effective in both the 1 and 20% O/W emulsions. In both the 1 and 20% O/W emulsion, OS packaging prevented both lipid hydroperoxide and TBARS formation during the entire storage study. These results indicate that the OS packaging was able to effectively scavenge oxygen even in the presence of higher amounts of fats that would create another reservoir of oxygen that would need to be removed to inhibit lipid oxidation.
Salt has been reported to accelerate lipid oxidation in O/W emulsions at pH 7.0 (Cui et al. 2016). Any factors that accelerate oxidation could compromise the effectiveness of the OS packaging because of the 1-2 day delay in oxygen scavenging when headspace is present. This would occur because in a rapidly oxidizing emulsion, the initial 2 days of sufficient oxygen concentration could result in oxidation of antioxidant and a decrease in oxidative stability. The impact of NaCl (250 mM, which is on the same order of the amount in commercial O/W emulsion), on the effectiveness of OS packaging was determined by quantifying primary (lipid hydroperoxides) and secondary (TBARS) lipid oxidation products during storage of the pH 3.0 fish O/W emulsion at 32°C in the dark (Figure 6.8). O/W emulsions, both with and without NaCl, exhibited similar oxidative stability with a lag phase of 2 days before lipid hydroperoxide and TBARS formation occurred. In the OS packaging, O/W emulsions (both with and without NaCl) showed significant inhibition of lipid oxidation as neither lipid hydroperoxides nor TBARS increased during the entire storage study. It was somewhat surprising that the NaCl did not decrease lipid oxidation lag phase, but this study was conducted at pH 3 with Tween80® whereas the prooxidant activity of NaCl was reported at pH 7.0 with Tween20®.
Figure 6.8. Formation of (A) lipid hydroperoxides and (B) thiobarbituric acid reactive substances (TBARS) in 1% fish oil-in-water emulsions (buffered at pH 3) in the presence and absence of 250 mM NaCl stored in control and oxygen scavenging (OS) packaging at 32°C in the dark. Dotted lines represent an emulsion samples that was opened at day 8 to exposed to oxygen and the re-stored in control packaging. Values represent averages and standard deviation of n=3.
A potential limitation of the OS packaging is that once it’s opened, protection is lost. To verify this limitation, samples were opened on Day 8 and transferred to control packaging for storage. The dotted line in **Figure 6.8** shows that oxygen re-introduction resulted an increased in both lipid hydroperoxides and TBARS after an additional 6 days of storage. Thus, OS packaging strategies that reduce oxygen are likely to be effective in the sealed container which could make them effective single serve applications. These results also highlight the fact that additional antioxidant strategies might be needed in combination with OS packaging to protect the food after opening.

**6.4.3 Dissolved Oxygen Content and Oxidative Stability of Salt Containing O/W Emulsions**

Consumption of dissolved oxygen was quantified in O/W emulsions packaged in control and OS packaging in the presence and absence of salt (250 mM NaCl; **Figure 6.9**). NaCl did not significantly (p>0.05) alter the ability of the OS packaging to scavenge...
oxygen. As previously reported (Johnson et al., 2016), 58% oxygen reduction was the minimum level to slow lipid oxidation. OS packaging showed an initial sharp decrease in dissolved oxygen after 2 days the oxygen reduction 65±8 and 73±10% (Figure 9) in the presence and absence of NaCl, respectively, suggesting that oxidation reactions should be starting to slow down. By day 4, oxygen concentrations were reduced by 91±2 and 88±8% in the presence and absence of NaCl, respectively, meaning that lipid oxidation would be substantially inhibited and explains why no increase oxidation was observed in the OS packaging system (Figure 6.8). In the control package in Figure 6.2, no reduction in oxygen was observed for ≥10 days (data not shown). However, in the O/W emulsion oxygen started to decreased sharply after 2 days of storage, which correlates with the end of the lag phase of both lipid hydroperoxide and hexanal formation. This oxygen decrease can be attributed to oxidizing fatty acids consuming the oxygen in the closed package.

6.4.4 Ability of Active Oxygen Scavenging Packaging to Prevent Vitamin Degradation

Ascorbic acid has the ability to scavenge oxygen and has been proposed as an antioxidant technology. However, previous work has shown that ascorbic acid alone (at pH 3), at concentrations practical to foods, does not have the capacity to lower oxygen concentrations to levels needed to inhibit lipid oxidation (Johnson et al. 2016). However, the use of ascorbic acid in combination with OS packaging in buffered solutions could have dual benefits in increasing oxygen reduction rates as well as inhibiting oxygen dependent ascorbic acid (1 mM) degradation in foods. Ascorbic acid oxygen scavenging and degradation is highly pH dependent with both oxygen scavenging and resulting
ascorbic acid degradation increasing with increasing pH (Buettner & Jurkiewicz 1996)).

**Figure 6.10a** shows ascorbic acid degradation rates in buffered solutions at pH 3 & 7 during storage at 32°C in the dark. Regression analysis demonstrated that ascorbic acid samples stored in control packaging, at both pH 3 and 7, declined linearly ($r^2=0.97$ and $r^2=0.79$, respectively) over the course of the study. Ascorbic acid concentrations in control packaging at pH 7 declined faster than pH 3, with statistical difference from time 0 being seen at 2 h for pH 7 and 2 days for pH 3. In OS packaging samples at pH 3, ascorbic acid concentrations remained constant over the 7 day experiment with no statistical difference from time 0. This lack of ascorbic acid degradation was likely due to ascorbic acid’s slower degradation at pH 3 which allowed the OS packaging to reduce oxygen (90%±7 reduction after 1 day, fig 9b) to levels where further oxygen dependent degradation was prevented.

At pH 7, ascorbic acid by itself was able to decrease oxygen levels by 44%±8 after 24 h (**Figure 6.10b**). Ascorbic acid concentrations decreased 18%±3 in the OS packaging during this same time period after which further degradation did not occur. This initial degradation probably occurred because of the decreased stability of ascorbic acid at pH 7 and the inability of the OS packaging to reduce oxygen concentrations fast enough to prevent all ascorbic acid-oxygen interactions. Interestingly, dissolved oxygen levels decreased faster in the OS packaging with ascorbic acid at pH 7 compared to pH 3. This increased oxygen consumption rate is likely due to the combined oxygen scavenging activity of ascorbic acid and the active packaging. This suggests that in situations where rapid initial oxygen reduction is needed and ascorbic acid concentrations do not need to be preserved, a combination of ascorbic acid (pH 7) and OS packaging could be useful as
the ascorbic acid would increase initial oxygen removal and the OS packaging would provide the capacity to remove more oxygen than the ascorbic acid can alone. Taken together, OS packaging was able to significantly extend the stability of ascorbic acid in buffered solutions (pH 3 & 7).

Figure 6.10. (A) Degradation of ascorbic acid (1.0 mM) in 10mM phosphate or acetate buffer (pH 7 & 3, respectively) stored at 32°C in the dark in control and oxygen scavenging (OS) packaging. (B) Dissolved oxygen scavenging by either ascorbic acid alone or in combination with oxygen scavenging (OS) packaging.
OS packaging could also be useful for stabilizing oxidatively liable fat-soluble nutrients through its ability to decrease the formation of reactive oxygen species. To test this, the ability of OS packaging to stabilize α-tocopherol in 1% O/W emulsions was determined (Figure 6.11). α-Tocopherol (188 ± 25 μM of the oil phase), that was naturally present in the oil, decreased in both control and OS packaging during storage at 32°C in the dark. Compared to day 0, α-tocopherol concentration in control packaging was statistically (p≤0.05) lower by day 6 with over 95% degradation by day 14. In the OS packaging α-tocopherol concentration was not significantly different than time 0 until day 14 with 65%±28 α-tocopherol remaining at the end of storage.

Figure 6.11. Tocopherol loss in 1% fish O/W emulsion (buffered at pH 3) in oxygen scavenging (OS) and control packaging stored at 32°C in the dark.

In the fish oil-in-water emulsions, α-tocopherol most likely decreased as reactive oxygen species such as alkoxyl and peroxyl radicals produced by lipid oxidation consumed the free radical scavenger antioxidant. Active packaging’s ability to reduce
oxygen resulted in decreased lipid oxidation rates (Figures 6.6, 6.7, 6.8) by decreasing the production of reactive oxygen species that could degrade α-tocopherol. Tocopherol protection could be important in tocopherol fortified foods. In addition, preservation of tocopherol levels would mean that more tocopherol would be in the product throughout the distribution chain. Thus, tocopherol levels would be greater allowing for increased protection of the product upon opening and reintroduction of oxygen into the product.

6.5 Conclusions

Results from this study suggest that OS packaging can significantly reduced the amount of packaged oxygen in liquid systems under the conditions expected in foods (e.g. pH, salt concentration, and range of fat concentrations). This oxygen reduction can inhibit lipid oxidation and nutrient degradation. This would be a benefit to food manufacturers in that it would increase shelf life and extend the nutritional quality of foods containing ascorbic acid and α-tocopherol. The major limitation of this active oxygen scavenging packaging is the initial 1-2 h delay in oxygen scavenging as the water from the food penetrates the inner most layer of film and activates the iron-based oxygen scavenging system. This could limit some application where oxidation reactions are very rapid (e.g. ascorbic acid degradation at high pH). Applications are also limited by the opaque nature of active oxygen scavenging portion of the packaging and the flexible film properties that might not be compatible with all foods and packaging lines. In addition, in these experiments, oxygen scavenging capacity is greater than the amount of oxygen in foods even when 50% headspace was included in the package, as was seen by the eventual near complete oxygen removal in all systems tested. Results suggests that the technology could be used to create other innovative packaging systems where this
technology is incorporated into only part of the package (e.g. lid, or bottom of the container) where visualization of the food could occur while still providing enough oxygen scavenging capacity to protect the food. Overall, the OS oxygen reducing packaging system can offer a ‘clean label’ solution to decrease the oxidative degradation of liquid foods susceptible to lipid oxidation and oxygen based nutrient degradation.

6.6 References


CHAPTER 7

FINAL CONCLUSIONS AND FUTURE WORK

This work highlights the importance that an understanding of both oxygen concentration and active packaging can provide oxidative stability for lipid-containing foods. Novel solutions, that do not involve the addition of exogenous antioxidants, are needed for the food industry as consumer demands increase for transparency and ‘clean’ labels. These solutions are especially needed in susceptible O/W emulsions systems commonly stabilized by synthetic additives, such as EDTA.

Much of O/W emulsion oxidation occurs from interactions at the oil-water interface that are greatly accelerated by transition metals (e.g., iron) ubiquitously found in foods. Thus, development of active packaging that can replace synthetic additives and chelate these prooxidant metals has been a recent area of interest. Progress has been made within this work to graft effective biomimetic iron chelating groups from a more robust packaging material, PET, as well as without the need for toxic photoinitiators (Chapter 3). Developing new technology is important for expansion of active packaging category to provide solutions for the food industry.

Unfortunately, the efficacy of metal chelating packaging alone is not enough to maintain the level protection against lipid oxidation provided by synthetic additives. A further understanding of how other alternative antioxidant solutions, such as oxygen reduction, can inhibit lipid oxidation was needed. Indeed, researchers have long known that removing oxygen from food packages can inhibit lipid oxidation. Yet, little research has been done on the actual amount of oxygen reduction needed to meaningfully increase oxidative stability. To overcome these past challenges, the impact of a wide range of
oxygen concentrations was studied (Chapter 4). Results showed that only high levels of oxygen removal, ≥93%, are needed to meaningfully extend a product’s shelf life. However, reaching these really low levels by traditional methods (e.g., nitrogen flushing or vacuum packing) has proven difficult (Chapter 4). More practical intermediate oxygen reductions were then combined with naturally derived antioxidants to determine if their antioxidant effect could be enhanced under reduced oxygen atmospheres (Chapter 5). It was found that 60-70% oxygen reduction did not synergistically enhance the activity of the antioxidants tested. Although antioxidants had the ability to extend oxidative stability, near complete oxygen removal is suggested.

Since really low concentrations of oxygen in emulsions are difficult to reach in processing conditions, attention was turned back to active packaging. Over the last 15 years, a lot of growth has taken place in the area of oxygen scavenging in packaging (Chapter 6). Advances include embedding the oxygen scavenging technology into the packaging so that the consumer does not see undesirable sachets and label inserts. An added benefit is that this technology allows for an opportunity to remove oxygen from liquid systems, like emulsions, that had been previously incompatible with oxygen scavengers. The application of the active oxygen packaging was thus characterized and for the first time its ability to inhibit oxidation of O/W emulsions and the degradation of oxygen-sensitive vitamins was demonstrated.

Innovative approaches to stabilize foods against oxidative deterioration will likely continue to be met by advances in food packaging. As expected, more studies are needed in order to scale-up metal chelation packaging from proof-of-concept studies to commercialization. This includes consumer safety studies as well as feasibility in current
plastic manufacturing. Another potential area for growth is the expansion of active oxygen packaging (Chapter 7) into more traditional bottle package formats or incorporation into existing packages is expected. Transparency of the package and traditional plastic shapes could allow successful application of this technology to susceptible foods like salad dressings and mayonnaise that currently rely on synthetic additives. Results from the research presented here shows promise that active packaging and a more detailed understanding of food systems can drive unique and consumer friendly solutions.
APPENDIX

THE IMPACT OF OXYGEN CONCENTRATION AND VITAMIN C ON LIPID OXIDATION IN BULK FISH OIL

1 Abstract

The quality and shelf life of foods such as bulk oils are largely dependent on the rate and extent of lipid oxidation. Strategies to prevent the oxidation of bulk oil in storage and in food processing have traditionally included the removal of oxygen. Surprisingly, little research has been done on the amount of oxygen that needs to be removed in order to have an antioxidant effect on bulk oil. In the present work, the impact of a range of oxygen concentrations on bulk fish oil storage was determined to understand the level of oxygen reduction necessary to have an antioxidant effect. Results indicate that any oxygen removal can be helpful in limiting overall oxidation products, but practical application of oxygen removal techniques requires >90% oxygen removal. The ability of ascorbic acid or ascorbyl palmitate to reduce dissolved oxygen in bulk oil was also measured. Dissolved oxygen coupled with lipid oxidation analysis revealed that both ascorbic acid and ascorbyl palmitate had an antioxidant effect on bulk oil, however this antioxidant activity does not appear related to the ability to scavenge oxygen in oil systems.

2 Introduction

Decreasing oxygen concentrations during processing and storage of bulk oil is a common strategy to extend product shelf life without the addition of exogenous antioxidants. Recently, our group has worked to define the level of oxygen reduction necessary in emulsions to drastically improve oxidative stability (Johnson et al. 2016). However, very few studies have similarly evaluated the impact of a broad range of
oxygen concentrations on bulk oil oxidation. Andersson et al. 1998 & 1999 clearly showed that low oxygen concentrations in the headspace above bulk rapeseed oil inhibited lipid oxidation, especially at concentrations below 0.5% headspace oxygen (~98% reduction). However in those studies, surface area was maximized by placing a small amount of oil (0.5 mL) on cotton and stored in 500 mL containers as a means to minimize the impact of oxygen diffusion. While that research has provided important guidelines that such low levels of oxygen can provide oxidative protection, it does not well represent oxidation of bulk oil during storage. One study did analyze the impact of initial dissolved oxygen on the oxidation of a representative bulk oil system, however the experimental procedures conducted likely resulted in contamination with oxygen (Min & Wen 1983). Further, to the author’s best knowledge no previous studies have accounted for both dissolved and headspace oxygen in bulk oil. Dissolved oxygen is likely to play a larger role in the lipid oxidation of bulk oil because solubility of oxygen in bulk oil is 3-10x greater than in aqueous systems (Johnson & Decker 2015). Therefore, it is important to define what levels of total oxygen reduction could provide guidelines that manufacturers need to reach in order to protect labile fatty acids.

Practically speaking, significant removal of oxygen from the system may not be feasible for manufacturers. One strategy that the food industry has at their disposal is the addition of edible oxygen scavengers such as ascorbic acid (water soluble) and ascorbyl palmitate (lipid soluble derivative) to potentially reduce oxygen. Ascorbates are attractive for use in foods because they possess vitamin C activity and are recognized by consumers on food package labels. Ascorbates can inhibit lipid oxidation by multiple antioxidant mechanisms such as: metal chelation, free radical scavenging, regeneration of tocopherol,
and oxygen scavenging (Cort 1982). Indeed, the ability of ascorbic acid to reduce the level of dissolved and headspace oxygen is well known and has been shown in buffered solutions and orange juice (Cort 1982; Johnson et al. 2016; Kennedy et al. 1992). However, it is uncertain whether ascorbates can be effective at reducing oxygen in bulk oil where solubility is a limiting factor and the amount of oxygen consumed is insufficient (Frankel 2005). The more hydrophobic ascorbyl palmitate is often used because of its greater solubility in oil and also has been shown to inhibit lipid oxidation in bulk fish oil when used in combination with lecithin (Hamilton et al. 1998). Despite difficulties in oil solubility, research has suggested that the water-soluble ascorbic acid to be more effective in preventing oxidative degradation in bulk oil than ascorbyl palmitate, however the mechanism of action is not well understood (Frankel et al. 1994). Therefore, determining if oxygen scavenging is responsible for antioxidant activity can help food scientists design systems where ascorbates are most effective.

3 Materials and Methods

3.1 Materials

Commercial fish oil without added antioxidants was obtained from DSM Food Specialties B.V. (The Netherlands) and stored in 1 L aluminum bottles at -80°C in the dark until use. Commercial, Wesson soybean oil was purchased from a local grocer (Big Y, Amherst, MA). 2-Propanol, isooctance, 1-butanol, methanol, sodium hydroxide, hydrochloric acid, L-ascorbic acid, and ammonium thiocyanate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Trichloroacetic acid and ascorbyl palmitate were purchased from Acros Organics (Morris Plains, NJ, USA). Thiobarbituric acid, 4-hydroxymethyl-2,6-diteriarybutylphenol (BHT), ferrous sulfate heptahydrate, barium
chloride dihydrate, and cumene hydroperoxide (80% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). High purity custom nitrogen and oxygen gas blends (7.5, 5.0 and 2.5 mole % oxygen balanced with pure nitrogen) were obtained from AirGas Specialty Gases (Radnor, PA, USA). All chemicals and solvents were used without further purification.

3.2 Sample Preparation and Antioxidant Addition

The model system, 5 mL bulk fish, was stored in acid washed glass vials (total volume 12 mL) in the dark at 32°C. Oxygen removal was carried out as described in Johnson et al. (2016) to set up a broad range of total package oxygen from oxygen saturation to near complete oxygen removal. Briefly, headspace oxygen reduction headspace oxygen was reduced by 0, ~38, ~74, and ~95% by flushing custom blends of nitrogen/oxygen or pure nitrogen through a sealed septum and into the sample headspace. Headspace oxygen reduction of ~99% was carried out by purging, flow of gas through the liquid phase, until near complete oxygen removal.

To evaluate the ability of ascorbates to reduce oxygen in a bulk oil systems, ascorbic acid (200 ppm) and ascorbyl palmitate (200 ppm) were distributed in a bulk soybean oil by stirring for 30 min at 300 rpm in the dark. While stirring (to maintain an even ascorbate distribution), aliquots (5 mL) were placed into vials. The oil type tested was chosen to be soybean oil because of its stability against oxidation. This allowed for a more accurate depiction that the measurement of oxygen was due to the activity of ascorbates and not consumption by lipid oxidation.

3.3 Quantification of Lipid Oxidation Products in Bulk Oil and Oxygen Analysis
Both lipid hydroperoxides and dissolved oxygen were used to follow lipid oxidation. Lipid hydroperoxides were measured to determine the oxidative stability of bulk oil and were quantified by the method reported by Shantha & Decker (1994) as modified by Homma et al. (2016).

A NEOFOX oxygen phase fluorometer (Ocean Optics, Inc., Dunedin, FL) with fluorescent optical sensors coating that allows the diffusion of oxygen, was used to determine headspace and dissolved oxygen in the bulk oil system as described in Johnson et al. (2016).

3.4 Impact of Ascorbates on Oxygen Concentration and Oxidative Stability of Bulk Oil

The oxygen reduction capacity of ascorbyl palmitate (200 ppm) and ascorbic acid (200 ppm) in bulk oil was determined using measurements of dissolved oxygen as described previously (Johnson et al. 2016). Soybean oil was chosen for this portion of the study due to its relatively high resistance to lipid oxidation at accelerated temperatures. The accelerated temperature would potentially allow ascorbates to consume oxygen prior to lipid oxidation reactions consuming oxygen to confound the results.

3.5 Statistical Analysis

Results are presented as means including standard deviations for experiments conducted in triplicate, unless otherwise stated.

4 Results and Discussion

4.1 Modifications of Oxygen Concentrations and Storage of Bulk Oil with Different Oxygen Reductions

The impact of oxygen reduction on lipid oxidation in bulk fish oil was tested under saturated (i.e., atmospheric) conditions and systems where headspace oxygen was
reduced by 0, ~38, ~74, ~95, and ~99%. Dissolved and headspace oxygen measurements demonstrated that distinct levels of oxygen were created by flushing with different oxygen/nitrogen blends and it also served as an initial marker to monitor lipid oxidation (Johnson et al., 2016) (Figure 1 & 2).

**Figure 1.** Dissolved oxygen of bulk fish oil stored at 32°C in saturated and reduced oxygen environments over time. Values represent means ± standard deviation (n=3).

**Figure 2.** Headspace oxygen of bulk fish oil stored at 32°C in saturated and reduced oxygen environments over time. Values represent means ± standard deviation (n=3).
4.2 Impact of Oxygen Concentration on Lipid Oxidation in Bulk Fish Oil

The effect of oxygen reduction on the oxidative stability of fish was quantified by measuring primary oxidation products (lipid hydroperoxides) and supported by oxygen analysis. Once samples had clearly exited lag phase, as evidenced by measurable lipid hydroperoxides and near complete oxygen depletion of the samples, they were removed from the study and not further characterized. Bulk fish oil samples between ~38-74% reduced headspace oxygen did not exhibit a clear lag phase and steadily increased over time. Further decreasing headspace oxygen to ~95 and ~99% removal resulted in substantial extension of lag phase 21 days and > 26 days respectively. Since, it was difficult to determine relative impact of oxygen concentrations on lipid oxidation by the traditional tangent line method, an alternative determination was be made by quantifying the number of days to reach a mean value of lipid hydroperoxides 2.5 mmol/kg oil, which is representative of good quality fish oil that can be refined to have a bland flavor (Hamilton et al. 1998). In this case, ~38% and 74% reduced headspace oxygen provide a small increase in oxidative stability over 4 days for saturated oxygen (8 and 6 days, respectively). Further decreasing oxygen by 95% resulted in 14 days of stability and a ~99% oxygen reduction resulted in stability over the duration of the study (26 days).

Oxygen equilibration of the dissolved phase in ~74% and ~95% reduced oxygen samples were delayed (over the course of two days) compared to that of emulsified systems (which were almost immediate), suggesting that oxygen diffusion is slower through the oil phase likely due to increased viscosity or increased oxygen solubility in the oil (Johnson et al. 2016). Dissolved oxygen decreased first followed by decreases in headspace oxygen, presumably as lipid oxidation occurs. This is evidenced by sharp
drops in oxygen level in bulk oil of 74% and ~95% reduced oxygen samples between
time 0 and 2 days without substantial increase in lipid oxidation. Overall, a similar trend
to lipid oxidation under reduced oxygen levels in emulsified systems was observed

**Figure 3.** Lipid hydroperoxide formation in bulk fish oil stored at 32°C in saturated and reduced oxygen environments over time. Values represent means ± standard deviation (n=3).

4.3 Impact of Ascorbates on Oxygen Concentration and Oxidative Stability of Bulk Oil

The oxygen reduction capacity of ascorbyl palmitate (200 ppm) and ascorbic acid
(200 ppm) in bulk oil was determined using measurements of dissolved oxygen. As
expected, soybean oil with and without ascorbates did not show signs of oxygen
reduction when stored at accelerated temperature (55°C) for 6 days. Oxygen
measurements taken at 22 days showed that oxygen had decreased, although less so in
ascorbyl palmitate samples, likely this oxygen decrease came from lipid oxidation
reactions. Formation of lipid oxidation products (lipid hydroperoxides) (**Figure 5**)
showed that soybean oil containing ascorbates oxidized at a slower rate than soybean oil
alone.
The modes of antioxidant action of ascorbates are commonly referred to include oxygen scavenging in systems containing oil. However, we have previously demonstrated that in buffer (pH 3.0) the time to scavenge and amount of oxygen would not be sufficient to protect against lipid oxidation (Johnson et al. 2016). Since dissolved oxygen was not reduced by ascorbates in bulk soybean oil, the results suggest that ascorbates protected against oxidation likely from either free radical scavenging or the regeneration of the tocopherols naturally present within the oil.

**Figure A.4.** Dissolved oxygen of bulk soybean oil stored at 55°C in the presence and absence of ascorbyl palmitate (200 ppm) and ascorbic acid (200 ppm). Values represent means ± standard deviation (n=3).
Figure 5. Formation of lipid hydroperoxides in bulk soybean oil stored at 55°C in the presence and absence of ascorbyl palmitate (200 ppm) and ascorbic acid (200 ppm). Values represent means ± standard deviation (n=3).

5 Conclusion

As with previous research regarding oxygen concentrations in emulsions, it was found that a minimum amount of oxygen removal was needed to provide any antioxidant effect. Almost complete removal of total packaged oxygen was required to provide meaningful extension of oxidative stability of bulk fish oil. Further, results suggest that while ascorbic acid and ascorbyl palmitate had antioxidant effects at the concentrations tested, that this effect was not likely to be attributed to the oxygen scavenging capabilities. Previous research has suggested that use of ascorbic acid as an oxygen scavenger, in fish O/W emulsions, was insufficient to prevent the oxidation of fish O/W emulsions (Johnson et al. 2016). In fact, ascorbic acid not only was ineffective at reducing oxygen content to a level that would have a protective effect, but was prooxidative. The prooxidant effect was presumed to be because of ascorbic acid’s ability to reduce transition metals to their more reactive oxidation state, which in turn increases lipid
hydroperoxide decomposition into free radicals. This prooxidant metal recycling of ascorbic acid and its ester did not appear to be influential in bulk oil where iron has less of a prooxidant impact. The results presented herein suggest that ascorbates can function as more ‘natural’ antioxidant alternatives for bulk oil stabilization. However, this antioxidant activity is most likely not attributed to oxygen scavenging, but more likely radical scavenging or tocopherol regeneration. Understanding how ascorbates function as antioxidants can best direct efforts on how to best stabilize bioactive unsaturated fatty acids, like fish oils.

6 References


REFERENCES


