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Reactive Carbonyl Compounds: Their Control and Consequences in Foods

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**REACTIVE CARBONYL COMPOUNDS: THEIR CONTROL AND CONSEQUENCES
IN FOODS**

A Thesis Presented

by

MICHAEL A. FREUND

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

MAY 2018

Food Science

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ABSTRACT

REACTIVE CARBONYL COMPOUNDS: THEIR CONTROL AND CONSEQUENCES IN FOODS

MAY 2018

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Polyunsaturated omega-3 fatty acids (n-3 PUFAs) have been suggested to reduce risk for multiple diseases but animal studies on the beneficial effects of n-3 PUFAs are conflicting, possibly due to the presence of toxic lipid oxidation products in the oils used in these studies. In order to provide guidance for future research in n-3 PUFA supplementation, this study researched lipid oxidation and its inhibition in an animal feed system enriched with fish oil. Different storage conditions were tested, and it was found that samples stored at room temperature or above were at significant risk for oxidation with lag phases of propanal formation being 56, 8 and 2 days at 4°C, 23°C and 37°C. More than 65% removal of oxygen was needed to significantly decrease lipid oxidation. Greater than 65% removal of oxygen could be achieved in less than 1 minute of nitrogen flushing. Tocopherols were not strong antioxidants in the animal feed but Trolox was, suggesting that the fish oil enriched rodent feed acts similarly to bulk oil. Both ascorbic acid and ascorbyl palmitate were found to be ineffective, possibly due to their prooxidant activity. In a comparison of propyl gallate (PG), butylhydroxy toluene (BHT), and tert-butylhydroquinone (TBHQ), results were found similar to other low-moisture systems, with PG being prooxidative, BHT improving lag phase, and TBHQ having a significant impact on lag phase. These results suggest that lipid oxidation products can be present at the start of a dietary omega-3 fatty acid study if poor quality oils are used and that oxidation can occur in the feed

during storage times common to animal studies. These findings indicate that researchers should use antioxidant strategies to control oxidation in animal feeds to avoid potentially conflicting effects of lipid oxidation products in dietary omega-3 fatty acid studies.

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CHAPTER 1

THE HEALTH BENEFITS OF DIETARY OMEGA-3 FATTY ACIDS

1.1 Introduction

The reported health benefits of unsaturated fatty acids and recommendations to reduce the saturated fat content of foods has increased interest in consuming omega-3 polyunsaturated fatty acids (n-3 PUFAs). Of these fats, long-chain eicosapentaenoic acid (EPA) and docosahexanoic (DHA) fatty acids have been observed to have the strongest positive impact on human health. While work has been done on obtaining these fatty acids from other marine sources such as algae, their primary dietary source is fish. Other potentially beneficial omega-3 polyunsaturated fatty acids can be obtained from vegetable fats, such as flaxseed oil. A great deal of literature suggests that an increased intake of these lipids helps to reduce the risk for multiple disease, including cancers and cardiovascular disease, among others.

1.2 Dietary Omega-3 Polyunsaturated Fatty Acids and Cardiovascular Disease

The largest and most heavily supported beneficial effect associated with n-3 PUFAs is its impact on cardiovascular disease risk (Arab-Tehrany 2012, Mori 2017, Saravanan et al. 2010). The literature is strong enough that the American Heart Association released a scientific statement encouraging its consumption (Siscovik et al. 2017). Epidemiological evidence, while not consistent, generally suggests an inverse between n-3 PUFA consumption and cardiovascular disease risk. For example, while Hu et al. (2002) found that higher fish consumption led to a lower risk of coronary heart disease and a similar result was found for omega-3 fatty acid intake

after a 12-year follow-up, Osler et al. (2003) found no association between fish consumption and coronary heart disease. The supporting evidence is enough, though, that a great deal of research has been performed to elucidate these supposed beneficial effects.

1.2.1 Omega-3 Polyunsaturated Fatty Acids and Cardiovascular Disease in vitro and in Animals

Both *in vitro* and animal models allow insight into the potential mechanisms through which dietary n-3 PUFAs may be exerting their beneficial effects observed epidemiologically. De Caterina et al. (2000) linked the consumption of DHA with antiatherogenic and anti-inflammatory properties through the ability of DHA to alter the expression of adhesion molecules *in vitro*. Introduction of DHA to adult rat cardiomyocytes was observed to significantly reduce lysophosphatidylcholine-induced spontaneous contractions (Leifert et al. 2000). The authors suggested that the mechanism of this prevention was linked to changes of membrane fluidity induced by DHA (Leifert et al. 2000). In adult guinea pig ventricular myocytes with sodium ion currents blocked, Ferrier et al. (2002) showed that DHA was able to inhibit Ca²⁺ influx while preserving cardiac contractile function, providing an antiarrhythmic effect. Xiao et al. (2004) found that EPA inhibited outward and inward sodium-calcium exchanger current in HEK293t cells, showing one of the possible protective effects of EPA against fatal arrhythmias. In hydrogen peroxide induced DNA damage in human aortic and endothelial cells, both EPA and DHA treatment were able to reduce DNA damage and did so through upregulation of the NRF2-mediated antioxidant response (Sakai et al. 2007). In myocytes from both rabbits and patients with heart failure, fish oil superfusion was found to inhibit triggered arrhythmias (Ruijter et al. 2008).

Similar functionality has been observed in animals. Dhein et al. (2005) found an antiarrhythmic effect of dietary DHA and EPA when infusing the fatty acid in spontaneously beating isolated rabbit hearts. Similarly, Dujardin et al. (2008) showed that DHA was able provide an ultrafast sodium channel block, which protected against dofetilide-induced arrhythmia. In a porcine infarct model, pericardial infusion of DHA was shown to reduce malignant arrhythmias, heart rate, and infarct sizes (Xiao et al. 2008). Rats fed two different high fat n-3 PUFA rich sources, a microalgal source and a fish oil source, showed reduced insulinemia and serum lipid levels compared to a control, after 8 weeks of treatment (Haimeur et al. 2016). In type-2 diabetic and dyslipidemic primates given n-3 PUFAS, Hals et al. (2017) observed that n-3 PUFA treatment had a positive effect on cardiovascular disease risk factors, such as reducing total cholesterol, LDL-cholesterol, and triglycerides while increasing HDL-cholesterol.

The results are not consistent between studies. For example, in hearts isolated from pigs that had received a n-3 PUFA diet, Coronel et al. (2007) observed that the fish oil was proarrhythmic compared to the control group during regional ischemia. An important consideration for n-3 PUFA enrichment both in comparing different studies and for human therapy is the differences in cardiovascular health of the subject. In pigs, a fish oil diet was observed have both pro and antiarrhythmic effects (Verkerk et al. 2006). Thus, the authors concluded that the pathophysiological setting is important for determining the effectiveness of n-3 PUFA enrichment (Verkerk et al. 2006).

1.2.2 Omega-3 Polyunsaturated Fatty Acids and Cardiovascular Disease in Humans

There is strong experimental evidence that dietary intake of n-3 PUFAs reduces cardiovascular disease risk. In the JELIS Trial, 18645 Japanese hypercholesterolaemic patients were fed either 1800 mg of EPA daily with statin or just statin for five years (Yokoyama et al. 2007). The EPA treatment was found to reduce major coronary events, both fatal and non-fatal (Yokoyama et al 2007). Guillot et al. (2009) observed 12 healthy men given different concentrations of DHA to consume. During the course of the study, DHA was present in platelet phospholipids in a dose dependent manner and low consumption of DHA displayed an antioxidant effect (Guillot et al. 2009). Bannikoppa et al. (2017) also found a correlation between dietary n-3 PUFA and erythrocyte n-3 PUFA.

Ready incorporation of DHA and EPA into atherosclerotic plaques is one potential explanation for the beneficial effects of n-3 PUFAs on cardiovascular disease. In patients awaiting carotid endarterectomy, fish oil supplementation led to an increase in EPA and DHA in carotid plaque fractions, a decrease in the signs of inflammation, and fewer thick fibrous caps macrophages in the plaques (Thies et al. 2003). The positive effect of n-3 PUFAs on health has also been closely associated lipid metabolism. DHA supplementation for 45 days in hypertriglyceridemic men was found to decrease fasting triacylglycerol, large VLDL, IDL while increasing concentration of large LDL particles (Kelley et al. 2007). The authors concluded that this change can improve health by reducing triacylglycerol and small, dense LDL particles (Kelley et al. 2007). Similarly, Milte et al. (2008) found that DHA supplementation decreased serum triacylglycerol while increasing both HDL and LDL cholesterol in a dose dependent manner. Erythrocyte DHA concentration increased in a dose dependent manner. Beneficial decrease in triacylglycerol correlated with rise in erythrocyte DHA (Milde et al. 2008).

Not all human studies have found a beneficial effect of n-3 PUFAs consumption. In the Rotterdam Study, intakes of EPA, DHA, and fish had no association with atrial fibrillation risk, the most common sustained cardiac arrhythmia (Brouwer et al. 2006). Rauch et al. (2010) found that omega-3 fatty acid supplementation was unable to reduce the rate of sudden cardiac death and other clinical events in survivors of acute myocardial infarction who were also being given guideline-adjusted treatment. In a prospective cohort study in women, Rhee et al. (2017) found no association between risk of major cardiovascular disease and intake of tuna, dark fish, α -linolenic acid, or marine omega-3 fatty acids. Garcia-Alonso et al. (2012) fed healthy women tomato juice enriched with n-3 PUFAs and found no impact of the supplementation on lipid profile. Mozaffarian et al. (2012) observed the impact of perioperative n-3 PUFA supplementation in patients scheduled for cardiac surgery and found no influence of n-3 PUFAs on postoperative atrial fibrillation. Patients with dysglycemia and at high risk for cardiovascular events were given n-3 fatty acids, but the intervention had no significant impact on cardiovascular death, death from any cause, major vascular events, or death from arrhythmia (Bosch et al. 2012). In patients with multiple cardiovascular risk factors, daily treatment of n-3 fatty acids was shown to have no significant impact on cardiovascular mortality and morbidity (Roncaglioni et al. 2013). As observed in animal models, this difference in results may be due to the differing disease states being researched. Another possibility that has not been focused upon in the current literature is the possibility of the treatment fats being oxidized. Lipid oxidation can lead to the formation of toxic products which may lessen the potential benefits of n-3 PUFA supplementation (Esterbauer 1993, Guéraud et al. 2010, Turner et al. 2006, Vieira et al. 2017).

1.3 Dietary Omega-3 Polyunsaturated Fatty Acids and Cancer

1.3.1 Omega-3 Polyunsaturated Fatty Acids and Cancer *in vitro* and in Animals

In vitro research suggests that n-3 PUFAs are able to reduce the negative impact of cancer, primarily through induction of apoptosis and altering gene expression in cancer cells. The proapoptotic influence of n-3 PUFAs, specifically DHA, has been shown in many different cancer cell models. Treatment of human colorectal cancer cells with DHA induced apoptosis and was shown to do so through proteasomal regulation of β -catenin levels and expression of TCF- β -catenin target genes (Calviello et al. 2007). In lung cancer cells, DHA treatment was found to induce apoptosis and involved a phosphatase activity (Serini et al. 2008). The ability of DHA to increase apoptosis has also been observed in human melanoma cells, human breast cancer cells, and human prostate cancer cells (Serini et al. 2012, Sun et al. 2011, Hu et al. 2010). In human breast cancer cell lines, both DHA treatment and EPA treatment were shown to inhibit proliferation and invasion of breast cancer cells, but DHA was more effective than EPA (Rahman et al. 2013).

Most cancer research on the effect of n-3 PUFAs has been in rodents with tumorigenesis induced either by genetics or the introduction of carcinogens. N-3 PUFAs have been observed to have a positive effect in multiple different cancers in animals. For example, in azoxymethane-induced colon cancer in rats, rats on a diets of flaxseed oil showed a reduced incidence of aberrant crypt foci (Williams et al. 2007). Diets of omega-3 fatty acids also reduced tumor load in colon cancer induced rats (Gutt et al. 2007). In colorectal cancer induced mice, Hawcroft et al. (2012) observed that EPA supplementation reduced tumor weight, tumor cell proliferation, and metastasis. Other cancer models have found similar results. Bose et al. (2007) provided ApcMin/+ mice with diets containing 20% (w/w) fish oil and found a reduction in the number of

large tumors. Berquin et al. (2007) used prostate-specific Pten-knockout mice fed an omega-3 enriched diet and found that the diet reduced prostate tumor growth, slowed histopathological progression, and increased survival compared to the control group. A diet containing n-3 PUFAs was observed by Hu et al. (2010) to lessen the progression of prostate cancer malignancy in mice. Strouch et al. (2011) provided (EL)-Kras transgenic mice with a high omega-3 fat diet and the diet reduced markers of cell proliferation in precancerous tumors of induced pancreatic cancer. Gleissmann et al. (2011) found that DHA by gavage was able to inhibit growth of established neuroblastomas in nude rats but dietary DHA was unable to, likely due to a slower accumulation of DHA when obtained from food. In athymic mice with intra-cardiac injection of breast cancer cells, DHA treatment significantly lowered breast cancer cell metastasis (Rahman et al. 2013).

1.3.2 Omega-3 Polyunsaturated Fatty Acids and Cancer in Humans

Research on dietary omega-3s in humans is conflicting and limited compared to the research in other models. Thus, the impact of dietary n-3 PUFAs on cancer in humans is still unclear (Song et al. 2014). Arem et al. (2013) found that higher intakes of EPA and DHA were significantly associated with lower risk for endometrial cancer, as was the use of fish oil supplements. In a 9-year follow-up study, Sasazuki et al. (2013) found associations that suggested marine n-3 PUFAs may be inversely related to colon cancer risk, but only in the proximal site of the large bowel. In a case-cohort analysis of women with or without breast cancer, Bassett et al. (2016) found that breast cancer risk was inversely associated with dietary DHA and EPA, while breast cancer risk was positively associated with saturated fat intake and omega-6 fatty acids. In women with newly diagnosed breast, cervical, or ovarian cancer,

Bannikoppa et al. (2017) found that patients with malignancies had significantly lower overall intakes of linoleic acid and long chain n-3 PUFAs. In addition, the erythrocytes of the cancer group had lower linoleic acid compared to controls (Bannikoppa et al. 2017). Contrasting with this evidence, Chajés et al. (1999) found no association between serum n-3 PUFAs and breast cancer risk in a comparison of the pre-diagnostic sera of women who later developed breast cancer. Song et al. (2014) found no overall association between fish, omega-3, or omega-6 PUFAs and colorectal cancer risk in a 26-year long follow-up study. But, marine omega-3 PUFA did have an inverse association with risk of distal colon and rectal cancer in men (Song et al. 2014).

In addition to associations between dietary n-3 PUFAs and cancer development, patients recovering from or currently diagnosed with cancers have been a target for research. Similar to other cancer literature, the results are not consistent (Eltweri et al. 2017, Gleissman et al. 2010, Mourouti et al. 2015). In colorectal cancer patients undergoing chemotherapy, supplementation with fish oil for 9 weeks positively impacted markers for inflammatory and nutritional status (Silva et al. 2012). Fish oil was found to prevent decreases in neutropenia in patients receiving chemotherapy after surgical tumor removal (Bonatto et al. 2012). Torrinhas et al. (2013) found that fish oil-based lipid emulsions provided post-operative immunological benefits to gastrointestinal cancer patients prior to surgery. This result conflicts, however, with observations by Makay et al. (2011), who found that supplementation with n-3 PUFAs after major gastric cancer surgery had no impact on biochemical parameters, complications, length of hospital stays, or mortality. Sultan et al. (2012) also observed no change clinical outcomes for oesophagogastric cancer patients given a n-3 PUFA enteral immunoenhancing diet 7 days before and after surgery. (Sultan et al. 2012).

1.4 Dietary Omega-3 Polyunsaturated Fatty Acids and Other Diseases

1.4.1 Epidemiological Evidence for Omega-3 Polyunsaturated Fatty Acids and Various Diseases

While there has been considerable research in cardiovascular disease and cancer, other diseases have been studied as well, guided by epidemiological evidence. For example, n-3 PUFAs may positively affect health by increasing insulin sensitivity. Rats fed fish oil and algal oil have been observed to have reduced insulinemia (Haimeur et al. 2016). In a follow-up study in normoglycemic elderly men and women, higher fish intake was associated with a significantly reduced incidence of glucose intolerance (Feskens et al. 1991). This observation is supported by a study by Browning et al. (2007), where individuals with a raised inflammatory status were given DHA and EPA supplements. The authors found that the n-3 PUFA treatment increased insulin sensitivity (Browning et al. 2007). However, in a dietary intervention study where saturated fat was replaced isoenergetically with long chain n-3 PUFAs, Tierney et al. (2010) found that there was no effect on insulin sensitivity in weight-stable individuals but did find a decrease in plasma triglycerides.

n-3 PUFAs have also been linked to depressive disorders. In the Hordaland health study, Raeder et al. (2007) found that users of cod liver oil supplements had a lower likelihood of having depressive symptoms. In a metaanalysis, Kraguljac et al. (2009) concluded that omega-3 fatty acids are a potential treatment for depressive disorders, but not for mania. Compared to healthy elderly subjects, those with depression were observed to have significantly lower n-3 PUFA concentration in their erythrocyte membranes (Rizzo et al. 2012). Supporting these observations, Rizzo et al. (2012) showed that n-3 PUFA supplementation reduced depression symptoms in elderly subjects.

Similarly, n-3 PUFAs have been suggested to have an impact on cognitive impairment and Alzheimer's Disease (Fotuhi et al. 2009, McNamara 2016). Low serum DHA has been associated with an increased risk for the development of Alzheimer's Disease (Kyle et al. 1999). High fish consumption has been associated with a decrease in cognitive impairment, a decrease in cognitive decline, and a decreased risk for the development of Alzheimer's Disease (Larrieu et al. 2004). The use of omega-3 rich oils has also been associated with a decreased risk for all-cause dementia (Barberger-Gateau et al. 2007). Milte et al. (2011) found that older adults with mild cognitive impairment had lower erythrocyte EPA. The current literature is in conflict, however, as to the applicability of these observations. While human subjects with mild cognitive dysfunction given arachidonic acid and DHA supplementation for 90 days showed improvement in immediate memory and attention according to Kotani et al. (2006), van de Rest et al. (2008) observed no benefit of DHA and EPA supplementation in cognitively healthy individuals.

1.4.2 The Anti-inflammatory Effect of Omega-3 Polyunsaturated Fatty Acids as a Disease Reduction Mechanism

The inflammatory response is implicated in the progression of many diseases. The potential antiinflammatory effect of n-3 PUFAs is one explanation of its ability to delay the onset of multiple diseases (Calder 2013, Rangel-Huerta et al. 2012, Singer et al. 2008). *In vitro* evidence strongly supports the antiinflammatory effect of n-3 PUFAs. In ultraviolet-B radiation induced inflammation in mouse skin, topical application of DHA lowered inflammation by inhibiting the activation of NF- κ B (nuclear factor κ B) and the expression of COX-2 and NOX-4, all of which play important roles in provoking inflammation and inducing oxidative stress (Rahman et al. 2011). After initiating the inflammatory response in human macrophages with lipopolysaccharide, Matinez-Micaelo et al. (2012) found that DHA prevented the activation of

the NF- κ B cellular cascade, decreasing the release of proinflammatory NF- κ B-regulated agents such as cytokines and prostaglandins. Oliver et al. (2012) pretreated macrophages with either EPA or DHA before exposing the cells to lipopolysaccharide to induce inflammation. The authors found that DHA was more effective than EPA and that both reduced the activation of NF- κ B activation and tumor necrosis factor (TNF) α secretion.

Positive results have been observed for n-3 PUFA supplementation and inflammatory markers, primarily in individuals with a disease state. In overweight women with high levels of inflammation, n-3 PUFA treatment increased plasma EPA and DHA, lowered serum triglycerides, and lowered inflammatory markers (Browning et al. 2007). Zhao et al. (2009) displayed that in patients with heart failure, n-3 PUFA supplementation for 3 months was able to reduce multiple inflammatory markers significantly. In patients with systemic inflammatory response syndrome, Barbosa et al. (2010) showed that fish oil administered to the peritoneum increased plasma EPA, modified inflammatory cytokine concentration, and decreased hospital stay length. As n-3 PUFAs impact inflammatory pathways, they may have a positive impact on reducing the negative effects of multiple diseases.

1.5 Conclusions

There is a considerable amount of research investigating the beneficial effects of omega-3 polyunsaturated fatty acids, especially on the impact of EPA and DHA. While observations in humans are conflicting, there is a great deal of research that shows a beneficial effect of dietary n-3 PUFAs on lipid metabolism, which in turn leads to a decrease in cardiovascular disease risk. Mechanisms have been researched as well, elucidating the effect of n-3 PUFAs on lipid metabolism and arrhythmias. Their ingestion may also have an impact on decreasing the risk for

cancer and other diseases. Dietary omega-3 polyunsaturated fatty acids have been linked heavily with an anti-inflammatory effect, which may explain its multiple health benefits. Within human and animal studies there is some conflicting evidence. Variation in model, especially in disease state, in dose volume, time frame, and fatty acid profile are all factors that can lead to this conflict. The presence of lipid oxidation products in the n-3 PUFA supplementation is another potential explanation, however, that has not been heavily addressed in the current literature. More research is needed in order to better elucidate the benefits, their mechanisms, and proper dosage in humans.

CHAPTER 2

LIPID OXIDATION AND ITS BIOLOGICAL IMPLICATIONS

2.1 Introduction

Highly unsaturated oils, especially those from marine sources, are an important topic of research. The U.S. Department of Health and Human Services and U.S. Department of Agriculture 2015-2020 dietary guidelines suggest replacing solid fats with unsaturated oils. Consumption of polyunsaturated fats, especially omega-3 polyunsaturated fatty acids (n-3 PUFAs), is recommended due to the potential health benefits. Thus, there is a great desire to introduce more unsaturated fats into the diet, through both food products and dietary supplements. The main downside of using highly unsaturated fats is that they are significantly more prone to lipid oxidation. In addition to causing problems with consumer acceptance and overall quality, lipid oxidation may lessen the health benefits of n-3 PUFAs and could even lead to toxicity.

2.2 Mechanisms of Lipid Oxidation

The process of lipid oxidation is a thoroughly researched and reviewed group of chemical reactions, thoroughly described in other works (Brady 2012, Girotti 1985, McClements and Decker 2008). In summary, lipid oxidation is defined by three phases: initiation, propagation, and termination. The first step of the process, initiation, occurs when a hydrogen is removed from an unsaturated fatty acid, leading to the production of an alkyl radical ($R\bullet$). The susceptibility of a fatty acid to hydrogen removal increases as the degree of unsaturation increases (Holman and Elmer 1947, Martín-Polvillo et al. 2004). For example, Martín-Polvillo et

al. (2004) tested sunflower oils of differing unsaturation degree and found that the rate of oxidized triglyceride monomers and peroxide formation and the amount of total oxidized triglycerides and peroxides were higher the more unsaturation was present. Thus, the likelihood of an oil becoming oxidized increases with increased double bonds. Lipid oxidation is also expedited by factors such as transition metals, enzymes, photosensitizers, high temperatures, and irradiation, all of which tend to promote the creation of free radicals or reactive oxygen species.

After the removal of a hydrogen, the fatty acid isomerizes to form a conjugated double bond. Afterward, propagation begins as atmospheric oxygen is added and forms a peroxy radical ($\text{ROO}\bullet$). Peroxy radicals are reactive enough to remove hydrogens from other unsaturated fatty acids, leading to a new alkyl radical ($\text{R}\bullet$) and a lipid hydroperoxide (ROOH). β -scission reactions lead to the decomposition of lipid hydroperoxides into alkoxy radicals ($\text{RO}\bullet$). These new radicals introduce another means of attacking unsaturated fatty acids and causes an exponential increase in oxidation rate. β -scission reactions break covalent bonds within the hydrocarbon chain and form low molecular weight compounds such as aldehydes, shorter hydrocarbons, and ketones. These smaller products are volatile and cause rancid odors. Other reactions follow β -scission reactions as well, including reacting with other components of the food, performing Michael additions, or Schiff's base reactions.

Termination occurs when two radicals react with one another to form a non-radical product. This reaction is often not relevant in foods. A food is already rancid before these reactions take place. The period prior to the exponential gain of lipid oxidation products is defined as the lag phase, or as the period before which there is no significant change in oxidation product amount. This period, where oxidation products are accumulating slowly, is the primary target of research and observation, and the focus of research in many foods and other oxidizable

systems is to extend the lag phase. It is during lag phase that products responsible for rancid odors are below sensory thresholds. As food is effectively rancid when the lag phase ends, it is the best representation of shelf life.

2.3 Lipid Oxidation Products in *in vitro* Studies

In vitro research suggests that lipid oxidation products are able to be taken up by intestinal cells and can exert an effect on lipid metabolism. Using human colonic CaCo-2 cells, Wang et al. (2000) found that subtoxic concentrations of lipid hydroperoxides increased cell apoptosis and disrupted redox homeostasis. Addition of hydroperoxy fatty acids to a CaCo-2 cell monolayer was shown to disrupt lipid metabolism and allowed their incorporation into more complex lipids, potentially leading to oxidizable lipoproteins (Muller et al. 2002). Penumetcha et al. (2002) showed that the presence of an oxidation product of linoleic acid enhanced the serum solubility of cholesterol and its uptake in CaCo-2 cells. As lipid oxidation products have an impact on cellular metabolism, they may affect human health.

2.4 Lipid Oxidation Products in Animal Studies

Ingestion of lipid oxidation products has been associated with multiple diseases in animals including inflammation, certain cancers, and cardiovascular disease (Turner et al. 2006). The largest impact of oxidized lipid ingestion has been observed in lipid metabolism and atherosclerosis. In both control and diabetic rats, feeding of oxidized lipids was observed to increase the quantity of peroxides in serum lipoproteins, and the effect was more dramatic when the animals were diabetic (Staprans et al. 1993). In low density lipoprotein (LDL) receptor and apolipoprotein E deficient mice fed either cholesterol or cholesterol with 5% oxidized cholesterol, Staprans et al. (2000) observed that the diets with oxidized cholesterol led to the

appearance of oxidized cholesterol in the serum. In addition, the authors found an increase in fatty streak lesions in mice fed oxidized cholesterol. In rats, Penumetcha et al. (2002) found that an oxidation product of linoleic acid increased the uptake of cholesterol and thus could lead to hypercholesterolemia and atherosclerosis. Keller et al. (2004) showed that erythrocytes of Guinea pigs fed oxidized fat were more susceptible to haemolysis *in vitro* and had reduced antioxidant content compared to a control diet. These studies suggest that not only are oxidized lipids absorbable from the diet, they cause a significant impact on lipid metabolism. The increased uptake of cholesterol and presence of oxidized lipids in chylomicrons suggests that lipid oxidation can play a role in atherogenesis, likely through a buildup of cholesterol and an increase in LDL oxidation.

Specific lipid oxidation products have been researched in animals as well. Crotonaldehyde, a representative α , β -unsaturated carbonyl compound, is considered to be genotoxic, mutagenic, and carcinogenic (Eder and Budiawan 2001). For example, crotonaldehyde was shown to significantly increased liver tumors in rats when added to drinking water (Chung et al. 1986). After 4 weeks of intake of crotonaldehyde in food in rats, Eder and Budaiwan (2001) found that 1,N2-propanodeoxyguanosine DNA adduct increased and persisted to some extent. Acrolein is another α , β -unsaturated carbonyl compound produced in foods from lipid oxidation. Mice given oral acrolein showed a significant increase in myocardial infarct size and acrolein was found to target mitochondria (Wang et al. 2008). Acrolein fed mice were also shown to have increased signs of myocardial oxidative stress, nitrative stress and in the heart, signs of physical translocation of ingested acrolein (Ismahil et al. 2011).

Some animal studies, however, have found a lack of oxidized lipid inducing general toxic symptoms. In miniature pigs fed thermally oxidized lipids, Eder and Stagl (2000) found no

difference in plasma clinicochemical variables. Eder et al. (2003) gave rats diets of fresh or heat oxidized fat and found that LDL in heat treated fat diet were more susceptible to oxidation, but the uptake of LDL by macrophages were unaffected and thus did not adversely affect the lipoprotein profile.

2.5 Lipid Oxidation Products in Human Studies

Research in humans suggests that lipid oxidation products are absorbed into the serum and may affect health. Human chylomicrons obtained after ingestion of thermally oxidized fat were shown to be rich in lipid peroxides and the authors observed an accumulation of cholesterol when the chylomicrons introduced to murine macrophages (Naruszewicz et al. 1987). Staprans et al. (1994) researched the impact of oxidized lipid ingestion on oxidized lipid level of postprandial serum chylomicrons and found that conjugated diene content increased significantly in chylomicrons after the consumption of highly oxidized corn oil. Partially oxidized oil also led to an increase of conjugated diene content, but not as large of an increase (Staprans et al. 1994). In addition, consumption of a meal containing used cooking fat, a source of lipid oxidation products, was found to increase in indicators of impaired arterial endothelial function compared to meal of fresh fat and a low-fat meal (Williams et al. 1999). In a study of healthy women aged roughly 40 years given fat containing hydroxy or dihydroxy triglycerides to consume, Wilson et al. (2002) showed that the lipid oxidation products were absorbed into the serum. In a comparison of postprandial inflammation markers after consumption of either pan fried beef or sous vide beef, Nuora et al. (2015) concluded that oxidized lipids have the ability to impact postprandial oxidative stress and inflammation. As oxidized lipids may be taken up by the small

intestine and packaged into chylomicrons, they can further lead to oxidized LDL, which are more atherogenic (Esterbauer 1993).

While many studies suggest that lipid oxidation products are absorbed and influence oxidative stress and other metabolic factors, the results are not consistent (Baynes 2007). One such study was performed by Ottestad et al. (2012), where the authors measured markers of oxidative stress, lipid peroxidation, inflammation, and plasma omega-3 fatty acids in humans after intake of 8g/day oxidized or unoxidized fish oil and found no change in these markers between the two groups after consumption for 3 or 7 weeks.

Due to the conflicts in animal and human studies, the toxicity of lipid oxidation remains an active area of research. There is, however, a great deal of evidence that suggests that lipid oxidation products are absorbed, and mechanisms have been displayed for their negative impact on health. Thus, the toxicity of lipid oxidation is still a concern, especially in products enriched with unsaturated fats, where lipid oxidation is more common. They are also a concern in studies on the health impact of dietary n-3 PUFAs, as their presence may cause negative health effects if not controlled for.

2.5 Conclusions

As the desire to consume polyunsaturated fats for their health benefits increases, so has the amount of research on lipid oxidation. Lipid oxidation presents a huge problem as the polyunsaturated fatty acid content of food and dietary supplements increases. As these fats are far more susceptible to lipid oxidation, understanding the mechanisms of lipid oxidation and its control are important. Lipid oxidation is influenced by many factors, including antioxidant addition, temperature, and oxygen concentration, which have received a great deal of research.

While there is substantial evidence that suggests that lipid oxidation products may exert a toxic effect in both animals and humans, the overall results are unclear. More research is needed in the field of lipid oxidation in order to safely recommend dietary enrichment of polyunsaturated fats, especially when obtaining those fats from food, as research in low moisture food systems is limited.

CHAPTER 3

LIPID OXIDATION IN ANIMAL FEED ENRICHED WITH FISH OIL

3.1 Introduction

Omega-3 fatty acids have been touted for their potential health benefits including reducing risk for cardiovascular disease, inflammation, and cancer. However, studies on the extent of these benefits in animal models are often conflicting (Fotuhi et al. 2009, McNamara 2016, Verkerk et al. 2006). Lipid oxidation rates increase with increasing level of unsaturation, thus omega-3 fatty acids with their 3-6 double bonds are extremely susceptible to oxidation. Lipid oxidation leads to the destruction of vital vitamins, such as vitamin A and E (Kubow 1992) and also results in the formation of numerous potentially harmful lipid oxidation products. As lipid oxidation could potentially cause a negative impact on animal health, it may be a confounding variable in dietary omega-3 fatty acids animal studies, making data interpretation in animal models difficult if precautions are not taken to insure both good starting oil quality and that lipid oxidation does not occur during feeding studies.

There is a dearth of research on the biological activities of the lipid oxidation products of n-3 PUFAs and ingestion of lipid oxidation products has been observed to have a negative impact on health (for review see Wang et al. 2017). Lipid oxidation products can survive digestion and thus they may be absorbed from a meal containing both unoxidized or oxidized omega 3 fatty acids (Goicoechea et al. 2011). Many different oxygenated α , β -unsaturated aldehydes are formed from lipid oxidation and they have been shown to have various degrees of toxicity. Examples lipid oxidation products formed from n-3 PUFAs include 4-hydroxy-2-hexenal (HHE) and 4-oxo-2-hexenal (OHE). Long et al. (2008) found HHE to be toxic to cerebral cortical neurons, supporting the reported correlation between neurodegenerative

diseases and oxidized brain DHA. Rats given 4-oxo-2-hexenal intragastrically for five weeks had significantly higher rates of mortality compared to control rats (Takasu et al. 2007). Awada et al. (2012) fed mice a high fat diet containing HHE and found an increased presence of HHE in the serum and an enhancement of both proinflammatory markers and nuclear factor kappa- β .

One of the largest effects of lipid oxidation products observed is their potential genotoxicity (for review see Ecki and Bresgen 2017). Thermally oxidized fish oil was observed to have skin cancer tumor initiating activity and slight tumor promoting activity when applied to the skin of mice (Pandey and Das 2006). In addition, HHE and 2-hexenal were both found to induce mutations in the Ames test (Grúz et al. 2017). OHE was found in DNA adducts in human autopsy tissues and may be a source of mutations which could contribute to cancer formation (Chou et al. 2010). After oral OHE administration to mice, Kawai et al. (2010) found an increase in the amount of DNA adducts detected in the stomach and large intestine. These results suggest adduct formation occurred from direct exposure to the stomach and large intestine as adducts were not found in the liver (Kawai et al. 2010).

Thus, one of the potential causes of the variability between omega-3 dietary enrichment studies may be that the oil is oxidized prior to feeding, hereby lessening, negating, or even reversing any potential benefit that could be obtained by omega-3 fatty acid consumption. Albert et al. (2015) measured lipid oxidation in 32 different commercially available fish oil supplements that were within their “best-before” consumption date and compared them to international standards for fish oil supplements. These voluntary safety standards, as set by the Global Organization for EPA and DHA Omega-3s and the Council for Responsible Nutrition, and International Fish Oil Standards (IFOS), can be defined as a hydroperoxide value lower than 5 mEq/kg, anisidine value lower than 20 mEq/kg or a total oxidation products measurement

(Totox) lower than 26 mEq/kg (Jackowski et al. 2015). Albert et al. (2015) found that 83% of the fish oil supplements exceeded recommended levels of hydroperoxide value and 50% exceeded the recommended Totox levels. Similarly, Mason and Sherratt (2017) investigated the three top selling fish oil dietary supplements in the United States of America and found that hydroperoxide value, anisidine value, and Totox levels exceeded the maximum levels established by international standards. Even if the amount of oxidation products in the oil before the start of a study is low, the oxidation could occur during the storage of the animal diets during the experimental time frame, which is often greater than 9 weeks. As an example of the oxidative susceptibility of omega-3 fatty acids, oil-in-water emulsions have been shown to oxidize in 6 days at 32°C (Johnson et al. 2016).

The objective of this study was to determine the susceptibility of omega-3 fatty acids to oxidation in a typical animal feed. In these studies, good quality oils, with low amounts of oxidation products were added to powdered animal feed and oxidation was monitored during storage. The influence of different storage conditions and antioxidant treatments were evaluated to determine effective strategies to control oxidation. Thus, the overall goal of this study was to provide techniques by which omega-3 fatty acid investigators may strengthen their findings by reducing the presence of lipid oxidation products in their studies, therefore allowing for a greater focus on the biological effects of omega-3 fatty acids.

3.2 Materials and Methods

3.2.1 Materials

Modified AJN-93G Purified Rodent Diet was obtained from Dyets Inc. (Bethlehem, PA) and fish oil was obtained from DSM Nutritional Products LLC, 1B1/X1.2/250/13 /F/ BVT 202131/TOU (Columbia, MD) and stored at -80C until use. The fatty acid composition was

found to be 6.73% C14:0, 1.08% C15:0, 19.65% C16:0, 8.15% C16:1, 0.89% C17:0, 4.33% C18:0, 17.97% C18:1 n9, 2.47% C18:1 n7, 2.38% C18:2 n6, 1.27% C18:3 n3, 1.81% C18:4 n3, 0.88% C20:0, 1.02% C20:1, 0.77% C20:4 n6, 9.27% C20:5 n3, 0.80% C22:1, and 12.56% C22:6 n3 by gas chromatography (Floch et al. 1997, Park et al. 2001). Menhaden fish oil was purchased from Sigma-Aldrich (St. Louis, MO).

Propanal, trichloroacetic acid (TCA), ascorbyl palmitate, and tert-butylhydroquinone (TBHQ) were purchased from ACROS Organic (Morris Plains, NJ). Methanol, ethanol, hexane, ascorbic acid, and chloroform were obtained from Fisher Scientific (Waltham, MA). 2-Thiobarbituric acid (TBA), ammonium thiocyanate, barium chloride, iron (II) sulfate heptahydrate, hydrochloric acid, propyl gallate (PG), butylhydroxy toluene (BHT), α -tocopherol, mixed tocopherols, 6-hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid (Trolox), 1,1,3,3-tetraethoxypropane (TEP), dioxane and cumene hydroperoxide, were bought from Sigma-Aldrich (St. Louis, MO). High purity nitrogen and nitrogen/oxygen gas blends (5.0 and 2.5 mol% oxygen in nitrogen) were obtained from AirGas Specialty Gases (Radnor, PA). Oxygen sensing patches were obtained from Ocean Optics Inc., 2015 (Dunedin, FL). Double distilled water was used to prepare all solutions and all solvents and reagents were of analytical grade or higher.

3.2.2 Sample Preparation

Samples were prepared via mixing 85% rodent feed and 15% fish oil by mass. One-gram samples were stored in acid-washed, 10-ml glass gas chromatograph (GC) vials (Supelco Analytical; Bellefonte, PA), closed with aluminum screw caps containing PTFE/silicone septa

(Supelco Analytical; Bellefonte, PA) and stored at -20 to 37°C in the dark, unless otherwise stated.

3.2.3 Measurement of Lipid Oxidation Products

3.2.3.1 Hydroperoxide Analysis

Hydroperoxide concentrations were measured with a procedure adapted from Shantha and Decker (1994). Samples (0.1 g) were placed into a test tube and vortexed with 10 mL of chloroform:methanol (2:1 v/v) . The samples were centrifuged at 2,500 rpm for 10 minutes and 0.2 mL of the supernatant was placed in a new test tube and mixed with 3.14 mL of the chloroform-methanol solvent, after which 34.5 μ L a 1:1 solution of 0.144 mM ferrous sulfate in water and 0.132 M barium chloride dissolved in 0.4 N hydrochloric acid was added alongside the same volume of 3.94 M ammonium thiocyanate. After 20 minutes, spectrophotometric analysis was performed at 500 nm on a Genesys 20 spectrophotometer (ThermoSpectronic; Waltham, MA). Hydroperoxide concentration was calculated using a cumene hydroperoxide standard curve.

3.2.3.2 Headspace Propanal Analysis

One gram of sample in the aforementioned glass gas chromatograph vials were analyzed using a GC2010 Plus Gas Chromatograph (GC) (Shimadzu; Kyoto, Japan). After samples were incubated at 45°C for 10 minutes, a 50/30 μ m divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) Stableflex solid phase microextraction (SPME) fiber (Supelco Analytical; Bellefonte, PA) was exposed to the sample headspace for 2 minutes. The volatiles were desorbed

for three minutes in the GC injector at 250°C at a split ratio of 1:7. Volatile compounds were separated using an Equity-1 fused-silica capillary column (30 m × 0.32 mm i.d. × 1 µm) coated with 100% polydimethylsiloxane (Supelco Analytical; Bellefonte, PA). The flame ionization detector was set to 250°C while the oven was at 65°C. The sample run time was 10 minutes and the peak for propanal was found at 1.6 minutes. Peak integration was calculated using Shimadzu Lab Solutions version 5.87 (2016).

A standard curve was created using fresh feed samples spiked with propanal in order to calculate the concentrations.

3.2.3.3 Thiobarbituric Acid Reactive Substances Analysis

The Thiobarbituric Acid Reactive Substances (TBARS) assay was used as a method for secondary oxidation products, using a procedure adapted from (Johnson et al. 2016). Two mL of TBA reagent (15%, w/v TCA, 0.375%, w/v TBA in 0.25 M HCl mixed with 2% w/v BHT in ethanol) was added to 1 gram of sample. The mixture was suspended in 10 mL of water and after 15 minutes of room temperature incubation, the sample was filtered using Whatman 4 qualitative circles (Sigma-Aldrich; St. Louis, MO). The filtrate was stored in the dark at room temperature for 24 hours to allow for the reaction of TBA with the oxidation products before measuring the absorbance at 532 nM on a Genesys 20 spectrophotometer (ThermoSpectronic; Waltham, 61 MA).

A standard curve was created using fresh samples spiked with 1,1,3,3 tetraethoxypropane (TEP) in order to calculate the concentrations of TBARS.

3.2.4 Impact of Temperature on Lipid Oxidation Rates

Samples were mixed and stored in triplicate as described above and stored in the GC vials at 37°C, 23°C, 4°C and -20°C in the dark. The sample stored at 37°C was measured for oxidation products every day, 23°C every other day, and 4°C and -20°C once a week.

3.2.5 Impact of Oxygen Removal on Lipid Oxidation

Samples were stored in acid washed crimp cap glass vials with teflon/rubber septa (ThermoFischer Scientific; Rockwood, TN) and in the dark at 37°C. Crimp cap vials were used in these experiments as they are more oxygen impermeable than screw capped vials. Oxygen sensing fluorescent patches (Ocean Optics Inc.; Dunedin, FL) were placed in the headspace of the vial and used to measure oxygen concentration, following a similar method to Johnson et al. (2016). Oxygen was removed by flushing the vials with nitrogen or nitrogen/oxygen blends for 3 minutes before crimping the vials closed. Oxygen concentration, expressed as the percentage of oxygen removal compared to atmospheric oxygen, was measured every day along with lipid hydroperoxides and TBARS.

3.2.6 Rate of Oxygen Removal

Feed samples were placed into a 5" x 8.25" vacuum sealable bag (Winpack; Winnipeg, Canada). The florescent patches, mentioned previously, were used to measure oxygen concentration both in the headspace and the section of the bag that contained sample. Nitrogen (>99%) was introduced through silicone tubing (5 mm diameter; MasterFlex, Gelsenkirchen, Germany) to flush the headspace at the maximum flow rate that did not cause the sample to be

blown into the air, approximately 2 L/min. The percent of oxygen removal was recorded every 5 seconds.

3.2.7 Impact of Antioxidants on Lipid Oxidation

Triplicate samples were mixed in triplicate with 200 μ moles of the respective antioxidant per kg of sample. The antioxidants tested included BHT, TBHQ, PG, α -tocopherol, mixed tocopherols, Trolox, ascorbic acid and ascorbyl palmitate. Every day, after storage at 37°C in the dark in the aforementioned capped gas chromatograph vials, lipid hydroperoxide, headspace propanal, and TBARS concentrations were measured.

3.2.8 Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison was conducted in order to determine statistical significance. The calculations were performed using Minitab 18.1 Statistical Software. All samples were run in triplicate. Lag phase was determined as the period before which there is no significant change in oxidation product amount.

3.3 Results and Discussion

Fifty published papers from the past five years on the evaluation of dietary omega-3 fatty acids in animal studies were reviewed, and these studies and their methods of oxidation control are summarized in Table 1. Of these 50 papers, 35 did not mention any form of oxidation control. The most common method of control used in the literature was through temperature, storing omega-3 enriched feed at -20°C or below, with 8 of the 50 sources mentioning these

storage conditions. Three studies used encapsulation of the oil as their primary method of control. Kemese et al. (2014) used previously encapsulated oil, MaxEPA (Merck Darmstadt, Germany), Eilati et al. (2013) filled fish oil into gelatin capsules, and Coulombe et al. (2018) used oil that was microencapsulated by DSM Nutritional Products (Columbia, MD). No data was provided on the effectiveness of encapsulation on oxidative stability during storage. Only 3 studies enriched the diets with antioxidants to inhibit oxidation. Njoroge et al. (2015) used vitamin A, vitamin C, vitamin E, and selenium. Madingou et al. (2016) used unspecified antioxidants during feed preparation, and the oil used by Thang et al. (2013) contained TBHQ. In the studies that used oxidation control strategies, no data to verify the effectiveness of these methods was presented. Only 1 of the 50 studies described measuring lipid oxidation at any point in the study and did so in the form of hydroperoxides at the beginning of the feeding study (Ostermann et al. 2017). As the amount of toxic oxidation product formation in the majority of these studies is unknown, their effect may be the confounding factor leading to differing results.

3.3.1 Oxidation Products in Commercially Available Fish Oils

Menhaden oil from Sigma and the fish oil from DSM Nutritional Products had hydroperoxide values lower than the limit of detection, 0.2 mmoles hydroperoxide/ kg sample, when analyzed on freshly received samples. Menhaden oil had significantly more headspace propanal, indicating that the menhaden oil was more oxidized than the fish oil (Figure 1). Lipid hydroperoxides are unstable and thus may have decomposed during storage or they may have been decreased during refining operation steps, such as steam distillation (Decker et al. 2012). Therefore, even though an oil can have low hydroperoxides, the presence of other oxidation products, such as propanal, indicates that the menhaden oil was rancid. Balogun et al. (2013)

used menhaden fish oil from Sigma in their research on omega-3 enrichment in mice without mentioning any measurements of lipid oxidation products or methods of lipid oxidation control, and thus may have used oxidized oil. The presence of lipid oxidation products in the commercial products, such as menhaden oil, suggests that some investigators could be starting with oxidized oils.

3.3.2 Impact of Temperature on Lipid Oxidation

It has been well documented that lipid oxidation decreases significantly with decreasing temperature (Gómez-Alonso et al. 2004, Ragnarsson and Labuza 1977). For example, in spaghetti enriched with fish oil, Verardo et al. (2009) found that one day of storage at 55°C caused an equivalent level of lipid oxidation to 18 days of storage at room temperature. In crackers, another low moisture food, a reduction of temperature from 35°C to 25°C increased the lag period from 10 weeks to 17 (Berenzon et al. 1998). Even though temperature reduction is practical method to inhibit lipid oxidation only 8 of the 50 animal studies reviewed mentioned using this precaution in their studies.

Figure 2 shows that under frozen storage at -20°C, neither lipid hydroperoxides nor headspace propanal were significantly higher than day 0 values after 63 days of storage. Refrigeration storage was also quite effective, with neither lipid hydroperoxides nor headspace propanal becoming significantly higher than day 0 values until day 49 and 56 days of storage, respectively. The lag phase for lipid hydroperoxide formation was shorter than propanal formation, which can be explained by the fact that propanal is formed from the decomposition of lipid hydroperoxides. Such large differences in the lag phases of lipid hydroperoxide and aldehyde formation has also been observed in a cracker model system and has been postulated to be due to the low diffusion and thus low reactivity of metals in low moisture systems (Barden et

al. 2015b). Both lipid hydroperoxide and propanal formation were extremely rapid at 37°C, with a lag phase of 2 days. At 23°C, the lag phases of lipid hydroperoxide and propanal formation were longer, at 6 and 8 days, respectively. The rapid oxidation observed at 23 and 37°C suggests that oxidation products could be formed during the course of a feeding study, especially if ambient temperature gets above 23°C since most feeding studies exceed one week (Table 1).

3.3.3 Impact of Oxygen Reduction on Lipid Oxidation

Oxygen is an essential substrate for lipid oxidation as its bi-radical nature allows for the rapid formation of peroxy radicals upon interaction with alkyl radicals. The alkyl radical-oxygen radical reaction rates are close to diffusion limited and thus only small amounts of oxygen are needed to promote lipid oxidation (For review, see Johnson and Decker 2015). Despite this, the positive effect of oxygen removal has been observed in other low moisture systems. In hazelnuts stored under 99% nitrogen and 1% oxygen, Ghiradello et al. (2013) found that the modified atmosphere reduced hydroperoxide value by 78% after 12 months of storage compared to a control with atmospheric oxygen. Reduction of oxygen (<1% oxygen) through the use of oxygen absorbers has been shown to increase the lag phase of crackers to past 44 weeks, compared to 17 weeks at atmospheric oxygen (Berenzon et al. 1998). Marasca et al. (2016) found that potato crisps had 30% lower hydroperoxide concentrations at 4 weeks of storage and 91% lower headspace hexanal at 5 weeks of storage when stored under nitrogen with a residual oxygen content below 2% compared to chips stored under ambient conditions. Jensen et al. (2011b) found that storing whole wheat bread in vacuum bags with nitrogen as the filler gas caused a “small, but significant” increase in oxidative stability. Thus, oxygen reduction has been

shown to reduce lipid oxidation in low moisture systems, although the effects have been observed almost exclusively with high degrees of oxygen removal.

In this study, headspace vials and nitrogen-oxygen blends were used to create 55, 65 and 80% reduced oxygen headspace conditions. The results of the storage study at 37°C are shown in Figure 3. The control sample (atmospheric oxygen) had a lag phase of 3 days, both as measured by lipid hydroperoxides and TBARS (headspace propanal could not be measured because the septum in the crimped vials could not be penetrated by the SPME needle). Reduction of oxygen to 55% increased the lipid hydroperoxide lag phase by a day but caused no change to the TBARS lag phase. Reduction of oxygen to 65% produced a lag phase of 5 days for both lipid hydroperoxides and TBARS while 80% oxygen removal led to a lag phase of 9 days for both lipid hydroperoxides and TBARS.

It is therefore possible that the lag phase of lipid oxidation product formation could be extended by oxygen removal if investigators flushed the feed with nitrogen long enough to get at least 65% oxygen reduction and stored samples in airtight containers. To determine if this was feasible, nitrogen was flushed into vacuum sealable bags containing fish oil enriched feed. Oxygen levels were determined using oxygen sensitive patches that were located in the headspace and within the feed. Headspace oxygen decreased rapidly, achieving 90% oxygen reduction within 5 seconds (Figure 4). Oxygen levels within the feed took 20 seconds to reach 75% oxygen removal after which no further statistically significant reduction was reached. This is a much faster rate of oxygen removal than seen in oil-in-water emulsions, where it takes a significant time (>60 minutes) to remove 65% of the total oxygen by nitrogen flushing (Johnson et al. 2016). This suggests that oxygen removal could be a viable method to slow lipid oxidation rates as long as the packaging material is oxygen impenetrable and oxygen does not reenter the

feed during storage. Container type is an important consideration if this antioxidant strategy is applied as Ziplock bags, which are made of either low or high-density poly(ethylene), have been shown to have an oxygen transmission rate of 6509 cm³/m²day (Rhee et al. 1999), compared to 0.36 cm³/m²day for vacuum packaging bags (López-Rubio et al. 2005) meaning that oxygen could be reintroduced into the feed during storage.

3.3.4 Impact of Antioxidants on Lipid Oxidation

Antioxidants are a viable method to control lipid oxidation however, many antioxidants have biological activity (e.g. tocopherols) so they could make data interpretation more difficult and thus may not be suitable to protect omega-3 fatty acids in animal studies. Metal chelators are not strong antioxidants in low moisture systems, presumably due to the lack of reactivity of transition metals due to their low mobility (Barden et al. 2015b). Free radical scavenging antioxidants display varying levels of effectiveness due to differences in their ability to scavenge free radicals, the energy level of the resulting antioxidant radical which can impact promotion of oxidation, their reducing potential which can reduce transition metals and increase prooxidant activity, and their polarity which can impact their partitioning into the lipid and thus their ability to interact with lipid radicals (McClements and Decker 2000). Therefore, several classes of antioxidants were tested to determine the properties of free radical scavenging antioxidants that would be most effective in animal feed.

Tocopherols and Trolox are useful tools to determine how the polarity of antioxidants impact their ability to inhibit lipid oxidation in a food or feed system since both have similar free radical scavenging activity but very different polarities. Both hydrophobic α -tocopherol and a commercially available mixture of tocopherols homologs (mixed tocopherols) were not very

effective at inhibiting lipid oxidation in the feed as determined by lipid hydroperoxides, TBARS, and headspace propanal (Figure 5). Conversely, Trolox was a much more effective antioxidant with lipid hydroperoxides, TBARS, and headspace propanal not exiting the lag phase for the 8-day duration of the storage study. Trolox has been reported to be more effective in bulk oils than tocopherols (Frankel 1996). In the feed system used in these studies, oil was mixed into a dried feed with no further processing. The oil is simply distributed in the dry powder and thus may have properties of a bulk oil, explaining why Trolox was observed to be more effective than tocopherols.

Ascorbic acid and ascorbyl palmitate also have similar free radical scavenging activity and different polarities but differ in that their reducing potential is much greater than phenolic antioxidants and thus can sometimes lead to prooxidant activity by metal reduction (Mäkinen et al. 2001). Ascorbic acid was not able to substantially inhibit lipid hydroperoxides, TBARS and headspace propanal formation (Figure 6). Ascorbyl palmitate slightly increased lipid hydroperoxide formation lag by 1 day and was not able to change the lag phase of headspace propanal formation or TBARS formation. Overall, ascorbic acid and ascorbyl palmitate were much less effective than Trolox, possibly due to their ability to promote oxidation.

Synthetic antioxidants such as propyl gallate (PG), TBHQ and BHT can also be used to inhibit lipid oxidation. These antioxidants differ in polarity, PG>TBHQ>BHT (most to least polar). The results of the comparison between PG, TBHQ and BHT are shown in Figure 7. Overall, propyl gallate was ineffective or prooxidative, reducing the lag phase by 1 day for lipid hydroperoxides, TBARS, and headspace propanal compared to the control. BHT did not change the lag phase for lipid hydroperoxides but increased the lag phase for TBARS by 3 days, and the

headspace propanal lag phase increased by 1 compared to the control. Samples containing TBHQ did not exit lag phase for the 10-day duration of the study.

These results are similar to those found in other low moisture food systems. In fried fish crackers, Ahmad and Augustin (1985) found that incorporation of TBHQ into either the cracker dough and the frying medium were able to reduce the rate of lipid oxidation. Sharma et al. (1997) treated fried potato crisps, banana chips and fried Bengalgram dhal with butylated hydroxyanisole (BHA), BHT and TBHQ. BHA provided the least protection while TBHQ provided the most. Barden et al. (2015a) found that in a model cracker system, antioxidant effectiveness was in the order of propyl gallate < BHT < TBHQ.

3.4 Conclusion

A potential explanation for the conflicting research in the field of omega-3 supplementation in animals is the presence of potentially toxic lipid oxidation products and a lack of control of lipid oxidation during storage of feeds. The majority of omega-3 animal feeding studies over the past 5 years do not provide information on what, if any, measures were taken to control the formation of toxic lipid oxidation products, nor do they assess the initial oxidative status of the oils. This study demonstrated that oxidation of omega-3 fatty acids in animal feed can occur under conditions commonly used for the storage of animal feed.

Researchers performing omega-3 animal feeding studies have several options to minimize the presence of lipid oxidation products in their studies. First, they should insure that the starting oils are of very high quality and has low levels of both primary (e.g. hydroperoxides) and secondary (e.g. aldehydes). For example, Global Organization for EPA and DHA Omega-3s, the Council for Responsible Nutrition, and International Fish Oil Standards (IFOS), define

acceptable oxidation levels as a hydroperoxide value lower than 5 mEq/kg, anisidine value lower than 20 mEq/kg and a total oxidation products measurement (Totox) lower than 26 mEq/kg (Jackowski et al. 2015). Even if an investigator starts with a high-quality oil, they must ensure that it does not decompose during storage. This can be done by keeping the high-quality oil or oil fortified feed at refrigeration temperatures for studies under 30-35 days and at least -20°C for longer studies. If the oil is kept under optimum conditions, one option to insure high quality is to prepare the feed daily. If this cannot be done, the feed can be refrigerated or frozen depending on the length of the feeding study. Alternately, the feed can be stored under reduced oxygen (at least 65% reduction) and stored in low oxygen permeable packaging or be treated with antioxidants. Both Trolox and TBHQ were the most effective antioxidants among those tested. Investigators may wish to be cautious about preserving the feed with antioxidants as the antioxidant could have biological implication in their feeding study and the antioxidant concentration in the feed will decrease during storage as they are consumed by lipid radicals.

While this study provides some guidelines for the optimal conditions for storage of omega-3 fortified animal feed, investigators should be aware that other components incorporated into the feed could impact oxidation rates. Compounds such as metal, free fatty acids, carotenoids, singlet oxygen generators (e.g. riboflavin), and components that impact water activity (e.g. sugars and salts) will impact lipid oxidation rates. In these situations, as well as a precaution in all omega-3 feeding studies, investigators should monitor lipid oxidation rates in both the starting oil and during storage of the feed. Both primary and secondary oxidation products should be monitored since there are situations where one marker could be low (e.g. in situations where lipid hydroperoxides stability is low) and provide misleading evidence that oxidation has not occurred.

3.5 Future Directions

Further research is needed to elucidate the optimal conditions for lowering lipid oxidation in omega-3 enriched feed for animal studies. Antioxidants and oxygen reduction used in combination may show a synergistic effect in lowering lipid oxidation and warrant further study. A benefit to synergism is that less antioxidant overall is needed, potentially lowering their confounding impact in test animals. Other methods of oxygen reduction, such as scavengers and vacuum, should be researched as well. One of the negatives of nitrogen flushing is that it is not practical for removing all of the oxygen in a system, especially in porous samples. In their research of modified atmosphere and potato crisps, Silva et al. (2004) found that an oxygen scavenger was more effective than nitrogen headspace at reducing lipid oxidation at 3 months of storage. Finally, while this research was performed in dry powder, experimental animal feed is also found in the form of extruded pellets. Providing feed in pellet form opens up different opportunities for inhibiting lipid oxidation. Pellets, unlike dry powder, can be encapsulated in films. Food films can provide protection against lipid oxidation by providing an oxygen impermeable membrane or can contain antioxidants themselves. But, extruded pellets are also porous, which would make removing oxygen through nitrogen flushing less efficient. Encapsulation of the oil is another method through which oxidation may be reduced and has been used in some of the recent literature. Future research should delve into other antioxidants, alternative methods of oxygen reduction, and other physical forms of feed and combination of these technologies in order to optimize oxidation reduction for research in dietary omega-3s in animal feed.

**APPENDIX
TABLE AND FIGURES**

Table 1: Methods of Oxidation Control in Published Dietary Omega-3 Enrichment Animal Studies

Citation	Animal	Enrichment	Control	Study Length	Rate of refreshment of feed
Madingou et al. 2016	Rat	EPA and DHA	Antioxidant addition	2 weeks	N/A
Njoroge et al. 2015	Mouse	emulsion form, 2.4 g/L DHA and EPA triglycerides	Antioxidant addition	2 weeks	Daily
Kemse et al. 2014	Rat	Fish Oil	Encapsulation	3 weeks	N/A
Eilati et al. 2013	Hens	Fish Oil	Encapsulation in gelatin	3 weeks	N/A
Ostermann et al. 2017	Mouse	EPA and DHA as ethyl esters	Measurement of Peroxide Value, Storage at -20°C	5 weeks	2-3 days
Coulombe et al. 2018	Mouse	DHA	Microencapsulation	40 weeks	N/A
Ross et al. 2016	Rat	EPA, and/or DHA	Storage -20°C	8 weeks	N/A
Diest et al. 2015	Rat	Tuna Oil	Storage at -20°C	4 weeks	7 days
Koivisto et al. 2014	Mouse	Fish Oil	Storage at -20°C	32 weeks	N/A
Li et al. 2017	Mouse	ALA	Storage at -20°C	8 weeks	N/A
Maditz et al. 2014	Rat	ALA, salmon oil	Storage at -20°C	12 weeks	Daily
Bates et al. 2016	Mouse	Microalgal Oil containing DHA	Storage at -20°C, new feed mixed biweekly, TBHQ	17 weeks	2 days
Figuroa et al. 2014	Rat	Fish Oil	Storage at 4°C	8 weeks	N/A
Tillander et al. 2014	Mice	Fish Oil or Krill Oil	Storage in airtight bags and frozen until use	6 weeks	N/A
Thang et al. 2013	Mouse	Menhaden Fish Oil	Storage in Ziploc bags at -80°C, TBHQ	10 weeks	Daily
Assis et al. 2015	Rat	Fish Oil	N/A	4 weeks	N/A
Balogun et al. 2013	Mouse	Menhaden Fish Oil	N/A	16 weeks	2 days

Bernhard et al. 2016	Mouse	Marine Oils	N/A	4 weeks	3 times per week
Caron et al. 2015	Mouse	DHA and EPA	N/A	5 weeks	N/A
Castellano et al. 2014	Pig	Conjugated ALA or n-3 fatty acids	N/A	17 days	N/A
Cheshmehkani et al. 2015	Rat	Fish Oil	N/A	8 weeks	7 days
Dasilva et al. 2017	Rat	Fish Oil	N/A	25 weeks	N/A
Dehkordi et al. 2015	Rat	omega-3 fatty acids	N/A	30 days	Daily
Dinnetz et al. 2013	Horse	EPA and DHA	N/A	8 weeks	N/A
Enos et al. 2015	Mouse	Multiple sources of fat	N/A	20 weeks	N/A
Fink et al. 2014	Rat	Walnut Oil	N/A	10 weeks	N/A
Freitas et al. 2016	Mouse	Fish Oil	N/A	3 weeks	N/A
Fu et al. 2015	Mouse	Omega-3 or Omega-6 fatty acids	N/A	17 days	N/A
Galmiche et al. 2016	Wistar rats	ALA or Omega-3 Long Chain Fatty Acids	N/A	4 weeks	7 days
Henderson et al. 2014	Mouse	Oleic Acid or ALA	N/A	5 weeks	8 days
Jacometo et al. 2014	Rat	Flaxseed Oil	N/A	3 weeks	N/A
Janssen et al. 2015	Mouse	ALA and/or DHA	N/A	60 days	N/A
Jones et al. 2013	Rat	Omega-3 Long Chain Fatty Acids	N/A	3 weeks	N/A
Jump et al. 2015	Mouse	DHA or EPA	N/A	16 weeks	N/A
Kemse et al. 2016	Rat	Omega-3 Fatty Acids	N/A	3 weeks	N/A
Lopez-Dominguez et al. 2016	Mouse	Fish Oil	N/A	32 weeks	N/A
MacLennan et al. 2013	Mouse	Menhaden Oil	N/A	3 weeks	N/A

Mark et al. 2014	Rat	Omega-3 Long Chain Fatty Acids	N/A	21 weeks	N/A
Pietropaolo et al. 2014	Mouse	Tuna Oil	N/A	N/A	N/A
Shoda et al. 2015	Mouse	Omega 3 or 6 Long Chain Fatty Acids	N/A	4 weeks	N/A
Sopian et al. 2015	Mouse	Fish Oil	N/A	3 weeks	N/A
Su et al. 2016	Mouse	Flaxseed Oil	N/A	14 weeks	N/A
Trevizol et al. 2013	Rat	Fish Oil	N/A	90 days	N/A
Turbitt et al. 2015	Mouse	Menhaden Fish Oil	N/A	16 weeks	2-3 days
Vigerust et al. 2013	Mouse	Krill Oil	N/A	6 weeks	N/A
Wandersee et al. 2015	Mouse	DHA	N/A	8 weeks	N/A
Wang et al. 2013	Rat	Fish Oil	N/A	6 weeks	N/A
Yip et al. 2013	Mouse	EPA	N/A	8 weeks	N/A
Zarei et al. 2016	Rat	Omega-3 Fatty Acid	N/A	4 weeks	Daily
Zulkafli et al. 2013	Rat	Omega-3 Fatty Acid	N/A	30 days	N/A

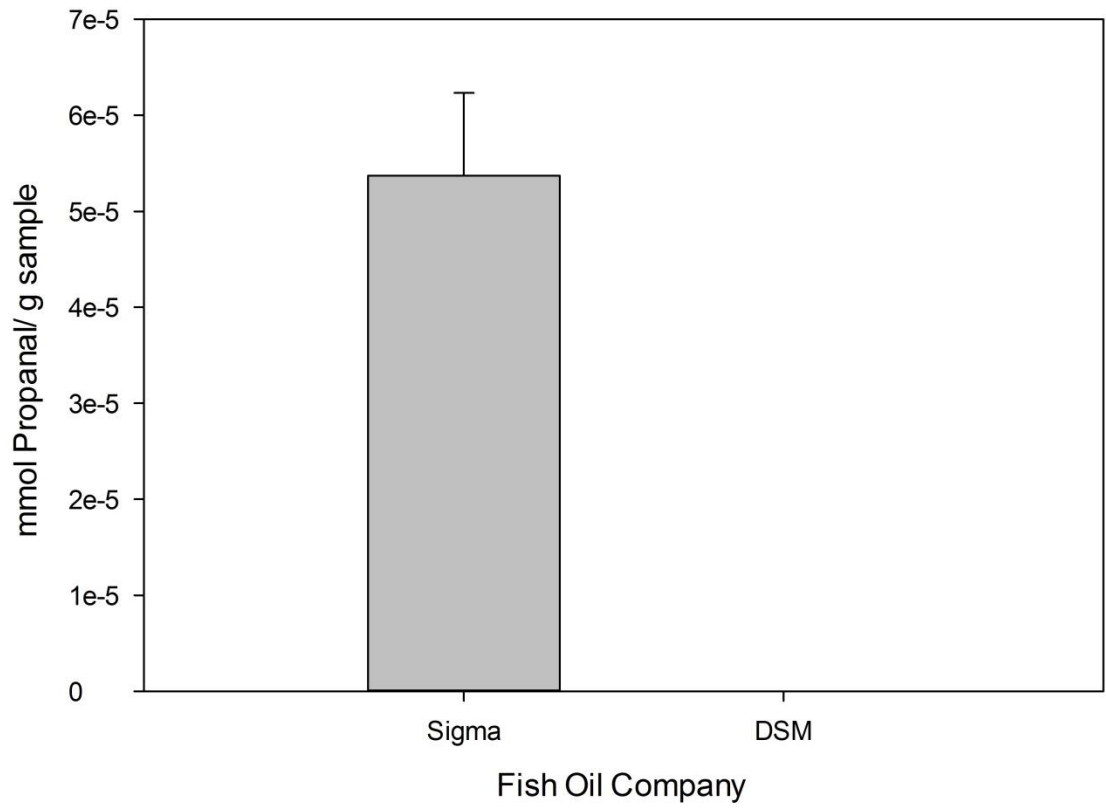


Figure 1: Headspace Propanal of Different Fish Oils

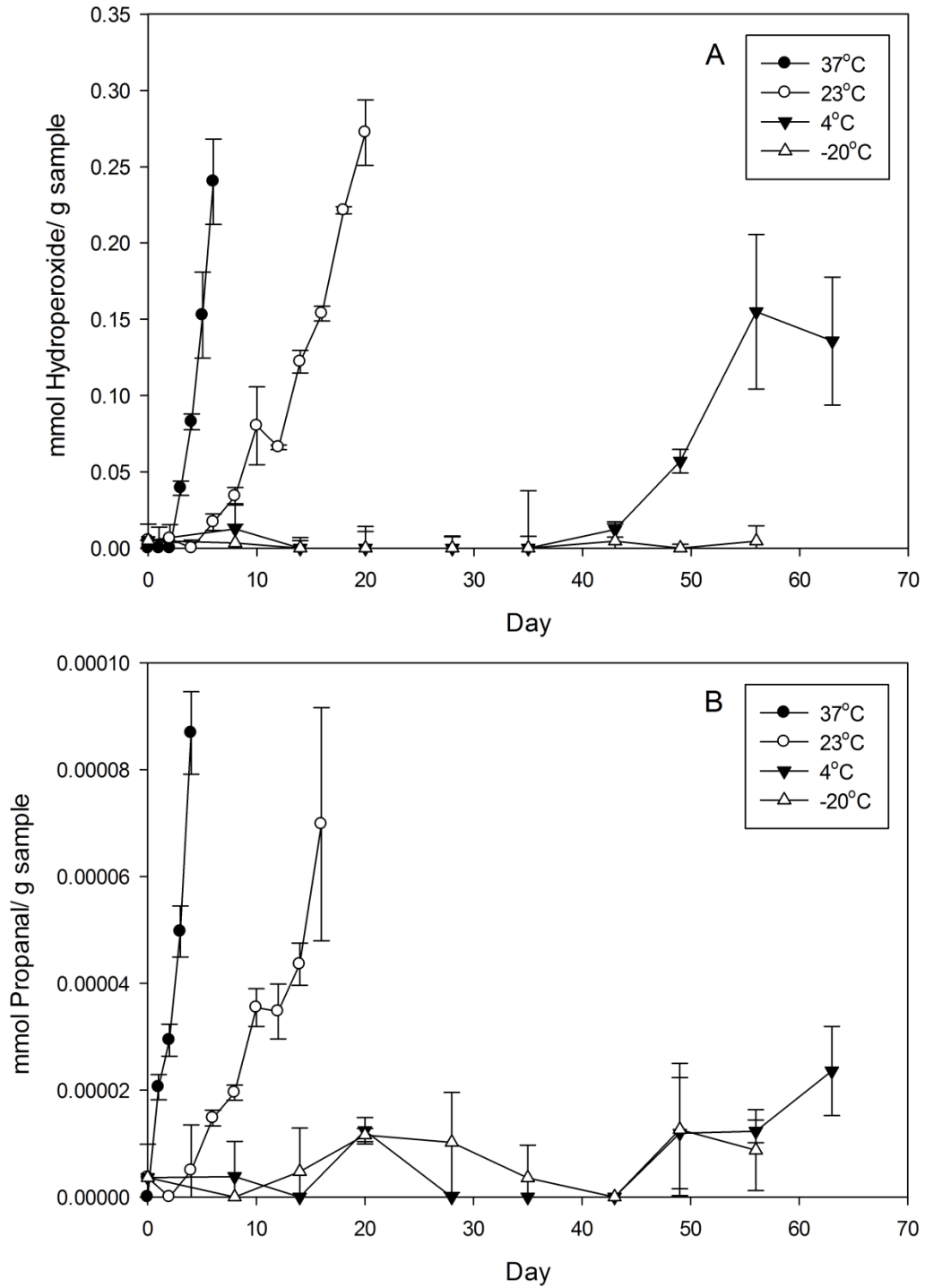


Figure 2: Hydroperoxide Value (A) and Headspace Propanal (B) of Samples Stored at Different Temperatures

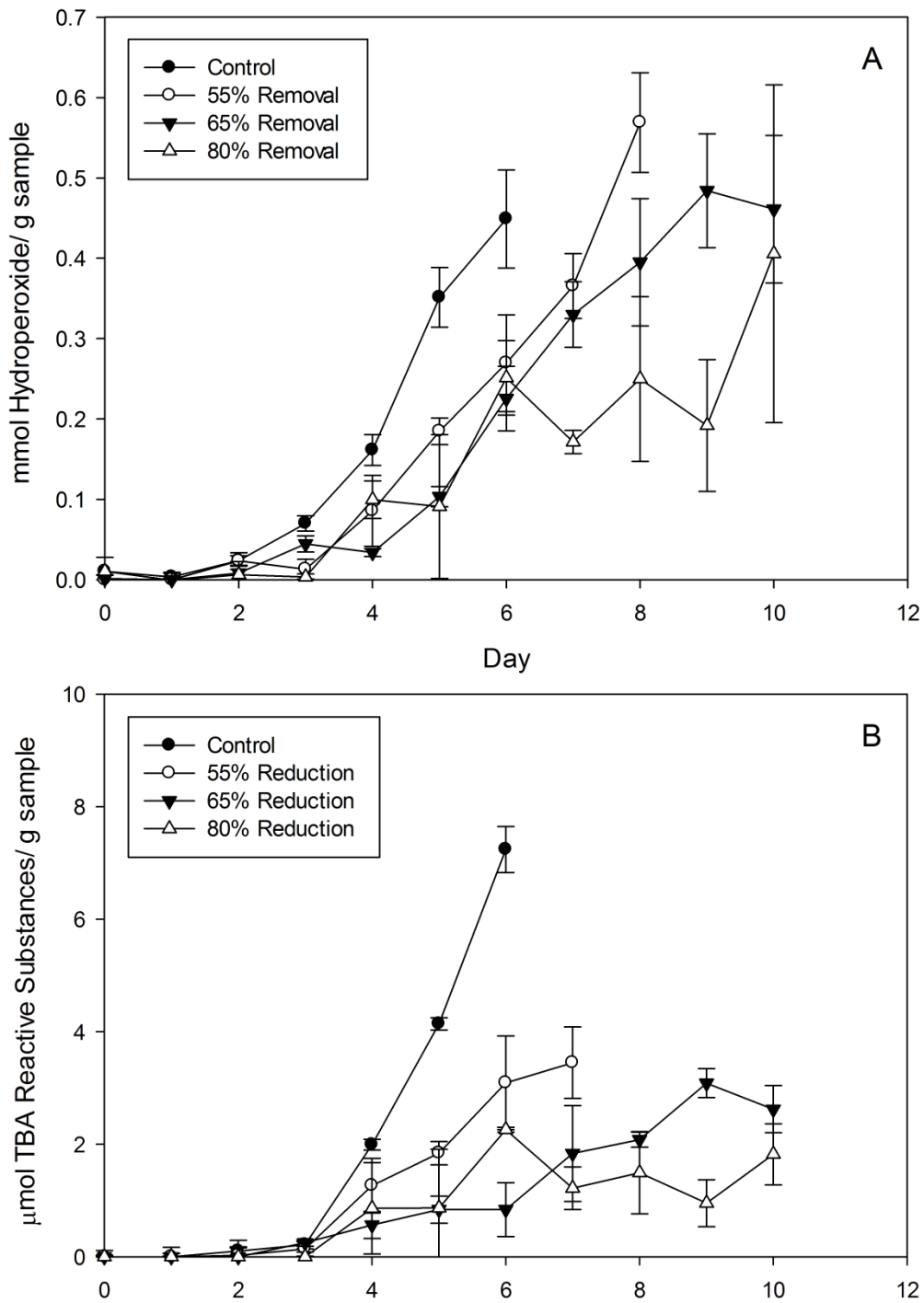


Figure 3: Hydroperoxide Value (A) and Thiobarbituric Reactive Substances (B) of Samples Stored at Different Oxygen Concentrations

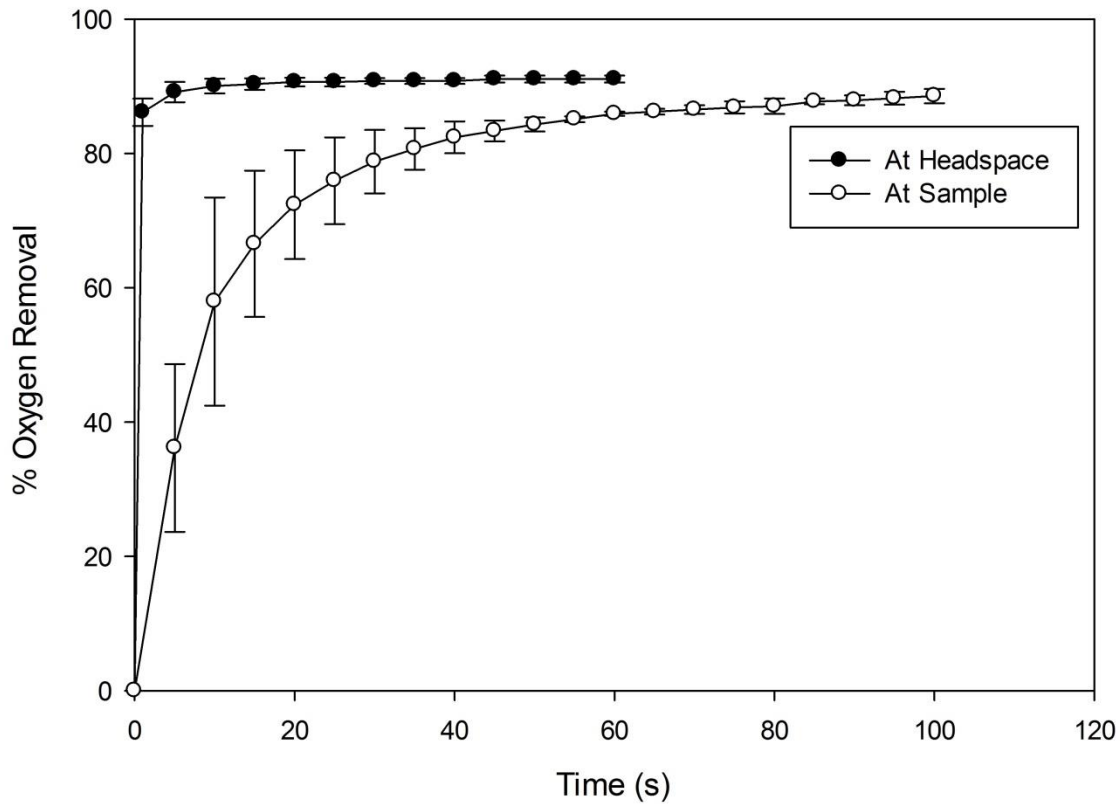


Figure 4: Rate of Oxygen Removal using Nitrogen Flushing of Animal Feed

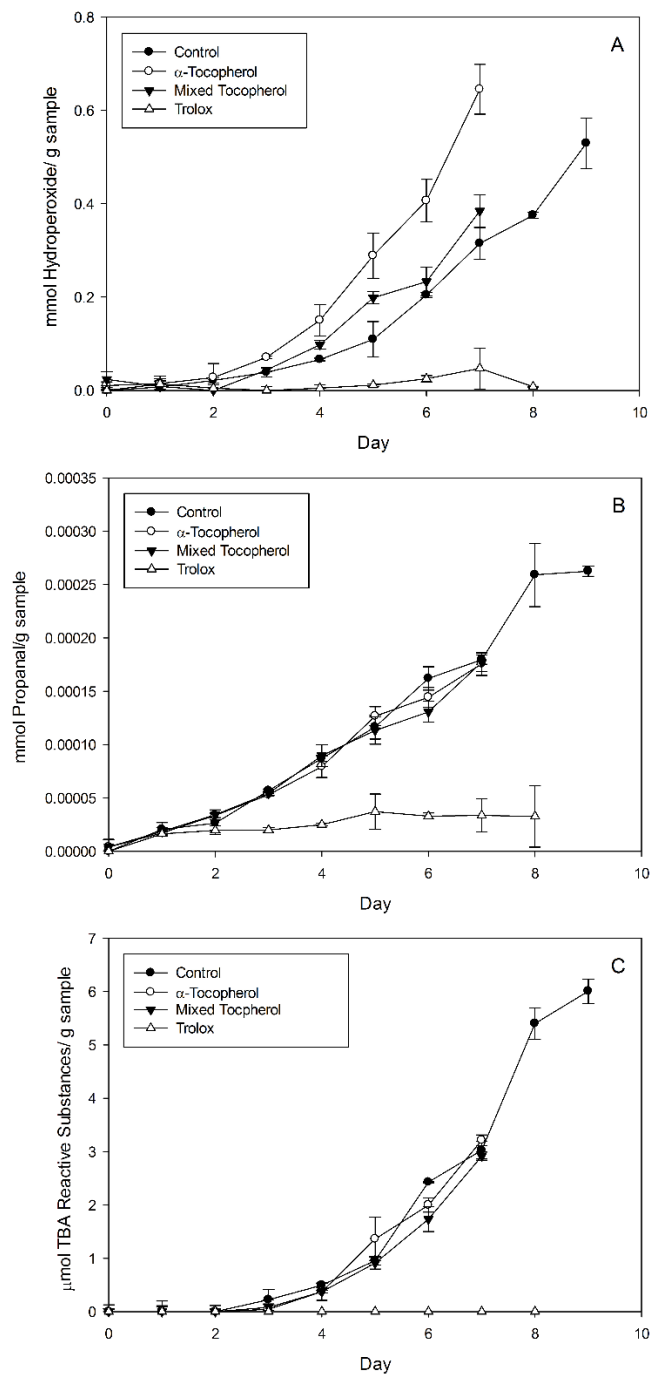


Figure 5: Hydroperoxide Value (A), Headspace Propanal (B), and Thiobarbituric Reactive Substances (C) of Samples with Tocopherols or Trolox

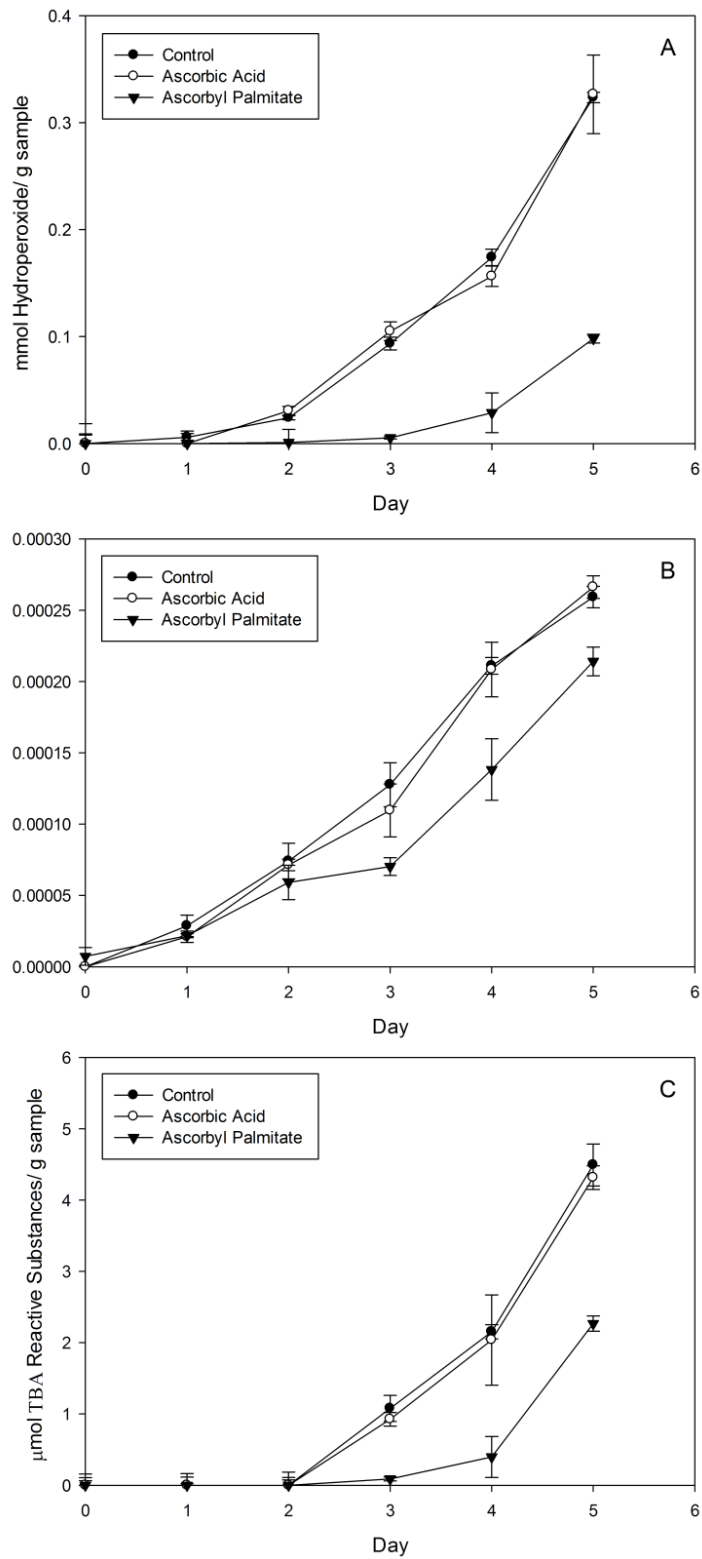


Figure 6: Hydroperoxide Value (A), Headspace Propanal (B), and Thiobarbituric Reactive Substances (C) of Samples with Ascorbic Acid or Ascorbyl Palmitate

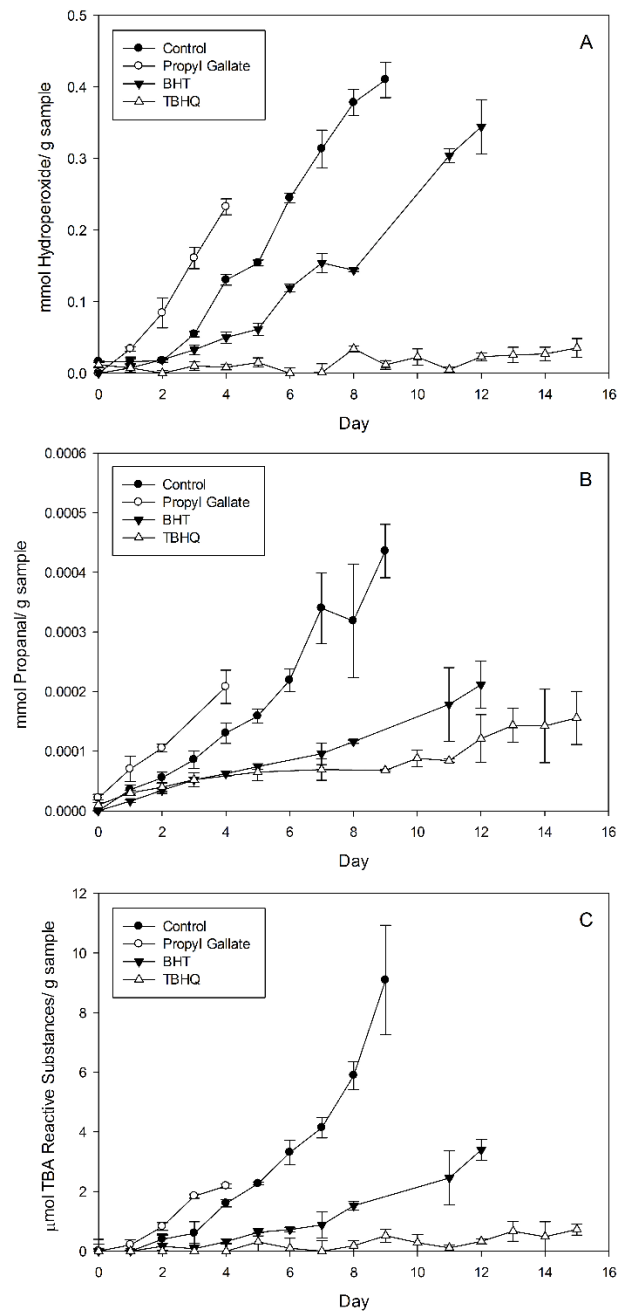


Figure 7: Hydroperoxide Value (A), Headspace Propanal (B), and Thiobarbituric Reactive Substances (C) of Samples with Propyl Gallate, Butylhydroxy Toluene (BHT), or Tert-butylhydroquinone (TBHQ)

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