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Effects of Solid Fat Content, Synthetic Antioxidants and Headspace Oxygen Reduction on the Rates of Oxidation in Surface and Total Lipids of Crackers

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A Thesis Presented

by

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ABSTRACT

EFFECTS OF SOLID FAT CONTENT, SYNTHETIC ANTIOXIDANTS AND HEADSPACE OXYGEN REDUCTION ON THE RATES OF OXIDATION IN SURFACE AND TOTAL LIPIDS OF CRACKERS

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The objective of this thesis was to determine if there is a significant variance in rates of oxidation between surface and total lipids in crackers and how solid fat content, synthetic antioxidants, and reduction of headspace oxygen affect those rates. Oxidation in low moisture foods has enormous financial and public health implications. It was hypothesized that lipids on the surface of a cracker would be more prone to oxidation than those on the interior. Existing methods were modified to observe oxidation at both levels. Three treatments were considered in this study, crackers formulated with oils of different solid fat contents (SFC), crackers prepared with the addition of synthetic antioxidants, and crackers flushed with varying percentages of nitrogen gas.

The first experiment consisted of two separate parallel treatments, one to monitor total lipid oxidation and one to monitor surface lipid oxidation. Crackers were formulated with soy oils of varying solid fat content. It was hypothesized that oils of lower SFC would oxidize first. Three differently processed soy oils were used. The first was an interesterified soy oil with a SFC of 14.2% at 55°C. The second was a refined, bleached, and deodorized soybean oil with a 0% SFC at 55°C. The third oil

used as a negative control was a hydrogenated soybean oil with a 100% SFC at 55°C. These fat contents were chosen in order to test the extremes and to capture a broad view of the implications of solid fat content on oxidative stability. Results showed that surface lipids oxidized 3 days prior to the total lipids, and indicated that the surface wash methods developed were viable. For surface lipids, the 0% and 14.2% SFC oils exited the lag phase and began to produce hydroperoxides at day 6 and day 9, respectively. For total lipids, these same oils did not begin to produce hydroperoxides until day 9. Subsequently for total lipids, the same two oils exited secondary lag phase and began producing hexanals after day 27. Conversely, the 100% SFC oil remained in lag phase for both hydroperoxides and hexanals throughout the full 45-day experimentation period.

The second experiment utilized the same cracker formula to monitor the effects of two synthetic antioxidants of varying hydrophobicity on surface lipid oxidation and interior lipid oxidation. The two antioxidants were butylated hydroxy toluene (BHT) and tert-butyl hydroxy quinone (TBHQ). Based on previous research, it was hypothesized that antioxidants of greater hydrophobicity would prove more effective in prolonging lag phases of oxidation in crackers. The antioxidants were added to the model system in two different ways. In the first, the antioxidant was dissolved in the lipid prior to making the crackers. In the second, the antioxidant was dissolved in 5 mL of solvent and added later, during the dry-ingredients addition stage. The BHT added in both lipid and dough phases exited the lag phase and began to produce hydroperoxides after day 12. The anisidine test was performed on all crackers to monitor secondary oxidation products. None of the

BHT samples exited lag phase for aldehydes. This was consistent in both the surface and interior lipids, and indicates that there was no difference in antioxidant partitioning. On the contrary, TBHQ was more effective at extension of the hydroperoxide lag phase on the surface than in the interior. Moreover, TBHQ showed increased efficacy when added to the lipid phase, extending the aldehyde lag phase beyond 45 days; compared to 36 days when added in the dough phase.

The third experiment involved flushing the headspace of cracker storage vials with different blends of nitrogen and oxygen gas. Overall reductions from atmospheric oxygen of 46%, 52%, 71%, and 97% were achieved. Based on previous research, it was hypothesized that greater than 58% total reduction would be necessary to significantly extend the lag phase of secondary oxidation products. Oxygen concentration is measured non-destructively using fluorescence technology that penetrates the glass wall of the storage vials and does not require the sample to be un-sealed. Oxygen content and oxidation products were measured in triplicate every three days for the duration of the 45 day experiment. Crackers exited the lag phases for both primary and secondary oxidation products in sequential order of increased oxygen reduction. Oxidative stability of the crackers was only achieved with 71% and 97% oxygen reduction, resulting in a prolonged lag phase for secondary oxidation products of 36 days and >45 days, respectively.

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CHAPTER 1

LIPID OXIDATION IN BAKERY FOODS

1.1 Introduction

Food trends and research in recent years have tended toward a focus on unsaturated fats over saturated ones in efforts to improve consumer health. Food spoilage in all its forms accounts for product and revenue loss, as well as quality complaints and, at worst, public health concerns. Food manufacturers are faced with the problem of finding a replacement for saturated fats in low moisture foods while maintaining a profitable shelf life. This problem is amplified when considered on an industrial scale.

Evidence shows that the consumption of saturated fats increases the risk for cardiovascular disease, the world's leading cause of death (WHO, 2014). This is causing a push in the food industry to remove them from foods. There are consequences, however; the unsaturated fatty acids being used as replacements are more prone to lipid oxidation. The increased rate of oxidation in unsaturated fats as compared to saturated ones, results in a shorter shelf life and higher costs attributed to product loss for food manufacturers. Thus, consumer trends are changing. The goal is to strike a balance between food safety and quality as well as nutritional value.

Internationally, for over a decade, cardiovascular disease (CVD) has claimed more lives than any other single cause. Annually, an estimated 7.4 million people succumb to some type of CVD (WHO, 2014). There is much evidence proving that saturated fat contributes to this growing number (Subramaniam, 2004; FDA, 2015).

There are negligible quantities of free fatty acids found in the body. When consumed, fatty acid binding proteins (FABPs) will bind the lipids to red blood cell membranes (Borchers and Spener, 1994). This can potentially result in high blood pressure, clotting, stroke or heart attack. In America, bakery products are among the top three contributors to saturated fat (NCI, 2013b). This includes items such as cookies, cakes, crackers, biscuits, and granola bars; to name a few. Due to this epidemic of heart disease, there is a push from third party health advocates, as well as from some government bodies, to remove saturated fats from processed or manufactured foods.

The functionality of lipids is unmatched in the baking world. No other ingredient can offer the unique capabilities of a fat, while maintaining the quality characteristics of the finished product. Lipids contribute to the elasticity, extensibility, and viscosity of doughs and batters, all aiding in the machinability of bakery foods (Carr, 1992; Sandrou and Arvanitoyannis, 2000). Other products have been tested for replacement of saturated fat, such as the use of corn, oat, or other plant fibers (Stoll et al., 2015; Shukla, 2005). These ingredients have immense water binding properties and mimic the mouthfeel of fat, but vastly increase the viscosity of a dough or batter. This makes it difficult to process in a manufacturing facility. Numerous protein isolates have also been used, such as wheat, pea, and whey (Ofosu et al., 2009; Zoulias et al., 2002). Lastly, gums such as xanthan, arabic, locust bean, and konjac have all been tested (Romanchik-Cerpovicz et al., 2002; Sandrou and Arvanitoyannis, 2000). However, none of these proposed solutions have been

completely successful. It is very difficult to mimic the exact mouthfeel and flavor of fat.

A more plausible alternative is the use of unsaturated fats as a replacement. These maintain the palatability of bakery products equitable to their saturated counterparts. They also closely mimic the rheological properties of doughs and batters made with saturated fats, with little deviation. (Marconi et al., 2014; Škrbić and Filipčev, 2008; Kolanowski and Laufenberg, 2006). Furthermore, unsaturated fats, namely those of the polyunsaturated nature, have been studied and found to actually decrease the risk of CVD (Mozaffarian et al., 2010; Hu and Willett, 2002). In most regards, it appears the replacement of saturated fats with unsaturated varieties is the most logical option. Yet there is one major problem; unsaturated fats are far more prone to lipid oxidation.

Due to the composition of unsaturated fatty acids, i.e. the fundamental double carbon bond in the hydrocarbon tail, they are configured in such a way that the molecule is bent. The presence of a double bond between carbons decreases the bond energy with the associated hydrogen. This is especially true in poly-unsaturated fatty acids, those containing two or more double bonds (Labuza et al., 1971; Frankel, 2005). This seemingly minor change in composition has a profound effect on the rates of oxidation of fatty acids (Barden et al., 2014). What this ultimately amounts to is a decrease in shelf life of the product. It also contributes to health concerns if shelf stability cannot be assured and products turn rancid. Adding to the concern, the FDA recently revoked the Generally Recognized as Safe (GRAS)

status of trans-fats in manufactured foods due to their contributions to CVD (FDA, 2017). By consequence, their use in the food industry dropped drastically.

Antioxidants may be a viable solution for controlling lipid oxidation and thus may help make unsaturated fats industrially applicable. Many additives have strong antioxidant properties but may be *a)* strictly regulated in their content, as is the case with BHT and other synthetic antioxidants (CFR, 2015), *b)* have strong flavors, such as various flavonoids found in grapes, berries, red wine, and other foods (Lesschaeve, 2005), or *c)* be unstable at high temperatures for extended periods of time, like the conditions in an oven, an example of this would be Vitamin C (Leskova, 2006).

Other potential solutions for reducing lipid oxidation include lowering the storage temperature of foods (Labuza et al., 1971). This would mean using refrigerated warehouses and trucks, and retailing in the refrigerated foods section. Packaging foods in opaque materials, which reduces oxidation through reduction of ultraviolet (UV) radiation, is also widely used.

Another solution is to use modified atmosphere packaging, as is already done with high fat foods like potato chips, which are highly prone to oxidation. Filling the headspace of a package with inert nitrogen gas may reduce another contributing factor to lipid oxidation. The presence of metal ions in a food are also a known contributor to product rancidity, adding chelators, such as EDTA, to bind the metal ions and prevent them from taking place in reactions may reduce this factor as well (Hart, 2000).

Given the parameters discussed above, the scope of this thesis was to focus on lipid oxidation in low moisture food products, specifically bakery products. Areas of investigation include the effects of solid fat content and the effects of two different industrial antioxidants. These two criteria are discussed in separate studies, but utilize the same control model so that aggregate effects can be examined and assessed. Rates of surface and interior lipid oxidation in the experiments were monitored. Crackers were used as a model in all experiments.

CHAPTER 2

LITERATURE REVIEW

2.1 Mechanism of Lipid Oxidation

The three phases of lipid oxidation are initiation, propagation, and termination; as defined by others (Labuza et al., 1971; Kamal - Eldin, 2003; Frankel, 2005; McClements and Decker, 2008). When a double-bonded hydrogen is abstracted from a fatty acid an alkyl radical (R•) is created. These radicals are capable of initiating the oxidation process. Abstraction is the process by which these free radicals bond with unsaturated fatty acids. Most often these reactions occur at a methylene-interrupted carbon of an unsaturated fatty acid. This is due to the relatively weaker covalent bond strength of hydrogen and its methylene carbon. An unsaturated fatty acid that has more double-bonds has more reaction sites and will be more susceptible to oxidation. This increase in vulnerability is significant, as researchers have shown that the oxidation rate is at least doubled by the addition of a single double-bond to an unsaturated fatty acid (Holman and Elmer, 1947; Bolland, 1948; Buttery, 1961). So, it can be stated that as the saturation level of a fatty acid decreases, the rate at which the fatty acid oxidizes will increase.

The alkyl radical produced during the abstraction process will almost immediately react with atmospheric oxygen, resulting in the formation of a peroxyl radical (ROO•). This resultant peroxyl radical will have enough energy to propagate hydrogen from another unsaturated fatty acid. Propagation is the description of this process and results in the formation of another alkyl radical, as well as a lipid

hydroperoxide (ROOH). These lipid hydroperoxides are not volatile, and therefore do not contribute to off-odors of oxidized foods.

A corollary part of the propagation phase of lipid oxidation is β - Scission reactions. These reactions cause further oxidation of the lipid hydroperoxides, forming alkyl radicals (RO•). At this point the oxidation rates begin to increase exponentially as the creation of this additional alkyl radical can react with another unsaturated fatty acid. These high-energy alkyl radicals are also responsible for generating the low molecular weight volatiles that produce rancid smells by breaking the aliphatic chain of the fatty acid. Both propagation and β - Scission reactions are monitored by measuring hydroperoxide content and aldehyde content, respectively.

The third and final phase of the lipid oxidation process is termination, in which two high-energy radicals bond to become one stable polymer, such as acid dimers, trimers, and fatty acids. Most foods will be deemed rancid or spoiled before termination reactions come into play due to the predominance of scission reactions in a more oxygen rich environment. Lipids however, may enter the termination phase before the offensive compounds formed by scission reactions have taken over.

The lipid oxidation process involves a lag phase in the beginning, which represents the formation of free radicals before the propagation phase begins. During this time, the accumulation of oxidized lipid products is slow. The primary generators of oxidized products, hydroperoxides and β - scission products, are not yet significantly present. Many lipids may also have antioxidants present naturally

or as additives that will contribute to lag phase longevity by scavenging free radicals. These will oxidize before the fatty acids. Since there is a significant increase in production of deleterious products after the lag phase, resulting in food spoilage, we can define shelf life as the time from when a lipid-containing food product is packaged, to the time lag phase ends (Labuza et al., 1971; Kamal - Eldin, 2003; Frankel, 2005; McClements and Decker, 2008).

2.2 Measuring Lipid Oxidation

2.2.1 Primary Lipid Oxidation Products

2.2.1.1 Hydroperoxides

Peroxide value is expressed as millimoles of hydroperixde per kilogram of lipid (mmol/kg) and is one of the most widely accepted markers for measuring oxidized products (Nawar et al., 1996; Frankel, 2005). One approach to measuring peroxide value is called the Ferric Thiocyanate Method. This uses a relatively small sample size and is more sensitive than other methods. Using spectrophotometry, this method analyzes the presence of ferric irons in the form of ferric thiocyanate (Shantha and Decker, 1994). Spectrophotometric techniques, which are employed to measure lipid oxidation, rely on measuring ratios of specific products of oxidation within a sample (Nawar et al., 1996). This process can be used on bulk oils or on those extracted by various methods from different foods (Frankel, 2005).

Peroxide value is a useful measure because it can be tracked from the beginning of the experiment and monitored throughout. During the early stages of oxidation peroxide production will be relatively low, then peaking before finally

slowing down (Nawar et al., 1996, Frankel, 2005). Because of the presence of more double bonds within polyunsaturated fats, whose lower energy makes them susceptible to free radicals, these lipids will reach peak peroxide production more rapidly than their saturated counterparts. Here the presence of antioxidants, as well as environmental conditions like heat and light can cause increases or decreases in the rates of oxidation (Frankel, 2005).

2.2.2 Secondary Lipid Oxidation Products

2.2.2.1 Hexanal

Volatile secondary products are measured using gas chromatography (GC) via the dynamic headspace method. The dynamic headspace method involves 1) placing a sample in a sealed container and 2) collecting vaporized volatiles - in this case hexanals - from sample headspace while maintaining an elevated temperature. The hexanals are then 3) desorbed from their holding chamber and fed into the GC. 4) The GC separates and measures compounds present in the cracker. All measurements are assessed based on a pre-established standard curve. Temperature control is essential to the validity of results gathered using this method as fluctuations can greatly affect yields (Frankel, 2005).

2.2.2.2 Aldehydes

The Anisidine value test is useful in determining β - scission products that are produced during secondary oxidation in lipids. By measuring aldehyde and/or ketone levels, the oxidation process can be tracked. The standard value of p-

anisidine is defined as 100 times the optical density with measurements at 350 nm in a 1 cm cuvette of solution containing 1.00 g of the sample lipid in 100 mL of a solvent and reagent solution. By allowing the ketones and/or aldehydes in the sample to react with the prepared solution containing p-anisidine, a color change will occur whose absorbance is then measured at 350 nm.

2.3 Extraction of Surface Free Fat – Milk Powders

The percentage of fat that can be extracted by means of organic solvents under standardized condition is known as free-fat. This is a standard measure used to determine the fat content of dairy powders. It is believed that free fat exists primarily on the powder surface. This has lead to the hypothesis that free-fat content may affect the flowability of dairy powder (Buma, 1971b; Onwulata et al., 1994). However, even when free-fat was expressed per-unit of surface area in experiments using different fat powders with similar mean-particle sizes, no correlation was found between the free-fat content and flowability. This may point to a flaw in the free-fat extraction method as reported by researchers who believe that the solvent employed in the method actually penetrates the crevices and pores of a sample, thus extracting interior as well as free-fat (Buma, 1971c; Buchheim, 1982, 1978). Therefore, researchers needed an additional technique to determine exact levels of surface fat without contamination from the accidental collection of interior fats.

In order to extract free-fat in this experiment, procedures were employed similar to those used by Buma (1971a). His method added one gallon of milk

powder to 40 mL of petroleum ether. Over a 48 hour period, the mixture was shaken by hand at intervals and then, using filter paper, the solvent and powder were separated (Whatman #4, Maidstone, Kent, UK). Next, the powder residue was treated with a 2x2 mL solvent and the filtrate allowed to evaporate. The resultant fat residue was measured once a constant weight was achieved. The amount of extracted fat was then expressed as g. free-fat/g. fresh powder.

It has been shown that brief exposure to an organic solvent will dissolve free-fat on the surface of a powder much more rapidly than it will from the interior of the particles (Pisecky, 1970; Buchheim, 1976). A sample of powder was washed multiple times with 5mL of organic solvent until the free-fat extraction reached a plateau. This required four washings. To ensure that only free-fat was collected, and not interior or surface fat, a second study was done using only one wash. One gram of milk powder was measured and washed with 5 mL of petroleum ether using filter paper (Whatman #4, Maidstone, Kent, UK). The wash process lasted 10 seconds on average. After drying the powder residue under a vacuum at room temperature and allowing extracted fat to reach a constant weight, the fat value was recorded as g. surface free-fat/g. fresh milk powder. This procedure was performed in triplicate to obtain consistent results.

2.4 Glass Transition Phase

Food stability may also be measured using glass transition concepts, particularly in low moisture foods. This is because water activity cannot fully predict lipid stability in all circumstances (Sun et al., 1996; Rahman, 2009; Ergun et

al., 2010). It has been well established that increased water content in glass state foods will decrease the glass transition temperature through plasticization, thus decreasing the shelf life of the food by increasing oxidation (Slade and Levine, 1991). Yet the relevance of these findings must be tempered by the fact that most foods are not homogenous glasses, but only contain within them glassy areas (Peleg, 1992; 1996). Using a freeze-dried emulsion of rapeseed oil, electron spin resonance was employed to show that the glassy matrix of the frozen oil was effective in trapping hydrophilic radicals and nullifying their oxidizing effects (Orlien et al., 2000). Meanwhile, the same radicals contained in non-glassy oil oxidized as normal. In low moisture foods in particular, establishing and identifying glassy regions and maintaining them via temperature control, may be the most relevant predictor of shelf life; given that water activity is kept stable.

CHAPTER 3

EFFECTS OF SOLID FAT CONTENT, SYNTHETIC ANTIOXIDANTS AND HEADSPACE OXYGEN REDUCTION ON THE RATES OF OXIDATION IN SURFACE AND TOTAL LIPIDS OF CRACKERS

3.1 Materials

The ingredients used in cracker preparation (Table 1) were purchased from a regional grocery store. One oil selected for this project was an interesterified soybean oil (#762400), suggested for low moisture foods from Archer Daniels Midland (Decatur IL, USA). Two additional oils were used to explore the effects of higher and lower solid fat content on lipid oxidation rates. The first was a refined, bleached, and deodorized soybean oil (#860700) which has a 0% SFC at 55°C. The second was a hydrogenated soybean oil (#866980) which has a 100% SFC at 55°C. Both of these oils were from Archer Daniels Midland (Decatur IL, USA). All oils were aliquoted into dark-walled containers, sealed and kept frozen (-20°C) until the day of use.

Table 1: Control cracker formula

Ingredient	Supplier / Manufacturer	Batch Size (grams)	Percent (w/w)
Flour	Pillsbury	62.50	50.6
Baking Soda	Arm & Hammer	0.58	0.5
Iodized Salt	Morton	1.50	1.2
Interesterified Soybean Oil	ADM	10.00	8.1
Double Deionized Water	Fisher Model	39.00	31.5
Flour (Sheeting)	Pillsbury	10.00	8.1
Total		123.58	100.0

Both antioxidants (BHT and TBHQ) used were of >99% purity and were purchased from Sigma-Aldrich company (St. Louis MO, USA). These antioxidants were chosen due to their variances in hydrophobicity. Antioxidants were added to the crackers at a concentration of 27.2 micromolar, calculated based on oil weight. This concentration relates to 400 parts per million (PPM).

Custom oxygen and nitrogen blends were purchased from AirGas Company (Springfield MA, USA). The blends consisted of 2.5% oxygen, 5.0% oxygen, and 7.5% oxygen; and used nitrogen as the balance. All other chemicals henceforth mentioned were purchased from Sigma-Aldrich (St. Louis MO, USA) and stored according to supplier recommendations.

3.2 Methods

3.2.1 Cracker Preparation

A Kitchen Aid mixer (model KSM95, Mississauga ON, CA) was used in the preparation of the crackers. The Kitchen Aid was equipped with a whisk attachment and a seven-quart stainless steel bowl. A Kitchen Aid dough-sheeting accessory was also utilized in cracker preparation. A 2,000 watt residential electric oven was used to bake the crackers (Kenmore, Hoffman Estates IL, USA; Product #316904486).

The formulation in Table 1 was used to make the crackers. Flour, baking soda and salt were sifted together. Frozen oil was added to the mixing bowl and blended with the whisk attachment on low speed for two minutes or until it was completely thawed. The flour, baking soda and salt mixture was added to the oil and blended on low speed for one minute. The resulting mixture was scrapped off the sides of the

bowl and re-blended for one minute on low speed. Water was gradually added to the mixture during blending with the whisk attachment for 30 seconds. The dough was collected from the mixing bowl and whisk and kneaded by hand continuously for four minutes, gradually adding half of the sheeting flour to the dough during the kneading process. The dough was rolled into a ball and allowed it to rest for two minutes during which the mixing bowl and whisk were removed and the dough-sheeting accessory was attached. The sheeting accessory thickness was set to two (~3mm) and ~30 gram pieces of the dough was passed through the sheeter. The rolled dough was folded in half and passed twice more through the sheeter, dusting lightly with the remaining sheeting flour if the dough became sticky. When all of the dough had been sheeted, a knife was used to form 0.5 cm square dough pieces which were baked on a metallic sheet pan at 163°C for 21 minutes.

3.2.2 Variations When Using Different Solid Fat Contents

It was not necessary to alter the process for cracker production for the interesterified and liquid oils. However, hydrogenated oil will remain solid at room temperature so the first stage of blending was used to reduce the particle size of the fat and allow the fat to be evenly distributed throughout the dough. Note that there was a variation in dough consistency and texture due to different solid fat contents of the oils, however this did not affect the overall machinability of the dough. All oils melt completely when the crackers are baked and will distribute themselves throughout the structure of the final cracker.

3.2.3 Variations When Using Synthetic Antioxidants

For the results denoting "antioxidant in lipid," the antioxidant was added to the oil in the first stage of mixing (e.g. oil and antioxidant were blended together with the whisk). For results denoting "antioxidant in dough," the antioxidant was dissolved in 5 mL ethanol and was incorporated into the cracker dough after the flour, baking soda, and salt had been blended with the oil, but prior to the addition of the water. Ethanol was chosen for its ability to rapidly solubilize both BHT and TBHQ. Additionally, ethanol has a low vaporization temperature and flashes off during the baking cycle.

3.3 Cracker Storage

After baking, all crackers were allowed to cool to room temperature before handling. Whole crackers [0.5 x 0.5 cm; approximately one gram (1.00 +/- 0.05 g.)] were placed in 5 mL volume glass vials and sealed with silicone/PTFE septa screw caps for the solid fat content and synthetic antioxidant studies. The oxygen reduction studies utilized crimp cap vials fitted with rubber/teflon septas (Supelco, Bellefonte PA, USA). Vials were incubated at 55°C in corrugated cardboard containers. Use of light-protected carriers was crucial to avoid the increased effects of UV/Vis radiation on lipid oxidation rates. Experimental period lasted for a maximum of 45 days with testing occurring every 3 days.

3.4 Lipid Extraction

3.4.1 Extraction of Total Lipids

Total lipids were extracted by pulverizing one-gram of cracker in a ceramic mortar with a pestle and then transferring to a large test tube (16x125mm, Fisher Scientific). To the same test tube, 5 mL of chloroform : methanol (2:1) was added and vortexed briefly. The crackers were centrifuged at 2,500 RPM for 5 minutes. After the cycle was completed, the top layer was extracted using a hand pipette and transferred to another set of test tubes for subsequent analysis of oxidation parameters.

3.4.2 Extraction of Surface Lipids

A new method was developed for the extraction and quantification of surface lipids from crackers for this study. This method was based upon practices proposed for dry powders by Kim (2009). Filter paper (Whatman #4, Maidstone, Kent, UK) was selected based upon pore size, allowing for the passage of solubilized lipids while inhibiting the passage of solid cracker residues. A single filter paper was folded and set in a conical funnel and the funnel was placed inside a large test tube. The one-gram cracker sample was placed in the filter paper and then rinsed with 5 mL of High Performance Liquid Chromatography (HPLC) grade hexanes. Hexane was used due to its low polarity, which can quickly extract the lipids on the exterior of the cracker. The solvent with extracted lipids was captured in the test tube below where it was subsequently evaporated under nitrogen, minimizing the effects of

oxidation on the extracted lipid. The difference of weight in the test tube before and after lipid extraction, was used to quantify the amount of surface lipid extracted.

3.4.3 Extraction of Interior Lipids

Crackers washed with hexane were removed from the filter paper and placed in small plastic weigh boats where the solvent was allowed to evaporate from the surface, approximately 45 minutes to 1 hour. When a constant weight was achieved, the crackers were crushed using a porcelain pestle and the cracker was transferred into a new test tube (16x125mm). To the test tube, 5 mL of HPLC grade hexanes were added and vortexed briefly. Crackers were centrifuged at 2,500 RPM for 5 minutes. Using a pipette, the top layer of solvent was extracted for interior lipid oxidation analysis.

3.5 Modification of Headspace Oxygen

The headspace of the control samples were not modified in any way. Other sample subsets utilized one of three nitrogen/oxygen blends or pure nitrogen purchased from AirGas Company (Springfield MA, USA). The blends were 2.5% oxygen, 5.0% oxygen and 7.5% oxygen, balanced with nitrogen. Using a needle attachment connected to the hose, gas was allowed to flush the vial for 30 seconds, completely exchanging the atmosphere with the defined gas blends. Vials were capped and sealed immediately. The blends resulted in a total oxygen reduction from the control equal to 71% (2.5% oxygen gas source), 52% (5.0% oxygen gas

source), and 46% (2.5% oxygen gas source). A 97% reduction was achieved by using 100% nitrogen to flush the vials.

3.6 Measurement of Lipid Oxidation Products

The solubilized oil extracted from the total lipid, surface lipid and interior lipid were used for analysis of oxidation products. This combination of lipid sources provided insights into oxidation at both the surface level and the interior of the cracker.

Lipid hydroperoxides were determined using the method of Shantha and Decker (1994). Reagent A was comprised of a 50:50 mixture (2 mL's per part) of two constituents. Constituent one was 0.1 grams ferrous sulfate dissolved in 10 mL double deionized water. Constituent two was 0.2 grams barium chloride dissolved in 25 mL of 0.4 N HCl. Reagent B was comprised of 7.5 grams of ammonium thiocyanate dissolved in 25 mL double deionized water. Constituent two of reagent A and reagent B may be prepared ahead of time and kept in the refrigerator for later use. Constituent one of reagent A must be prepared the same day that oxidation measurements are taken.

Before analyzing the cracker samples, a blank was prepared for calibration of the spectrophotometer. The blank consisted of 200 μL hexane, 3.13 mL chloroform : methanol (2:1), and 16.7 μL of reagents A and B. To measure hydroperoxides in the lipids extracted from the crackers, 200 μL of each sample of solubilized oil was placed into individual test tubes. Then 3.13 mL of chloroform : methanol (2:1) was added to each tube and vortexed approximately 5 seconds. Then 16.7 μL of Reagent

A was added to each sample followed by 16.7 µL of Reagent B, vortexing and incubation for 20 minutes.

Spectrophotometric properties were evaluated on a Genesys 20 Spectrophotometer (ThermoSpecrtronic; Watham, MA) outfitted with a 1.4 mL quartz cuvette (FireFlySci, Staten Isl., NY) and set at 500 nanometers (nm). The spectrophotometer was warmed up for a minimum 15 minutes. The spectrophotometer was zeroed using the sample blank. The cuvette was rinsed with chloroform : methanol (2:1) between samples.

Gas chromatography (GC) was utilized to quantify headspace hexanal, a secondary product of lipid oxidation. A Shimadzu GC 2014 equipped with an AOC 5000 auto-sampler and a flame ionization detector (Shimadzu, Kyoto, Japan) was used in this experiment. The parameters used were as follows: samples were incubated for 10 minutes at 55°C in their respective sealed containers. A solid phase microextraction syringe fiber (SPME, Supelco Bellefonte PA; USA) then pierced the silicone/PTFE septa to a depth of 22mm and absorbed the headspace for 2 minutes. The SPME fiber transferred the headspace to the injection port (250°C) in the GC. The GC was equipped with a fused silica capillary column (30m x 0.32mm inner diameter x 1 µm) from Supelco (Bellefonte PA, USA). The column was held at a temperature of 65°C. The sample was desorbed into the column for 3 minutes. The flame ionization detector and oven were both set to 250°C. The GC run time was 10 minutes. After the run was complete, values were recorded as the area under the curve. This data was then plotted using a standard curve to convert area to millimoles (mmol) of hexanal per kilogram of cracker.

P - Ansidine value (p-AnV) was also used to detect secondary lipid oxidation aldehydes. This test was used in experiments where headspace hexanal could not be collected (e.g. surface lipids). Anisidine value is a widely accepted American Oil Chemist Society (AOCS) method (cD 18-90) and has been adapted here for use with HPLC grade hexanes as the solvent and small quantities of oil.

In both cases of surface and interior oxidation, the following procedure was used. First, the p-anisidine solution was prepared by adding 25 mg of crystallized p-anisidine to 10 mL of acetic acid, and vortexed into solution. The crystallized anisidine should be stored in brown glass with a sealed top in the refrigerator. Surface or interior lipid extracts (0.836 mL) were vortexed with the p-anisidine solution (164 μL). Samples were incubated for 10 minutes. UV rays will damage the color compound so samples are incubated in the dark. Color change was measured spectrophotometrically at 350 nm (Ultrospec 3000 pro, Biochrom, Cambridge, UK). Samples were measured in 1.4 mL methacrylate cuvettes. It was imperative to use methacrylate, or other non-reactive materials such as quartz, otherwise a reaction will occur with the acetic acid solution that will begin to decompose the cuvette. Notably, do not use other disposable cuvettes such as polystyrene. The blank (1.0 mL) in each set was used to zero the absorbency reading before each sample measurement.

Once the samples were run and values recorded for each sample and blank, the following formula was used to convert to the Anisidine Value:

$$\text{p-AnV} = 10 * 1.2 * ((As - Ab)/m))$$

Where *m* is the mass of the oil used in the test portion in grams and As and Ab were the absorbance of the sample and blank, respectively. It was determined that an average of 40 mg of oil was collected per sample, so *m* = .04 grams. Anisidine value is an arbitrary measurement so levels of oxidation do not have specific units.

3.7 Antioxidant Consumption Over Time

Antioxidants are detected using a Shimadzu (Kyoto, Japan) HPLC equipped with a normal phase column. The HPLC consisted of a system controller, diode array detector (SCL – 10A VP), a chromatographic pump (LC – 20 AD), a degasser (DGU – 14A), and a fluorescence detector (RF 20A xs). The surface and interior extracted lipids were solubilized in HPLC grade hexanes, filtered with a 25μm syringe filter to prevent particle contamination of the column and injected into the column, using a volume of 25 μl per sample. The column used was a Supelcosil LC-DIOL (#137611-04, Supelco, Bellefonte PA, USA) with the following dimensions: 25 cm L x 4mm D x 5 μm ID. The mobile phase used in these experiments consisted of 95% HPLC grade hexanes with 5% HPLC grade Dioxane, and was pumped through the column at a flow rate of 1 mL per minute. The HPLC was run in isocratic mode with an 8 minute run time. Analysis was done every six days of storage of the crackers. Antioxidants were quantitated with the fluorescence detector by comparing the area under the curve with standard curves made from each antioxidant.

3.8 Monitoring Oxygen Content

An oxygen monitoring system, NEOFOX, was purchased from Ocean Optics (Dundedin FL, USA). The system was described in detail by Johnson et al. (2017). Fluorescent optical sensor (FOSPOR) patches were placed on the inside wall of the glass vials before the crackers were added. An LED light was used to excite a coating on the patch while simultaneously reading emission. In the presence of oxygen the FOSPOR patch will produce a light signal that is captured by the LED probe. Through a series of automatic calculations done by the NEOFOX software the signal is translated to percent oxygen.

3.9 Statistical Calculation of Lag Phase

All experiments were conducted in triplicate. All calculations compare the means by analysis of variance (ANOVA) and use a significance level $p<0.05$. The lag phase of lipid oxidation is determined by statistical analysis (SPSS Statistical Software, IBM; North Castle NY, USA). The end of the lag phase was defined as the first time point after which the concentration of the oxidation products were significantly greater than time zero, and where each time-point thereafter were also greater than time zero.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Solid Fat Content

The impact of solid fat content on oxidation rates was determined using soybean oil with a SFC of 0% at 55°C, an interesterified soybean oil with a SFC of 14.2% at 55°C and a totally hydrogenated soybean oil with a SFC of 100% at 55°C. The hydrogenated soybean oil represented a negative control since saturated fatty acids do not oxidize. As expected, hydroperoxide and hexanal formation was very low in crackers made with the hydrogenated oil. Small amounts of hexanal were observed in these samples at the end of storage, which could be due to the oxidation of lipids inherent to the flour.

The lag phase was the same for both total lipid hydroperoxides (9 days) and hexanal formation (27 days) in the crackers containing either the liquid or interesterified soybean oil. This was somewhat surprising since the liquid oil had more unsaturated fatty acids than the interesterified oil. However, both had greater than 50% unsaturated fatty acids suggesting that fatty acid concentrations were not limiting lipid oxidation rates in either fat source.

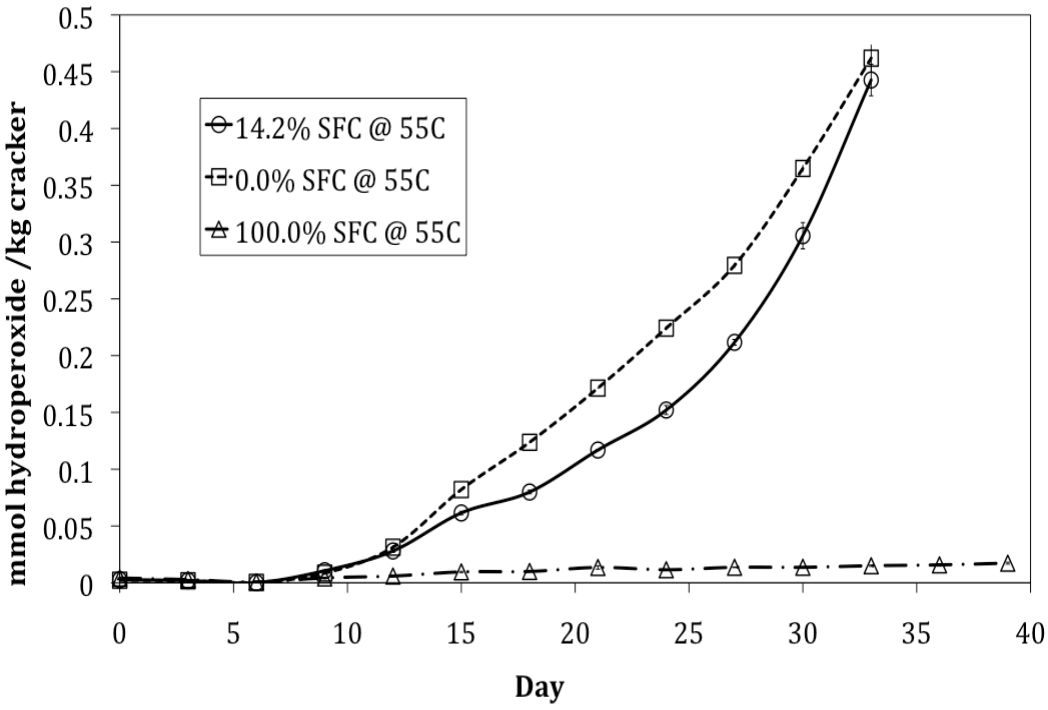

Figure 1a. Lipid Hydroperoxides formed by total lipids

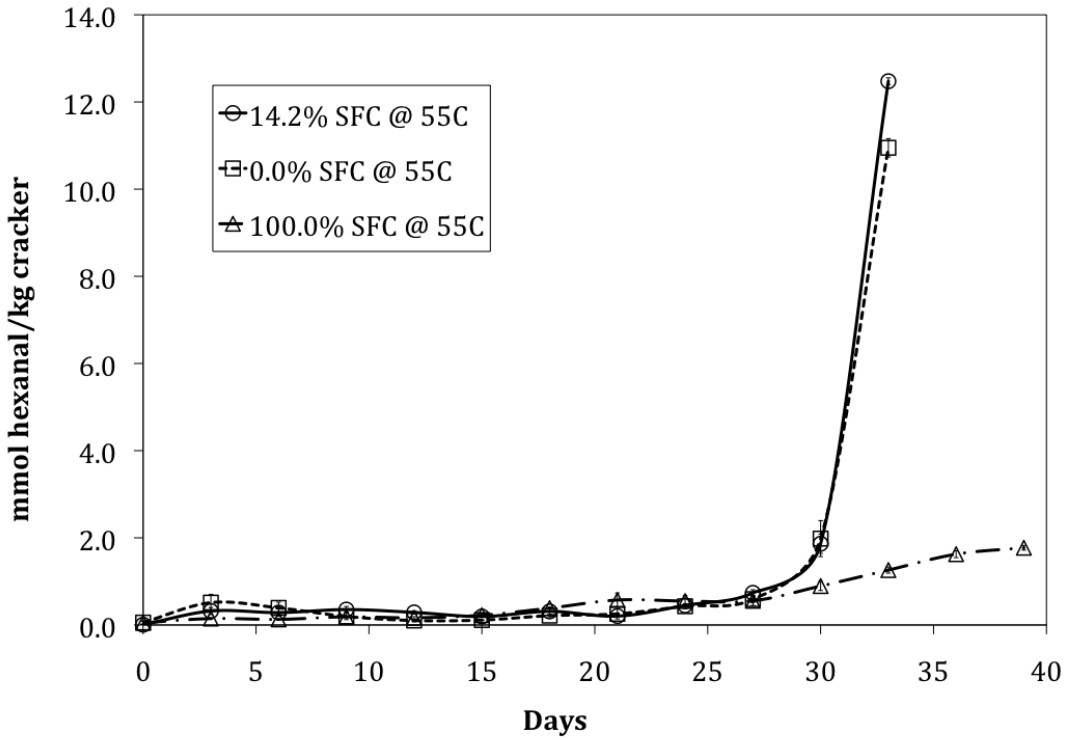

Figure 1b. Hexanal formed by total lipids

Table 2. Fatty Acid profiles of soybean oils:

Fatty Acid	Refined Soybean Oil	Interesterified Soybean Oil
14:0	0.2	0.0
16:0	11.4	11.3
18:0	4.1	31.3
16:1	0.2	0.1
18:1	21.4	14.6
18:2	52.3	35.7
18:3	7.0	4.9

The composition of the oils was determined by gas chromatography. All values are expressed as a percent of the total. The total does not reach 100% due to trace amounts of unknown compounds.

In spray dried powders, lipids on the surface of the powders are known to be the most susceptible to oxidation because they are not encased in the glassy carbohydrate matrix and thus are exposed to oxygen. This could also be the situation in crackers since some of the lipids are embedded within the starch granules (Barden et al., 2014) and some are on the surface. These two populations could oxidize at different rates. If this was the case, technologies could be developed to protect the surface lipids (e.g. surface application of antioxidants) and thus prolong shelf-life. The amount of surface lipids on the crackers made from liquid soybean oil and interesterified soybean oil were similar at an average of 10.7% of the total fat (data not shown).

The surface lipids in crackers made with both soybean oil and interesterified soybean oil developed lipid hydroperoxides after 6 and 9 days of storage (hexanal could not be measured since some if it would be lost during lipid extraction). This compares to a hydroperoxide lag phase of 9 days for both soybean oil and interesterified soybean oil for lipids extracted from the entire cracker suggesting that surface lipids were slightly more susceptible to lipid oxidation.

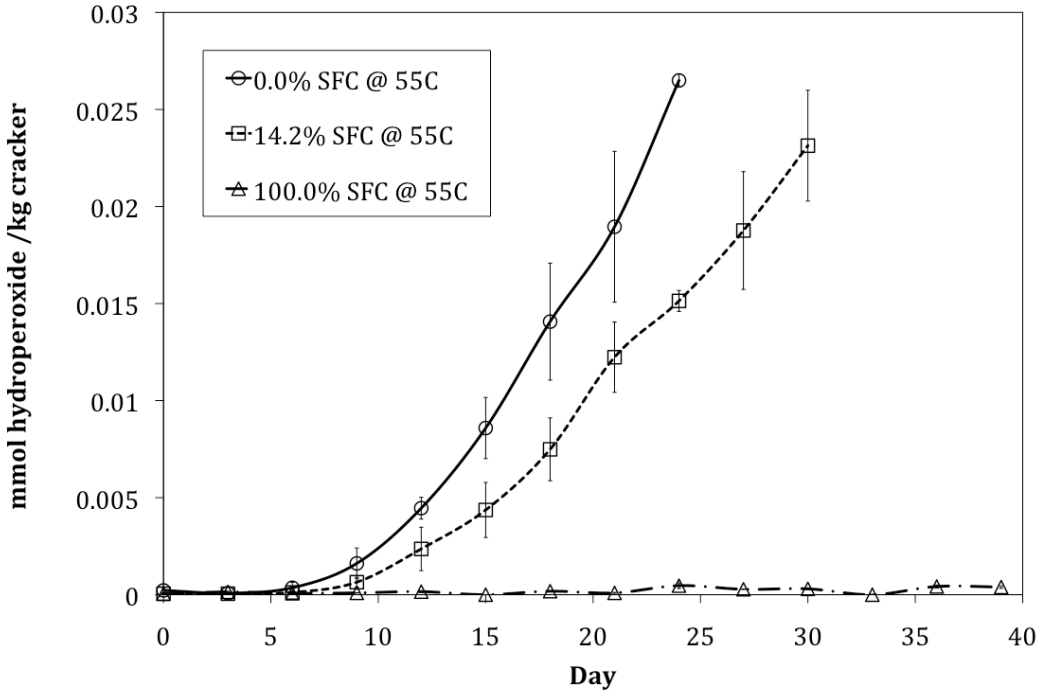

Figure 2. Lipid Hydroperoxides formed by surface lipids

4.2 Synthetic Antioxidants

Barden et al. (2014) found that when 12 and 20 carbon rosmarinic acid esters were added to the lipid before cracker production they were more effective antioxidants than when they were added with the water during dough mixing. This was most notable for the 12 carbon ester of rosmarinic acid which inhibited

oxidation when added to the lipid but was ineffective when added to the dough. The activity of the 20 carbon ester was decreased from a lag phase of 55 days when added to the lipid, to a lag phase of 49 days when added to the dough. It was thought that the difference in antioxidant activity from the order of addition was due to differences in the physical location of antioxidants since the effectiveness of an antioxidant is greatest when they partition at the same location as the oxidizing lipids. The Barden et al. (2014) study also showed that TBHQ and BHT were effective antioxidants in crackers. However, in this study, oxidation was not monitored long enough to determine which antioxidant was more effective since neither of the crackers with the antioxidant in the lipid phase exited the lag phase.

This experiment examined the ability of TBHQ and BHT to inhibit oxidation for both surface and interior lipids as a function of when they were added to the crackers. Surface and interior lipids in control crackers both oxidized at similar rates with both lipid sources having a hydroperoxide lag phase of 9 days and an anisidine lag phase of 33 days. One potential reason for why surface lipids did not oxidize first as they did in the previous experiment with surface and total lipids (Figures 4.1a and 4.2) was that that the interior lipids are collected after the surface lipids. This double extraction could have led to further oxidation of the interior lipid resulting in a shorter lag phase compared to the total lipids.

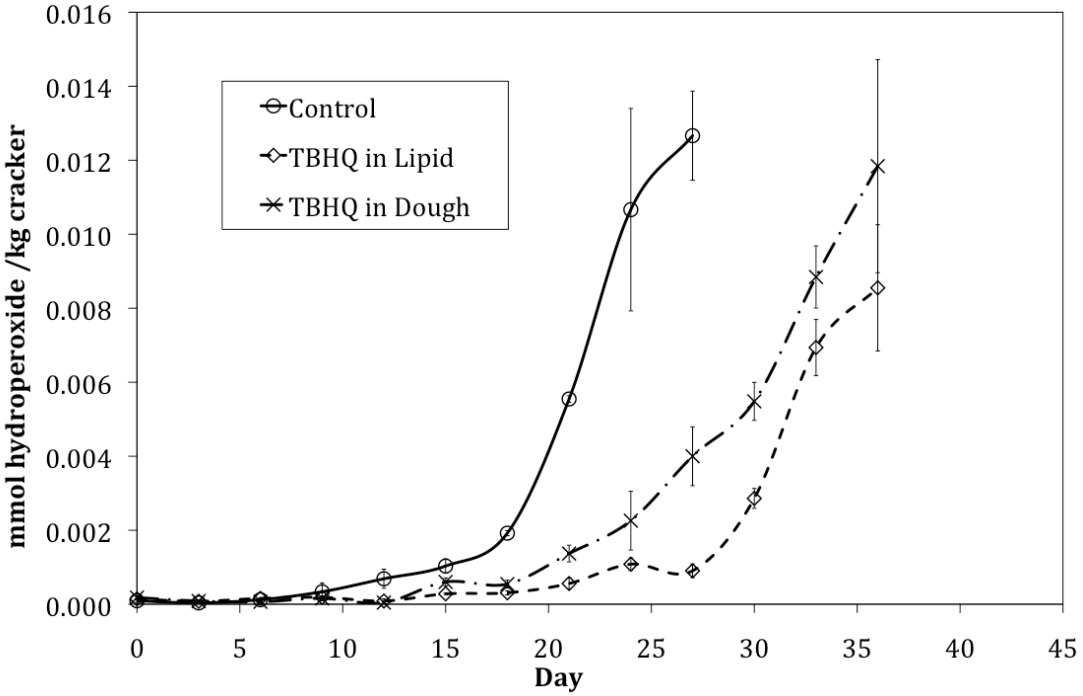

Figure 3a. Lipid Hydroperoxides formed by surface lipids of crackers with TBHQ

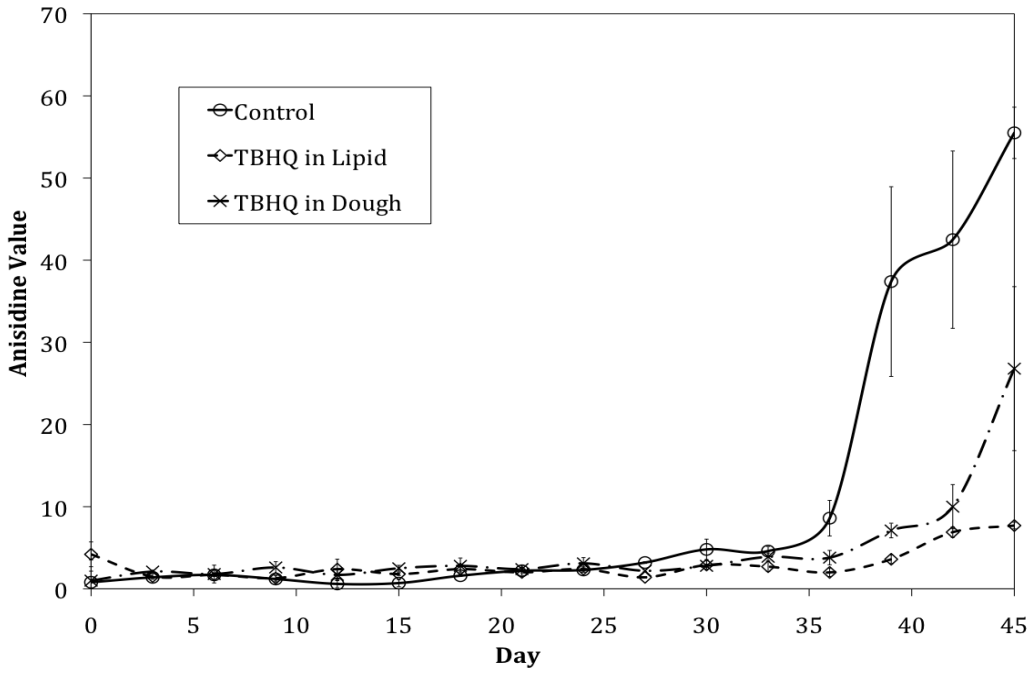

Figure 3b. Aldehydes Formed by surface lipids of crackers with TBHQ

TBHQ was used in this study as an intermediate polarity antioxidant similar to the 12 carbon rosmarinic acid ester used in the Barden et al. (2014) study. In general, TBHQ was able to inhibit both hydroperoxide and aldehyde formation with the TBHQ being more effective when added to the lipid than when added to the dough. When added to the lipid, TBHQ was more effective at inhibiting hydroperoxide formation in the surface lipids (lag phase 21 days) than the interior lipids (lag phase 18 days). When added to the dough, TBHQ was equally effective at inhibiting aldehyde formation (lag phase 36 days) in surface and interior lipids. When TBHQ was added to the lipid, surface and interior lipids did not exit the lag phase. This suggests that TBHQ was able to inhibit hydroperoxide formation in the surface lipid but was not as effective at inhibiting hydroperoxide degradation into secondary lipid oxidation products that react with anisidine. Conversely, in the interior lipids, TBHQ was less effective at inhibiting hydroperoxide formation but was more effective at inhibiting hydroperoxide degradation into secondary lipid oxidation products. These differences could be due to differences in antioxidant concentrations in the different lipid fractions. High antioxidant concentration can result in iron reduction, which cause hydroperoxide degradation. If TBHQ was preferentially partitioning into the surface lipid at concentrations that could promote iron reduction, this could cause rapid formation of secondary lipid oxidation products.

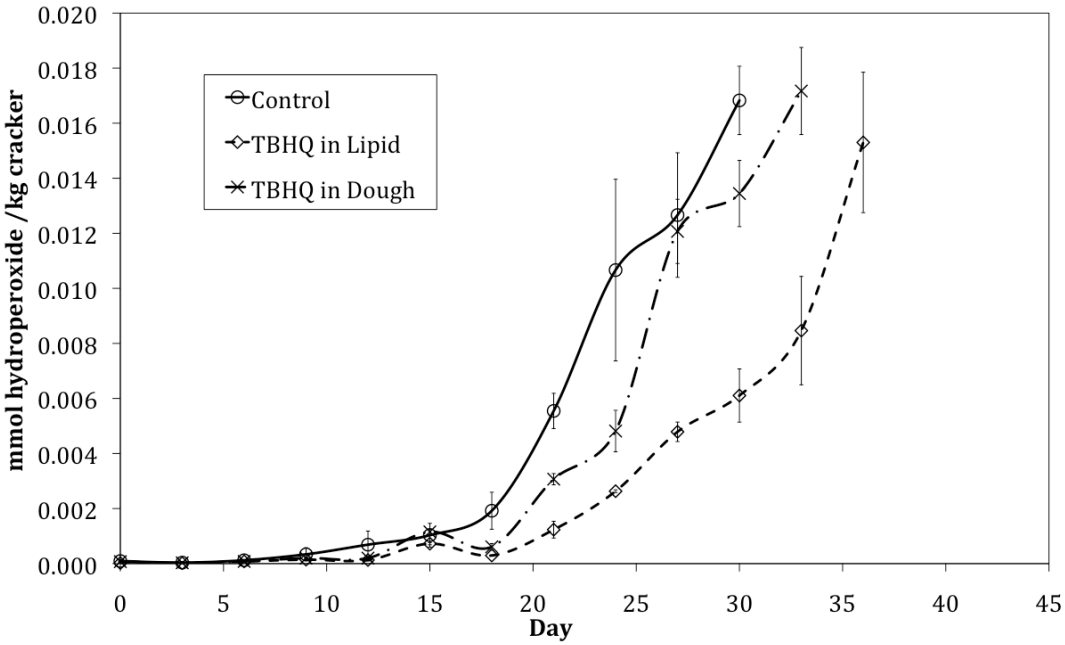

Figure 4a. Lipid Hydroperoxides formed by interior lipids of crackers with TBHQ

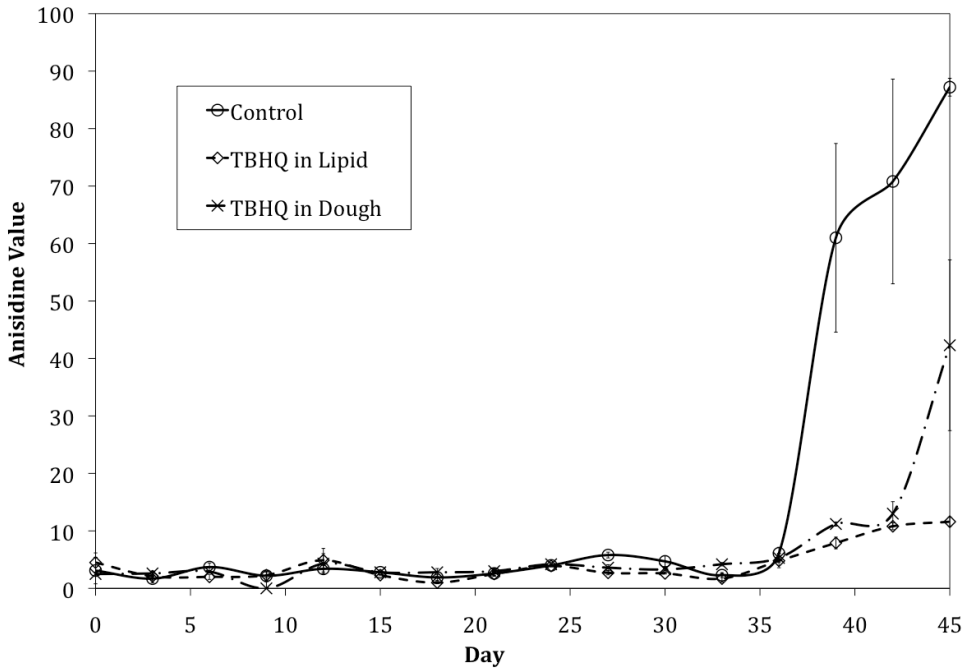

Figure 4b. Aldehydes formed by interior lipids of crackers with TBHQ

BHT had very similar activity in both the surface and interior lipids regardless if it was added to the lipid or the dough. Overall, BHT was more effective than TBHQ at inhibiting formation of secondary lipid oxidation products that react with anisidine. This suggests that BHT is partitioning equally in the surface and interior lipids. Figure 4.7 shows that BHT depletion is slower than TBHQ depletion during cracker storage. This means that BHT concentrations are higher than TBHQ during storage. This greater concentration would lead to greater oxidative protection, explaining why BHT is more effective than TBHQ.

Barden et al. (2014) found that the 20 carbon ester of rosmarinic acid was more effective than the 12 carbon ester. This is similar to these results where the more nonpolar BHT was more effective than TBHQ. These two studies agree that nonpolar antioxidants are the most effective in crackers. This is unlike emulsions where surface active antioxidants are the most effective, and in bulk oils where polar antioxidants are most effective (Barden et al., 2014). Many food manufacturers do not want to use synthetic antioxidants like BHT, and rosmarinic acid esters are generally not available as commercial antioxidants. An alternative is tocopherol homologs, which are very non-polar. An interesting study would be to compare the differences in antioxidant activity of the tocopherol homologs since α-tocopherol is more nonpolar than δ-tocopherol. If the trend holds true, they would be a more effective antioxidant in crackers. As there are only a few nonpolar natural antioxidants available, it might be useful to identify other nonpolar antioxidants in nature to see if they would be effective in low moisture foods like crackers.

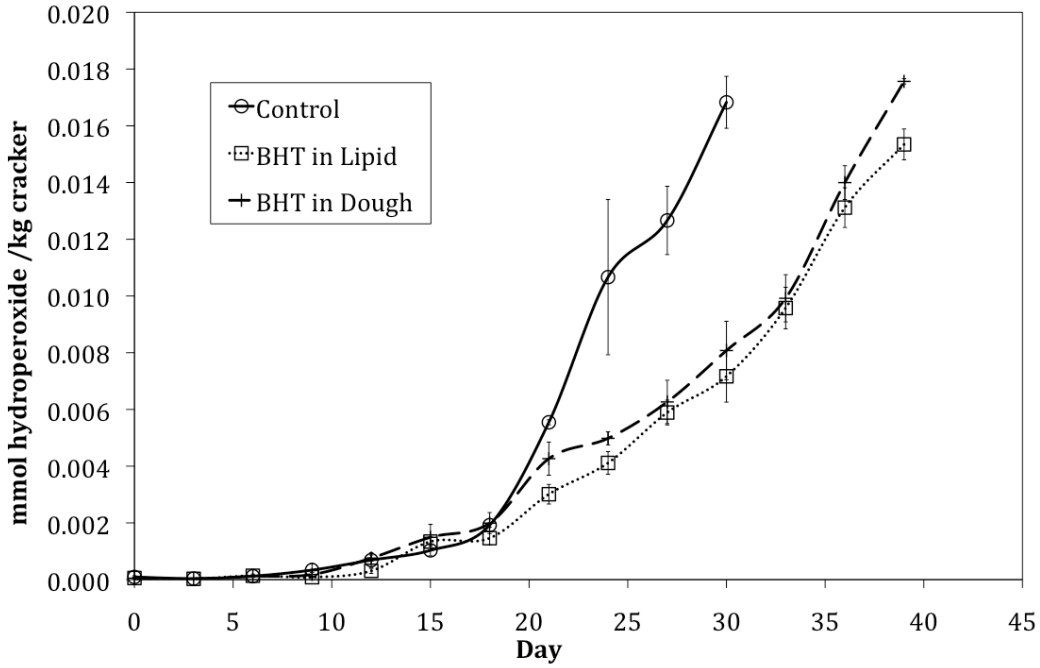

Figure 5a. Lipid Hydroperoxides formed by surface lipids of crackers with BHT

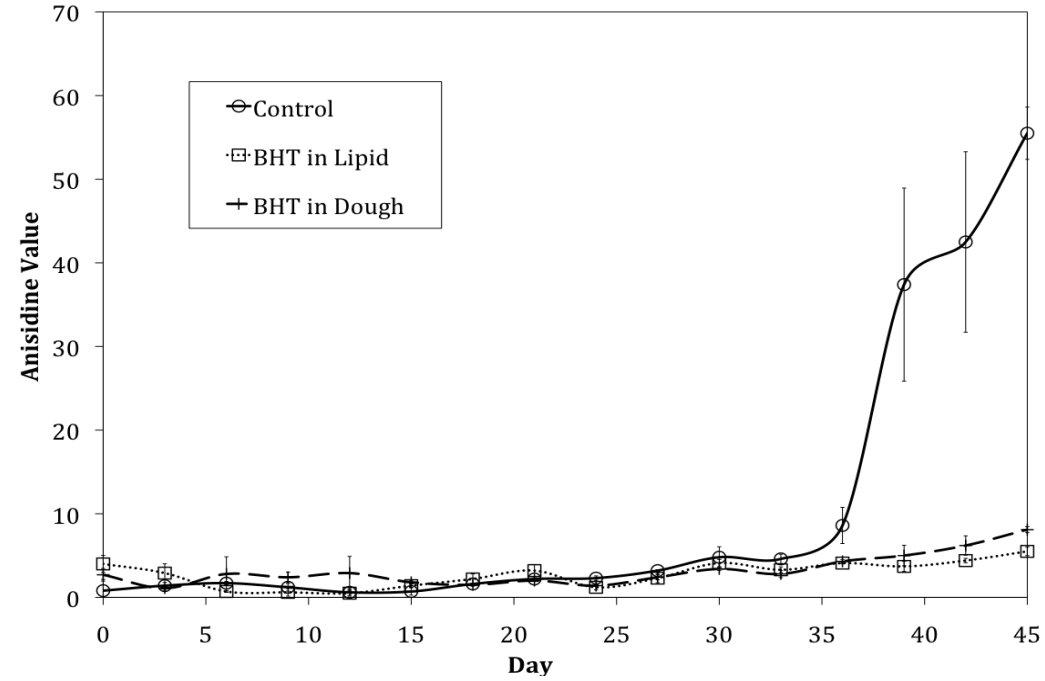

Figure 5b. Aldehydes formed by surface lipids of crackers with BHT

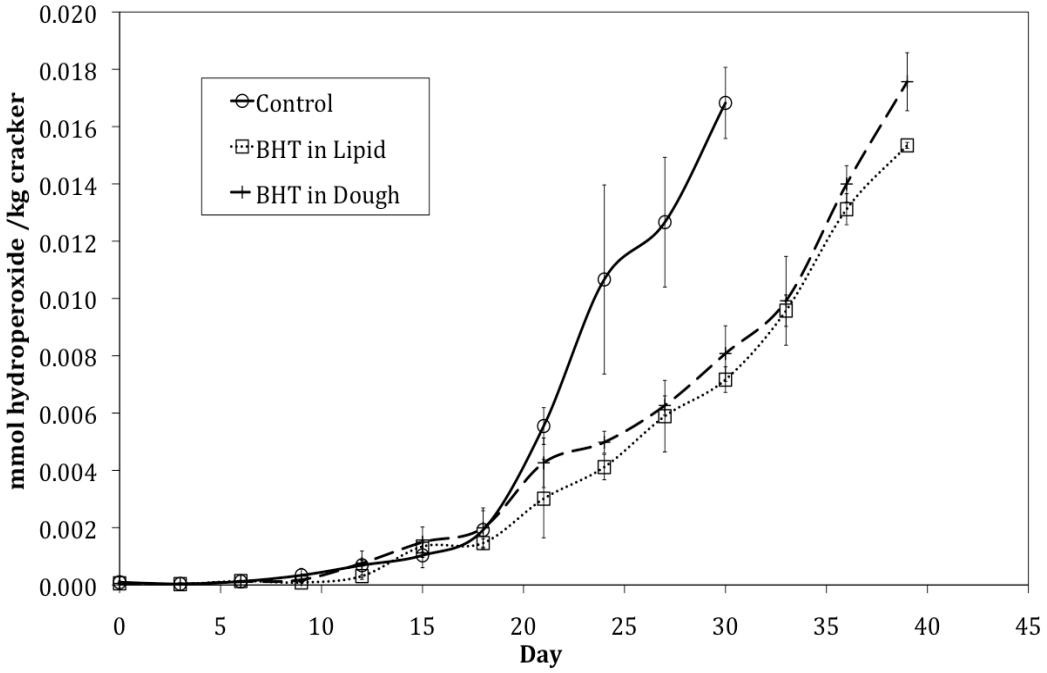

Figure 6a. Lipid Hydroperoxides formed by interior lipids of crackers with BHT

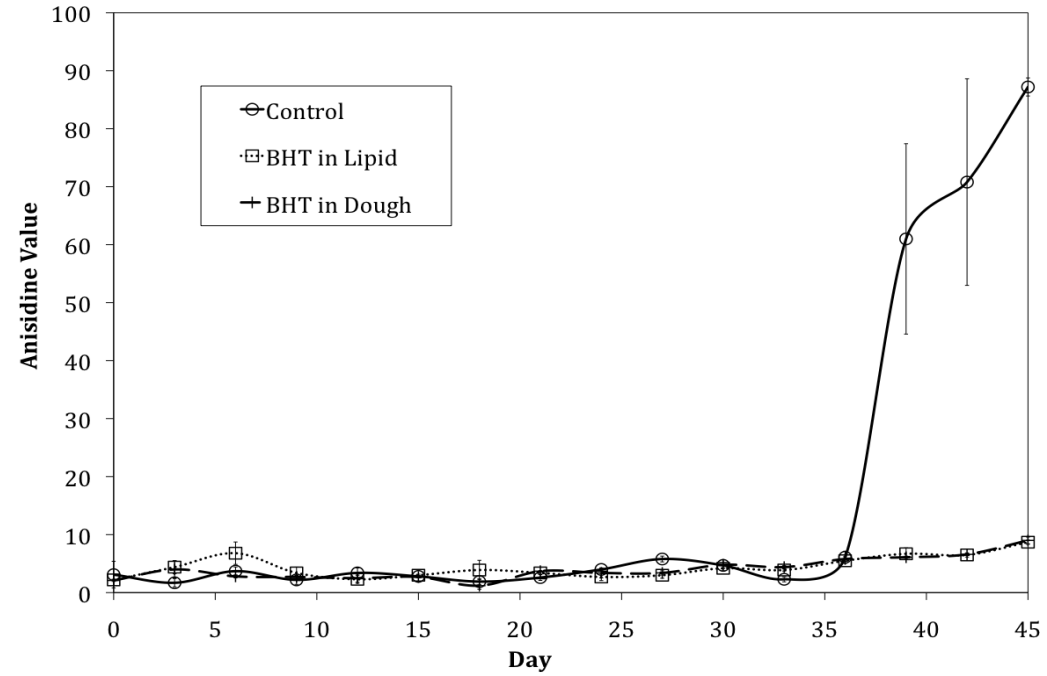

Figure 6b. Aldehydes formed by interior lipids of crackers with BHT

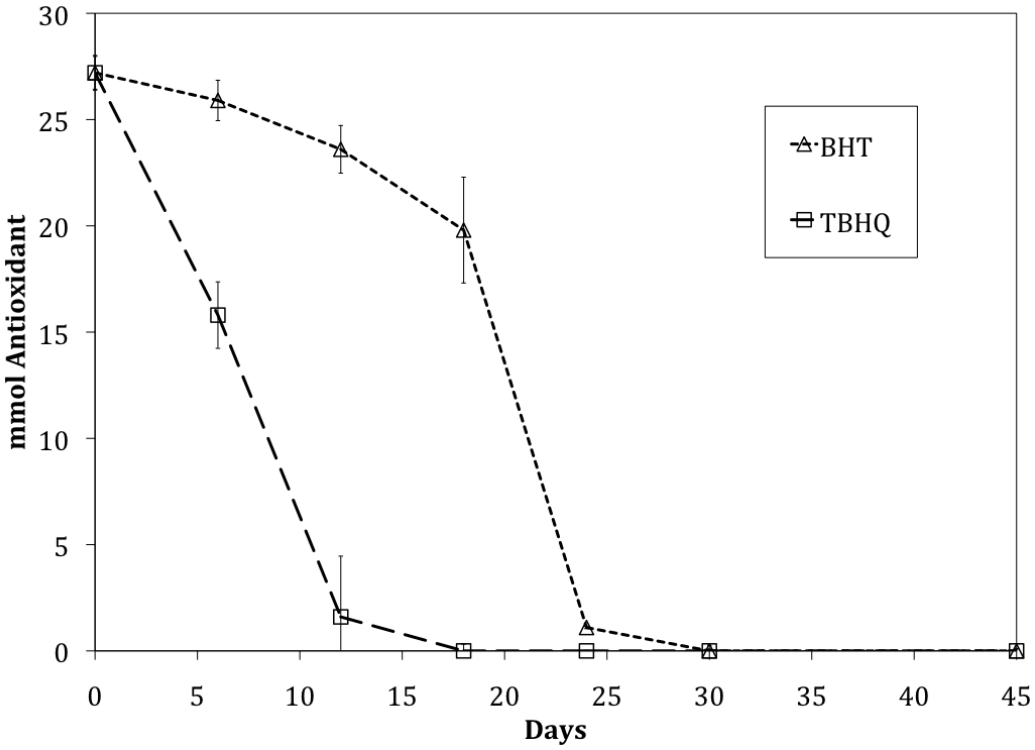

Figure 7. Antioxidant consumption over time by interior lipids

4.3 Headspace Oxygen Reduction

The oxygen reduction study utilized unmodified crackers for determining the impact of oxygen concentrations on lipid oxidation kinetics. Control samples (atmospheric oxygen) had a lag phase for aldehydes of 27 days. Oxygen reduction provided increased oxidative stability for all samples. A reduction of 97% resulted in the extension of aldehyde lag phase beyond the 45 day experiment period. This was consistent with the findings of Johnson et al. (2017) who discovered that 98% oxygen reduction in O/W emulsions conferred total oxidative stability during their shelf-life study. Oxidative stability was also achieved with the 71% reduction,

prolonging the lag phase for aldehydes to 36 days. The 52% oxygen reduced crackers exited the lag phase for aldehydes at 33 days, and the 46% reduced oxygen resulted in an aldehyde lag phase of 30 days. Significant extension of aldehyde lag phase was observed at oxygen reduction levels of 71% and 97%. This is contrary to the findings of Johnson et al. (2017) where meaningful oxidative stability was not shown until at least 93% of the oxygen was removed.

Figure 4.9 shows the consumption of oxygen during storage. A visible drop is seen from day 6 to day 9 in crackers where headspace oxygen was reduced by 0%, 46%, and 51%. This drop occurred at a similar time as the initial formation of primary oxidation products. Consumption continues until day 33 where it drops sharply to <1% total oxygen, after which secondary oxidation products are formed.

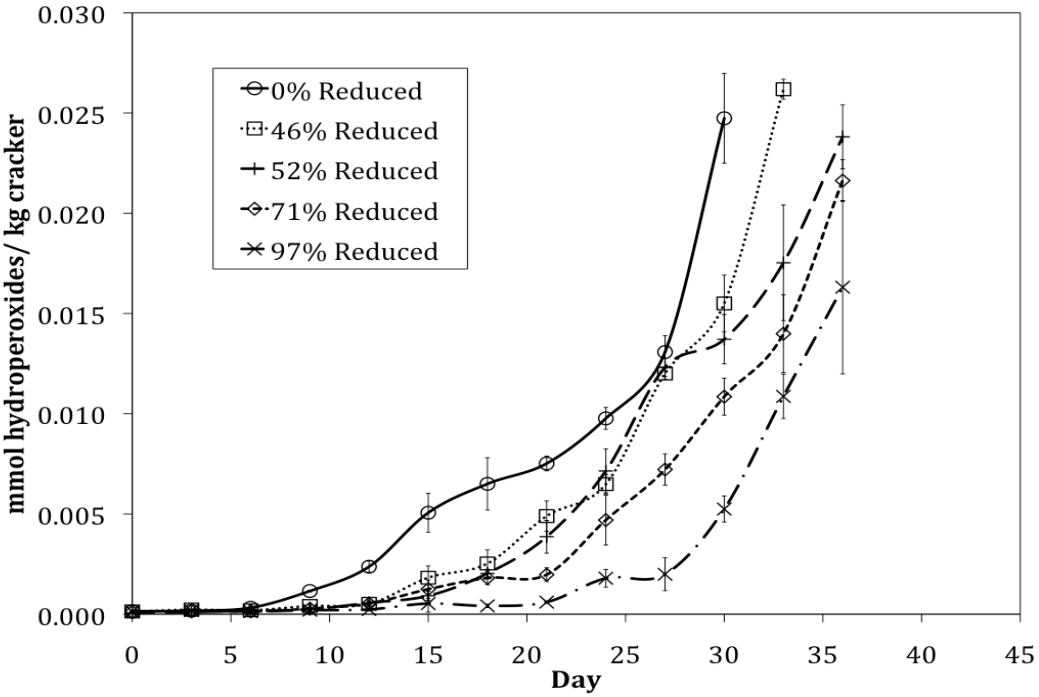

Figure 8a. Lipid Hydroperoxides formed by total lipids of crackers with modified headspace

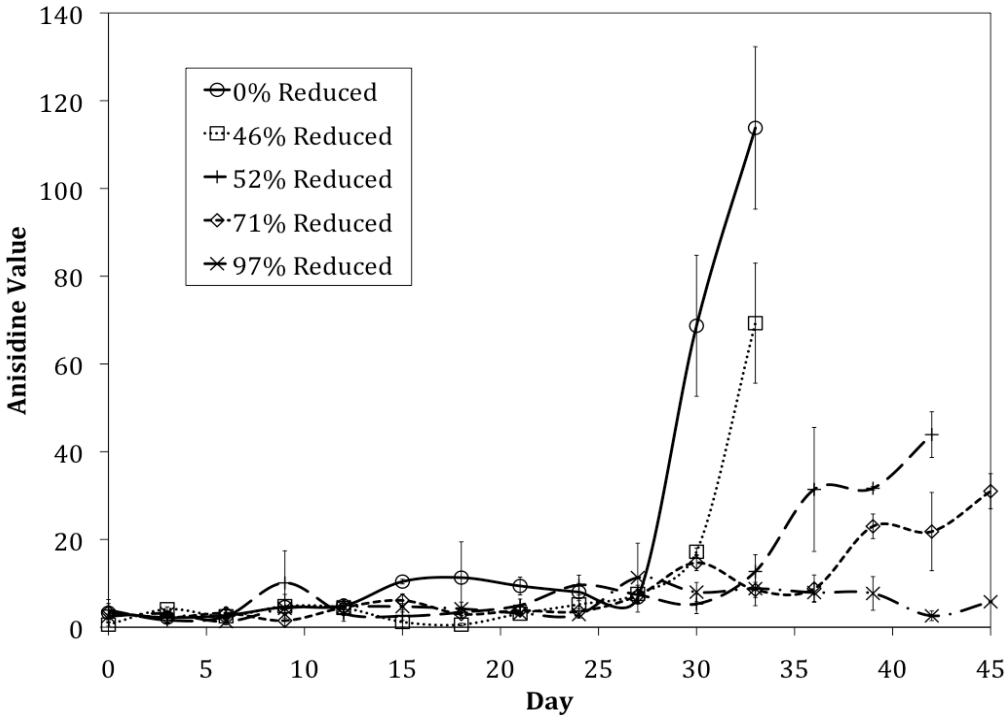

Figure 8b. Aldehydes formed by total lipids of crackers with modified headspace

The remaining oxygen that was present in the 97% reduced samples was entirely consumed by day 36. This treatment exited the lag phase for hydroperoxides on day 24, but did not exit the lag phase for aldehydes during the entire storage study. Overall, it was observed that formation of hydroperoxides was delayed as oxygen levels decreased. Once aldehyde formation was observed, the samples had no headspace oxygen. Thus, the aldehydes originated from the decomposition of the original hydroperoxides and not from additional oxidation products, as the samples were essentially anaerobic once aldehyde formation was observed. This is an important factor to consider if oxygen reduction is to be a viable antioxidant strategy for solid foods.

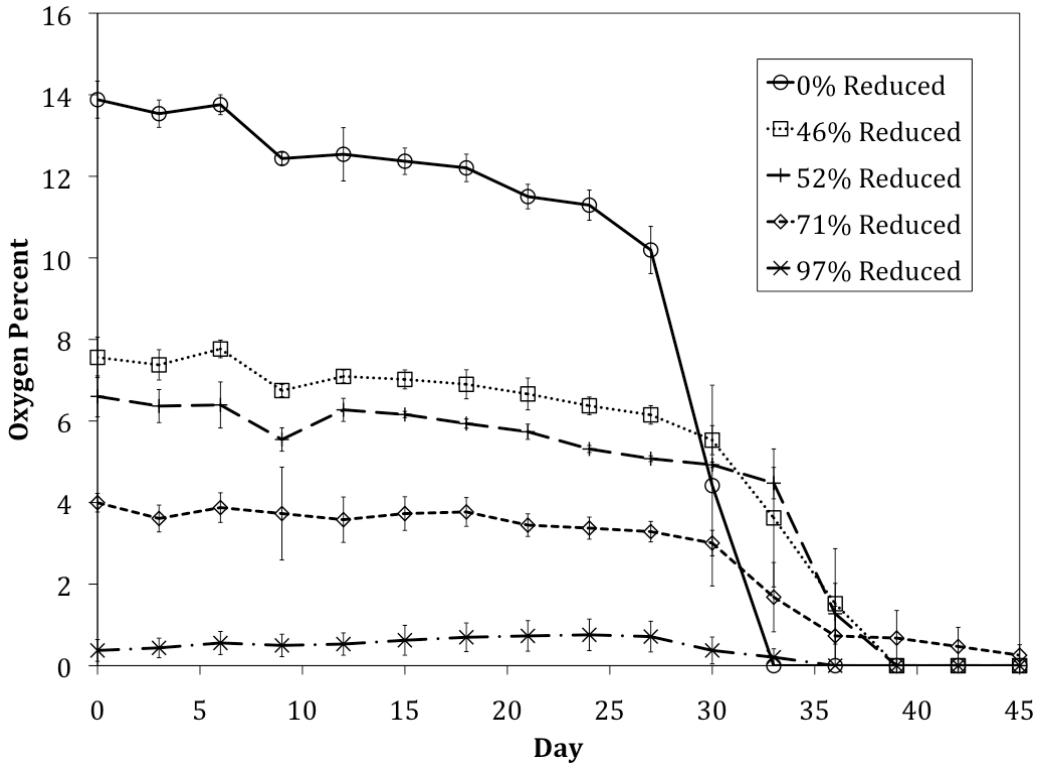

Figure 9. Oxygen consumption over time by total lipids

CHAPTER 5

CONCLUSIONS

5.1 General Conclusions

It is generally understood that the more highly unsaturated a fatty acid is, the more prone to oxidation it will be (Labuza et al., 1971; Frankel, 2005). With each double bond that is added to the fatty acid, bond strength weakens and susceptibility to free radicals increases. This has adverse effects on shelf life and food safety because these fatty acids will oxidize more rapidly. Yet these same unsaturated fats are also the most desirable to counter heart disease and promote good health (NCI, 2013a). There are many factors such as antioxidant presence, temperature levels, exposure to oxygen, fat content, and UV exposure that contribute to the rate at which oxidation, and therefore spoilage, occurs in fatty acids. This thesis focused on three of these factors, solid fat content, antioxidant treatment, and the effects of oxygen reduction.

The first experiment monitored the effects of solid fat content on rates of oxidation. Two variables were tested, an interesterified soybean oil with a SFC of 14.2% at 55°C and liquid oil with a SFC of 0% at 55°C. It was found that an increase in solid fat content did not confer increased oxidative stability to the crackers. In the first experiment, surface lipids oxidized faster than total lipids. However, this difference was small and no significant difference was observed in the oxidation rates of surface vs. total lipids. Overall, this suggested that surface lipids were not more susceptible to oxidation as they are in spray-dried powders. This could be because of the high porous nature of the crackers where oxygen can quickly

penetrate the matrix and reach all lipids, compared to spray dried powders where the carbohydrates are in the glassy state and provide an effective oxygen barrier.

The second experiment monitored the effects of antioxidants on rates of lipid oxidation. Two synthetic antioxidants were tested with varying degrees of hydrophobicity, BHT and TBHQ. Both antioxidants were shown to be effective but the more hydrophobic BHT was a better antioxidant than TBHQ. This is in agreement with studies with fatty acids esters of rosmarinic acid where antioxidant activity increased with increasing hydrophobicity. More worked is needed to verify whether other nonpolar antioxidants are the most effective in low moisture foods like crackers.

The third experiment monitored the effects of headspace oxygen reduction on rates of lipid oxidation. Crackers were flushed with nitrogen/oxygen blends to achieve varying degrees of reduction. Every subset that was reduced showed an extension in lag phase for primary and secondary oxidation products over the control. However a substantial antioxidant effect was not observed until a minimum reduction of ≥71% was achieved. Employing oxygen monitoring strategies to industrial processed foods that are nitrogen flushed would be interesting to see if the levels of oxygen reduction that are achieved are viable antioxidant methods.

CHAPTER 6

FUTURE WORK AND CRITICISMS

6.1 Theories and Suggestions

Researchers theorized that there may be potential increased availability of oxygen on the surface of a food, and that the encapsulation of the food in a type of antioxidant barrier may provide stability. This suggests that a study be performed which observes the effects of a solid fat coating on the crackers as well as an antioxidant coating. Minor work was done to spray the crackers with melted fat through an atomizer, however more work is needed to refine the methods. The method suggested for application of an antioxidant coating is to mix a solution of desired strength in a pure intermediate, such as ethanol. This will allow for rapid volatilization of the solvent, reduced solubilization of surface lipids, and an even distribution of the antioxidant at a known concentration.

Tertiary verification of antioxidant presence and oxidation levels in crackers as a whole would improve accuracy and validity of findings thus far. Utilization of fluorescence microscopy in surface lipid studies would increase power by providing this reference point for the effective removal of surface lipids and potential visualization of antioxidant partitioning. Improved methods of fluorescence analysis of solid foods need to be employed to view surface lipids in crackers.

Antioxidants such as butylated hydroxy toluene or tert-butyl hydroxy quinone are becoming more controversial among consumers. Companies are beginning to recognize this trend and have released prohibitory lists to their suppliers naming these compounds (WFM, 2017). Natural antioxidants in low

moisture foods may provide one viable alternative, as consumer perceptions of natural preservatives are more positive. Research into industrially viable natural antioxidants may be beneficial.

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