Evaluation of Formulations and Oxidative Stability of Coconut Oil Blends

By

EMILY NERING

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ABSTRACT OF THE THESIS

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BY EMILY NERING

Thesis Director:

Professor Karen M. Schaich, Sc.D.

Tropical fats such as coconut oil contain medium chain fatty acids which activate lipases, converting fats directly into energy rather than storage in adipose tissue. Implications that these fats may be helpful in reducing obesity and related diseases, and also replace trans fats in foods, have led to interest in developing "healthy" coconut oil-based products for use in table spreads, cooking, and baking applications.

This study evaluated effects of water quality, antioxidants, and oil components on thermal and oxidative stability of margarine-type blends (coconut oil, palm shortening, and high oleic sunflower oil with/without polyunsaturated canola and flax seed oils) developed for baking and sautéing applications. Thermal stability was determined by heating blends in an Oxipres^{TM} oxygen bomb at 80, 100, 120, and 150 °C to simulate baking, sautéing, and frying applications, respectively. Refrigerated shelf life was determined in blends stored in glass jars sealed under argon for up to one year. Samples were analyzed periodically for lipid oxidation by conjugated dienes and hydroperoxides (chemical analyses), carbonyls (dinitrophenylhydrazine reaction with high pressure liquid

chromatography detection and quantitation), and formation of volatile degradation products (gas chromatography).

Thermal and shelf stability varied with unsaturated fatty acid composition of the blends; products were detected from all unsaturated fatty acids present. Handling during preparation and storage, as well as quality of the water used in the blend, was also important in directing stability. No blend tested maintained acceptable peroxide levels when heated at 150°C. Blends with higher levels of canola and flax seed oil degraded unacceptably at 100 °C. Peroxide values did not accurately reflect degradation of tropical fat-based blends. Mixed tocopherols added at levels from 200 to 1000 ppm paradoxically increased peroxide and aldehyde levels during heating and storage. Maximum peroxides were <20 and only low levels of aldehydes were present, yet strong off-odors and flavors were present. Butanal, in particular, was produced in unusually high levels. These results can be explained by alternate oxidation pathways including peroxyl radical addition to double bonds and epoxide formation exceeding hydroperoxide formation and scission. Tocopherols stabilized hydroperoxides by hydrogen bonding and shifted oxidation back to standard pathways.

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LIST OF ABBREVIATIONS

TAG	Triacylglycerol
PV	Peroxide Value
ASE	Accelerated Solvent Extraction
CD	Conjugated Dienes
FFA	Free Fatty Acids
DNPH	2,4-Dinitrophenylhydrazine
GC	Gas Chromatography
FID	Flame Ionization Detector
HPLC	High Performance Liquid Chromatography
DAD	Diode Array Detector
MCFA	Medium Chain Fatty Acids
MCT	Medium Chain Triacylglycerols
LCFA	Long Chain Fatty Acids
LCT	Long Chain Triacylglycerols
PUFA	Polyunsaturated Fatty Acids
MUFA	Monounsaturated Fatty Acids
SFA	Saturated Fatty Acids

1. INTRODUCTION

The food industry often looks to research in food science and nutrition to create innovative products that have perceived health benefits, but also align with consumer trends. Currently, tropical fats, like coconut and palm oil, are being utilized increasingly by consumers in home cooking as well as by industry in consumer products due to emerging research indicating potential health benefits of these fats. In the past, tropical fats, particularly coconut oil, were demonized due to their high saturated fat content, which has long been considered to be detrimental to human health. According to the American Heart Association (AHA), saturated fats have been associated with high levels of LDL cholesterol in the blood, which in turn increase the risk of heart disease and stroke. However, re-examination of research on saturated fats and their effects on heart and cardiovascular health has suggested that saturated fats alone have no clear direct effects on heart or cardiovascular diseases or on diabetes and insulin resistance (Dias et al, 2014; Micha and Mozaffarian, 2010; DiNicolantonio et al, 2015). In fact, a critical review of studies found that saturated fatty acids increase blood lipids and cholesterol only when diets are deficient in essential fatty acids (Dias et al, 2014).

Based on recommendations from the AHA, as well as the U.S federal government, to reduce the intake of saturated fats in the diet, the food industry pushed to remove these fats from foods as much as possible. This led to the replacement of saturated fats with refined sugars and carbohydrates, salt, and trans fats in foods. According to DiNicolantonio et al (2015), diets generally high in refined carbohydrates and sugars create a 3-fold higher risk of cardiovascular disease than saturated fats as well, and even worse, they increase risk for other diseases like diabetes and fatty liver. Since the early 1990s, it was proposed by

Harvard School of Public Health researchers that trans fats have inherent toxicity that enhances buildup of plaques in the arteries, thereby increasing risk of coronary heart disease and heart attacks, and therefore need to be clearly labeled on food products (Ascherio et al, 1994; Willett et al, 1993). As a result of considerable pressure from trans fat critics, the U.S. Food and Drug Administration (FDA) no longer recognizes trans fats as safe (non-GRAS status) and is pushing to have all artificial trans fats, hydrogenated, or partially hydrogenated oils (PHOs) gradually removed from food products over the next three years (FDA, 2015). Trans fats can be found in a number of products on the market today, such as cookies, crackers, margarines, coffee creamer, canned frosting and refrigerated doughs (FDA, 2015). However, removal of trans fats and hydrogenated fats from processed foods also necessitates identification of other fats that can replace their texture, flavor and mouthfeel while also providing health benefits, or at the very least, no significant detriments to health.

As a result, many companies are re-evaluating tropical fats as a replacement to trans fats in processed foods. Tropical fats that are mainly composed of medium chain saturated fatty acids with moderately high metling points. Tropical fats, particularly coconut oil, have been eschewed in the U.S. market since the late 70s when they were attacked as enhancing cardiovascular disease. Recent research, however, suggests that these reports were erroneous and, more importantly, that medium chain fatty acids (MCFAs) have a number of unique biochemical properties that classify them as “healthful” (Babayan, 1985. 1987). MCFAs are absorbed directly from the stomach to the liver where they are metabolized for energy, rather than ending up in adipose tissues. Perhaps even more importantly, MCFAs activate lipases in tissues and stimulate breakdown of stored fat. This activity has

stimulated great interest in determining whether these fats may be effective in weight loss as well as preventing cardiovascular diseases and insulin resistance (Nagao, 2010; Bourque, 2003; Arunima, 2012; Clegg, 2010).

Clinical trials of margarine and shortening-type products composed of coconut oil and palm shortening plus other oils are underway to test whether such products can be useful in reducing childhood obesity (proprietary information). The idea for these products came from a company that sought to create a healthy tub-style margarine free from trans fats and common allergens (e.g. soy), plus another "stick-type" margarine for use in cooking or baking. The spreadable margarine contains high levels of essential fatty acids like omega-3s as well as healthy medium-chain fatty acids (MCFAs) from coconut oil. The stick-margarine was made to resist degradation in cooking applications and therefore have more MCFAs and monounsaturated fats but lower levels of very few essential unsaturated fatty acids.

Little is known about how tropical fats degrade, by what mechanisms they oxidize, or what products are formed. Most research on the lipid degradation and oxidation has been focused vegetable oils, especially soybean oil, due to cooperative government research with industry as well as the fact that it is the largest U.S. oilseed crop, increasing its popularity in home and industrial kitchens in the U.S (Dutton, 1981; Frankel, 1980; USDA, 2012). However, if tropical fat products are to be used in nutritional support, particularly with children, the stability and safety of the product must be assessed experimentally first. It is also important to identify factors that are critical for industry to control when developing products based on tropical fats, in order to prevent development of the off-flavors and off-odors that results in rejection of the product by consumers.

Lipid oxidation is a major problem that contributes to rancidity, off-flavors and off-odors in foods, resulting in loss of sensory quality and nutritional value (Fullana, 2004a; Chiang et al, 1997; Qu et al, 1992). Some lipid oxidation products are also toxic (Chiang et al, 1997; Qu et al, 1992) so safety also becomes an issue. Lipids in emulsions, including margarine, oxidize differently in a bulk oil, and indeed are more susceptible to oxidation due to increased surface area on the lipid droplets in contact with the water phase. Extensive research documents that the interfacial layer between oil and water phases drives lipid oxidation in the system and the presence of water increases the occurrence of lipid oxidation in emulsions compared to bulk oils (McClements and Weiss, 2005; Mei et al, 1998; Coupland, 1996; Mosca et al, 2013; Frankel, 1994, 1996, 2012; Schaich et al, 2013). Thus, components of the water phase must be a focus for stabilizing any food emulsions.

For products that will be used in any form of cooking, thermal degradation is also a limitation. Heat breaks bonds in lipid chains, leading to formation of radicals that add oxygen and initiate a cascade of reactions (Labuza, 1971; Marcuse and Fredriksson, 1968; Schaich et al, 2013). Saturated fats have lower smoke points than unsaturated oils, meaning they begin decomposing at lower temperatures (Man and Hussin, 1998). While thermal degradation of unsaturated vegetable oils has been studied extensively, relatively little information is available tracking thermal degradation processes in saturated fats, particularly tropical fats.

This thesis examined the oxidative and thermal stability of coconut oil blended with other vegetable and seed oils to create margarine-type products. Appropriateness of oil formulations in the blends were evaluated by shelf life studies and thermal stability studies. Oxidation was followed in shelf life studies of blends stored at refrigerated temperatures

for up to one year. Thermal degradation was determined in blends heated in an Oxipres™ oxygen bomb. For both processes, early products in lipid oxidation were analyzed by standard chemical assays of conjugated dienes and hydroperoxides. Secondary degradation products were identified and/or quantified using gas chromatography (GC) of volatile products and high performance liquid chromatography (HPLC)-dinitrophenylhydrazone analyses of non-volatile carbonyls. Effects of water quality and levels of added mixed tocopherols were examined in both shelf life and thermal stability studies. Informal taste tests were also conducted in order to better identify potential problems with the blends.

Research on oxidative and thermal degradation in tropical fats is timely in that the FDA rulings mandating removal of partially hydrogenated oils from foods are imminent and alternative ingredients thus must be evaluated. Coconut oil and other tropical fats like palm oil are being utilized increasingly in the food industry and their patterns of degradation and oxidation need to be documented in order to create palatable food products from these oils.

2. BACKGROUND

2.1 Lipid Oxidation

The main chemical reaction limiting shelf and quality of processed foods is lipid oxidation (Schaich et al, 2013; Frankel, 2012). Even though margarine-type products contain mostly saturated fatty acids, the unsaturated components are highly susceptible to oxidation which drives degradation of these products during storage. Lipid autoxidation has been viewed traditionally as a straight-forward free radical chain reaction with three stages of activity: initiation, propagation, and termination as shown in Figure 1 (Schaich, 2005; Schaich et al, 2013; Frankel, 2012). Once started, the process is self-perpetuating, which makes controlling lipid oxidation challenging.

Lipid oxidation is not a spontaneous reaction -- it needs a catalyst such as heat, metals, light, or radicals to remove an electron and create the *ab initio* lipid or oxygen radical that starts the radical chain (Schaich et al, 2013) (Reaction 1):

$$LH \longrightarrow L\bullet \qquad (1)$$

Lipid oxidation is difficult to prevent since catalysts are present ubiquitously in foods. However, initiation can be limited by reducing access to catalysts, particularly by protecting from heat and light, and by adding chelators to reduce metal activity. Initiation processes have been reviewed in detail by Schaich (2005, 2013) and interested readers are referred to those references.

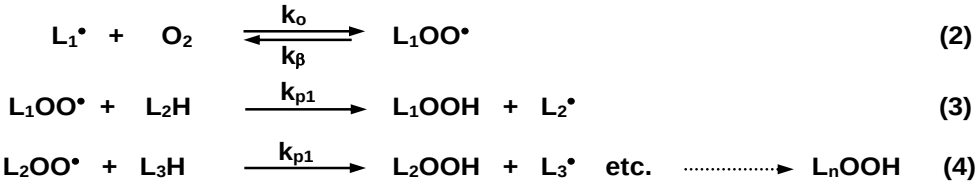
Propagation -- the second stage – establishes the chain of repetitive free radical reactions and keeps it going. In this stage, oxygen first adds to the lipid $L^{\bullet}$ radicals at diffusion-controlled rates (almost instantly when oxygen is not limited) to form reactive peroxyl radicals (Reaction 2) (Schaich et al, 2013):

CLASSICAL FREE RADICAL CHAIN REACTION MECHANISM OF LIPID OXIDATION

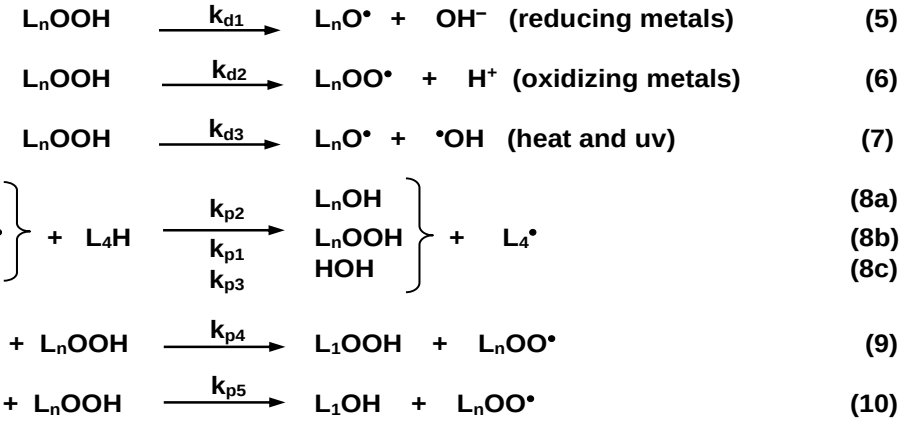
Initiation ***(formation of ab initio lipid free radical)***

Propagation

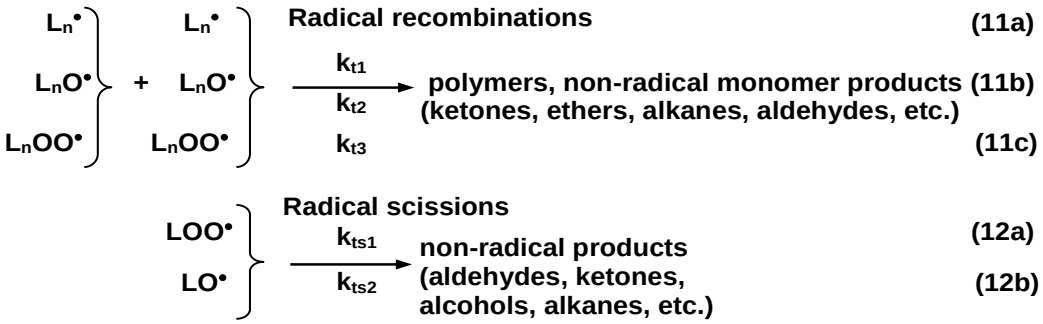
Free radical chain reaction established



Free radical chain branching (initiation of new chains)



Termination ***(formation of non-radical products)***



i - initiation; o-oxygenation; β-O_2 scission; p-propagation; d-dissociation; t-termination; ts-termination/scission

Figure 1. Traditional free radical chain reaction of lipid oxidation (Schaich, 2005).

$$L\bullet + O_2 \longrightarrow LOO\bullet \quad (2)$$

Peroxyl radicals then abstract hydrogen atoms from nearby lipid molecules to form hydroperoxides, in the process generating new lipid radicals that perpetuate the radical

$$L\bullet + O_2 \longrightarrow LOO\bullet \quad (3)$$

Peroxyl radicals then abstract hydrogen atoms from nearby lipid molecules to form hydroperoxides, in the process generating new lipid radicals that perpetuate the radical chain reaction $LOO\bullet + LH \quad LOOH + L\bullet$

(4).

$$LOO\bullet + LH \longrightarrow LOOH + L\bullet \quad (4)$$

The new L• radicals thus created add oxygen, form peroxyl radicals, and abstract hydrogen atoms to form more lipid radicals, repeating Reactions 2 and 3 indefinitely until no more hydrogens can be abstracted or radicals are quenched by other reactions.

Hydroperoxides are stable in the absence of catalysts, but rapidly decompose into alkoxyl and hydroxyl radicals in the presence of ultraviolet light, heat above 40 °C, metals, proteins, and radicals:

Metals: $LOOH \longrightarrow LO\bullet + -OH \quad (4a)$

UV light, heat: $LOOH \longrightarrow LO\bullet + \bullet OH \quad (4b)$

The alkoxyl and hydroxyl radicals can then abstract hydrogen atoms from nearby lipids to generate new radical chains (Reaction 5a, 5b):

$$LO\bullet + LH \longrightarrow LOH + L\bullet \quad (5a)$$

$$\bullet OH + LH \longrightarrow HOH + L\bullet \quad (5b)$$

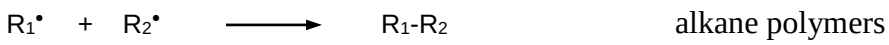
Lipid hydroperoxides can also be oxidized to peroxyl radicals, which then repeat hydrogen abstraction (Reaction 3) to generate more radicals:

Metals: $LOOH \longrightarrow LOO\bullet + H^{+}$ (6)

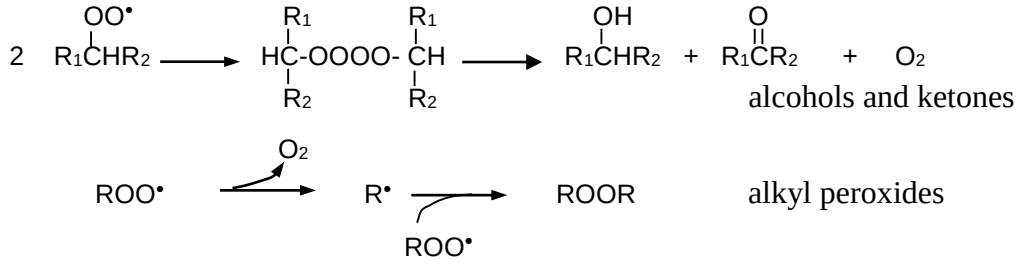
Termination is the stage of lipid oxidation that stops individual chain reactions (total lipid oxidation is nearly impossible to stop) by any of four mechanisms: radical recombinations, α and β scission reactions of alkoxyl radicals, co-oxidation of non-lipid molecules, and group eliminations (Schaich et al, 2013).

Radical recombinations form non-radical products like ketones, ether, alkanes, alcohols, and aldehydes (Figure 2). The exact products resulting from radical recombinations vary with temperature, oxygen pressure, and types of lipid radicals available.

Alkyl radicals:



Peroxyl radicals:



Alkoxyl radicals:

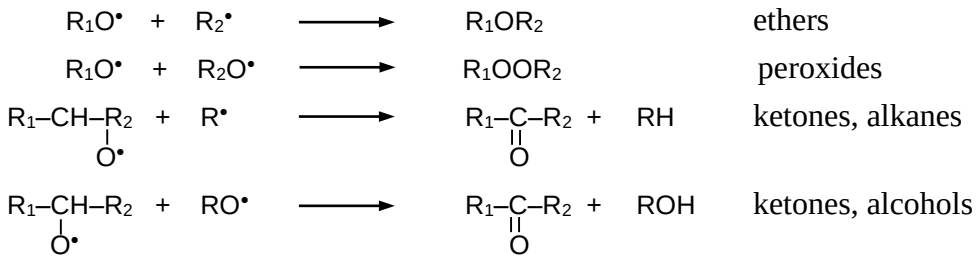
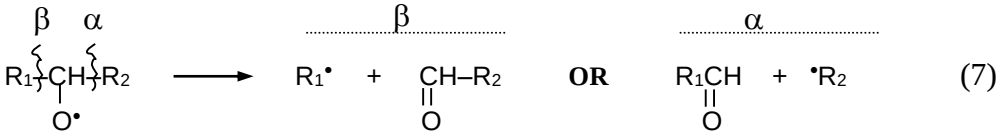


Figure 2. Examples of lipid oxidation products formed by radical recombinations (Schaich et al, 2013).

Scission reactions are generally recognized as the source of off-odors and off-flavors associated with "rancidity" (Schaich et al, 2013). Alkoxyl radicals undergo scissions on either side of the alkoxyl carbon to generate carbonyl products (aldehydes) and alkyl free radicals (Schaich et al, 2013):



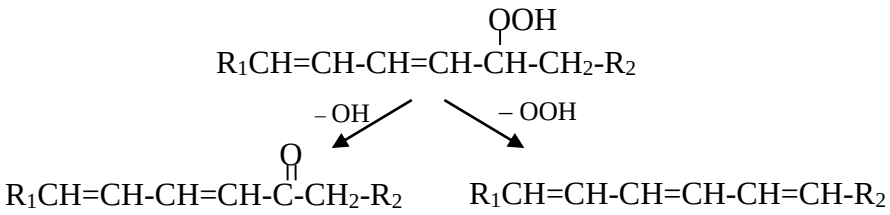
(7)

The radical fragments R• either anneal to alkanes or add oxygen and start new radical chains (Schaich et al, 2013). High heat and water are reportedly key drivers of radical

scissions (Frankel, 1982, 1984). Thus, scission products should be expected to dominate in margarine-type emulsions that are used in high temperature applications such as baking and sautéing.

Co-oxidations occur when lipid radicals abstract hydrogen atoms from non-lipid molecules, transferring radicals and oxidation potential to other food molecules such as proteins, antioxidants, vitamins, among others (Schaich, 2008; Schaich et al, 2013). The process may stop or slow an individual lipid radical chain, but at the same time it activates radical reactions in other molecules, greatly broadcasting oxidation beyond lipids (Pryor, 1978, 1989; Schaich, 1980, 2008; Borg and Schaich, 1984; Schaich et al, 2013).

Group eliminations are a minor form of termination, but can account for lipid oxidation products that are not easily formed by other mechanisms (Schaich et al, 2013). HO^- and HOO^- can both be eliminated from LOOH, yielding ketones and products with additional double bonds, respectively:



(8)

2.2 Issues affecting oil stability

Many factors contribute to oil stability and stability of oils in foods and must be considered when developing new food products or processing methods. These factors

provide critical control points in a food manufacturing plants to ensure that highest lipid quality is maintained in food products.

1.2.1 Fatty acid (FA) composition

While unsaturated fatty acids strongly impact food stability and sensory qualities, they also are crucial nutritional and functional components in foods (Budilarto and Kamal-Eldin, 2015; Shahidi and Zhong, 2010). Linoleic, linolenic, and arachidonic acids are essential fatty acids for humans (they cannot be synthesized physiologically), and 20:5 and 22:6 are now also considered essential because they are produced at such slow rates; they are particularly important as precursors of prostaglandins and leukotrienes that control many physiological processes (Jump et al, 2012; Brossard et al, 1996; Conquer and Holub, 1997). Polyunsaturated fats (PUFAs) reduce risks associated with cardiovascular disease and overall health (Zheng et al, 2014; Nestel et al, 2015; Lopes et al, 2015).

While it may seem that PUFAs provide a solution to many health issues with fats in diets, PUFAs also present a huge drawback as ingredients in foods because they are not stable. Omega-3 fatty acids, in particular, oxidize very rapidly, thus reducing their shelf life and limiting their applications in health foods without extreme protections (Budilarto and Kamal-Eldin, 2015; Schaich, 2005; Choe and Min, 2006; Chen et al, 2011). Foods lower in PUFAs generally have a much longer shelf life than those high in PUFAs; this important factor must be considered when determining food formulations.

1.2.2 Refining

Crude food oils consist mainly of TAGs with traces of other naturally occurring compounds like FFAs, metals, phospholipids, antioxidants, pigments, sterols, and proteins; some of these compounds reduce the oxidative stability and need to be removed, while

antioxidants should be retained to the greatest extent possible (Chen et al, 2011; Choe and Min, 2006). Refining applies degumming, neutralization, bleaching, and deodorization to remove as many pro-oxidants as possible and reduce their concentrations to low levels, but even small concentrations of pro-oxidants can still impact lipid oxidation.

Virgin pressed oils, such as the virgin coconut oil used in this study, do not undergo these refining steps and are instead usually cold-pressed and retain many of the minor components (both pro- and anti-oxidant) in the oil. Likely pro-oxidants from palm oil include beta-carotene and organic photosensitizers which oppose tocopherol activity (Meara and Weir, 1976; List and King, 2011).

1.2.3 Light

Ultraviolet (UV) light might not seem like an important factor when considering that food is typically prepared indoors, shielded from the sun. However, fluorescent lighting emits UV light and even a small doses can rapidly catalyze lipid oxidation, primarily by decomposing hydroperoxides to alkoxyl and hydroxyl radicals that greatly increase propagation rates. (Schaich, 2013):

$$\begin{matrix} \text{ROOH} \\ \text{HOOH} \\ \text{LOOH} \end{matrix} \xrightarrow{h\nu\ (uv)} \begin{matrix} \text{RO}^{\bullet} + {}^{\bullet}\text{OH} \\ \text{HO}^{\bullet} + {}^{\bullet}\text{OH} \\ \text{LO}^{\bullet} + {}^{\bullet}\text{OH} \end{matrix} \xrightarrow{2\,L_2H} \begin{matrix} \text{ROH} + H_2O \\ 2\,L_2^{\bullet} + 2\,H_2O \\ \text{LOH} + H_2O \end{matrix} \qquad (9)$$

where (ROOH is any organic hydroperoxide, HOOH is hydrogen peroxide, LOOH is a lipid hydroperoxide)

Transparent plastic containers without UV filters allow penetration of light, which increases oxidation. Functional groups that absorb UV light are carbonyls, double bonds, and peroxide bonds. Light effects on the rate of oxidation diminishes as temperature

increases and thermal degradation of hydroperoxides becomes more competitive (Velasco and Dobarganes, 2002).

Visible light at longer wavelengths does not have sufficient energy to initiate oxidation by producing radicals directly, but it does initiate oxidation indirectly through photosensitizers which transform the light energy into chemical energy (Schaich et al, 2013). In photosensitization, visible light excites sensitizers into higher energy states which then transfer energy either to a) bonds of lipids forming free radicals directly, Type 1, or b) oxygen to form singlet oxygen, Type 2, which adds to lipid double bonds to generate hydroperoxides without radicals (Figure 3) (Foote, 1976; Murray, 1979; Schaich et al, 2013). Common photosensitizers in foods are chlorophyll, food dyes, and hemes.

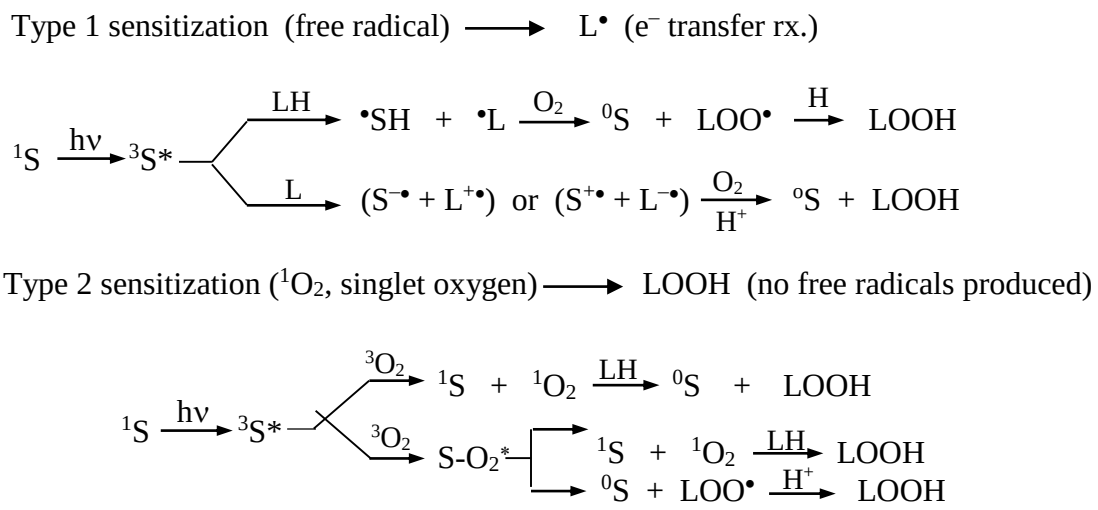


Figure 3. Photosensitization processes that initiate lipid oxidation; (Schaich et al, 2013).

1.2.4 Heat

Temperature is a critical factor in maintaining the oxidative stability of lipids. Even relatively small increases in temperatures can decrease shelf-life so temperature control at each stage of production must be addressed to reduce the impact of lipid oxidation on food products (Schaich et al, 2013). Heat affects lipid oxidation in three major ways: (1) Heat at low temperatures increases the rate of LOOH decomposition and initiation of secondary chains (Labuza, 1971; Marcuse and Fredriksson, 1968; Schaich et al, 2013). (2) Heat changes dominant mechanisms and resulting products (Schaich et al, 2013). (3) Heat at high (frying) temperatures induces thermal scissions of acyl chains creating radicals that add oxygen and can initiate autoxidation under air (Nawar, 1969, 1986; Schaich et al, 2013).

At the high temperatures endured during baking, cooking, and frying, the dominant degradation products generated are free fatty acids, short-chain alcohols, aldehydes, ketones, acids, hydrocarbons, mono- and di-glycerides, as well as cyclic and epoxy

compounds which result from hydrolysis, oxidation, isomerization and polymerization. All of these reactions and compounds affect each other and influence the dominant resulting products and mechanisms (Choe and Min, 2007; Velasco et al, 2009; Zhang et al, 2012; Pokorny, 1989; Rojo and Perkins, 1987). The interplay between thermal scissions and oxidation chains creates very complex reaction pathways which are not fully understood making it difficult to predict exactly what products will form (Zhang et al, 2012); this is compounded by the fact that each fatty acid and each type of oil is different and can be difficult to compare. Mechanisms of degradation of the oils while cooking differ depending on the oils or blends of oils used, the conditions of cooking, and other foods (i.e. high moisture, high protein) that may be present and cause other interactions or reactions during cooking.

Hydrolysis occurs most commonly when water is present when oils are heated. Water molecules react with the ester bonds of TAGs and produce FFAs, mono- and di-glycerides, and glycerol molecules; FFAs increase over the time the oil heated and are typically monitored in frying operations to determine how degraded the oil has become and whether or not the oil is still acceptable for cooking; FFA levels >1% are typically considered unacceptable in frying operations (Choe and Min, 2007; Chung et al, 2004). Hydrolysis is also favored greatly in the presence of oils with shorter chain fatty acids – short chain fatty acids increase water accessibility to glycerol ester bonds in triacylglycerols and they also have greater solubility than LCFAs in water (Nawar, 1969; Choe and Min, 2007). As FFAs are produced, they actually help to accelerate the additional hydrolysis within the oil, causing further degradation (Frega et al, 1999; Choe and Min, 2007).

Oxidation chain reactions (described in Section 2.1) increase their rates at elevated temperatures due to the increased energy that enhances reactions, including bond scission (Choe and Min, 2007; Min and Boff, 2002). Volatile and non-volatile termination products both collect in the system; when large amounts of water are present in the heated oil, fewer volatiles are retained in the system and are generally evolved from the oil from evaporation processes, though this is dependent on the type of oil and cooking conditions (Choe and Min, 2007; Wu and Chen, 1992). These termination products contribute greatly to the flavors and odors during cooking as well as contribute to off-flavors detected by consumers.

TAGs polymerize under heated conditions with or without oxygen, creating non-polar (-C-C-C- linkages) and polar polymers (-C-O-C- or –C-O-O-C- linkages) respectively through radical reactions (Zhang et al, 2012). The types of polymers (i.e. polar, non-polar, cyclic, etc.) created depend on the heating conditions and the type of oil used (Choe and Min, 2007; Zhang et al, 2012). Non-polar acyclic polymers are formed by the recombinations and double bond additions of allylic carbon-centered radicals (

Figure 4) (Choe and Min, 2007). Polar polymers are formed when TAGs react with oxygen and form either alkyl hydroperoxides or dialkyl peroxides which decompose to alkoxyl and peroxyl radicals; these radicals combine to produce dimers and eventually polymers (Figure 5) (Choe and Min, 2007). Polymers create problems with increased viscosity and darkened color in cooking oils (Tseng et al, 1996; Zhang et al, 2012). Under heated conditions over time, polymers are an inevitable degradation product.

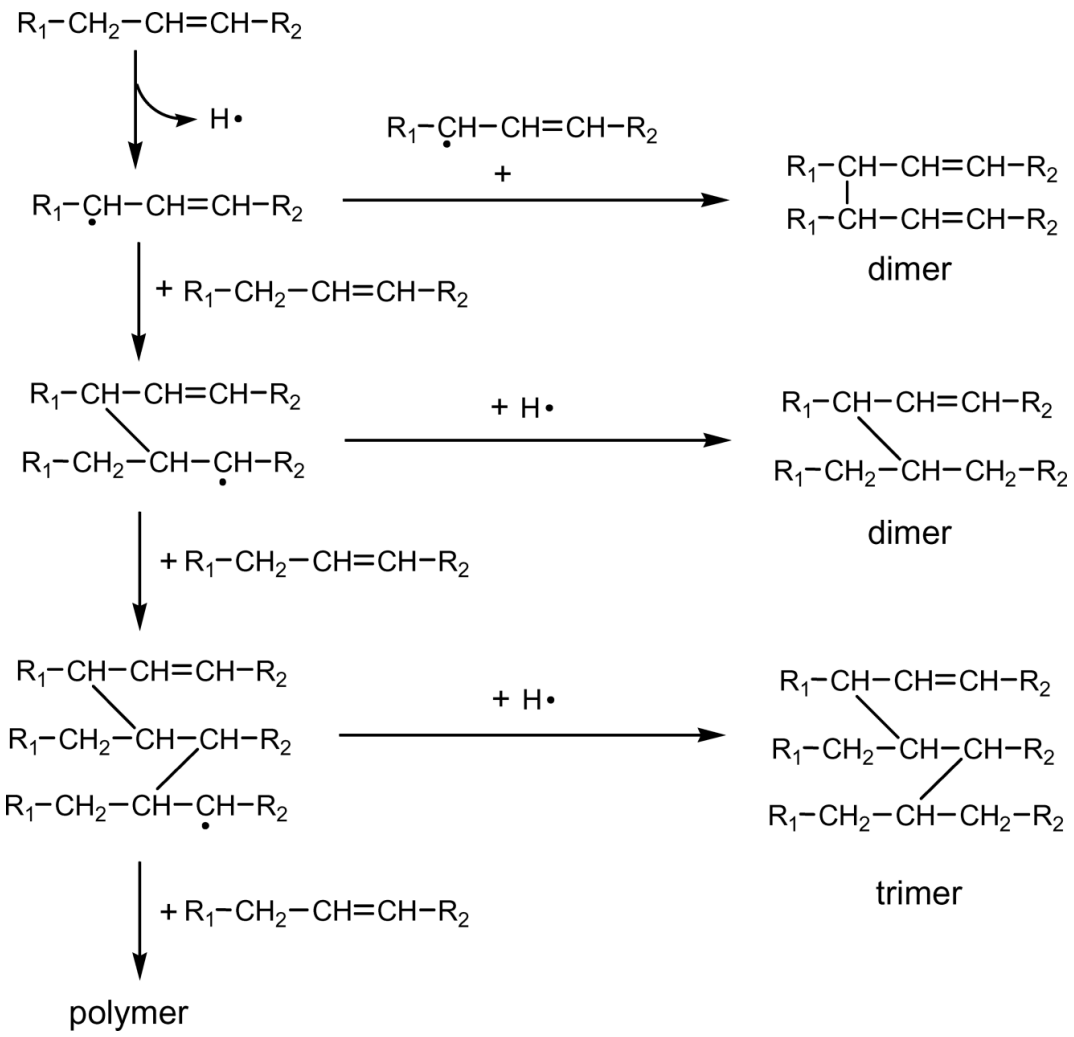
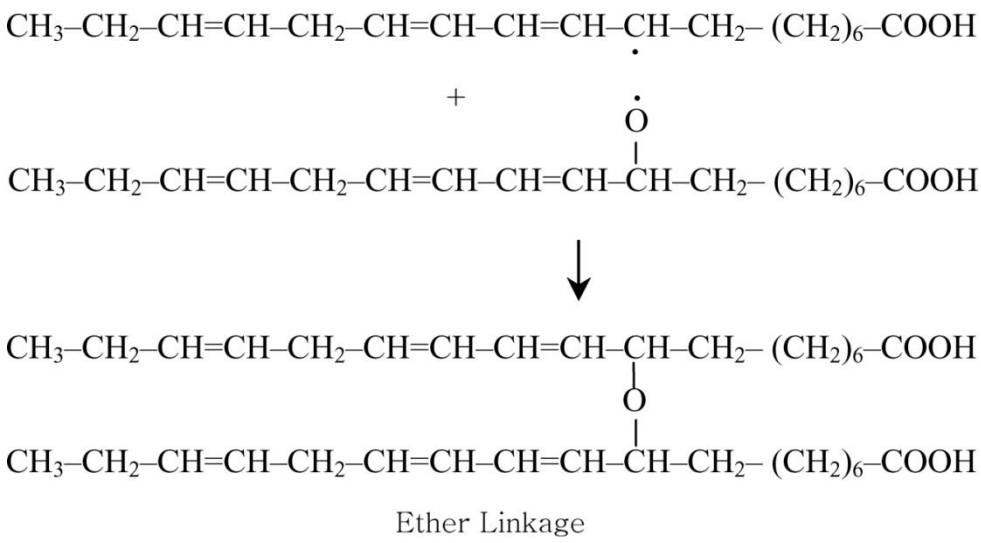


Figure 4. Polymer formation from oleic acid; (Choe and Min, 2007).



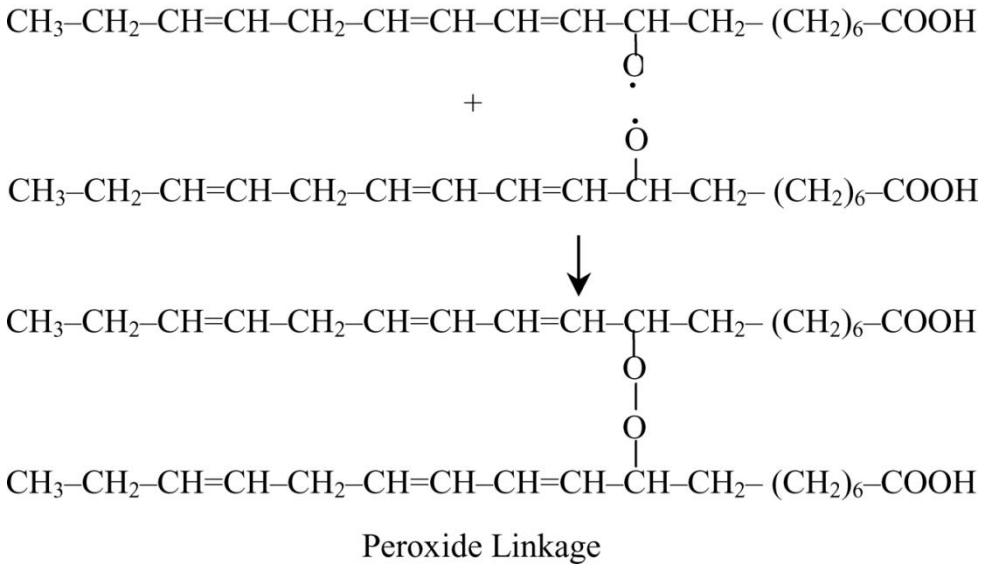


Figure 5. Formation of ether or peroxide linkages during polymerization of oils; (Choe and Min, 2007).

2.2.5 Metals

Transition metals are perhaps the most important and active catalysts of lipid oxidation in foods. Higher valence state metals (oxidants) react with lipids to form alkyl

radicals directly (Reaction 10), while lower valence state metals (reducing agents) reduce oxygen to hydroperoxyl (Reaction 11) or superoxide (Reaction 12) radicals or mediate lipid hydroperoxide decomposition to radicals (Reaction 13) (Schaich, 2005; Choe and Min, 2006).

Direct $RCH{=}CHR + M^{(n+1)+} \longrightarrow RCH{-}CHR + M^{n+} \longrightarrow L^{\bullet} + RH$ (10)

Indirect a) formation of active metal-oxygen complexes

$$M^{n+} + O_2 \longrightarrow [M^{(n+1)+}\ldots O_2^{-\bullet}] \xrightarrow{LH} L^{\bullet} + M^{n+} + HO_2^{\bullet} \quad (11)$$

$$HO_2^{\bullet} \xrightarrow{L'H} L'^{\bullet} + H_2O_2$$

b) autoxidation of reduced metals (active at moderate to high pO_2)

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^{-\bullet} \underset{}{\overset{H}{\rightleftharpoons}} HOO^{\bullet} \xrightarrow{L} L^{\bullet} + H_2O_2$$

$$O_2^{-\bullet} + HOO^{\bullet} \longrightarrow H_2O_2 \xrightarrow{Fe^{2+}} Fe^{3+} + HO^{-} + {}^{\bullet}OH \xrightarrow{LH} H_2O + L^{\bullet} \quad (12)$$

c) reduction or oxidation of hydroperoxides (ROOH, H_2O_2, LOOH)

(dominates with low metal, substrate, and oxygen concentrations)

$$ROOH + M^{n+} \xrightarrow{fast} RO^{\bullet} + {}^{-}OH \quad (13)$$

$$ROOH + M^{(n+1)+} \xrightarrow{slow} ROO^{\bullet} + H^{+} \quad (14)$$

1.2.6 Antioxidants

Antioxidants are used to limit oxidation by preventing initiation (metal chelators and singlet oxygen quenchers), quenching radicals (phenols and other compounds that transfer electrons or hydrogen atoms to radicals), and controlling environmental factors such as dark, cold, and low water that slow oxidation kinetics (Schaich et al, 2013). A combination of these antioxidant techniques is usually much more effective in reducing oxidation in food systems than any one factor alone (Frankel, 1994, 1996).

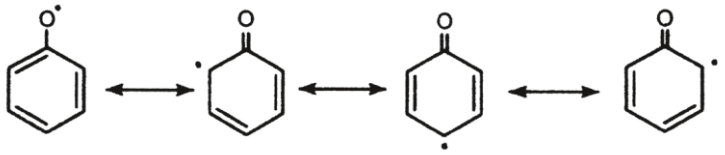
All edible oils contain some naturally-occurring antioxidants such as tocopherols, tocotrienols, carotenoids, phenolic compounds, and sterols (Choe and Min, 2006) that quench radicals by one of several mechanisms, including hydrogen atom transfer (15), single electron transfer (Reaction 16), and radical recombinations (Reaction 17) (Schaich et al, 2013) where A designates an antioxidant compound:

$$ROO\bullet + AH \longrightarrow ROOH + A\bullet \quad (15)$$

$$ROO\bullet + A \longrightarrow ROO^{+} + A^{-} \quad (16)$$

$$ROO\bullet + AH_2 \longrightarrow ROOH + AH\bullet \xrightarrow{R\bullet} RA \text{ or } ROOA + H^{+} \quad (17)$$

Reaction 15 suggests that any hydrogen donor may act as an antioxidant, but this is not the case. Antioxidant radicals must be stable and unreactive so as to not propagate radical chains themselves. This requirement is met primarily by phenolic compounds in which low O–H bond energy of the phenol facilitates abstraction of the H by lipid radicals. The resulting free electron then delocalizes from the phenolic oxygen over the entire aromatic ring (Borg and Schaich, 1989; Decker, 2002; Nawar, 1996), greatly reducing its reactivity and creating a stable resonance structure (Reaction 18):



(18)

Low reactivity of antioxidant radicals is only relative, however, and is not total absence of reactivity. Paradoxically, at high concentrations, antioxidant radicals even with low reactivity become competitive by mass action and convert to pro-oxidants (Borg and Schaich, 1989). Therefore, concentrations of antioxidants in oils, whether naturally present or added, are critical factors in oil stabilization. Too little antioxidant to handle the radical load will fail to inhibit lipid oxidation while excess antioxidant can actually catalyze the

degradation (Schaich et al, 2013; Bakir, et al, 2013; Chapman, T.M. et al, 2009; Naumov and Vasil'ev, 2003). If the radical load is too high, no amount of antioxidant will stabilize the system (Schaich et al, 2013).

The radical quenching effectiveness of phenolic antioxidants is partially dependent on the presence and concentrations of other added antioxidants (Considine, 1982). For example, it has been found that ascorbic acid is less effective as an antioxidant, and can act as a pro-oxidant in the presence of tocopherols. Here, high ascorbic acid concentrations donate H-atoms to tocopherol molecules to regenerate them, an antioxidant action, but in the process form reactive ascorbyl radicals (Scarborough, 1949). With the addition of higher concentrations of tocopherols, these antioxidants act more synergistically (Scarborough, 1949).

Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are synthetic phenols that have long been industry standards for reducing oxidation (Shahidi and Wanasandara, 2005). However, recognition of their toxicity at high levels, and consumer pressure as well, are forcing the food industry to reduce use of these antioxidants and whenever possible replace them with natural compounds. Tocopherols are the major phenols that occur naturally in vegetable oils such as canola, sunflower and palm oils, and they are also the major natural antioxidant added to foods. α-Tocopherol (vitamin E) and its congener structures are shown in

. α-Tocopherol has the highest biological activity *in vivo* while δ-tocopherol has the highest antioxidant activity in foods (Schaich et al, 2013). Because tocopherols are lipid-soluble, unlike most antioxidants from natural materials, they are the most important antioxidants in oils.

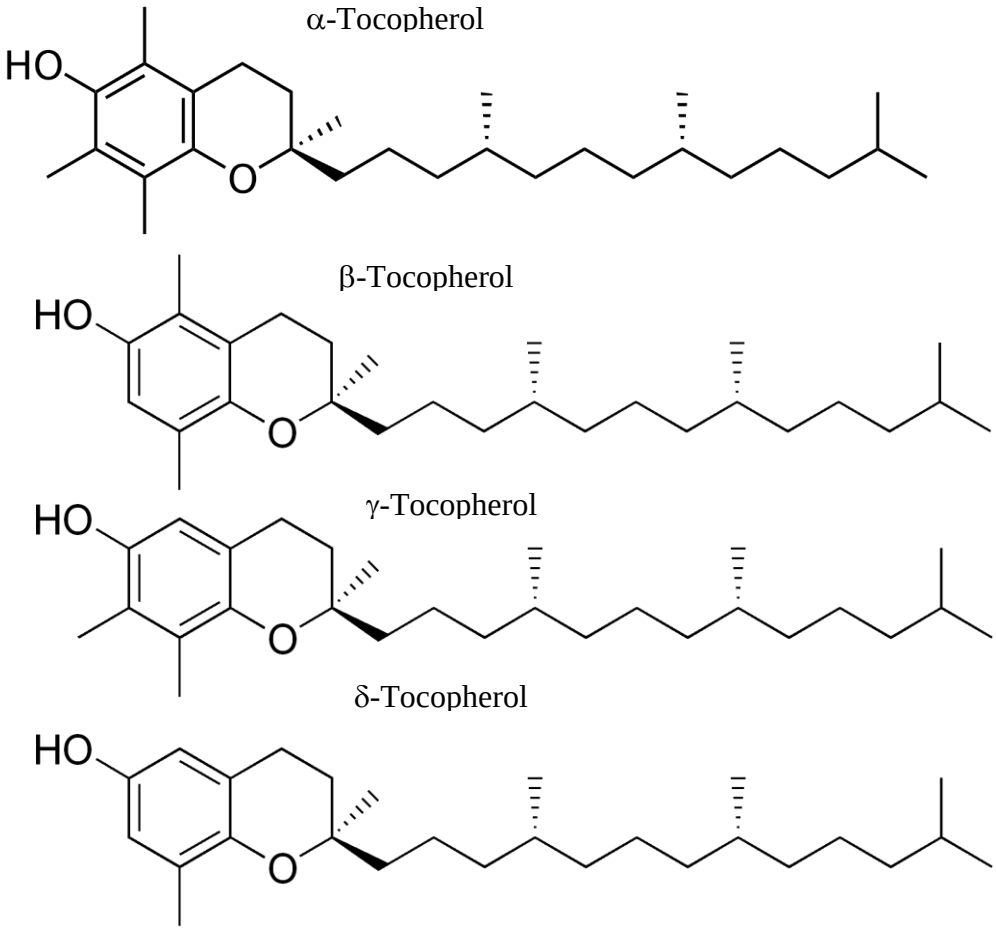


Figure 6. Structures of tocopherol isomers.

Antioxidant compounds other than phenols may also be incorporated into edible oils or packaging material to work in synergy with primary antioxidants (radical scavengers) and further slow oxidation. In particular, citric acid is routinely added to refined oils to complex metals such as iron and copper ions that are not removed during bleaching or are picked up during processing (Shahidi and Wanasandara, 2005).

2.3 Oils used in formulation of margarine-like Blends

Several oils were tested as blend components in this study, the most important of which were coconut oil, palm oil, high oleic sunflower oil, and canola oil. Preliminary tests showed that canola oil, while desirable for adding essential fatty acids, had to be limited due to rapid oxidation and destabilization of the Blends.

2.3.1 Coconut oil as an ingredient

Interest in coconut oil as a food ingredient is growing based on current research identifying its healthful qualities as well as its potential for replacing trans fats in food products.

2.3.1.1 Health implications of fatty acid compositions in Blends

Coconut oil, a tropical fat extracted from the coconut kernel grown primarily in the Philippines, Indonesia, and other tropical climates, is comprised of approximately 90% saturated fats, 9% monounsaturated fats, and 1% polyunsaturated fats (Considine, 1982). The fatty acid profile is shown in Table 1. Over half the saturated fats in coconut oil are medium chain fatty acids (MCFAs) that contain six to twelve carbons in their acyl chains. Medium chain fatty acids are beneficial physiologically because they are absorbed directly from the stomach and are carried via the portal vein to the liver where they are metabolized

directly for energy (Papamandjaris et al, 1998; Nagao and Yanagita, 2010). They are also are reportedly effective in weight management because they activate lipases in the body, which leads to deconstruction and mobilization of triacylglycerols in adipose tissue (Nagao and Yanagita, 2010). MCFAs have been used as dietary supplements since the 1950s for people suffering from malabsorption syndrome due to their ability to be utilized quickly in the body for energy (Babayan, 1987; Hashim and Tantibhedyangkul, 1987; Papamandjaris et al, 1998; Nagao and Yanagita, 2010). MCFAs also preserve insulin sensitivity, by raising levels of adiponectin (an adipose-specific secretory protein that enhances insulin sensitivity) in the blood (Nagao and Yanagita, 2010; Takeuchi et al, 2006; Okamoto et al, 2006). It also increases "good" HDL cholesterol and bile production, leading to lower total blood lipids (Nagao and Yanagita, 2010; Bourque, 2003; Arunima, 2012; Clegg, 2010). These behaviors have generated considerable interest in using MCFAs in coconut oil to combat obesity, diabetes, and related diseases (Nagao and Yanagita, 2010).

Table 1: Fatty acid profiles of source fats used in formulating Blends. The highlighted fatty acids are MCFAs. Sources: USDA Nutrient Database, estimations from suppliers, and (Koushki et al, 2015).

Fatty Acids	Formula	Coconut Oil	Palm Oil	H.O. Sunflower Oil	Canola Oil
Butyric	C 4:0	N/A	N/A	N/A	N/A
Caproic	C 6:0	N/A	N/A	N/A	N/A
Caprylic	C 8:0	5-9%	N/A	N/A	N/A
Capric	C 10:0	6-10%	N/A	N/A	N/A
Lauric	C 12:0	44-52%	0.1-0.5%	N/A	N/A
Myristic	C 14:0	13-19%	0.9-1.4%	N/A	0-0.2%
Palmitic	C 16:0	8-11%	37.9-41.7%	4.37%	2.5-7%
Palmitoleic	C 16:1	0-2.5%	0.1-0.4%	N/A	0-0.6%
Stearic	C 18:0	1-3%	4.0-4.8%	3.40%	0.8-3.0%
Oleic	C 18:1	5-8%	40.7-43.9%	80.07%	51-70%
Linoleic	C 18:2	0-1%	10.4-13.4%	10.96%	15-30%
Linolenic	C 18:3	N/A	0.1-0.6%	0.05%	5-14%
Arachidic	C 20:0	0-0.5%	0.2-0.5%	N/A	0.2-1.2%
Eicosenoic	C 20:1	N/A	N/A	N/A	0.1-4.3%
Behenic	C 22:0	N/A	N/A	N/A	<0.6%
Erucic	C 22:1	N/A	N/A	N/A	<7.0%

Lignocervic	C 24:0	N/A	N/A	N/A	N/A

Table 2. Tocopherol contents in coconut, canola, palm, and sunflower oils (Shahidi and Ambigaipalan, 2015). N/A=data not available.

Oil	**Tocopherol (mg/kg)**				**Tocotrienol (mg/kg)**			
	α	**β**	**γ**	**δ**	**α**	**β**	**γ**	**δ**
Coconut	5-10	N/A	5	5	5	trace	1-20	N/A
Canola	180-280	N/A	380	10-20	N/A	N/A	N/A	N/A
Palm	180-260	trace	320	70	120-150	20-40	260-300	70
Sunflower	350-700	N/A	10-50	1-10	N/A	N/A	N/A	N/A

That MCFAs are still saturated fats (SFAs) has led to numerous claims that they are unhealthy, including one from the American Heart Association which recommends limiting saturated fats in the diet, regardless of source. As noted in the Introduction, for years saturated fats were demonized and blamed for strokes, atherosclerosis, cardiovascular disease, high cholesterol, and elevated total blood lipids (Dias et al, 2014; Micha and Mozaffarian, 2010). However, a recent critical review of studies that examined links between dietary SFAs and disease risks found no clear association between SFA consumption and increased risk of cardiovascular disease, insulin resistance, diabetes, or stroke (Micha and Mozaffarian, 2010). Another critical review of epidemiological studies found that SFAs only increase total blood lipids and risk of cardiovascular diseases in diets deficient in essential fatty acids (Dias et al, 2014).

2.3.1.2 Thermal stability of coconut oil

Thermal degradation occurs as heat-induced scissions of acyl chains generate -C• free radicals, some of which recombine into dimers and polymers and some of which oxidize to volatile compounds that continuously evolve from heated oils (Nawar, 1969; Tian, 2013). Cooking applications are only suitable at temperatures below the smoke point for each specific oil, or perhaps even lower to prevent the formation of the toxic aldehydes acrolein and acetaldehyde in the cooking oils (Fullana et al, 2004b; Zhu et al, 2001; Umano and Shibamoto, 1987). Smoke points are the temperature at which thermal degradation of fats and oils begins, as evidenced by release of thin wispy smoke from heated oils (Belitz et al, 2009). Smoke points decrease with saturation (lower bond energy in C-C bonds) and as an oil is successively heated (Man and Hussin, 1998).

The smoke point of refined coconut oil is 163°C (Man and Hussin, 1998), relatively low compared to other vegetable oils and lower than deep frying temperatures for most materials. Thus, usefulness of coconut oil for high temperature frying, even in combination with other oils, should not be expected. Coconut oil itself has long been used as a solid fat in baked products ($T \leq 100°C$), but heating applications at baking and sautéing temperatures for blended products containing coconut oil still need to be tested.

Above the smoke point (180°C), coconut oil breaks down into mainly alkanals, with pentanal maintaining the highest levels followed by heptanal and then butanal as temperature increases (Katragadda et al, 2010). Alkenals and alkadienals are also generated, with 2-heptenal and 2,4-heptadienal predominating, respectively. Low enal levels are to be expected since coconut oil has very low proportions of unsaturated fatty acid precursors for these products.

2.3.1.3 Endogenous antioxidant compounds

Coconut oil contains significant levels of tocopherols, tocotrienols, phytosterols, and phenolic acids that all contribute to antioxidant activity (2.3.2 Palm Oil

Palm oil for food products is derived from the oil palm grown primarily in tropical countries located in Africa, South America, Southeast Asia, and the South Pacific (Koushki et al, 2015). The nearly 1:1 ratio of saturated to unsaturated fatty acids in this oil give it semi-solid properties that are ideal for products such as margarines and shortening (Koushki et al, 2015; Sambanthamurthi et al, 2000) This oil has a deep red color thought to be caused by high levels of carotenoids (Chen et al, 2011; Koushki et al, 2015). Palm oil, even after refining, retains high levels of endogenous tocopherols, 100-150 ppm, and tocotrienols, 620-650 ppm, (Chen et al, 2011; Choe and Min, 2009; Jung, 1989). Palm oil also has a considerable amount of chlorophyll, a potent photosensitizer that induces radical oxidation when exposed to light (Sambanthamurthi et al, 2000; Koushki et al, 2015).

Table 3) (Appaiah et al, 2014). Virgin coconut oil has higher contents of these important compounds than traditionally processed and refined coconut oil (Marina et al, 2009a,b).

2.3.2 Palm Oil

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Table 3. Antioxidant content of coconut oil (CO) (Appaiah et al, 2014).

Antioxidants	**Processing**	
	Refined CO	**Virgin CO**
Total phytosterols (mg/100 g)	33.31 ± 2.1	50.27 ± 1.5
Total Phenolics Content (mg/100 g)	1.1 ± 0.11	0.7 ± 0.02
Phenolic acids (µg/100 g)		
Gallic acid	30.3 ± 1.1	103.9 ± 2.2
Hydroxybenzoic acid	94.1 ± 0.9	127.4 ± 1.7
Vanillic acid	ND	ND
Syringic acid	ND	ND
Coumaric acid	11.2 ± 1.1	48.9 ± 0.0
Caffeic acid	4.9 ± 0.3	ND
Ferulic acid	ND	5.4 ± 0.4
Cinnamic acid	1.3 ± 0.6	9.8 ± 0.3
Total	141.8	291.4
Tocopherols (mg/100 g)		
α-Tocopherol	ND	3.6 ± 0.0
β + γ-Tocopherol	3.7 ± 0.0	ND
δ-Tocopherol	ND	ND
α-Tocotrienol	2.1 ± 0.2	0.1 ± 0.0
β + γ-Tocotrienol	0.9 ± 0.1	0.4 ± 0.0
δ-Tocotrienol	ND	0.3 ± 0.1
Total (Tocopherol + Tocotrienol) mg/100 g	6.7 ± 0.5	4.4 ± 0.8

2.3.3 Canola Oil

Canola oil is derived from rapeseeds bred for low erucic acid (22:1) which causes fatty deposits in the heart, muscles, and adrenal glands of humans, and for low glucosinate which can interfere with iodine uptake (Przybylski et al, 2005). Canola oil has a very low saturated fatty acid content and high proportions of unsaturated fatty acids. It is a particularly good source of essential omega-6 and omega-3 fatty acids. Linolenic acid (18:3) is present in high enough concentrations that it becomes the biggest factor limiting the oxidative stability of this oil. High amounts of chlorophyll also catalyze rapid photo-oxidation. These two factors together account for rapid breakdown of canola oil under light, during storage, and in high temperature applications (Przybylski et al, 2005). A high smoke point between 220-230°C characterizes canola oil as suitable for deep fat frying (180°C), but the rapid oxidation eliminates its use in frying. Although canola oil is used in some low-heat cooking applications, it is used preferentially in salad dressings, mayonnaises, and margarines (to boost EFA content) (Przybylski et al, 2005). Typical oxidation products of canola oil include alkanals (predominantly nonanal), alkenals (predominantly 2-heptenal), and alkadienals (predominantly 2,4-heptadienal) (Katragadda et al, 2010).

2.3.4 High Oleic Sunflower

Traditional sunflower oil has a typical fatty acid distribution as follows: ~12% SFA (palmitic and stearic), 66-72% PUFA (linoleic), and 16-20% MUFA (oleic) (Grompone, 2005). However, concern for the low oxidative stability and formation of potentially toxic products increasing health risks, sunflowers for oil seeds have been genetically modified for 80-90% oleic acid and greatly reduced PUFAs (Grompone, 2005). High levels of oleic acid make this oil good for not only salad dressings and spreadable margarines, but also

for cooking and frying due to its enhanced ability to resist thermal degradation. In addition, this oil can maintain a long shelf life without the need for even brush hydrogenation (Grompone, 2005).

2.4 Margarine-type Emulsions

Margarines and margarine-type products are water in oil (W/O) emulsions formed by dispersing the water phase and its components as tiny droplets in the oil phase (Schaich, 2011). The dispersed water droplets are stabilized with the addition of emulsifiers (amphiphilic molecules such as lecithin and mono- or di- glycerides) situated on the surface of the droplets to prevent re-association and increase interactions with the oil phase. In W/O emulsions, catalysts are carried most often in the water phase but they can also migrate into the oil phase or interact at the O/W interface. Antioxidants can be carried in either phase, depending on solubility.

2.4.1 Lipid oxidation in emulsions

Lipid oxidation in bulk oils has been extensively studied since the discovery of lipid oxidation as a radical chain reaction (Frankel, 1994, 2012), and most of our knowledge about lipid oxidation was derived from such studies. However, behavior in totally hydrophobic oils is not always representative of oxidation in emulsions where the surface area is very large and the system is non-homogeneous. Lipid oxidation in emulsions depends on many of the same factors as in bulk oils (structure of lipids, variety and concentration of anti- and pro-oxidants, temperature, quality of ingredients, oxygen concentration) but now major influences are the location of ingredients and interfacial characteristics which drive the interactions within the matrix (McClements and Weiss,

2005; Mei et al, 1998; Coupland, 1996). One key difference between emulsions and bulk oils is that radical scission reactions reportedly increase immensely in the presence of water, allowing secondary lipid oxidation products to accumulate faster than in bulk oils (Frankel, 1994). The sensory impact of off-flavor and off-odor compounds are amplified in emulsion systems where water facilitates flavor release so secondary oxidation products such as aldehydes are tasted at concentrations orders of magnitude lower than in bulk oils (Druaux and Voilley, 1997; Haahr, 2000). In addition, flavor perception varies drastically with the food system (Jacobsen, 1999). Both of these behaviors make the accumulation of secondary oxidation products in emulsions even more troublesome for shelf life and stability of food products. Therefore, it is even more important to apply appropriate interventions to prevent lipid oxidation when formulating emulsion products.

2.4.2 Oxidative stabilization of emulsions

There are several strategies to effectively reduce lipid oxidation in emulsions. Obviously, catalyst contamination of both water and oil phases must be avoided or severely limited. Addition of both hydrophilic and hydrophobic antioxidants, inclusion of metal chelators, controlling oxygen in the two phases and in the atmosphere during processing, and use of oxygen-impermeable packaging are all important considerations (Coupland, 1996; Mosca et al, 2013; Frankel, 1994, 1996). Also, the order of addition of ingredients can substantially reduce oxidation occurring at the interface. For example, incorporating antioxidants into the emulsifier before dissolving the emulsifier in oil positions the antioxidants in the interfacial layer and provides more effective scavenging of radicals in both phases (Coupland, 1996). Creative combination of these strategies is critical for maximum extension of shelf life in emulsion food products.

2.5 Nutritional quality and health effects of degraded lipids

Common oxidation compounds that are produced when edible oils are heated or oxidized include aldehydes, ketones, alcohols, epoxides, and acids which produce off-flavors and off-odors, reduce the nutritional quality of foods by loss of essential fatty acids and co-oxidation of other important food components, and cause detrimental health effects from inhalation and ingestion, including lung cancer and other mutagenic effects (Fullana, 2004a; Chiang et al, 1997; Qu et al, 1992). Aldehydes are major volatile products during cooking and can have an inherent toxicity, carcinogenic and mutagenic effects (Chiang et al, 1997; Qu et al, 1992); they also react with amino groups on proteins causing damage to foods and biological tissues (Fullana, 2004a; Fullana, 2004b; Schaich, 2008). Acrolein is a known carcinogenic aldehyde resulting from high temperature oxidation of the glycerol backbone from triacylglycerols. Very high levels of aldehydes can be released from oils during cooking (Fullana et al, 2004b; Zhu et al, 2001; Umano and Shibamoto, 1987), and inhaling these (e.g. in fast food operations) has been shown to cause lung cancer and exacerbate respiratory problems (Chiang et al, 1997; Teschke et al, 1989; Gere, 1982).

Repeated use of frying fats and oils is commonplace in commercial and food service industries to cut costs and avoid waste. However, this practice contributes to the temperature abuse of oils and results in consumption of degraded oils (Fullana et al, 2004b; Hamsi et al, 2015). Thermally-abused oils are well-known to mediate negative health effects, including increased blood pressure, athlerosclerosis and inflammation (Hamsi et al, 2015; Leong et al, 2009; Adam et al, 2009).

Hence, potential health benefits of fats and oils, including natural antioxidants, essential fatty acids and other healthy fatty acids, are destroyed after thermal and oxidative degradation. This needs to be taken into consideration when designing and formulating any product claiming health benefits from fats.

3. RESEARCH OBJECTIVES

Objectives of this research were to

1. investigate thermal and oxidative degradation processes of tropical fat-based margarine-type blends,
2. determine which analytical methods and analyses are necessary to properly assess lipid oxidation in unique oil blends,
3. determine useful lifetime and appropriate heating applications of blends, and
4. recommend formulation improvements to extend shelf life, increase thermal stability, and improve sensory qualities.

4. EXPERIMENTAL DESIGN, MATERIALS, AND METHODS

4.1 Overall Experimental Design

This study was undertaken in collaboration with a small entrepreneurial company to create a shelf and heat stable margarine and baking product based on tropical fats that are being clinically tested for enhanced fat metabolism in treatment and prevention of childhood obesity. Since the product is being fed to children, it is important to ensure that the product is thermally and oxidatively stable while still maintaining healthy lipids (essential fatty acids) in typical cooking applications. Heat, in particular, is a problem because it rapidly degrades the nutritional and functional quality of these fats (Schaich et al, 2013).

The industry collaborator had already marketed a margarine product created for use as a table spread, but desired additional products tailored for cooking applications.. Formulated with coconut oil and high levels of polyunsaturated fats, the original spread provided the “gold standard” mouth feel and taste for test new margarine and baking fat blends in this project.

This study was dynamic, so as each part of the study was completed, the data was analyzed and interpreted, then recommendations were made regarding changes to formulation and analyses needed to track lipid degradation accurately. Each part of the study influenced the samples and analyses in the next part. Over the course of this study, several formulations of the tropical fats blended with unsaturated oils content and other added ingredients were tested.

This study was executed in three parts:

(1) Initial testing: Initial studies compared thermal stability of three test blends with

different types and levels of unsaturated oils against the current commercialized product. One specific goal was to determine which polyunsaturated oils could be added to the tropical fats without compromising thermal and oxidative stability of the blends, as well as what levels of polyunsaturated components could be tolerated.

(2) Phase I: Use data from the initial study to reformulate blends for maximum stability. Perform long-term shelf life and short-term thermal degradation studies to assess and quantitate blend stability.

(3) Phase II: Further refine stabilization of blends by testing effects of the quality (presumably metal content) of water used in the blends and the level of antioxidant (mixed tocopherols) added. Water and antioxidant effects were evaluated in shelf-life and thermal studies similar to those used in Phase I.

4.2 Materials

4.2.1 Sample blend compositions

All margarine blends were water in oil emulsions prepared and provided by an entrepreneurial company seeking to create a healthy margarine spread and cooking blend. Initial goals were to investigate whether more unsaturated oils could be added to the formulation already in use without compromising shelf life and whether these new blends would be sufficiently stable for cooking applications. Blends 1, 2, and 3 were formulated for comparison to a base blend for evaluation. The detailed formulations are proprietary, so for the purposes of this thesis, the blends will be identified only their approximate lipid components:

Base blend: Virgin coconut oil, palm shortening, high oleic sunflower oil, canola oil, flaxseed oil. Approximate weight ratios 5:5:5:4:1.

Test Blend 1: Virgin coconut oil, palm shortening, high oleic sunflower oil, flaxseed oil. Approximate weight ratios 9.5:9:8.5:1

Test Blend 2: Virgin coconut oil, high oleic sunflower oil, palm shortening, flaxseed oil. Approximate weight ratios 10.5:10:9:1

Test Blend 3: Virgin coconut oil, high oleic sunflower oil, palm shortening, flaxseed oil. Approximate weight ratios 11.5:9:10:1

The evolution of these samples over the course of this study are shown in Figure 7. Fatty acid compositions of each blend are presented in Table 4. All other components of the blends were identical.

In Initial Testing, Test Blend 3 was identified as having greatest thermal and oxidative stability. However, even greater stability was desired since the new product was being designed for baking, so Test Blend 3 was reformulated without flaxseed oil to increase resistance to heat (Test Blend 3G) for further study in Phase I. In addition, significant charring was observed during heating of all blends in Initial Testing (Figure 8), resulting from burning of the butter flavoring added to the product. Thus, butter flavor was replaced with ghee (clarified butter) in the Base Blend and in Test Blend 3G to impart a natural butter flavor without charring. Resulting fatty acid compositions of these two blends are presented in Table 5.

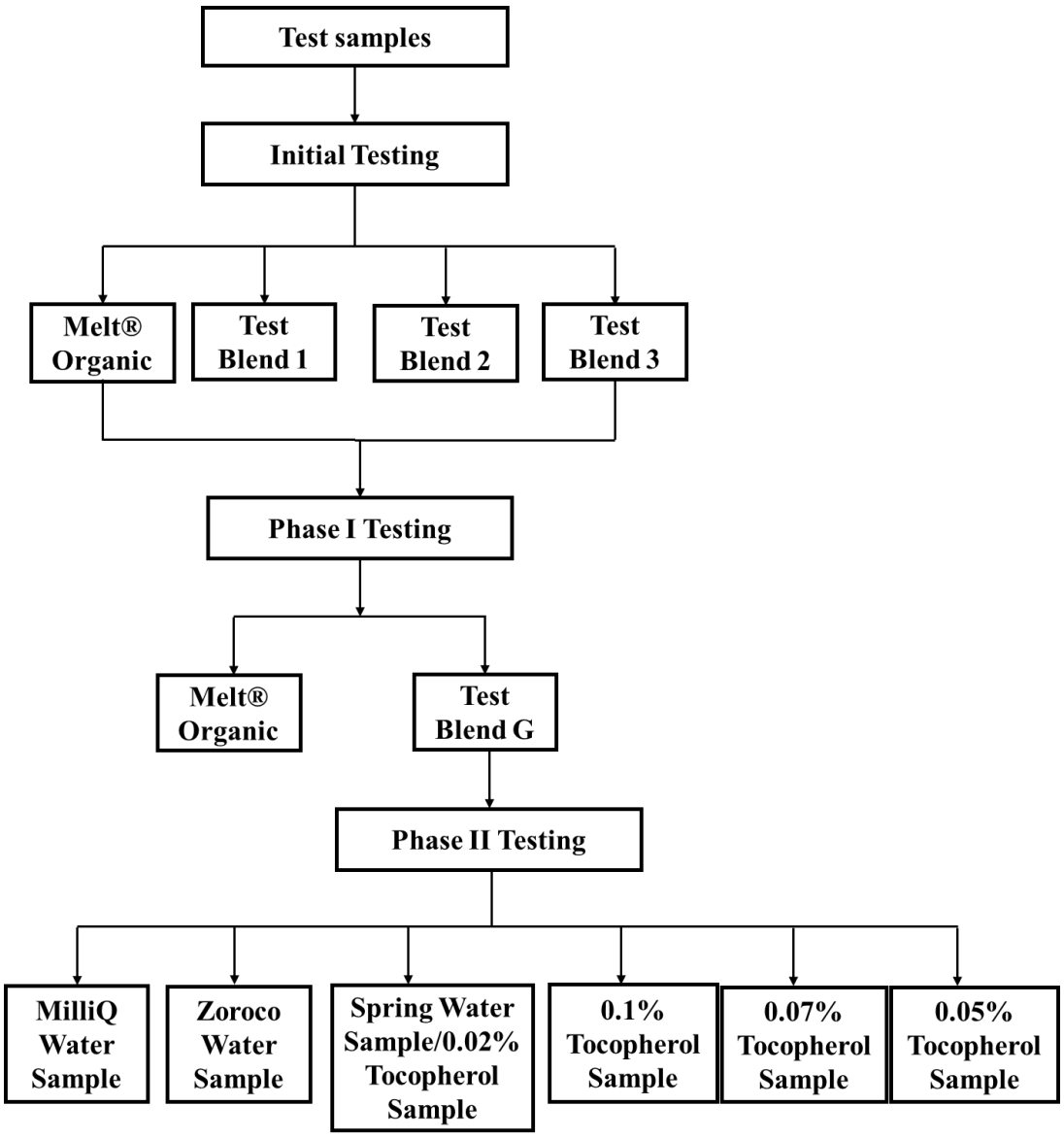


Figure 7. Scheme for testing blends over the course of the study.

Table 4. Fatty acid % composition of the initial test cooking blends.

		Base Blend		**Test Blend (1)**		**Test Blend (2)**		**Test Blend (3)**	
	Formula	Average	St dev	Average	St dev	Average	St dev	Average	St dev
SFA:PUFA:MUFA		41:15:44		43:18:39		50:15:35		54:09:37	
Short Chain Fatty Acids									
Butyric	C4:0	0.19	0.00	0.19	0.00	0.19	0.00	0.19	0.00
Medium Chain Fatty Acids									
Caproic	C6:0	0.22	0.10	0.22	0.11	0.24	0.12	0.27	0.15
Caprylic	C8:0	1.90	0.68	1.90	0.68	2.33	0.84	2.85	1.03
Capric	C10:0	1.93	0.65	1.93	0.65	2.35	0.81	2.85	1.00
Lauric	C12:0	12.79	1.56	12.79	1.56	15.82	1.94	19.33	2.36
Long Chain Fatty Acids									
Myristic	C14:0	4.97	1.18	4.95	1.16	6.03	1.44	7.20	1.70
Palmitic	C16:0	15.49	2.38	16.59	2.44	18.57	2.53	17.13	2.30
Palmitoleic	C16:1	0.33	0.27	0.26	0.20	0.31	0.25	0.34	0.28
Stearic	C18:0	3.77	1.54	4.42	1.57	4.27	1.63	4.11	1.65
Oleic	C18:1	42.31	5.14	38.17	4.68	35.98	4.11	36.40	3.92
Linoleic	C18:2	10.49	3.93	15.09	3.93	10.67	3.19	6.51	2.89
Linolenic	C18:3	5.05	1.33	2.98	0.41	2.95	0.37	2.90	0.33
Arachidic	C20:0	0.29	0.19	0.13	0.07	0.15	0.09	0.17	0.09
Eicosenoic	C20:1	0.56	0.52	0.05	0.04	0.05	0.04	0.08	0.05
Behenic	C22:0	0.10	0.03	0.00	0.00	0.00	0.00	0.00	0.00
Erucic	C22:1	0.83	0.83	0.03	0.03	0.03	0.03	0.04	0.04
Lignocervic	C24:0	0.05	0.05	0.05	0.05	0.05	0.05	0.06	0.06

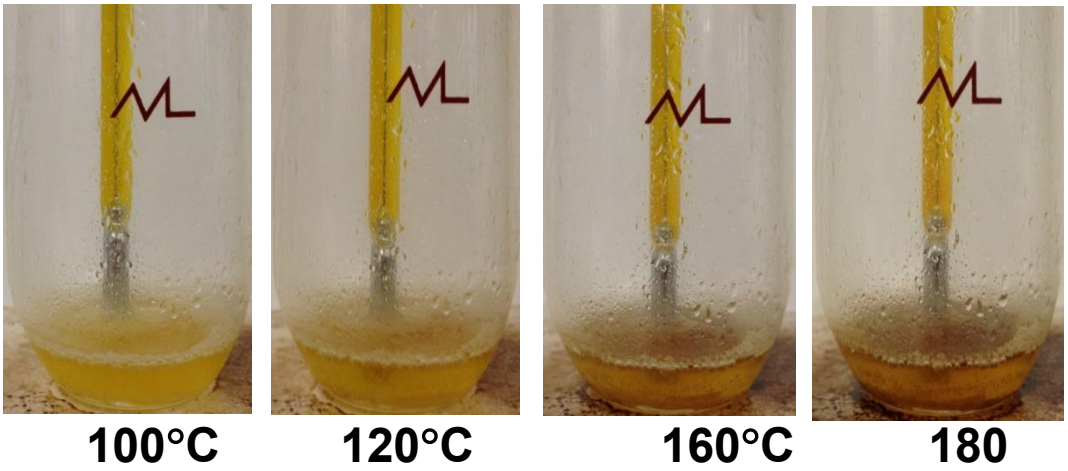


Figure 8. Charring of butter flavor in all initial blend formulations increased as temperature increased.

Table 5. Fatty acid composition of Base Blend and Test Blend 3G with ghee.

		Base Blend		**Test Blend (G)**	
Fatty Acids	Formula	Average (%)	±	Average (%)	±
Butyric	C 4:0	0.19	0	0.2	0
Caproic	C 6:0	0.22	0.1	0.27	0.14
Caprylic	C 8:0	1.9	0.68	2.66	0.96
Capric	C 10:0	1.93	0.65	2.67	0.92
Lauric	C 12:0	12.79	1.56	18.02	2.2
Myristic	C 14:0	4.97	1.18	6.78	1.61
Palmitic	C 16:0	15.49	2.38	17.6	2.28
Palmitoleic	C 16:1	0.33	0.27	0.33	0.28
Stearic	C 18:0	3.77	1.54	4.15	1.59
Oleic	C 18:1	42.31	5.14	40.73	4.13
Linoleic	C 18:2	10.49	3.93	6.37	3.13
Linolenic	C 18:3	5.05	1.33	0.21	0.14
Arachidic	C 20:0	0.29	0.19	0.19	0.1
Eicosenoic	C 20:1	0.56	0.52	0.09	0.06
Behenic	C 22:0	0.1	0.03	0	0
Erucic	C 22:1	0.83	0.83	0.05	0.05
Lignocervic	C 24:0	0.05	0.05	0.08	0.08
SFA:PUFA:MUFA		41:15:44		52:06:42	

Phase II sought to further optimize Test Blend 3G. The oil composition was maintained and effects of the water quality and added antioxidants were examined. To test water effects, blends were prepared identically except with water from three different sources:

1. Milli-Q™: high purity water (double distilled, deionized, purified to 18 megohm resistivity in a Milli-Q™ four cartridge water purification system with two ion exchange resin cartridges to remove metals).
2. "Packer" water: ground water from region near packing company (expected to have a high metal content).
3. Spring water supplied for water cooler used by a consulting company.

All of these samples contained 0.02% added mixed tocopherols. This group of samples will be referred to as the "water samples".

To assess whether addition of lipophilic mixed tocopherols as antioxidants could increase stability of the blends, four levels of mixed tocopherols (0.1, 0.07, 0.05. 0.02% antioxidant in oil, weight basis) were added to the oil phase of the blends before emulsification. This concentration range brackets the legal limits for tocopherol addition to oils. Spring water was the aqueous phase in the emulsion. These samples will be referred to as the "tocopherol samples".

Samples for initial work and Phase I were prepared by a commercial producer, packed into 1420 mL/48oz/6cup clear polypropylene (#5 plastic) containers with blue/clear "snap-on" lids, capped, and shipped to us. Samples for Phase II were prepared in small batches by a consulting laboratory. In the Initial Phase and Phase II, upon receipt, samples were immediately transferred to gas-impermeable glass 8 ounce Ball jars, flushed with

argon, sealed with lids and Parafilm®, and stored at −80 °C until use (Figure 9).

In Phase I, after being received, the samples were flushed with argon, plastic lids snapped on, and sealed with Parafilm®; this packaging was believed to be the "market packaging", and was used to provide a realistic view of the shelf life with this packaging. In addition to the margarine samples in Phase I, fresh canola oil, provided by the margarine company, was handled identically and tested alongside the blends in shelf-life analyses.

Figure 9. Pictures of the margarine blends after transfer to glass canning jars.

4.2.2 Chemicals Used in Assays

Peroxy-Safe™ and FASafe™ kits were purchased from MP Biochemicals, LLC (Solon, OH) to analyze hydroperoxides and free fatty acids, respectively, in the test blend samples. Iso-octane (HPLC Grade), chloroform (HPLC grade), acetonitrile (HPLC grade), isopropanol (HPLC grade), 2,4-dinitrophenylhydrazine (DNPH) (reagent grade, ≥ 97%), N,N-dimethyl formamide (HPLC grade, ≥ 99%), and sulfuric acid were purchased from Sigma Chemical Co. (St. Louis, MO). Aldehyde standards butanal (≥ 98%), pentanal (≥

97%), hexanal (≥ 98%), heptanal (≥ 95%), octanal (≥ 99%), nonanal (≥ 95%), decanal (≥ 98%), undecanal (≥ 97%), trans-2-pentenal (≥ 95%), trans-2-hexen-1-al (≥ 95%), cis-4-heptenal (≥ 98%), trans-2-octenal (≥ 95%), trans-2-nonenal (≥ 95%), trans-2-decenal (≥ 92%), trans,trans-2,4-heptandienal (≥ 88%), trans,trans-2,4-nonadienal (≥ 89%), and trans,trans-2,4-decadienal (≥ 89%) were purchased from Sigma Chemical Co. (St. Louis, MO). Hydromatrix™ (a pelletized diatomaceous earth material used in accelerated solvent extractions) was purchased from Perkin Elmer/Agilent, (Wilmington, DE).

The water used in all tests was double-distilled water further purified to 18 MegOhm (MΩ) resistivity by a four-cartridge Millipore Milli-Q Water Purification System (Millipore, Billerica, MA).

4.3 Experimental Methods

4.3.2 Initial testing – thermal stability of blends

Four oil blends (Base blend, Test Blends 1, 2, and 3) were analyzed to determine the effects of heat on degradation and stability of the blends.

Approximately 10 grams of each blend was heated at 180°C in an Oxipres™ oxygen bomb (Mikrolab Aarhus A/S, Højbjerg, Denmark) under two bars purified air pressure for three hours (see Section 2.3.4.1 for details). Samples were then cooled rapidly to room temperature in a -20°C freezer to minimize the effects of residual heat, and analyzed for conjugated dienes, hydroperoxides, and free fatty acids (Figure 10). All analyses were performed in triplicate.

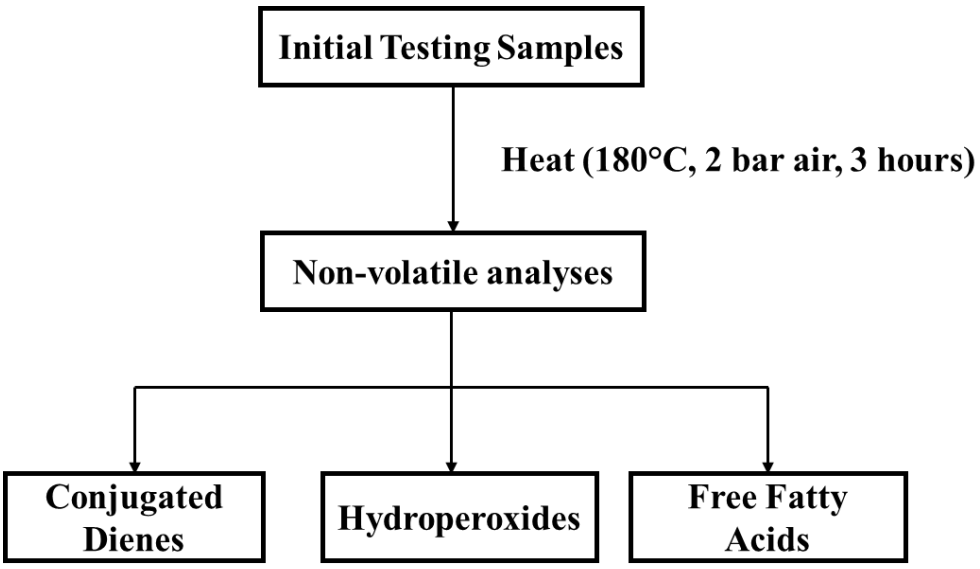


Figure 10. Experimental flow for evaluation of thermal stability in the initial test blends.

4.3.3 Phase I testing

The reformulated test blend (Test Blend 3G) and the commercial product (Base Blend) were tested for thermal stability in the same manner as in the Initial Testing and for long-term oxidative stability in a shelf life study. In the shelf life study, oxidative degradation of the two blends was followed in parallel with canola oil over the course of about one year. A flow chart for the thermal and shelf life studies is presented in Figure 11.

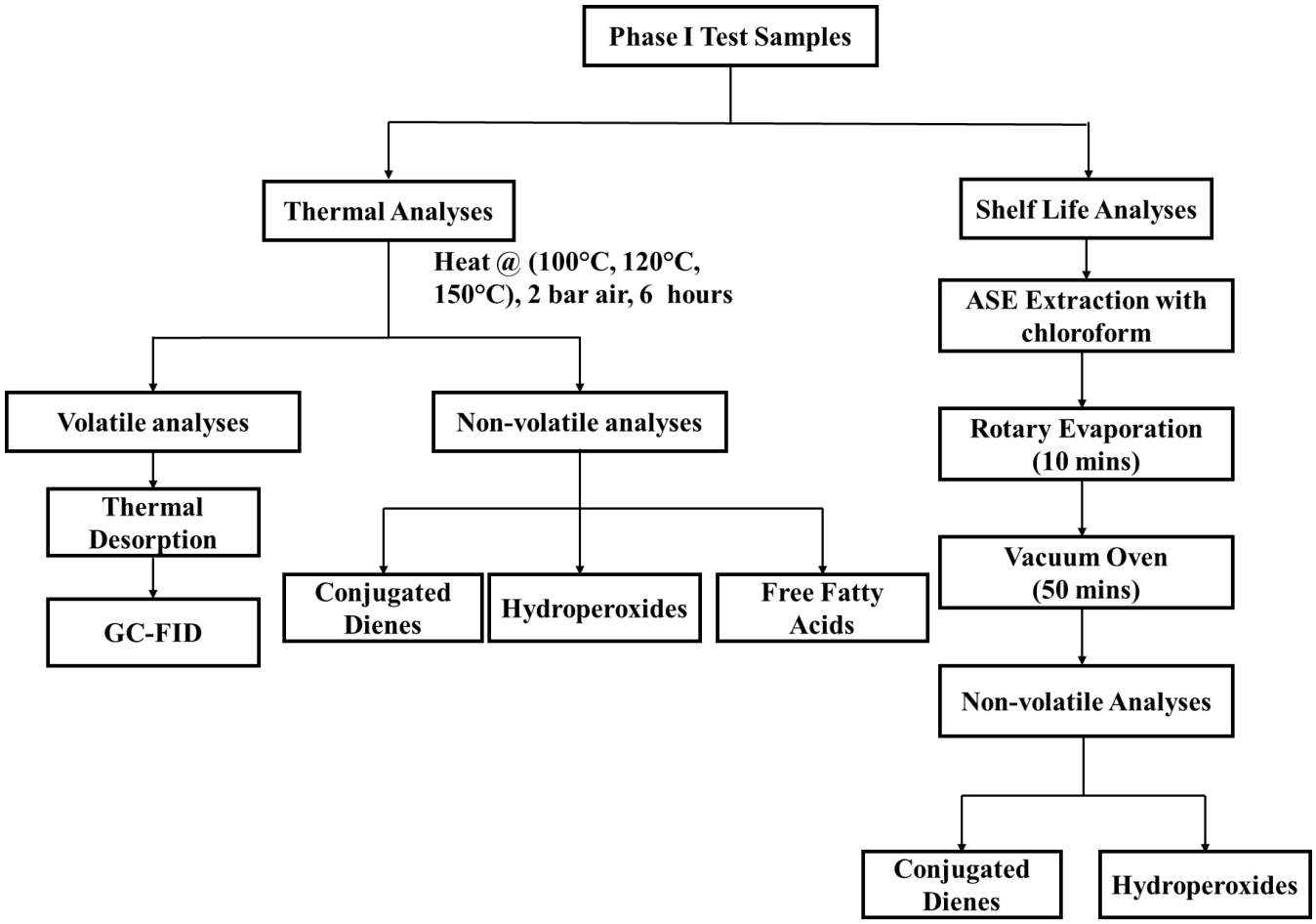


Figure 11. Experimental design for the Phase I evaluation of thermal and oxidative stability in test blends formulated with different oils.

4.3.3.1 Shelf-life Analyses

Blends were stored in a 4°C refrigerator over the course of the study. Each week, three ~1 gram samples were removed from under the surface of the blend in order to limit variability of surface exposure to air and light. After removal of samples, the blend container was flushed with argon, capped, re-sealed with Parafilm™, and returned to the 4°C refrigerator. Lipids were extracted from Blend samples for analysis by accelerated solvent extraction (ASE) using a Dionex™ ASE 350 Accelerated Solvent Extractor (Dionex Corp., Sunnyvale, CA).

Lipid samples extracted from the Blends were analyzed for conjugated dienes and hydroperoxides. Lipid hydroperoxides and conjugated dienes were also analyzed in canola oil stored under the same conditions and aged for the same amount of time as the blends.

4.3.3.2 Analyses of thermal stability

Thermal stability of the Blends was tested using only proportional blends of oil components, with and with tocopherol antioxidants, because preliminary investigations found that the high water levels in the Blends caused water splattering and vaporization during heating and interfered with performance of the pressure transducer in the Oxipres™ (see Section 4.3.5.1). Thus, thermal stability of the lipids in the blends was determined on component oils of the two blends in their respective formulations. This approach was valid since the goal of Phase 1 was to optimize the oils formulation in the blends.

Component oils and Blends were stored frozen at -80 °C until analysis. After warming to room temperature, the component oils of the Base Blend and the Test Blend 3G were combined in their respective formulations alone (one set with and one set without 0.02% added tocopherols). Approximately ten grams of each oil blend were weighed into

glass Oxipres™ sample flasks, placed in heating cells of an Oxipres™ oxygen bomb system (see Section 4.3.5.1), sealed and pressurized to 2 bars with purified air, then heated in oxygen bomb for 6 hours at 100°C, 120°C and 150°C to simulate commercial frying conditions. Each blend and oil were tested in triplicate at each temperature. At the end of the heating, Oxipres cells were vented through thermal desorption traps to collect volatile products which were subsequently analyzed by gas chromatography with flame ionization detection. Oil samples were removed, cooled, and analyzed for conjugated dienes, hydroperoxides and free fatty acids (Sections 4.3.6.1, 4.3.6.2, and 4.3.6.3, respectively).

4.3.4 Phase II testing

Seven test blends with three water formulations (Milli-Q water blend, Packer water sample blend, Spring water sample blend, all with 0.02% added mixed tocopherols) and four tocopherol formulations (0.02, 0.05. 0.07, and 0.1% tocopherol) prepared with spring water were evaluated for oxidative stability in shelf-life studies and thermal stability in the Oxipres™ oxygen bomb as shown in the flowchart in Figure 12. This specific blend of oils was designed for baking and stove cooking, which were mimicked by using the Oxipres® oxygen bomb system keeping the cell open to the ambient air rather than sealing and pressurizing it. This approach also allowed heating of the intact Blends rather than component oils.

4.3.4.1 Shelf-life Analyses

The shelf life study was performed in the manner described for Phase I testing, with the exception that the samples were analyzed monthly rather than weekly.

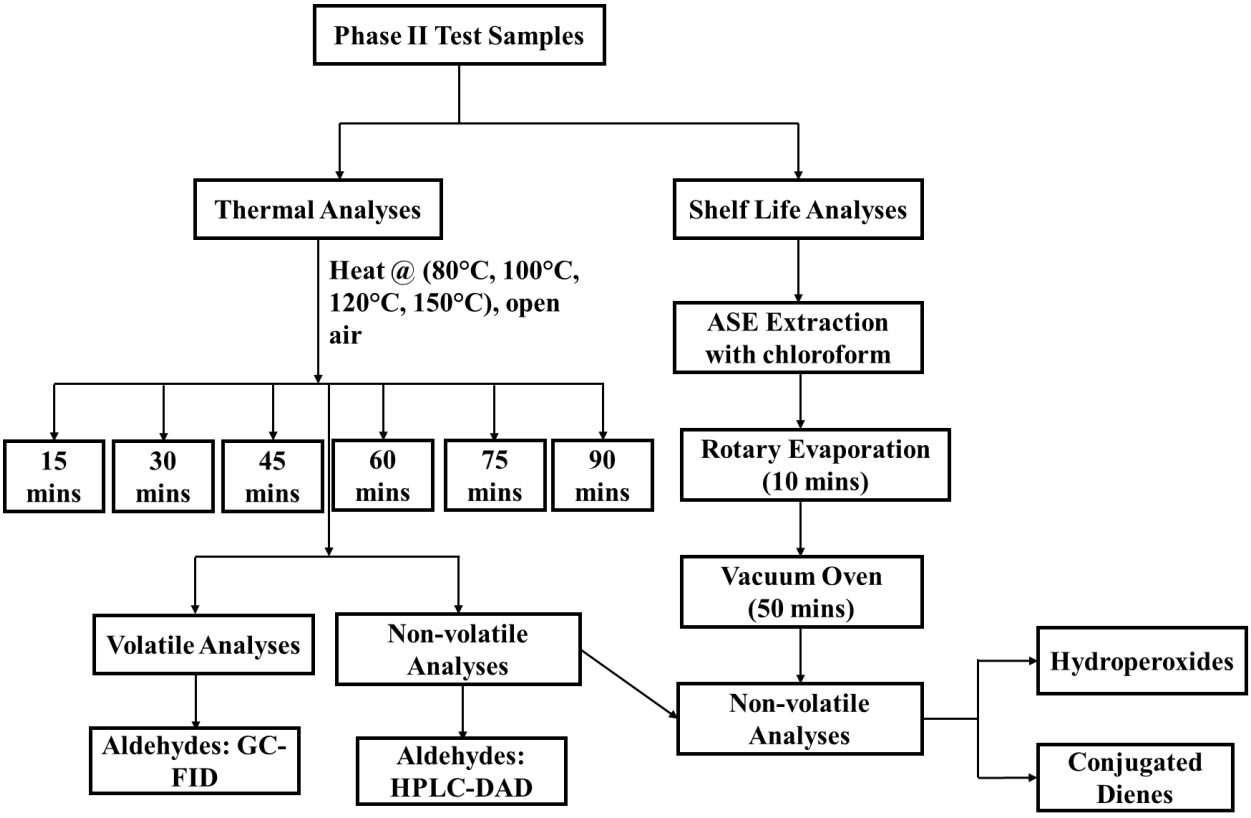


Figure 12. Experimental design for evaluating the effects of the water and the level of tocopherols on the chosen blend's thermal and shelf life stability.

4.3.4.2 Analyses of thermal stability

Thermal stability of the three water Blends and four tocopherol Blends was evaluated using the Oxipres™ open to the air to mimic cooking. Approximately 7.5 gram samples of the blends were placed into glass Oxipres™ containers and then transferred to the heating cells, which had already been equilibrated at the test temperatures (80°C, 100°C, 120°C or 150°C). Each temperature represents a different cooking application: 80°C and 100°C for baking and 120°C and 150°C for stove cooking. Every 15 minutes for 90 minutes, a sample was removed from the heated cell, placed immediately into a freezer

for approximately 5 minutes to stop further heating, then cooled to room temperature. Duplicates were analyzed for all samples.

Approximately 2 grams of the cooled blends were transferred to 20 ml headspace vials for analysis of volatiles by GC-FID (see Section 4.3.6.5). Each of these sample vials were flushed with argon for one minute to displace oxygen in the headspace, capped and sealed with a septa, wrapped with aluminum foil to prevent light penetration, and frozen at -80°C until ready for headspace analysis. The remaining sample was used for the chemical assays: conjugated dienes and lipid hydroperoxides (Sections 4.3.6.1 and 4.3.6.2). Any leftover sample was flushed with argon for one minute to displace oxygen in the headspace, capped and sealed, and frozen at -80°C until analysis of non-volatile aldehydes by DNPH-HPLC.

4.3.5 Instrumental Methods

4.3.5.1 Oxipres™ oxygen bomb system

The Oxipres™ oxygen bomb system was used in this study to heat samples under highly controlled conditions that mimic cooking. The Oxipres™ operates at elevated temperature and pressure to accelerate lipid oxidation mechanisms. Each Oxipres™ unit is composed of a control base and a two-position heating block (Figure 1313). Temperature and pressure are set manually for each sample vessel. The control unit connected to a computer provides precise control of experimental heating cycles and records pressure data (Tian, 2013).

For Initial Testing, the test margarine blends were heated in the oxygen bomb cells under two bars air pressure at 180°C for three hours. During these analyses, it was found

that the water in the margarine blends caused splattering within the oxygen bomb cell and the water vaporized from the samples caused damage to the pressure tranducer detectors. This problem was avoided in Phase I testing by heating only proportional mixtures of the test blend component oils (one set with and one set without added antioxidants) without water or any of the other added ingredients. This provided information about the innate stability of the oil components themselves. Phase I oil blends were heated to 100, 120, and 150 °C under two bars air for six hours. Volatile degradation products released from these blends were collected by venting the cells through thermal desorption traps after heating.

In Phase II, intact Test Blends in Oxipres™ cells in the Oxipres heating block open to the ambient air were heated to 80, 100, 120, and 150) for up to 90 minutes, with samples removed for analysis every 15 minutes.

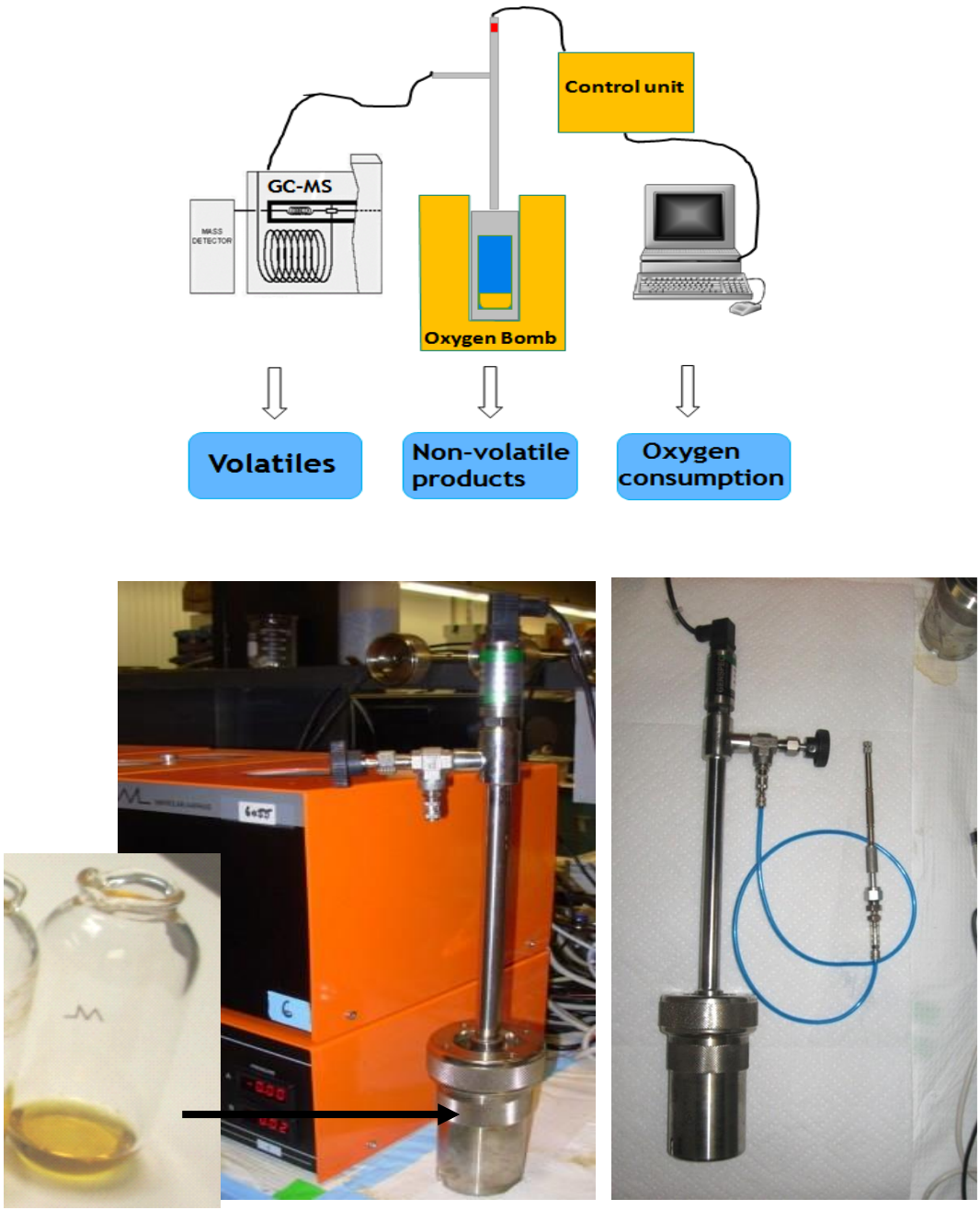


Figure 13: Oxipres(TM) setup.

4.3.5.2 Accelerated Solvent Extraction (ASE)

Chemical analysis of lipids in the Test Blends required separation of the oil phase from the water in the blends. This was accomplished using Accelerated Solvent Extraction (ASE) in a Dionex™ 350 Accelerated Solvent Extractor (ASE) (Dionex Corp, Sunnyvale, CA). One gram of each margarine blend was dispersed in 25 mL Hydromatrix, a high purity inert diatomaceous earth sorbent material, then funneled into a Dionex™ stainless steel cell plugged with a frit and cellulose filter.

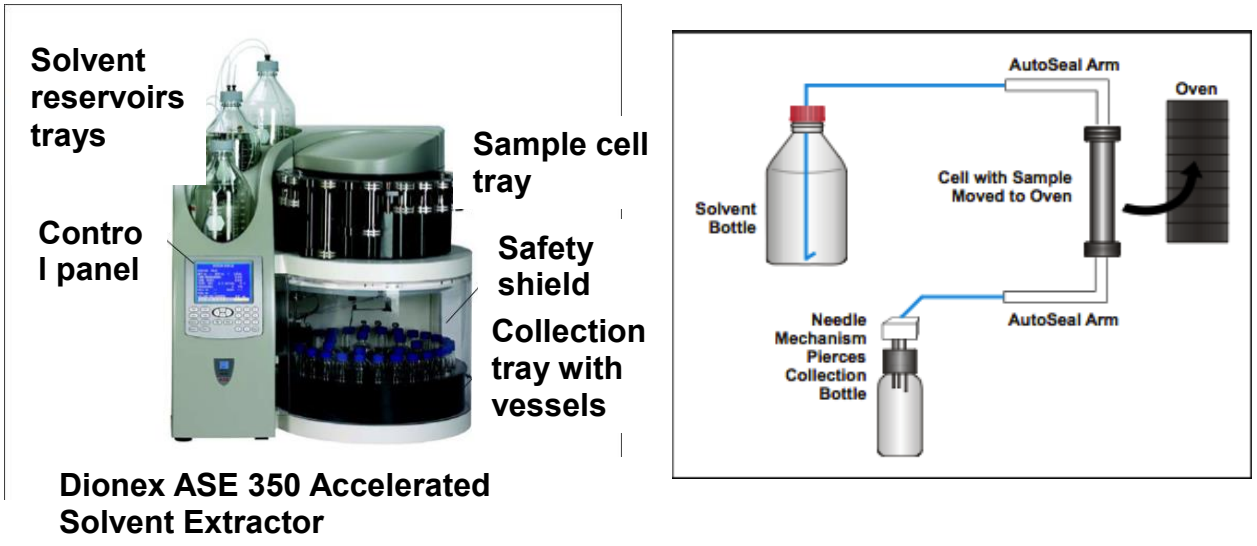


Figure 14: ASE setup.

Cells were heated to a temperature of 40°C under ~1500 psi nitrogen pressure and lipids were extracted from the Test Blend emulsions by HPLC-grade chloroform using one five minute static cycle and one rinse cycle with 75 mL of solvent.

Solvent with extracted lipid was collected in 125 ml sealed flasks, then transferred to 125 ml flat-bottomed evaporator flasks for removal of solvent. Solvent was evaporated from each sample using a rotary evaporator under 120 mm vacuum with water bath set at 38°C for ten minutes. Dry samples were removed from the rotary evaporator, wrapped in aluminum foil to prevent light exposure, flushed with argon for two minutes, capped and sealed, then stored in the refrigerator until all the samples were extracted. All samples together were then uncapped, covered with a Kimwipe™ over the opening (secured with a rubber band) to prevent contamination, then dried in a vacuum oven at room temperature for 50 minutes to remove final traces of the solvent.

4.3.6 Analysis of degradation products

4.3.6.1 Conjugated Dienes

Conjugated dienes (CD) were analyzed by a modification of the AOCS procedure Ti 1a-64 as adapted by (Tian, 2013). 30 µL of oil was dissolved in 10 mL HPLC grade iso-octane in a 15 mL test tube, capped, and inverted to distribute the oil in the solvent. 3 mL of this solution were transferred to a quartz cuvette, and the optical absorbance was read at 234 nm against an iso-octane blank in a Varian Cary 50 UV/Vis Spectrophotometer (Varian Inc., USA). Samples were diluted if absorbance was greater than 1.0, where accuracy of the spectrophotometer decreases. The concentration of conjugated dienes (in mmol) was then calculated from the Beer-Lambert Law and converted into mmol/mol.

4.3.6.2 Peroxide value

Peroxide values were determined using the PeroxySafe™ Standard Assay kit (MP Biomedical, Solon, OH) based on the xylenol orange reaction as adapted by Steltzer (Steltzer, 2012). Initial analyses were conducted using the optical analyzer supplied with the kit. In later analyses, methods were adapted to standard UV-Vis spectrometers.

SafTest optical analyzer. Oil samples had to be diluted with the PeroxySafe™ Prep Reagent before each analysis to fall in the 0.05-0.50 meq peroxide/kg fat detection range for the PeroxySafe™ assay (Steltzer, 2012). The dilution required increased with the extent of oxidation. Exactly 25 µL of diluted oil sample was dissolved in 1000 µL of PeroxySafe™ Reagent A in 10mm × 75 mm borosilicate test tubes, then 100 µL of PeroxySafe™ Reagent B and 160 µL of PeroxySafe™ Reagent C were added. The test tubes were capped and vortexed, then incubated at 25°C (controlled in a heating block) for 15 minutes, shielded from light with aluminum foil. Optical absorbance was measured in the SafeTest MicroChem II optical analyzer using the 570 nm filter and recorded by computer. Peroxide values in units of meq peroxide/kg fat reported by the PeroxySafe™ software were converted into mmol peroxide/mol TAG to compare all products on a common basis.

$$\left(\frac{PV\ measurement\ \frac{meq\ LOOH}{kg\ fat}}{1.13\ mol\ TAG}\right) \times sample\ dilution = \frac{mmol\ LOOH}{mol\ TAG}$$

The assay was calibrated every two weeks using the calibrators provided in the kit.

UV-Vis Method

Oil samples were diluted and the reaction was run as described in the previous section, but the optical absorbance was measured at 570 nm using the Varian Cary 50 UV-Vis

Spectrophotometer. Absorbances were converted into mmol peroxide/mol TAG using the calibration curves developed from the calibrators provided in the kit, as in the MicroChem analyses. The calibration curve is shown in Figure 17. Hydroperoxide concentrations were calculated using the equation, where 0.8016 and 0.12 are the values determined from the calibration curve:

Calculation: $\left(\frac{(Absorbance*0.8016)-0.12\frac{meq\ LOOH}{kg\ fat}}{1.13\ mol\ TAG}\right) \times sample\ dilution = \frac{mmol\ LOOH}{mol\ TAG}$

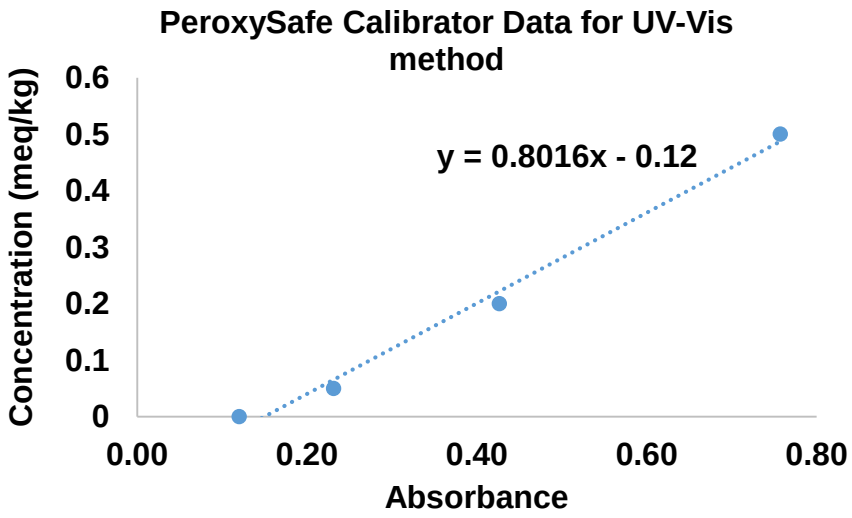


Figure 15. Calibration curve for PeroxySafe™ assay conducted on the Varian Cary 50 UV-Vis Spectrometer.

4.3.6.3 Free Fatty Acids

Free fatty acid concentrations were determined using the FASafe™ Standard Kit (MP Biomedical, Salon, OH) with calculations adapted for oils (Tian, 2013). Oil samples were diluted with FASafe™ Prep Reagent to a range of concentrations before analysis.

Exactly 50 µL of diluted oil sample was added to 1000 µL of FASafe™ Reagent A and 100 µL FASafe™ Reagent B in a 2 mL borosilicate test tube. The test tubes were capped, vortexed for 10 seconds, incubated for 10 minutes at 38°C in a heating block, and optical absorbance at 550 nm was recorded using the SafTest MicroChem II optical analyzer. Fatty acid concentrations were calculated by the SafeTest Software and reported in % FFA.

4.3.6.4 DNPH-HPLC Assay for Non-Volatile Lipid Carbonyls

Non-volatile aldehydes were identified and quantified using the HPLC- DNPH assay method of (Yao, 2015).

DNPH reagent: 40 mg 2,4-dinitrophenylhydrazine recrystallized from n-butanol was dissolved in 990 µL N,N-dimethylformamide acidified by 10 µL sulfuric acid (95%) and vortexed.

Reaction: 100 µL DNPH reagent were mixed with 100 µL isopropanol:acetonitrile (1:1) and 100 µL chloroform in a 10 mm × 75 mm borosilicate test tube. 50 µL of the sample oil blends (heated to make liquid) were added, the mixture was vortexed thoroughly and allowed to react for 20 minutes, then 650 µl mixed acetonitrile:isopropanol (1:1) was added to dilute the sample to 1 ml. The reaction mixture was filtered through a syringe filter into 1 mL HPLC sample vials. The vials were capped with a septa cap and placed into the auto-sampler of the HPLC.

Hydrazones were separated and detected on an Agilent (Santa Clara, CA) 1290 Infinity HPLC system equipped with 1200 Infinity Degasser, Quant Pump, HiP/ ALS Autosampler, column compartment and Diode Array Detector. The injection volume was 5 µl. Carbonyl hydrazones were separated on a Restek Ultra C18 (4.6 mm * 150 mm) column with the following elution gradient and a total run time of 45 minutes:

16.7% to 100% A in 17 min (1.2 ml/ min),

100% A 1.2 ml/ min to 1.4 ml/ min in 2 min,

hold at 100% A 1.4 ml/ min for 16 min,

return to 16.7% A 1.2 ml/ min in 5 min

hold 5 min.

Eluting solvents were A: acetonitrile/ isopropanol (50:50); B: acetonitrile/ isopropanol/ DI water (25:25:50). Organic solvents were added to the aqueous phase to avoid bubbles from two phases mixing. Carbonyl-DNPH peaks were detected at 360 nm. Aldehydes were identified by comparing retention times to those of standards (Table 6). Peaks were quantified using calibration curves developed for individual standard carbonyls (Table 7).

Table 6. Aldehyde standards and their retention times on the HPLC system described.

Retention Times	Compound
4.52	Butanal
4.57	Butanal
5.76	Pentanal
5.79	2-Pentenal
7.05	Hexanal
7.22	2-Hexen-1-al
7.30	2,4-Heptadienal
7.32	2-Hexen-1-al
7.47	2,4-Heptadienal
7.65	2,4-Heptadienal
8.31	Heptanal
9.51	Octanal
9.63	4-Heptanal
9.90	4-Heptenal
9.90	2,4-Nonadienal
10.21	2,4-Nonadienal
10.36	2,4-Nonadienal
10.86	Nonanal
10.93	2-Octenal
11.13	2,4-Decadienal
11.25	2-Nonenal
11.48	2,4-Decadienal
11.62	2,4-Decadienal
12.16	2-Decenal
12.47	Decanal
12.48	2-Decenal
13.64	Undecanal

Table 7. Regression equations (mM) for standard carbonyl hydrazones separation by HPLC as described. Data from (Yao, 2015).

Carbonyl	Molecular mass (g/mol)	Equations (Based on molarity)	R^2
Butanal	72.11	y = 4783.3x + 21.343	0.9998
2-Butanone	72.11	y = 3644.9x + 1.8328	0.9999
t-2-Hexenal	98.14	y = 2912.6x + 17.959	0.9998
Hexanal	100.16	y = 4339.7x + 14.555	0.9999
Heptanal	114.19	y = 2594.1x + 4.2909	0.9999
2-Heptanone	114.16	y = 3296.4x + 1.0884	0.9999
t-2-Octenal	126.2	y = 3008.1x + 13.143	0.9999
t-3-octen-2-one	126.1	y = 2714.5x + 3.4011	0.9999
Octanal	128.21	y = 3054.4x + 6.1784	0.9999
t,t-2,4-Nonadienal	138.21	y = 3158.4x + 7.1715	0.9998
Nonanal	142.24	y = 3314.9x + 5.2397	0.9998
t,t-2,4-decadienal	152.23	y = 3136.8x + 8.9496	0.9999
Decanal	156.27	y = 3076.3x - 12.905	0.9999
Undecanal	170.29	y = 2223.3x + 5.0198	0.9999
Dihexyl ketone	198.34	y = 2266.9x - 2.4865	0.9999

4.3.6.5 Gas chromatography analysis of volatile lipid oxidation products

Initial gas chromatography (GC) studies were performed on volatiles generated during heating of margarine test blends and oils in the Oxipres™ oxygen bomb. After results showed that high levels of water vapor from the blends interfered with oxygen

detection and volatiles analysis, volatiles analyses were shifted to a static headspace method.

Thermal desorption gas chromatography with flame ionization detection (GC-FID). After heating of blends and oils, pressure on Oxipres cells was released by venting the headspace through Tenax-Carboxen 569 (1:1, 50 mg each) traps connected to the cells. Traps were stored frozen (-20°C) until GC analyses.

Thermal desorption was performed using a Model TD-1 thermal desorption unit (Scientific Instrument Services, Ringoes, NJ) connected to a Hewlett Packard 5890 series gas chromatograph equipped with an FID detector, split/splitless injector and an Equity-5 fused capillary column (60 m x 32 mm x 1.0 µm film thickness, Supelco, Bellefonte, PA, USA). Following procedures of Bogusz (2015), loaded thermal desorption traps were connected to the TD-1 via a 35 mm seal-autodesorb needle, and the side with the serial number was capped. The thermal desorption controller was set to 10 seconds to allow carrier gas (helium) to flush the needle before injection. The needle was injected through the injection port for 30 seconds to re-equilibrate the inlet pressure. The heating block at 250 ºC was then closed and the trap was desorbed onto the column for 5 minutes. Helium carrier gas was passed through the column at 1.0 mL/minute under constant pressure mode for both desorption and chromatography.

For chromatography, the initial GC temperature was set to -20 ºC with dry ice for 5 minutes to ensure resolution of small volatiles; the temperature was then increased to 280 ºC at a rate of 10 °C/min. The FID temperature was 300 °C and inlet temperature was 250 °C. Purge time was five minutes. The peak data was stored and integrated using

Chemstation software. Peaks were identified by comparing sample retention times to those of authentic standards.

Static headspace GC-FID. In Phase II of the study, volatiles from thermal and oxidative decomposition of Blend samples were detected and analyzed by static headspace GC-FID. 2 grams of heated samples were weighed into 10 mL glass headspace vials, flushed with argon, sealed with a septum cap, wrapped in aluminum foil, and stored at -80°C until analyses were performed, up to 4 weeks.

For headspace analysis, samples were removed from the freezer and warmed at 40 °C for 15 minutes. A 2-mL gas-tight syringe was equilibrated at 40°C before insertion so as to not cause condensation of the headspace within the system and to ensure that an accurate sample of headspace was collected. The warmed syringe was inserted into the septa of the vial and positioned in the middle of the headspace of the sample, making sure that the needle did not touch the sides of the vial or the sample. 2 mL of headspace were removed from the vial and injected into the GC.

Volatiles were analyzed using a Hewlett-Packard 6890N Gas Chromatograph (Agilent, Santa Clara, CA) equipped with a Flame Ionization Detector (FID) and a fused silica capillary column 60 m × 0.32 mm × 1.0µm film thickness. Inlet temperature, 275 °C: FID temperature, 315 °C. The oven temp ramp was set at 40°C and increased 5°C/min until 200°C was reached, then increased 10°C/min until 315 was reached. Helium was used as the carrier gas at a flow rate of 1.3 mL/min. Chromatograms were collected and peak areas were integrated and analyzed using Chemstation computer software. Peaks from typical lipid oxidation products were identified using the standards shown in Table 8.

Table 8. Retention times of volatile standards on the GC-FID system described.

Standard	Retention Times
1-pentene	7.34
pentane	7.43
butanal	9.25
pentanal	12.14
2-pentenal	14.06
Hexanal	15.61
2-hexenal	17.53
4-heptenal	19.11
Heptanal	19.21
hexanoic acid	21.47
2-pentyl furan	22.56
Octanal	22.71
2,4-Heptadienal	23.05
2-octenal	24.60
2,4,6-Octatrienal	24.68
Nonanal	26.03
Octanoic Acid	27.52
2-nonenal	27.83
2,4-Nonadienal	28.91
Decanal	29.20
2,4-Nonadienal	29.54
2-decenal	30.86
Undecanal	32.06
2,4-Decadienal	32.48
decanol	33.22
butanoic acid	34.40

5. RESULTS AND DISCUSSION

5.1 Initial testing

Initial test blends were all analyzed fresh upon receipt to establish baseline degradation levels before heating (Table 9). The fresh Base Blend had the highest levels of starting conjugated dienes and hydroperoxides. This was expected since the Base Blend has the lowest levels of saturated fatty acids and highest levels of unsaturated fatty acids. Test Blend 3 was the opposite, with lowest unsaturated fat content and highest saturated fat content of the Blends, hence lowest initial degradation.

Initial oxygen bomb studies of blends heated for 3 h at 180°C revealed problems with browning and burning of particles from milk solids present in the organic butter flavor (

Figure *8*). The burning started at 120 °C and increased with temperature. Replacing butter flavor with ghee (clarified butter oil) eliminated the problem.

Table 9: Lipid degradation products in fresh blends. (CD: Conjugated dienes; PV: Peroxide value; FFA: Free fatty acids).

Sample	**CD (mmol/mol TAG)**	St dev	**PV (mmol/mol TAG)**	St dev	**FFA (%)**	St dev
Base Blend	8.28	1.12	0.70	0.43	ND (<0.04%)	N/A
Test Blend 1	7.90	0.40	0.25	0.02	ND	N/A
Test Blend 2	5.73	0.07	0.22	0.01	ND	N/A
Test Blend 3	5.29	0.12	0.23	0.09	ND	N/A

Heating tests were repeated on the samples reformulated with ghee. Low levels of oxidation products in the oils demonstrated limited degradation (

Table *10*). Although conjugated dienes (CD), peroxide values (PV) and free fatty acids (FFA) increased measurably after heating, final concentrations still fell within ranges considered acceptable for food use: <5 PV and <1% FFA. Importantly, levels of lipid degradation products were also lower than observed for typical frying oils analyzed under identical conditions (Tian, 2013).

Table 10: Lipid degradation products in blends formulated with ghee heated at 180°C for 3 hours. Values are averages of three independent heating replicates.

Sample	**CD (mmol/mol TAG)**	St dev	**PV (mmol/mol TAG)**	St dev	**FFA (%)**	St dev
Base Blend	33.52	0.28	3.08	0.51	0.63	0.05
Test Blend 1	24.53	0.15	1.57	0.81	0.52	0.05
Test Blend 2	24.56	0.16	2.74	0.31	0.55	0.05
Test Blend 3	20.10	0.29	3.88	0.33	0.60	0.06

Test blend 3 had the lowest conjugated dienes indicating lowest initiation, reflecting the high saturated and mono-unsaturated fatty acid contents. Surprisingly, test blend 3 also had the highest peroxide values. This most likely occurs because saturated fatty acids undergo thermal scissions at lower temperatures than unsaturated fatty acids (reflected in smoke points). The scission radicals thus formed that add oxygen to form terminal hydroperoxides different than the allylic hydroperoxides normally formed from unsaturated fatty acids (Reaction 19):

$$\text{-CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-} \rightarrow \text{-CH}_2\text{CH}_2^{\bullet} + {}^{\bullet}\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-} \rightarrow \quad (19)$$

$$\text{-CH}_2\text{CH}_2\text{OO}^{\bullet} + {}^{\bullet}\text{OOCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{-} \rightarrow$$

$$-CH_2CH_2OOH \quad + \quad HOOCH_2CH_2CH_2CH_2CH_2-$$

Because these blends were being designed for cooking applications lower than deep frying, saturated fatty acids were less susceptible to oxidation, and the multiple double bonds of unsaturated components were targets for attack by scission radicals, the company developing the blends decided to reformulate blend 3 without flax seed oil to reduce the extent of unsaturation and increase stability. This modified blend, designated 3.2, also contained ghee in place of butter flavor and was used in all subsequent testing. It was also designated as "not to be used for deep frying".

5.2 Phase I testing

Standard Blend 3G reformulated without flaxseed oil and with ghee replacing butter flavor was tested for thermal stability at lower temperatures (80, 100, 120, and 150 °C) and for shelf life at refrigerated temperatures (normal storage temperature for these products).

5.2.1 Shelf life analyses

A 12-month storage study was designed to determine how inclusion of canola oil for nutritional support would affect storage stability and whether the Base Blend would meet the market goal of minimum 12-month shelf life. The Base Blend was compared against pure canola oil and against Test Blend 3G, which contained no canola or flax seed oil.

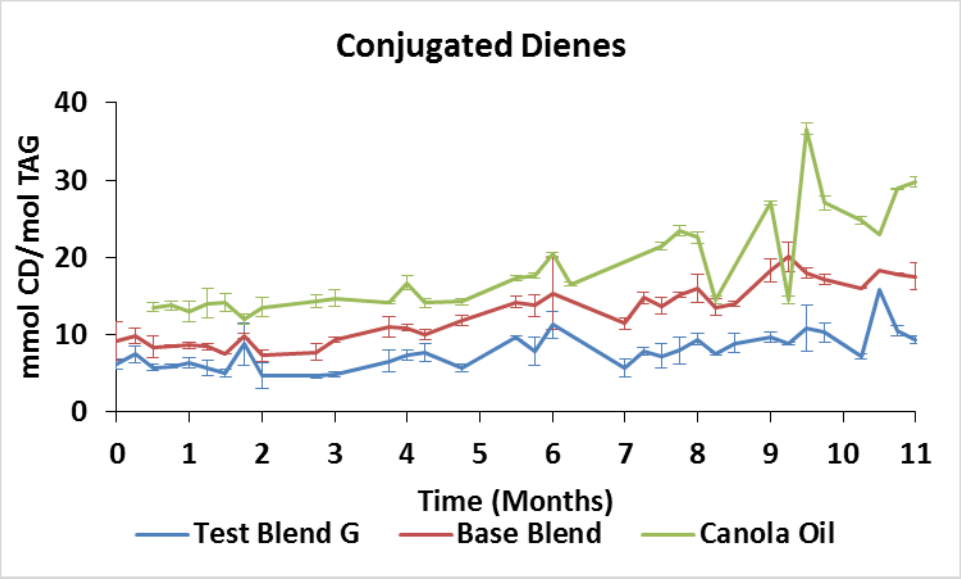


Figure *16* shows that conjugated diene (CD) levels were reasonably stable for the first 4-5 months for all three materials, although the starting oxidation levels were highest in canola, followed by Base Blend and then Blend 3G. Differences between samples became noticeable after this point. Not surprisingly, CD increases in canola oil itself were most dramatic; they were also most variable because the extensive polyunsaturation (>30%, Table 11) allows for many secondary reactions that remove the conjugation at the same time that new radical chains (and conjugation) are being generated. The balance between the two actions constantly changes. The Base Blend containing canola oil oxidized slightly faster than Blend 3G due to higher content of polyunsaturated fatty acids, specifically ~11% linoleic acid and ~5% linolenic acid (Table 5). Test Blend 3G, based on coconut oil with no canola oil, oxidized most slowly, showing little change until near the end of the year's incubation. The most obvious explanation for this stability is that Test Blend 3G had much lower content of polyunsaturated fatty acids in which conjugated dienes form (Table 11). However, endogenous levels of tocopherols and tocotrienols, as well as other antioxidants need to be determined before this simplistic explanation can be validated.

It is important to note that conjugated dienes can only be formed in fatty acids with two or more double bonds (Reaction 20):

$$\text{-CH=CH-CH}_2\text{-CH=CH-} \rightarrow \text{-CH=CH-CH=CH-C}^{\bullet}\text{H-} \quad (20)$$

Nonconjugated starting material Conjugated oxidized material

Consequently, coconut oil should naturally show low CDs due to its lack of polyunsaturated fatty acids. This also means that conjugated dienes probably do not fully reflect oxidation in coconut-oil dominant Blends and one or more additional products must be measured.

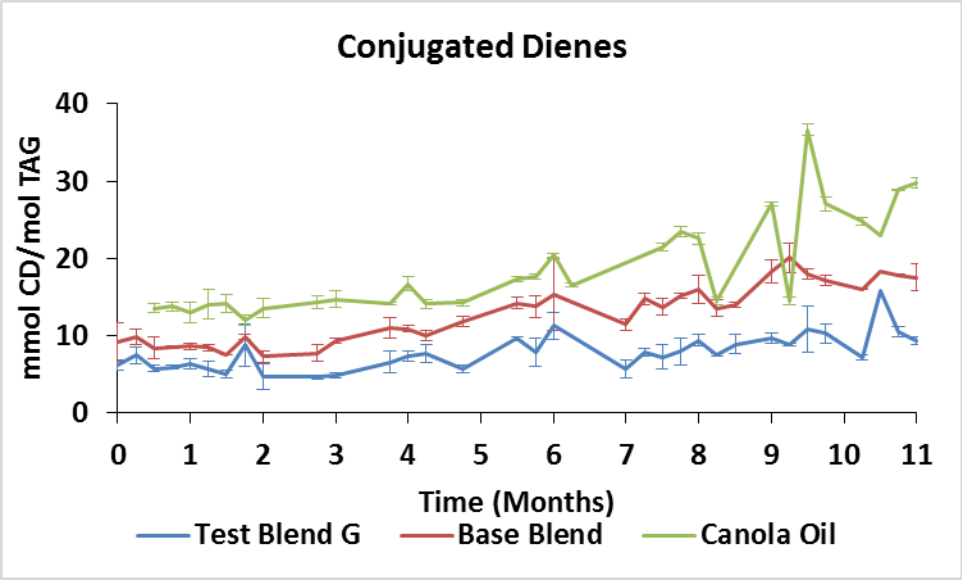


Figure 16: Conjugated dienes of Test Blend 3G, Base Blend, and canola oil samples over the course of 11 months at refrigerated temperature (4°C). Analyses were conducted with three replicates.

Table 11: Fatty acid profiles of the major components of the blends. From Bailey's Industrial Fats and Oils and USDA National Nutrient Database.

Fatty Acid Profiles of the Major Components of the Blends				
Fatty Acids	**Coconut Oil**	**Canola Oil**	**Palm Oil**	**Sunflower Oil (High Oleic)**
Caprylic Acid (8:0)	6%	--	--	--
Capric Acid (10:0)	5%	--	--	--
Lauric Acid (12:0)	45%	--	--	--
Myristic Acid (14:0)	20%	--	--	--
Palmitic Acid (16:0)	10%	4%	45%	4%
Palmitoleic Acid (16:1)	3%	--	--	--
Stearic Acid (18:0)	7%	2%	4%	5%
Oleic Acid (18:1)	1%	61%	35%	82%
Linoleic Acid (18:2)	--	21%	10%	9%
Linolenic Acid (18:3)	--	10%	--	--

For this study, the alternate product determined was peroxide values. Lipid hydroperoxides increased gradually in all test materials over the course of the one year study (

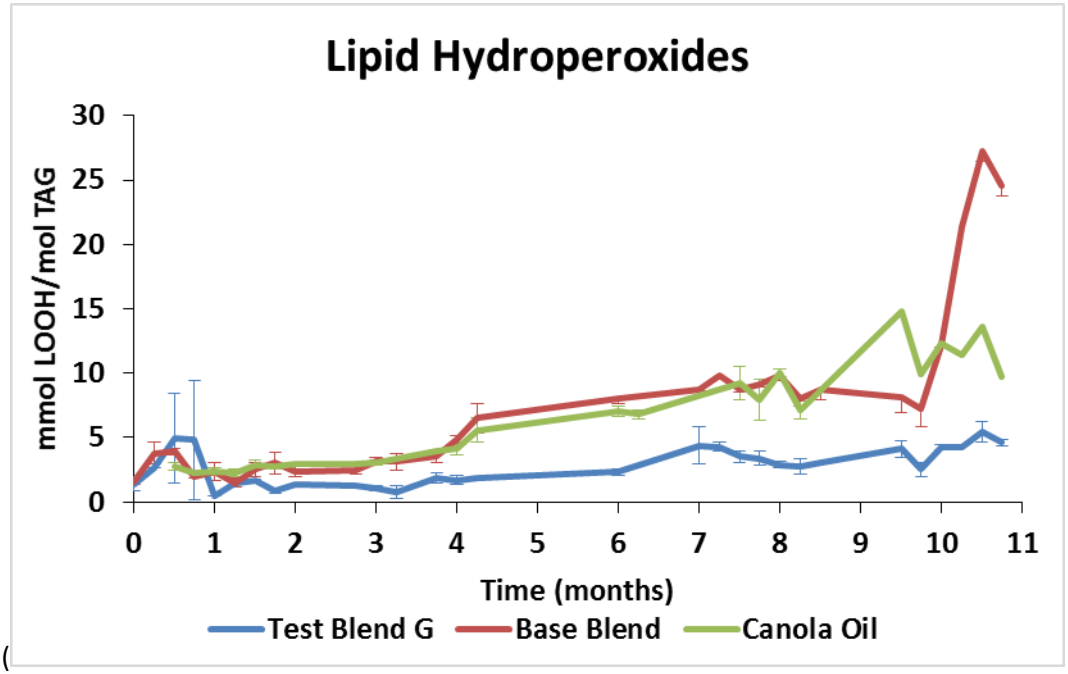


Figure *17*). Hydroperoxides in Blend 3G remained low (<5) throughout the storage period, which is expected from its low level of unsaturated fatty acids. In contrast, the Base Blend with coconut oil plus canola oil reached a peroxide value of 5 after only four months

and by the end of the study reached a value of over 25. Based on these results, the Base Blend shelf life is rather short and the target shelf life of one year at refrigeration temperatures cannot be attained. Development of hydroperoxides in canola oil closely paralleled that of the Base Blend until about 9 months, at which time the canola oil PV jumped to over 10, which is considered a reject level for consumer products. This kind of delayed development of rapid oxidation is normal with unsaturated oils, where oxidation remains low as long as endogenous antioxidants are present and active, then rapidly accelerates after the antioxidants have been fully consumed.

There is little reason to expect that a blend composed of mostly coconut oil and a small percent of canola oil should in the end oxidize much more rapidly than pure canola oil except for handling-induced contamination with catalysts. Coconut oil does contain some natural antioxidants (tocols and tocotrienols) but does not usually have citric acid added. The blends are exposed to more handling during preparation and water was added as a second phase in the emulsion. Either handling or water, or both, could introduce sufficient oxygen and metal contaminants to catalyze lipid oxidation, particularly if citric acid is not present. More detailed information about contents of endogenous antioxidants would help clarify reasons for differences between canola oil and the coconut oil and canola base blend.

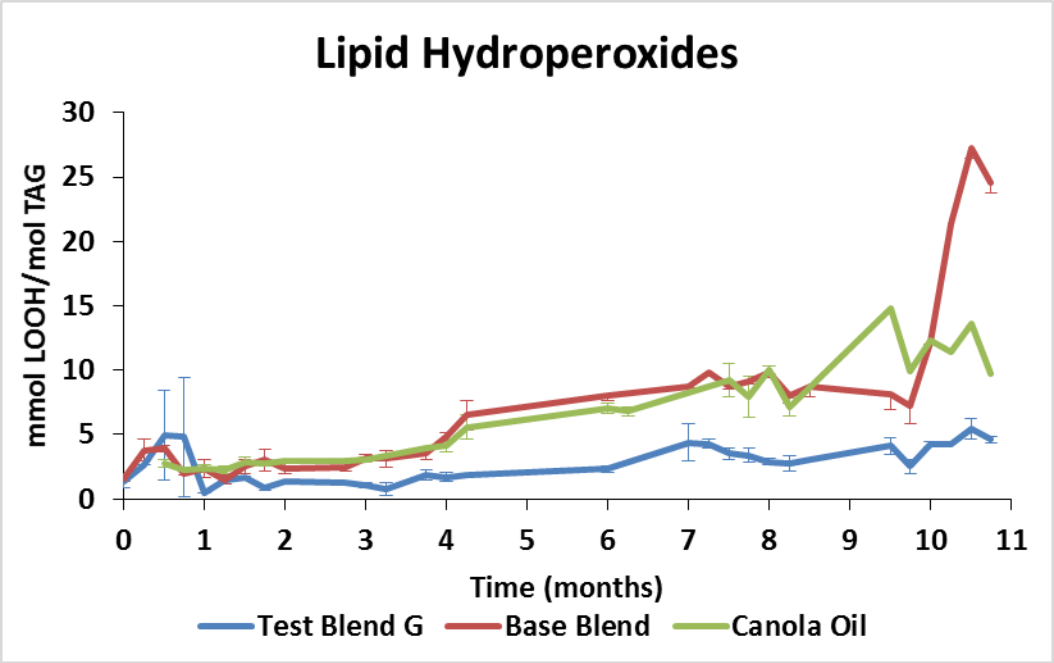


Figure 17: Peroxide values of Test Blend 3G, Base Blend, and canola oil samples over the course of 11 months at refrigeration temperature (4°C). Analyses were conducted with three replicates.

Information about antioxidant contents of the canola oil was either not available or not provided by the suppliers. Nevertheless, in general, oil refining attempts to leave behind as much natural antioxidant (e.g. tocopherols and perhaps carotenes) as possible, and citric acid is added as a metal chelator and oxidation inhibitor. With the high unsaturation of canola oil, it is not surprising that antioxidants are consumed faster, allowing active oxidation to commence earlier than with either of the blends. At the same time, this rapid oxidation may be controlled by higher endogenous antioxidant levels and citric acid, leading to final peroxide levels lower than Base Blend.

Blends were packaged and stored in a single container of thin clear plastic that is oxygen permeable and lacks a UV filter; each package was opened for sampling then

resealed (flushed with argon after each opening) multiple times during the shelf life study period. Packaging such as glass and thicker plastics (NalgeneTM, HDPE) with lower oxygen permeability and inclusion of a UV filter or opacity in the packaging would greatly limit oxidation. Furthermore, these products were manufactured with tap water, whereas using high purity water in the emulsion would help to prevent oxidation by reducing metal contents.

5.2.2 Analyses of thermal stability of Blends

Thermal stability of the Blends was tested using an Oxipres oxygen bomb for controlled heating and atmosphere. To eliminate interference of water with the pressure transducer and volatiles collected on thermal desorption traps, these heating experiments were conducted on pure component oils mixed in the same proportions as the Base Blend and Test Blend 3G reformulated with ghee, both alone and with tocopherols added at the same concentrations as the formulation. Oil blends were heated at 100, 120, and 150 °C to test thermal stability under conditions that simulated baking (100°C), sautéing (120°C) and deep frying (150 °C).

Conjugated diene, hydroperoxide, and free fatty acid levels in the two blends measured at the end of the heating time are shown in Table 12 (no antioxidants) and Table 13 (200 ppm added mixed tocopherols. Results showed that the Base Blend alone retained low peroxide levels at 100°C without tocopherols, but at higher temperatures peroxides exceeded the PV=10 acceptable limit even with added tocopherols.

That high peroxide levels accumulate is especially problematic since it indicates new hydroperoxide formation is faster than decomposition by the high temperatures. Thus, the Base Blend might be used in baking applications where temperatures stay below 100 °C, but not frying. Test Blend 3G maintained low peroxide levels even at 120 °C, reflecting the lower

unsaturates, but then degraded excessively at 150 °C. These results suggest that Blend 3G might be used in baking and low temperature sautéing applications but, again, not high temperature frying. Oxipres oxygen consumption curves supported this judgment, showing only slow reaction at 100 °C but notably increasing rates of oxidation at higher temperatures (data not shown).

Table 12: Lipid degradation in test blends without added antioxidant, fresh and after heating for 6 hours under 2 bar air. All values are significantly different.

Sample	CD (mmol/mol TAG)	stdev	PV (mmol/mol TAG)	stdev	FFA (%)	stdev	Total Volatiles (AUC)
Base Blend							
Fresh	7.91		1.22		ND (<0.04%)		N/A
100°C	10.22	0.39	3.97	0.09	ND		2.53E+06
120°C	14.41	0.12	12.26	0.15	ND		4.90E+06
150°C	29.91	0.59	15.63	0.74	1.62	0.26	7.17E+07
Test Blend 3G							
Fresh	5.08		0.65		ND		N/A
100°C	5.91	0.09	1.40	0.03	ND		2.27E+06
120°C	8.08	0.25	3.78	0.03	ND		2.24E+06
150°C	18.22	0.34	23.33	1.34	0.73	0.21	4.25E+07

Table 13: Lipid degradation in test blends with 200 ppm mixed tocopherols, fresh and after heating for 6 hours under 2 bar air. All values are significantly different.

Sample	**CD (mmol/mol TAG)**	stdev	**PV (mmol/mol TAG)**	stdev	**FFA (%)**	stdev	**Total Volatiles (AUC)**
Base Blend							
Fresh	8.39		2.77		ND (<0.04%)		N/A
100°C	2.84	0.43	10.08	0.83	ND		3.33E+06
120°C	8.55	0.97	15.88	4.32	ND		6.64E+06
150°C	20.45	2.67	33.63	4.98	0.62	0.13	4.59E+07
Test Blend 3G							
Fresh	6.06		1.74		ND		N/A
100°C	1.60	0.31	2.87	0.67	ND		2.72E+06
120°C	6.49	0.27	5.88	0.76	ND		1.52E+06
150°C	13.41	1.92	46.58	7.21	0.29	0.07	1.91E+07

Effects of tocopherol on thermal stability were not clear. Added tocopherols decreased conjugated dienes in both Blends heated at 100, 120 and 150 °C, but peroxides were substantially higher (Table 13). There are two possible explanations for this pattern. If all initial radicals are converted to hydroperoxides, conjugated dienes and peroxide levels should be comparable. That CD < PV with added tocopherol suggests that some peroxyl radicals were being rerouted to oxidation pathways away from hydroperoxide formation, e.g. addition or epoxide formation. At the same time, increased hydroperoxides could result from stabilization by tocopherols rather than enhanced oxidation. Hydrogen bonding of tocopherol phenolic groups to hydroperoxides (Figure 18) blocks hydroperoxide decomposition and H abstractions that initiate secondary autoxidation chains, thereby reducing levels of secondary products and new conjugated dienes (Hui, 2005). Peroxide stabilization has also been observed in other projects in our laboratory.

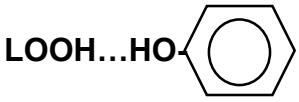


Figure 18: Lipid hydroperoxide stabilized by hydrogen-bonding to the hydroxyl group of a phenol, e.g. tocopherol.

Higher levels of tocopherols also donate H atoms to increase formation of hydroperoxides at the expense of internal rearrangement to epoxides. However, this seems less likely than other mechanisms since CD levels are not retained. More detailed chemical analyses will be required to distinguish which option, if either, is active.

Gas chromatograms of dominant volatiles collected after heating by venting the bomb through a thermal desorption trap showed that major products were pentane, pentanal, butanal, and hexanal – all products expected from linoleic acid (10.5% in Base Blend and 6.5% in Test Blend 3) – and decanal, a common product from oleic acid (~42% in both blends) (Figure 22). Butanal may also be generated by linolenic acid. Peak patterns were generally comparable in the two samples, just lower concentrations in Blend 3G. If oxidation pathways are being diverted, different volatile products should be detected. If hydroperoxide stabilization is active in Blend 3G, presumably lower levels of the same volatiles will be generated. The latter was, in fact, observed. That tocopherols decreased volatiles in the blends only at higher temperatures – in the Base Blend at 150 °C and in Test Blend 3G at 120 and 150 °C - where hydroperoxides were also highest (even though heat usually decomposes hydroperoxides rapidly) appears to support the hydroperoxide stabilization scenario, but cannot eliminate possibilities of other unmeasured product pathways being activated.

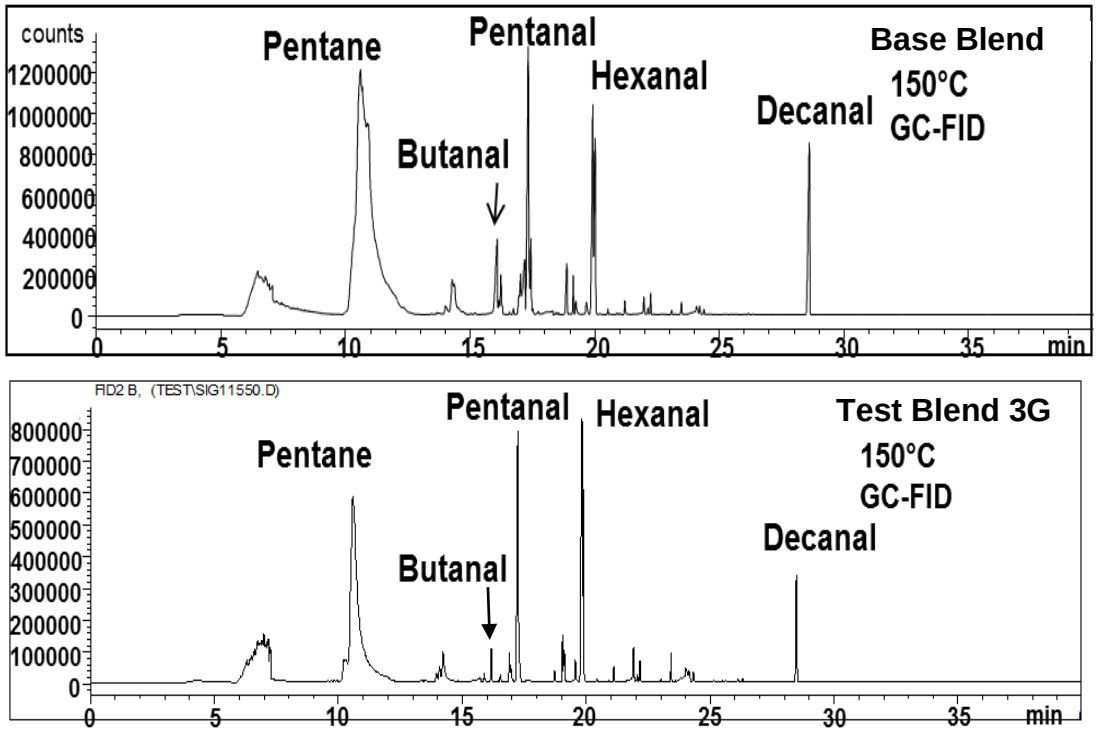


Figure 19: GC-FID chromatograms of the Base Blend (top) and the Test Blend 3G (bottom) heated at 150°C for six hours.

The medium chain saturated fatty acids in coconut oil and palm shortening may not oxidize but they are very sensitive to thermal scissions (i.e. they have a low smoke point). That short chain volatile thermal scission products (e.g. alkanes, aldehydes, and acids) from thermal scissions were not detected at identifiable levels suggests that under conditions of this experiment, non-volatile products such as dimers may dominate. Future studies of these Blends should analyze for a wide range of non-volatile products to ascertain thermal degradation pathways that are active in these Blends.

Overall, polyunsaturated fatty acids in the Base Blend show both thermal degradation

and oxidation. This prompts the recommendation to either remove PUFAs from the Base Blend or to not use this blend in heating applications. Degradation at 150°C suggests that the Test Blend 3G may not be appropriate for high-temperature frying, but may perform acceptably in baking or low temperature sautéing. Further tests are needed to verify this.

5.3 Phase II testing

Results of the initial shelf-life and thermal study raised questions about the potential role of water purity (especially metals) in the stability of the Blend emulsions. Thus, in Phase II three versions of Test Blend 3G were prepared by a consulting laboratory using water from different sources: Milli-Q (18 MΩ resistivity water; double distilled, deionized through two ion exchange cartridges and finish filtered through an Organex cartridge to remove trace organic contaminants), Packer (ground water from the packer locale), and a spring water marketed for drinking coolers (water source for consultants). A second series of test samples were also prepared with spring water and 0.02%, 0.05%, 0.07%, and 0.10% added mixed tocopherols to test whether endogenous antioxidants are sufficient to protect the blends, assess protective effects of added antioxidants, and determine what levels of added antioxidant may actually accelerate oxidation. Both series were tested for thermal stability and refrigerated shelf life.

5.3.1 Shelf life analyses

Effects of water. First, it must be noted that samples began with higher oxidation levels when received than had been seen in earlier Blends. Thus, effects of water and antioxidants may be confounded by handling in preparation of these experimental blends.

Higher starting oxidation levels inadvertently provide proof that handling during processing, packaging, and transport are also critical for starting quality, which in turn significantly affects stability during heating and storage.

Conjugated diene levels are perhaps most important since they reflect initiation rates and presence of catalysts (Frankel, 2012; Schaich, 2013). Conjugated dienes were generally higher in the spring water samples (Figure 23, top), but differences between samples at all time points were not significant (data not shown).

Peroxide values of the fresh Packer and spring water samples started at levels higher (PV>5) than would be acceptable in the food industry for fresh materials (

Figure 20, bottom). The Blend prepared with Milli-Q water was the only sample that started at low peroxide levels (PV~2), although even these were higher than customary target starting levels. Packer water samples developed significantly higher hydroperoxides than spring water and Milli-Q water samples during storage, demonstrating the importance of water quality in the Blend stability and corroborating the critical importance of quality of water used in formulating margarine and shortening blends. The peroxide values are not high on an absolute scale, but the differences (15 vs 10) are sufficient to make a distinct change in sensory perception of off-flavors.

Transition metals are the main catalysts of lipid oxidation in foods, and water is the greatest source of catalytic metals by far (Schaich, 2005; Frankel, 2012). Even traces of metals in glassware, solvents, and water can markedly accelerate and amplify lipid oxidation and move oxidation into pathways that generate different products. Packer

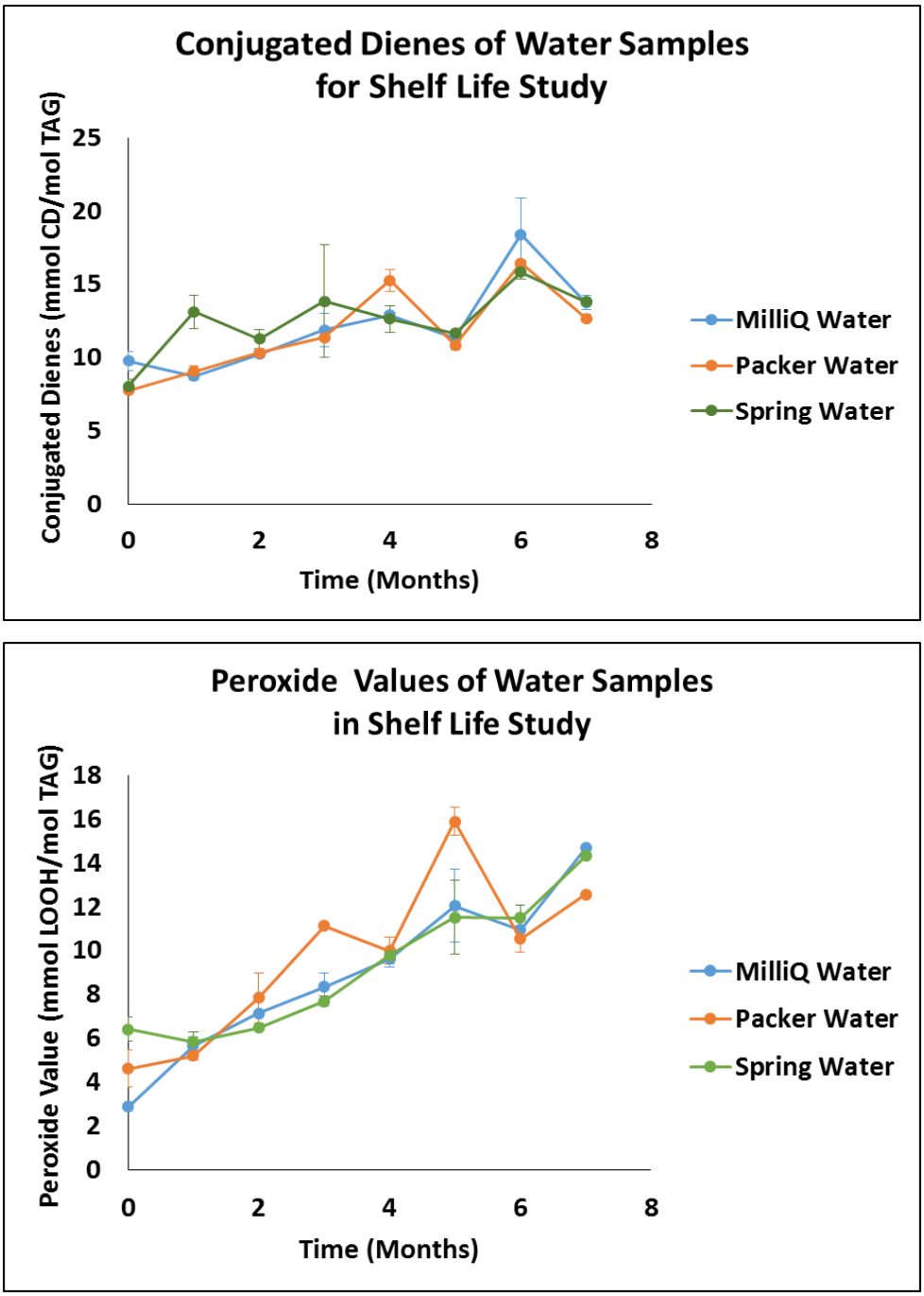
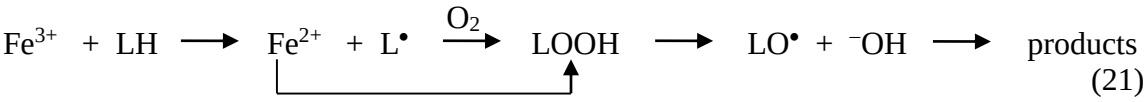


Figure 20: Conjugated dienes (top) and peroxide values (bottom) from Blend 3G formulated with different waters and incubated at 4 °C for seven months.

water is tap water (ground water) and therefore may be high in catalytic transition metals such as iron and copper (Azoulay et al, 2001). In this case, Packer water comes from an area near Mt. St. Helens, an active volcano, where high metal content is expected due to the geology of the area which has many igneous rock formations including basalt (high in iron and magnesium), andesite (high in iron and magnesium), and dacite (high in sodium, potassium, and silica) (USGS, 2015). In addition, many water pipes are made from copper and leach traces of copper ions into tap water. Despite this background, analyses provided by the Packer and the spring water supplier both claimed zero metals detectable at ppb levels, which is not believable.

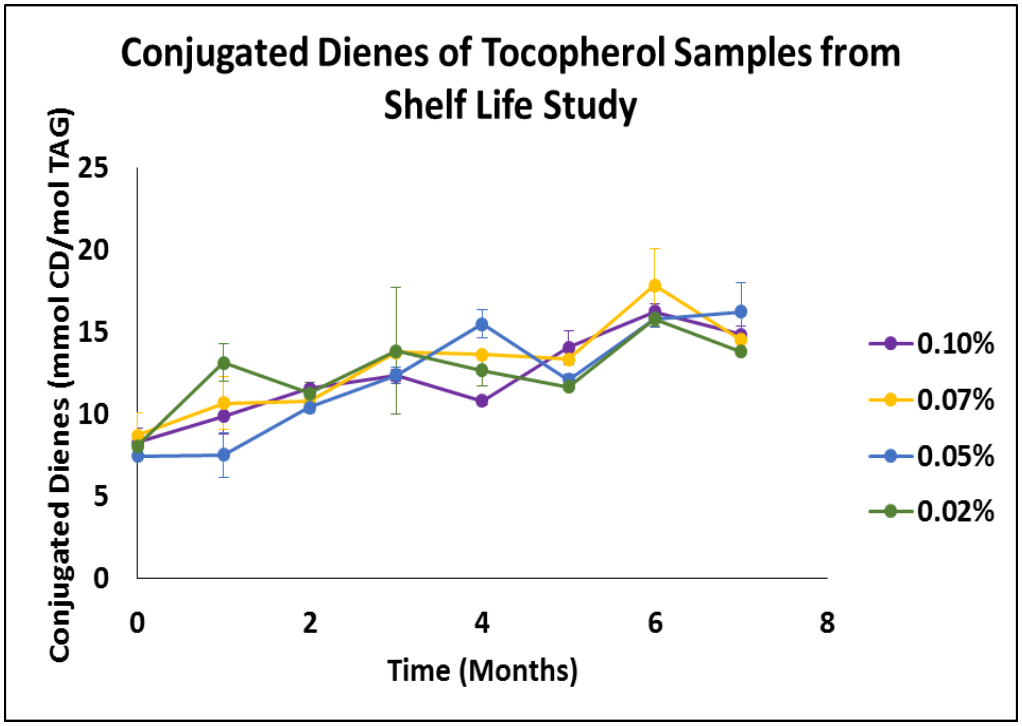
That spring water samples showed highest conjugated dienes but lower hydroperoxides may seem contradictory, but is in fact consistent with elevated metal levels which initiate oxidation chains then are present to decompose hydroperoxides (Reaction 21), and also reroute oxidation to alternate pathways not measured in this study (Schaich et al, 2013).



(21)

At the same time, for diene conjugation to be retained, the alkoxyl radical must not decompose or rearrange. Addition of LO• to a double bond or H-abstraction to form hydroxylipids would both satisfy these requirements. However, detailed product analysis is required to validate the speculation.

Effects of added tocopherols. Various levels of mixed tocopherols were added to Blend 3G prepared with spring water to assess whether higher levels of antioxidants could increase stability of the product. Oxidation product levels are shown in Figure 21.



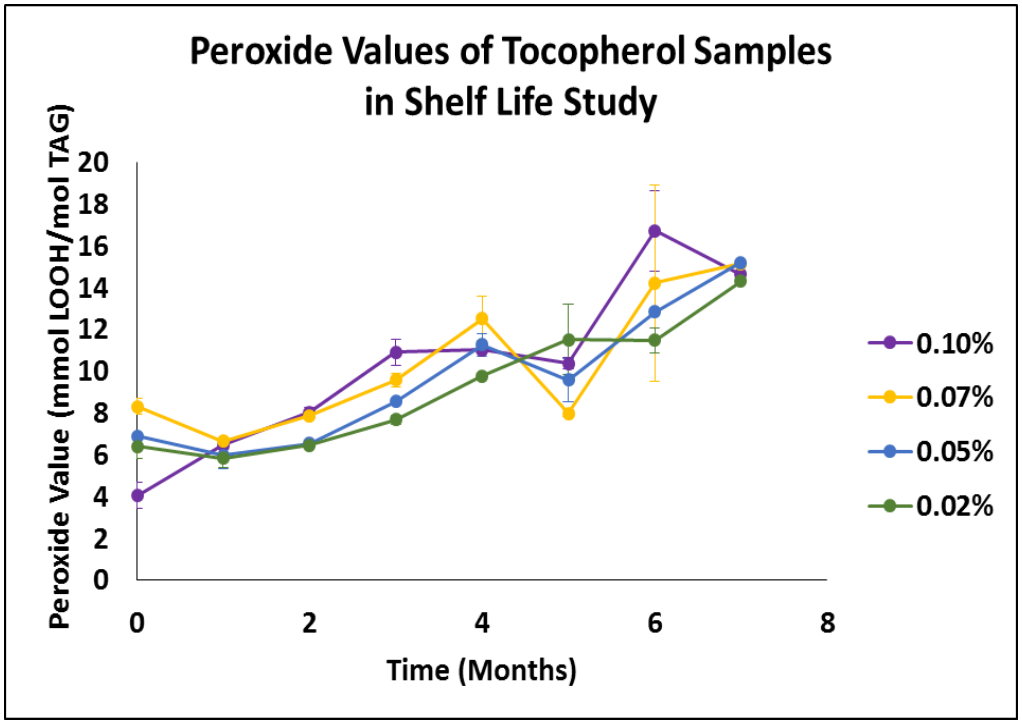


Figure 21: Conjugated dienes (top) and peroxide values (bottom) from Blend 3G formulated with spring water and different levels of mixed tocopherols and incubated at 10 °C for seven months.

Although there was no clear effect of tocopherol level on conjugated dienes, there was a tendency for hydroperoxides to increase with tocopherol level, consistent with hydroperoxide stabilization by hydrogen bonding to the phenol group. However, the differences between tocopherol levels were not significant, suggesting that increasing added tocopherol levels does not seem to add protection to these products.

There are two possible explanations for this ineffective inhibition of oxidation. First, the starting coconut oil and palm shortening may already have sufficiently high levels of tocopherol and tocotrienols that additional levels do not help, or even become more pro-oxidant. Endogenous antioxidant contents of starting materials were not available and our laboratory did not have the facilities to complete independent analyses. However, data from UN-FAO and one supplier of palm shortening indicates that from 0.04 to 0.15% tocopherols plus tocotrienols may be present in this component alone (UN-FAO, 1999). Thus, this system may already be saturated with antioxidants.

Alternatively, since conjugated dienes remain very close regardless of tocopherol content for the first three months of refrigerated storage, the initiating event in blends may not be accessible to tocopherol intervention. The most likely initiator in these systems is trace metals (Reaction 22):

$$-CH_2-CH=CH-CH_2- + Fe^{3+} \longrightarrow -CH_2-CH^{+}-{}^{\bullet}CH-CH_2- + Fe^{2+} \qquad (22)$$

$$-CH_2-CH^{+}-{}^{\bullet}CH-CH_2- + -CH=CH-CH_2-CH=CH- \longrightarrow \underset{\text{conjugated diene}}{-CH=CH-CH=CH-{}^{\bullet}CH-}$$

Since tocopherol does not react with carbon centered radicals, this process would not be stopped. Only secondary processes involving peroxyl radicals and downstream oxygen-centered radicals would be affected.

Peroxide values increased slowly during the first two months of storage, and although it perhaps seems counterintuitive, the peroxide levels increased with increasing tocopherols. This may be caused for two reasons: 1) higher levels of tocopherols hydrogen bond with hydroperoxides to prevent their decomposition, or 2) higher levels of tocopherols donate H atoms to $LOO^{\bullet}$ to increase formation of hydroperoxides at the expense of alternate pathways, e.g. addition to double bonds and subsequent decomposition to epoxides. We have seen epoxides as major products (>hydroperoxides) in pure oil (Xie, 2015), but also observed peroxide stabilization by phenols. More detailed chemical analyses will be required to distinguish which option, if either, is active. However, these results point out quite clearly the dangers of using single products to assess lipid oxidation.

Overall assessment: Results show that water purity does play a role in Blend stability but also suggest that general handling and packaging may be just as important. With a fatty acid composition that is predominantly saturated and mono-unsaturated, Blend 3G should have reasonably long shelf life, more than 6 months. Peroxide levels increased by only 6 to 8 mmol peroxide/kg fat over six months refrigerated storage, so if these samples had not started with high PV levels, they would still be acceptable products.

Results also suggest that the best compromise for shelf stability may be 0.05% tocopherols, but this level clearly cannot be claimed as "optimal". Running samples with zero and >0.10% tocopherols would provide some additional clues. However, this question can only be answered definitively in the context of total formulation and processing issues and the radical load created by them.

Determining actual acceptable life time for Blend 3G is not as straightforward as with soybean oil or similar polyunsaturated product where taste panel scores corresponding

to various oxidation levels are well established, and a peroxide value of 10 is considered a discard point in legal guidelines for frying and as a practical guideline for stored products (Matthaus, 2006; Nielson, 2003). Studies coordinating sensory qualities with lipid oxidation levels in tropical fats, which would make possible straightforward guidelines for the Blends, are lacking. The flavor and odor compounds released from saturated and monounsaturated fatty acids are different from the PUFAs in vegetable oils, so the consumer interpretation of a PV of 15 in the Blends is not known. Obtaining sensory evaluation data to match against oxidation levels would be helpful in determining consumer "reject" conditions for these products. Sensory descriptors and acceptability at different oxidation levels will establish the constraints for processing, product rejection points, and required testing protocols for quality control in tropical fat-based products.

If consumers find flavors acceptable in blends with PVs up to about 15, the current data suggests a refrigerated shelf life of 3 months. It must be noted, however, that this was with samples sealed in glass jars under argon, so must be viewed as close to a best case scenario. Samples stored in the common plastic containers and without at least nitrogen flushing will have shorter shelf life.

5.3.2 Thermal analyses

Water and tocopherol samples were heated at four different temperatures (80°C, 100°C, 120°C, and 150°C) for 90 minutes and analyzed for conjugated dienes (CDs), peroxide values (PVs), non-volatile carbonyls, and volatile oxidation compounds.

5.3.2.1 Effects of water source on thermal stability of Blend 3G

Water source appeared to have little effect on oxidation of samples heated at 80 °C. However, differences between water sources became more pronounced as heating

temperature was increased (Figures 22 and 23, CDs and PVs respectively). At 120°C, samples with Packer and Spring waters showed slightly higher CD levels than Milli-Q water samples, although the differences were not statistically significant (Figure 22). At 150°C, CDs were notably higher in Packer water samples than the Milli-Q and Spring water samples, and these differences were statistically significant ($p<0.05$). Milli-Q water samples had the lowest CDs with spring water sample values in between. This analysis shows clearly that the higher the heating temperature, the more important the water quality becomes. Since these Blends are being designed for cooking and baking applications, this issue must be addressed in manufacturing.

Peroxide values showed a very interesting pattern (Figure 23). Peroxides are poor indicator products for thermal degradation because heat induces LO–OH bond scission, releasing alkoxyl and hydroxyl radicals, $LO^{\bullet}$ and $^{\bullet}OH$, respectively. Peroxide values observed depend on which reaction dominates -- LOOH decomposition or initiation of secondary chains by the $LO^{\bullet}$ and $^{\bullet}OH$ radicals. At 80 °C, PVs decreased with heating time, indicating that LOOH decomposition dominated. At 100 °C, PVs were constant with heating time, suggesting that LOOH decomposition and formation of new chains were in balance. In contrast, at 120 °C PVs began to increase with heating time, and at 150 °C the increase was faster, showing that secondary chain propagation clearly exceeded LOOH decomposition. Except for a few time points at 80 °C, PVs of Packer and spring water samples were higher than Milli-Q samples, demonstrating that effects of water quality were definitely important and increased with temperature. Although PV levels reached normal discard levels of 10 only at 150 °C, the PVs at lower temperatures still stayed in the "danger zone" of 5 to 10 PV where small differences can often be distinguished by consumers. An

important side consideration here is how to interpret peroxide values in mostly saturated fats. Consumer correlations with peroxide values were determined using vegetable oils that are predominantly unsaturated. Blends based on tropical fats will have very low contents of unsaturated fatty acids so should be expected to exhibit low conjugated dienes and peroxides when calculated on a total fat basis. However, this grossly underestimates the degree of oxidation in the unsaturated fraction. Perhaps a more useful approach for mostly saturated blends is to calculate oxidation products based on unsaturated fatty acid content.

The heating behavior observed in the Blends supports error in the metal analyses indicating "no detectable transition metals" provided by the water suppliers. Higher conjugated dienes with Packer water are particularly important because they result from increased initiation of new oxidation chains, most likely by metals, and cannot result from mere organic contamination of the water. To the same point, samples prepared with the bottled spring water exhibited even higher PVs during heating than the other two waters. The high PVs with relatively stable CD values for the spring water is likely due to high metal content and organic contaminants typically found in bottled spring waters, and actually found in this specific brand of spring water (Azoulay et al, 2001; Diduch et al, 2011). Spring water, therefore, is clearly not optimal for use in these Blends.

These results demonstrate cogently that the water with which the Blends are prepared plays a significant role in product stability, probably as important as the fatty acid composition of the fats and oils used in the blends. In terms of practical applications, water composition and handling become even more critical at higher temperatures, i.e. for any Blend intended for baking, braising, or sautéing.

Furthermore, antioxidant levels required for stabilization of any product are determined largely by the radical load developed in the product by its treatments, composition, and reactions. Transition metal contaminants of water initiate oxidation and catalyze secondary decompositions, thus serving as a major contributor to radical load. When the radical load is too high, stabilization may not be achieved at any antioxidant concentration (Yam, 2009; Schaich et al, 2013). Conversely, optimum antioxidant concentrations for a Blend formulation cannot be determined without controlling initiators in water and handling.

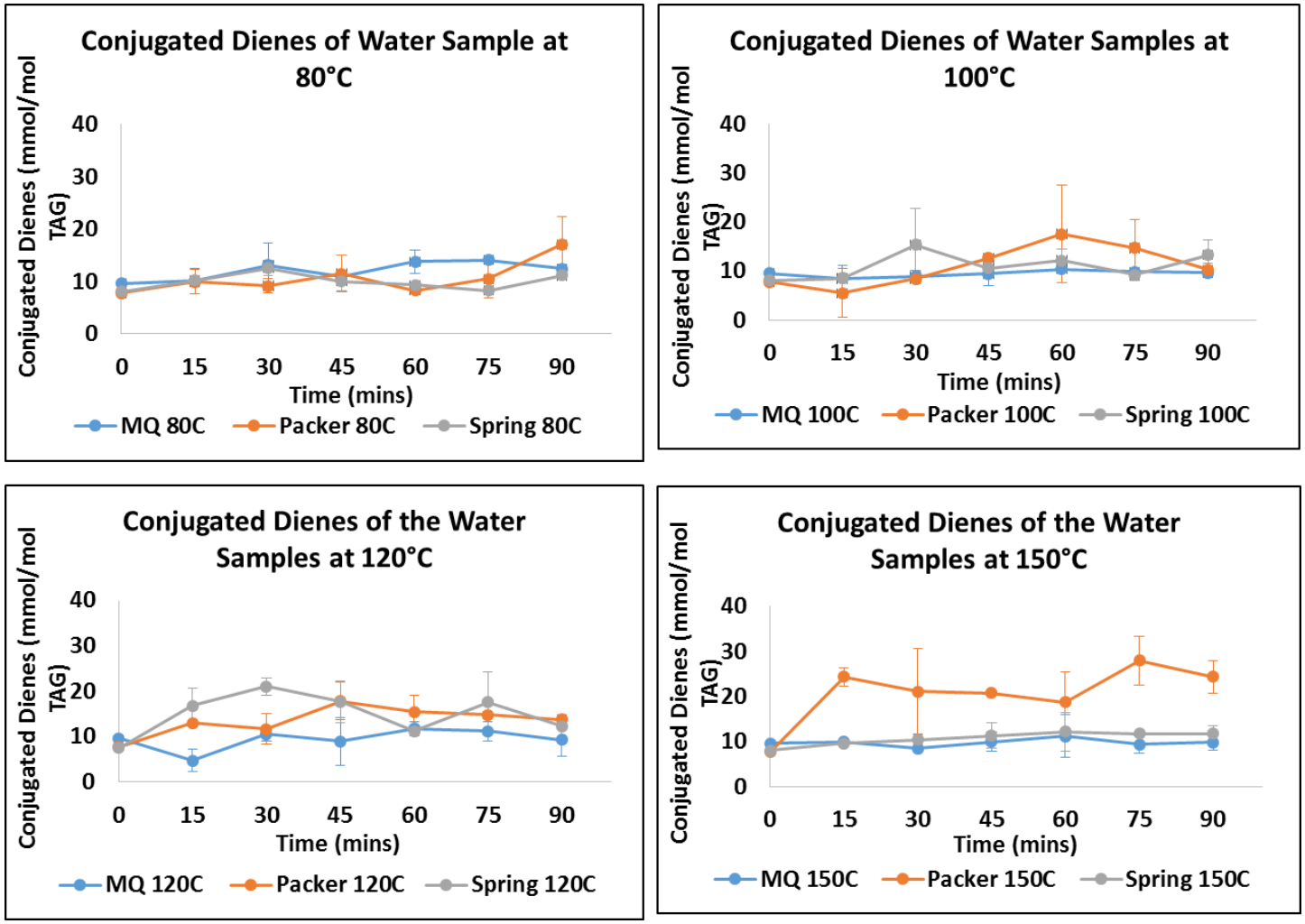


Figure 22: Effect of water source and temperature on conjugated diene formation in Blend 3G heated for 90 minutes.

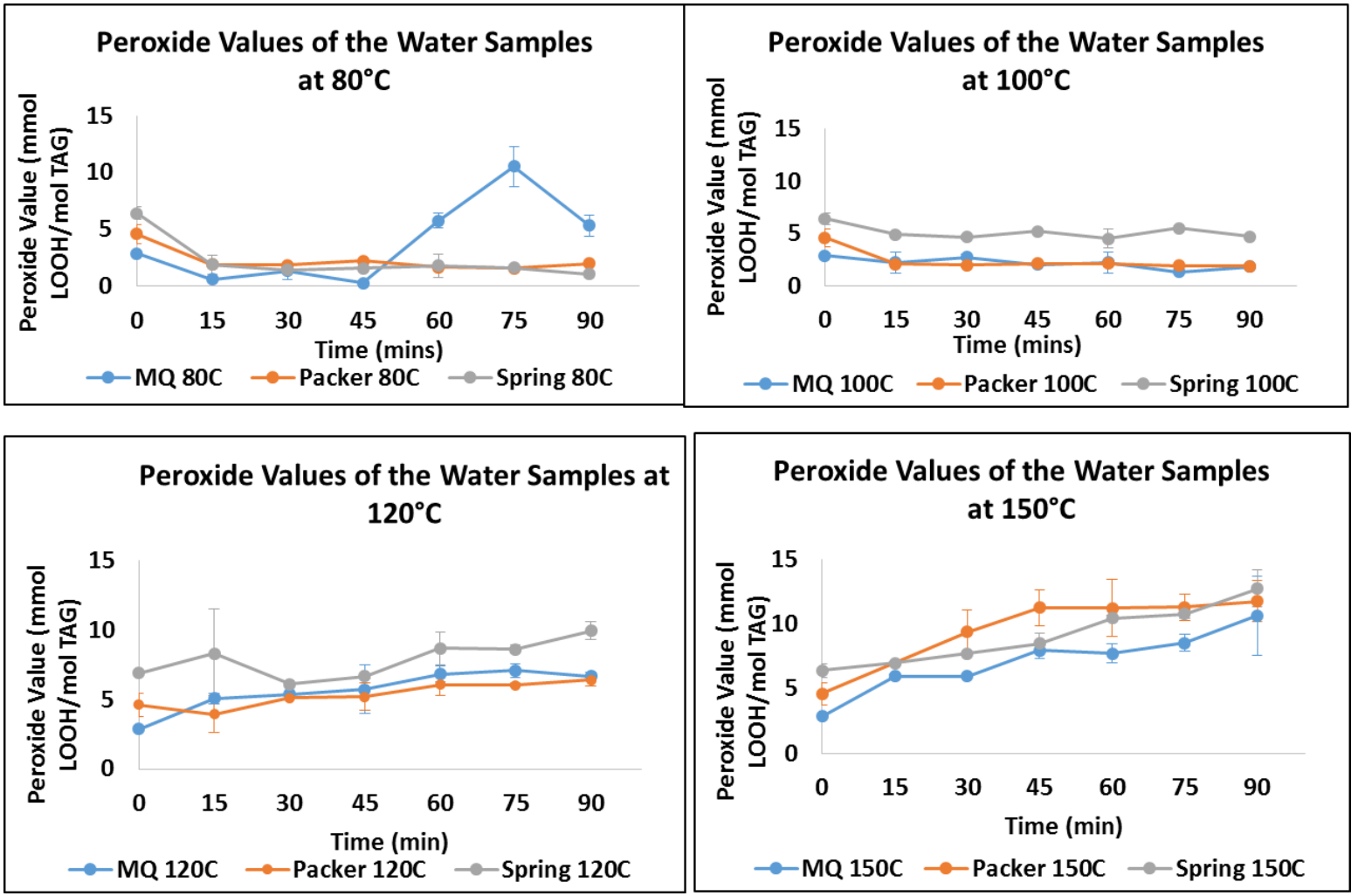


Figure 23: Effect of water source and temperature on hydroperoxide formation in Blend 3G heated for 90 minutes.

Furthermore, antioxidant levels required for stabilization of any product are determined largely by the radical load developed in the product by its treatments, composition, and reactions. Transition metal contaminants of water initiate oxidation and catalyze secondary decompositions, thus serving as a major contributor to radical load. When the radical load is too high, stabilization may not be achieved at any antioxidant concentration (Yam, 2009; Schaich et al, 2013). Conversely, optimum antioxidant concentrations for a Blend formulation cannot be determined without controlling initiators in water and handling.

5.3.2.2 Effects of added tocopherol on thermal stability of Blend

Mixed tocopherols exerted no consistent effect on thermal stability of Blend 3G. Conjugated dienes remained close in range and were not statistically different among the tocopherol levels across all four temperatures (Figure 24). There were some cyclical increases at 30 min and again at 75 min, but with no obvious pattern of tocopherol effects. This indicates that conjugated dienes are probably not good indicators of the active chemistry in the samples and that the tocopherols are not able to intervene in whatever reactions generated conjugated dienes.

PVs (

Figure 25) similarly showed close correspondence between tocopherol levels at all temperatures except at 100 °C where 0.02% samples had higher PVs and at 120 °C, where 0.07% tocopherol samples had notably higher PVs. These two differences were statistically significant ($p<0.05$); all others were not. As with the previous heating study, PVs decreased with time at 80 °C, were more constant at 100 °C, then gradually increased at higher

temperatures reflecting different balances between hydroperoxide decomposition and increased radical scissions.

This data seems to support earlier speculation that antioxidant levels are already saturated and/or that tocopherols are not able to effectively intervene in the thermal reactions active in these Blends. At this point, we also cannot eliminate sampling issues (where the sample was removed from the jar) as being the major source of variability, considering how close the CD and PV values are in the various samples. An impractically large number of samples would be required to test this possibility. If the data represents true scatter, we are clearly not measuring the right products or detecting the active pathways.

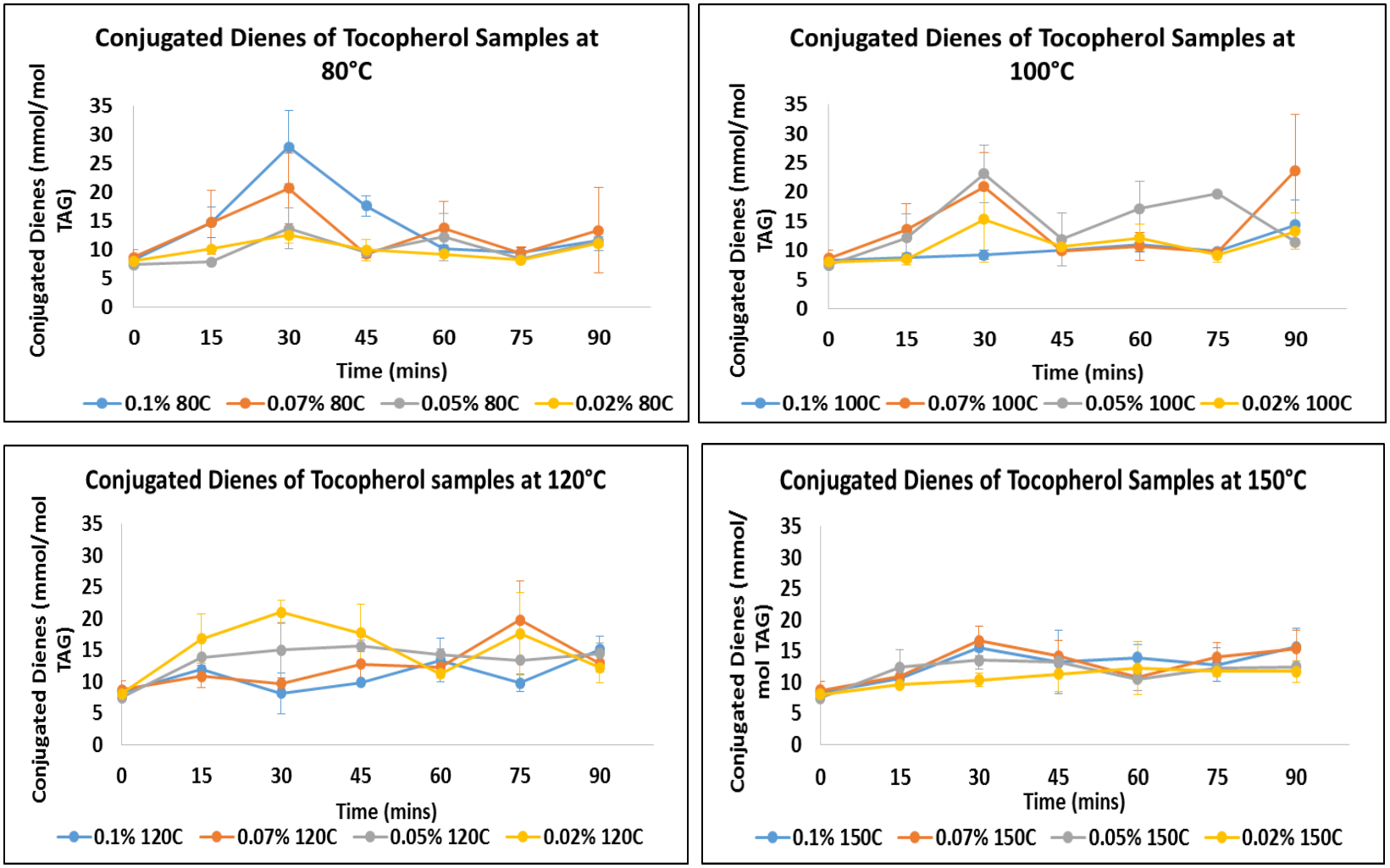


Figure 24: Effect of heating temperature and tocopherol level on conjugated dienes in Blend 3G (spring water) heated for 90 minutes.

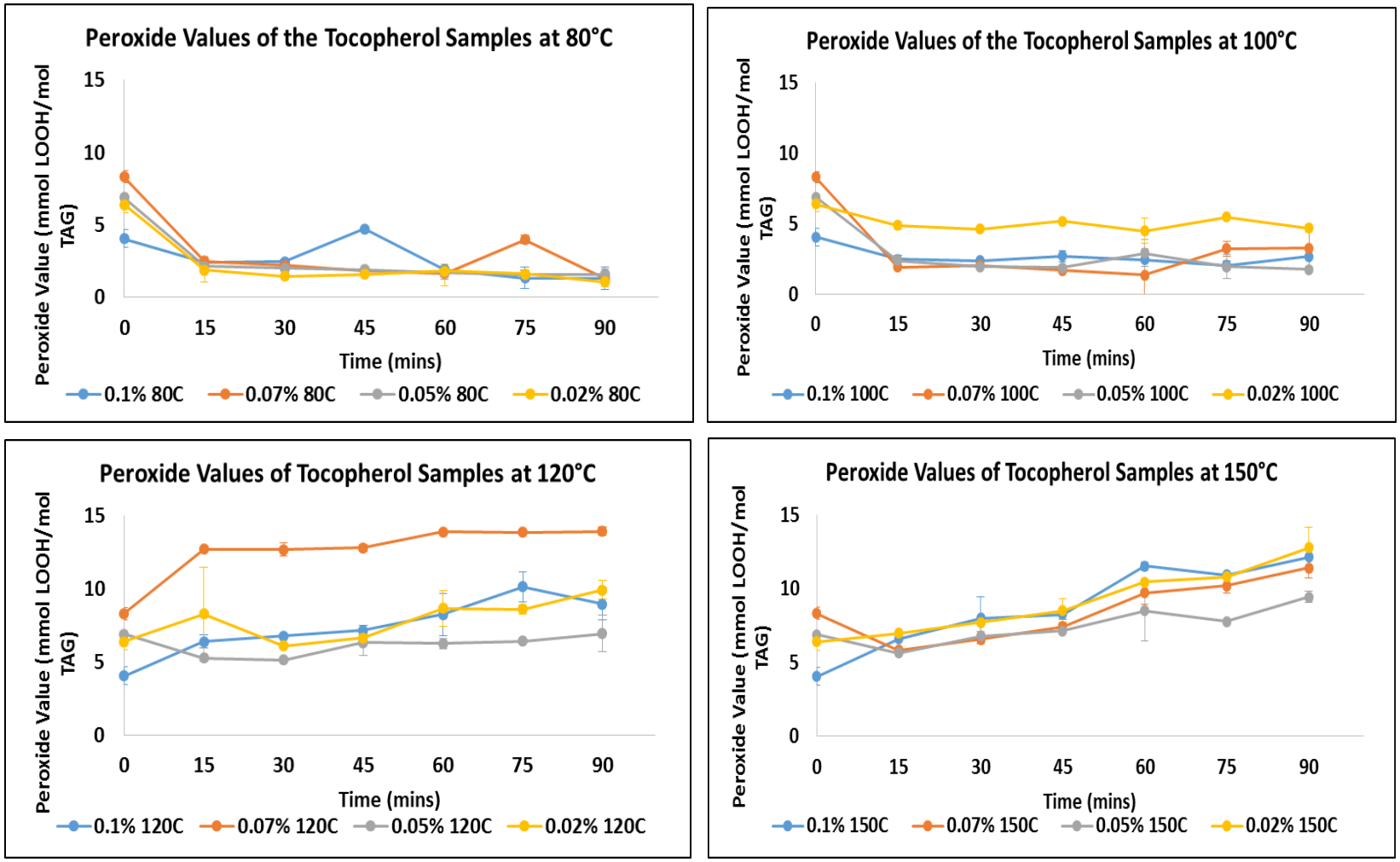


Figure 25: Effect of heating temperature and tocopherol level on peroxide levels in Blend 3G (spring water) heated for 90 minutes.

Overall assessment: Given the low level of unsaturation in Blend 3G, conjugated dienes and hydroperoxides appear to be of questionable use in evaluating oxidative stability of this product, the effects of tocopherol antioxidants, or progress of thermal degradation. In thermal degradation studies and when comparing different levels of tocopherols, changes were relatively small with both conditions and antioxidant. In shelf life studies, low CD and PV levels were not truly indicative of total degradation since they indicated acceptable products, yet the Blends developed noticeable odors and off-flavors. Thus, we are left with the question: is this low level of change due to actual stability, i.e. the saturated fatty acids are not affected during storage or heating and thus the tocopherols have no radical reactions to quench, or do degradation reactions proceed by different pathways and yield primarily products that were not measured? If the latter, other degradation products such as epoxides and co-oxidation of components other than fatty acids in the tropical oils must also be considered as potential sources of the off-odors and flavors.

Evaluation of the stability of this product cannot be completed without answering this question, which in turn requires analysis of additional oxidation products that may be formed independently of unsaturated components and hydroperoxides. To address this issue, secondary products were analyzed by two methods: non-volatile carbonyls that would contribute to off-flavors were analyzed by DNPH-HPLC assays, and volatile degradation products were analyzed by gas chromatography.

5.3.2.3 Gas chromatography analysis of volatile products generated in Blends

Gas chromatography analyses were performed to better understand what volatile products were evolving from the blends during cooking. At the levels shown in the previous experiments, lipid hydroperoxides and CDs cannot be tasted, but informal tasting and

smelling of the blends confirmed notable off-odors and off-flavors. Thus, PVs and CDs are insufficient to make a judgment on the quality of the blends, especially since they showed few statistically significant differences. The gross initiation of oxidation may be comparable, but differences in the metal contents between the blends will affect the mechanisms of degradation and the products produced. This initiated studies to analyze some of the secondary products produced via GC-FID.

After the samples were heated, approximately 2 grams of each blend was placed in a 20 mL clear glass headspace vial, flushed with argon, and sealed with a septa. The vials were wrapped in aluminum foil to prevent light penetration and stored in a -80°C freezer until analysis was performed. Before analysis, the vials were removed from the freezer and incubated at 40°C for 15 mins. Approximately 2 mL of headspace was taken from the vial using a static headspace syringe kept at 40°C, to prevent condensation of the volatiles, and injected into the GC-FID for analysis.

Equilibrium samples were taken using this method, although some of the volatiles were lost during test heating. Despite not being able to determine what volatiles evolved during cooking, this method is more representative as to what would be tasted and smelled in foods after preparation with the blends and is therefore more representative of the sample. 80°C (cakes) and 100°C (cookies) represent baking temperatures while 120°C (light sautéing) and 150°C (stir frying) represent stovetop applications.

The major unsaturated fatty acid in these samples is oleic acid (at about 40% of fat content in the blends), which will be the most likely suspect as a contributor to the proliferation of secondary oxidation products such as aldehydes. In Figure 26, the scission products of oleic acid are shown. However, there is also a significant amount of linoleic

acid (at about 7%), which could also contribute to secondary oxidation product accumulation; typical scission products are shown in Figure 27. Autoxidation secondary products for both fatty acids are shown in Figure 26. To fully understand the quality of the blends, off odors need to be also examined. While this study did not quantify specific odors nor were any formal sensory evaluations performed, odor characteristics of individual aldehydes and products shown in Table 15 can help predict likely sources of off-odors. Odor thresholds listed in Table 15 show that levels of volatile products detected in this study are in a range that can be perceived and recognized by consumers.

In analyzing this data, we evaluated both total levels of volatiles and levels and patterns of evolution for individual volatile products, the latter to identify active degradation pathways and fatty acids involved. Total volatiles in the water samples are compared in Figure 28; total volatiles in the tocopherol samples are compared in Figure 29. Individual volatiles identified are tabulated for water samples (Tables 16-19) and tocopherols (Tables 19-23) In the interest of space, individual product data is presented for tocopherol samples only at 80°C; all other data is available upon request.

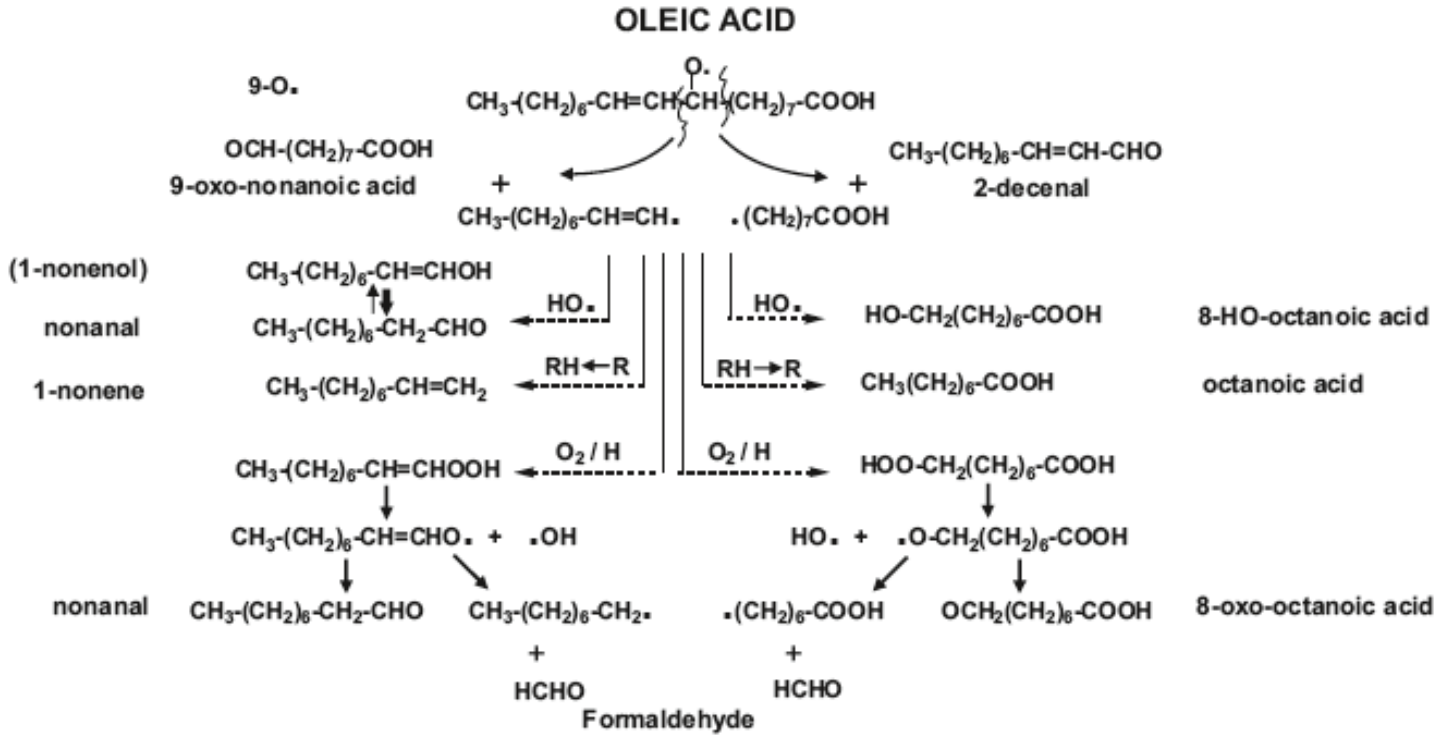
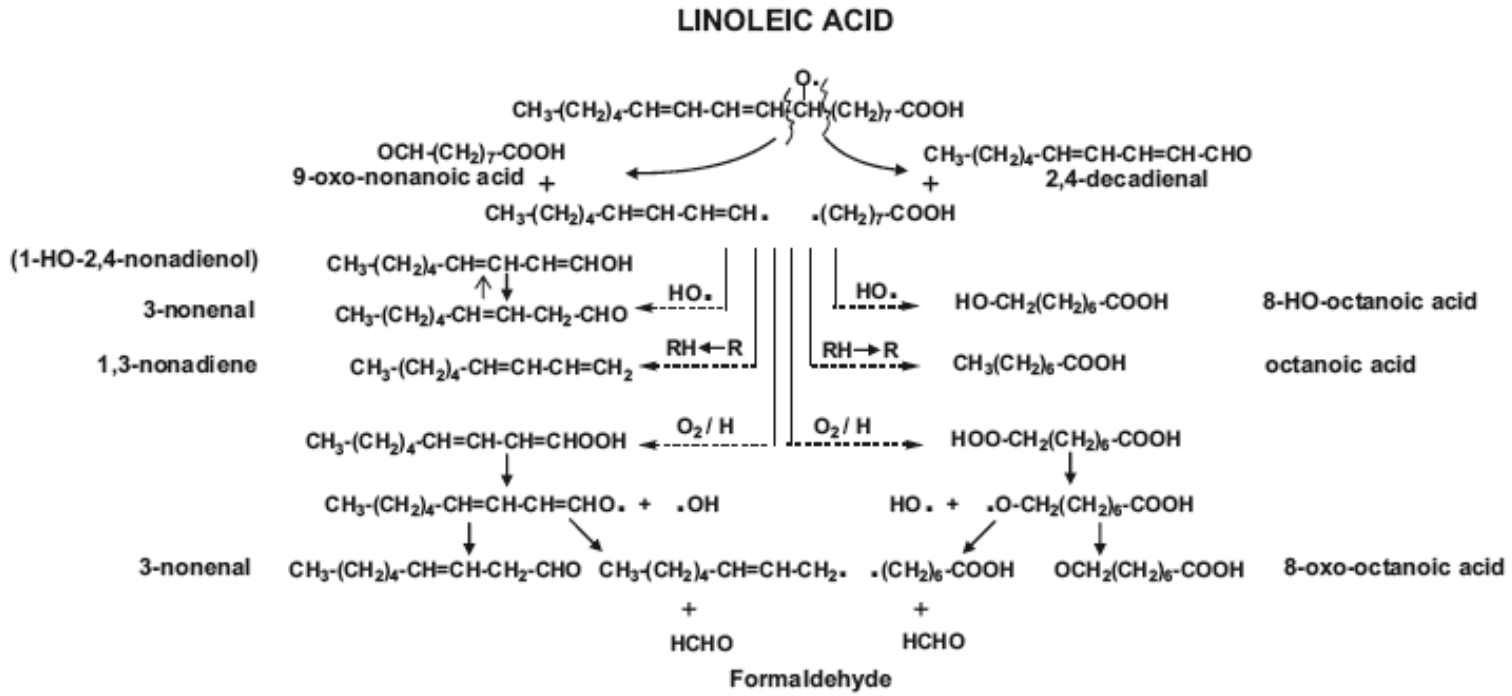


Figure 26: Scission pathways for oleic acid; adapted from Schaich, 2005.

Following the same fragmentation pattern –

	β-scission		*α-scission*	
8–O• →	8-oxo-octanoic acid	+ decanal 1-decene (nonanol nonane nonanal formaldehyde)	2-undecenal	+ 7-HO-heptanoic acid heptanoic acid 7-oxo-heptanoic acid (6-HO-hexanoic acid hexanoic acid 6-oxo-hexanoic acid formaldehyde)
10–O• →	10-oxo-8-decenoic acid	+ octanol octane octanal (heptanol heptane heptanal formaldehyde	nonanal	+ 9-oxo-nonanoic acid 8-nonenoic acid (octanol octane octanal formaldehyde)
11–O• →	11-oxo-9-undecenoic acid	+ heptanol heptane heptanal (hexanol hexane hexanal formaldehyde)	octanal	+ 10-oxo-decanoic acid 9-decenoic acid (nonanol nonane nonanal formaldehyde)

Figure 26, continued: Fragmentation patterns of scission products of oleic acid; adapted from Schaich, 2005.



Following the same fragmentation pattern –

<u>β-scission</u>

13–O• → 13-oxo-9,11-tridecadienoic acid
+ pentanol
pentane
pentanal
(butanol
butane
butanal
formaldehyde)

<u>*α-scission*</u>

hexanal + 12-oxo-9-dodecenoic acid
9,11-dodecadinoic acid
(11-HO-9-undecenoic acid
9-undecenoic acid
11-oxo-9-undecenoic acid
formaldehyde)

Figure 27: Scission pathways and fragmentation patterns for linoleic acid; adapted from Schaich, 2005.

Table 14: Volatile compounds formed by autoxidation of the unsaturated fatty acids; adapted from Belitz, 2009.

Oleic acid		Linoleic acid	
Heptanal	50	Pentane[b]	+[c]
Octanal	320	Pentanal	55
Nonanal	370	Hexanal	5,100
Decanal	80	Heptanal	50
(E)-2-Decenal	70	(E)-2-Heptenal	450
(E)-2-Undecenal	85	Octanal	45
		1-Octen-3-one	2
		1-Octen-3-hydroperoxide	+[c]
		(Z)-2-Octenal	990
		(E)-2-Octenal	420
		(Z)-3-Nonenal	30
		(E)-3-Nonenal	30
		(Z)-2-Nonenal	+[c]
		(E)-2-Nonenal	30
		(Z)-2-Decenal	20
		(E,E)-2,4-Nonadienal	30
		(E,Z)-2,4-Decadienal	250
		(E,E)-2,4-Decadienal	150
		trans-4,5-Epoxy-(E)-2-decenal	+[c]

Table 15: Sensory properties of the aroma components from lipid peroxidation; adapted from Belitz, 2009.

Compound	Flavor quality	Odor threshold (µg/kg) in oil	
		nasal	retronasal
Aldehydes			
2:0	fruity, pungent	0.22	7.1
3:0	fruity, pungent	9.4	68
5:0	pungent, like bitter almonds	240	150
6:0	tallowy, green leafy	320	75
7:0	oily, fatty	3200	50
8:0	oily, fatty, soapy	55	515
9:0	tallowy, soapy-fruity	13,500	260
10:0	orange peel like	300	75
5:1 (E-2)	pungent, apple	2300	600
6:1 (E-2)	apple	420	250
6:1 (Z-3)	green leafy	1.7	1.2
7:1 (E-2)	fatty, bitter almond	14,000	400
7:1 (Z-4)	cream, putty	2	1
8:1 (Z-2)	walnut	–	50
8:1 (E-2)	fatty, nutty	7000	125
9:1 (Z-2)	fatty, green leafy	4.5	0.6
9:1 (E-2)	tallowy, cucumber	900	65
9:1 (Z-3)	cucumber	250	35
10:1 (E-2)	tallowy, orange	33,800	150
7:2 (E,Z-2,4)	frying odor, tallowy	4000	50
7:2 (E,E-2,4)	fatty, oily	10,000	30
9:2 (E,E-2,4)	fatty, oily	2500	460
9:2 (E,Z-2,6)	like cucumber	4	1.5
9:2 (Z,Z-3,6)	fatty, green	–	–
9:3 (E,E,Z-2,4,6)	Oat flakes	–	–
10:2 (E,Z-2,4)	frying odor	10	–
10:2 (E,E-2,4)	frying odor	180	40
10:3 (E,Z,Z-2,4,7)	cut beans	–	24
trans-4,5-Epoxy-(E)-2-decenal	metallic	1.3	3

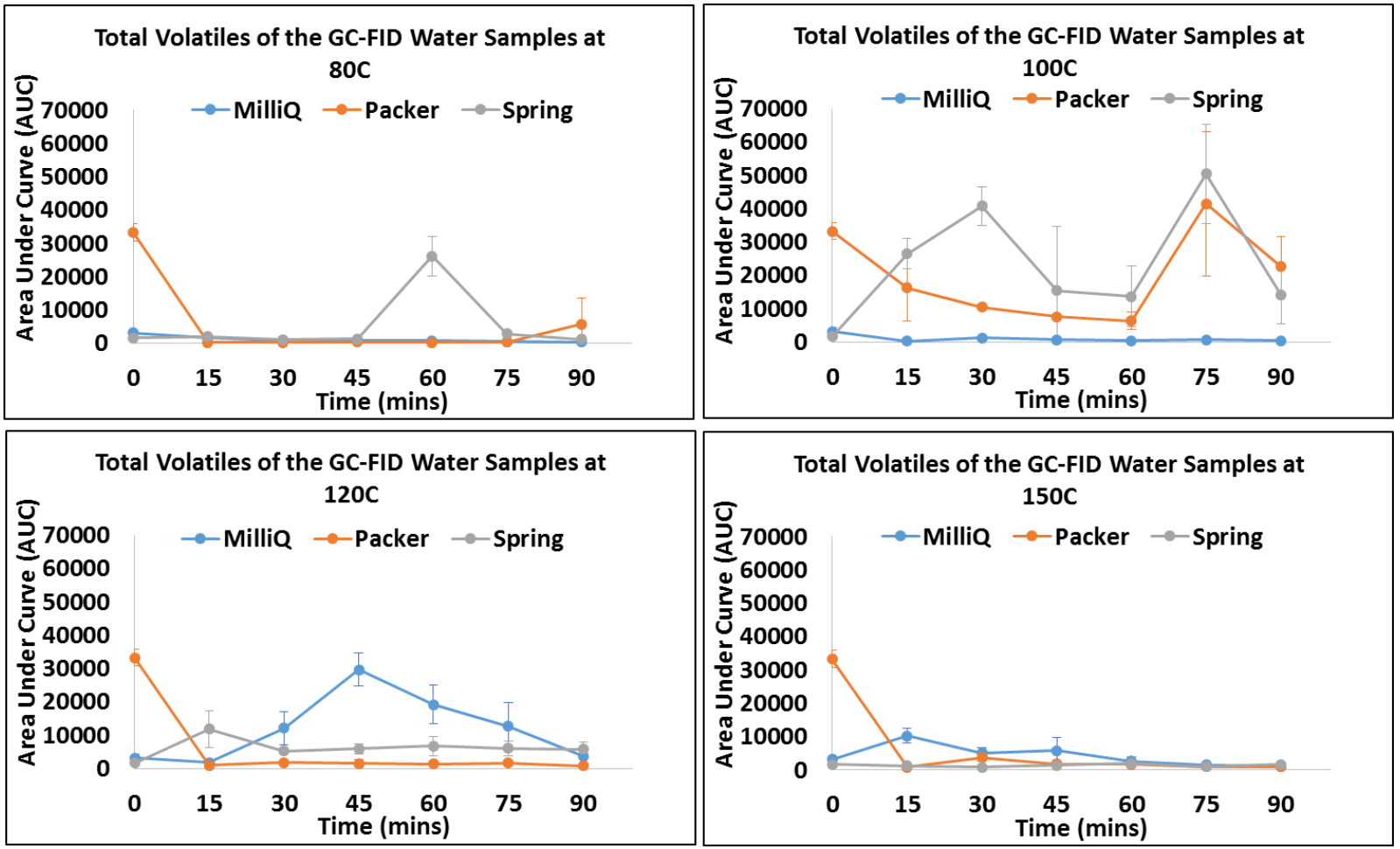


Figure 28: Area under the curve of the GC-FID analysis for the water samples at 80°C, 100°C, 120°C, and 150°C over time.

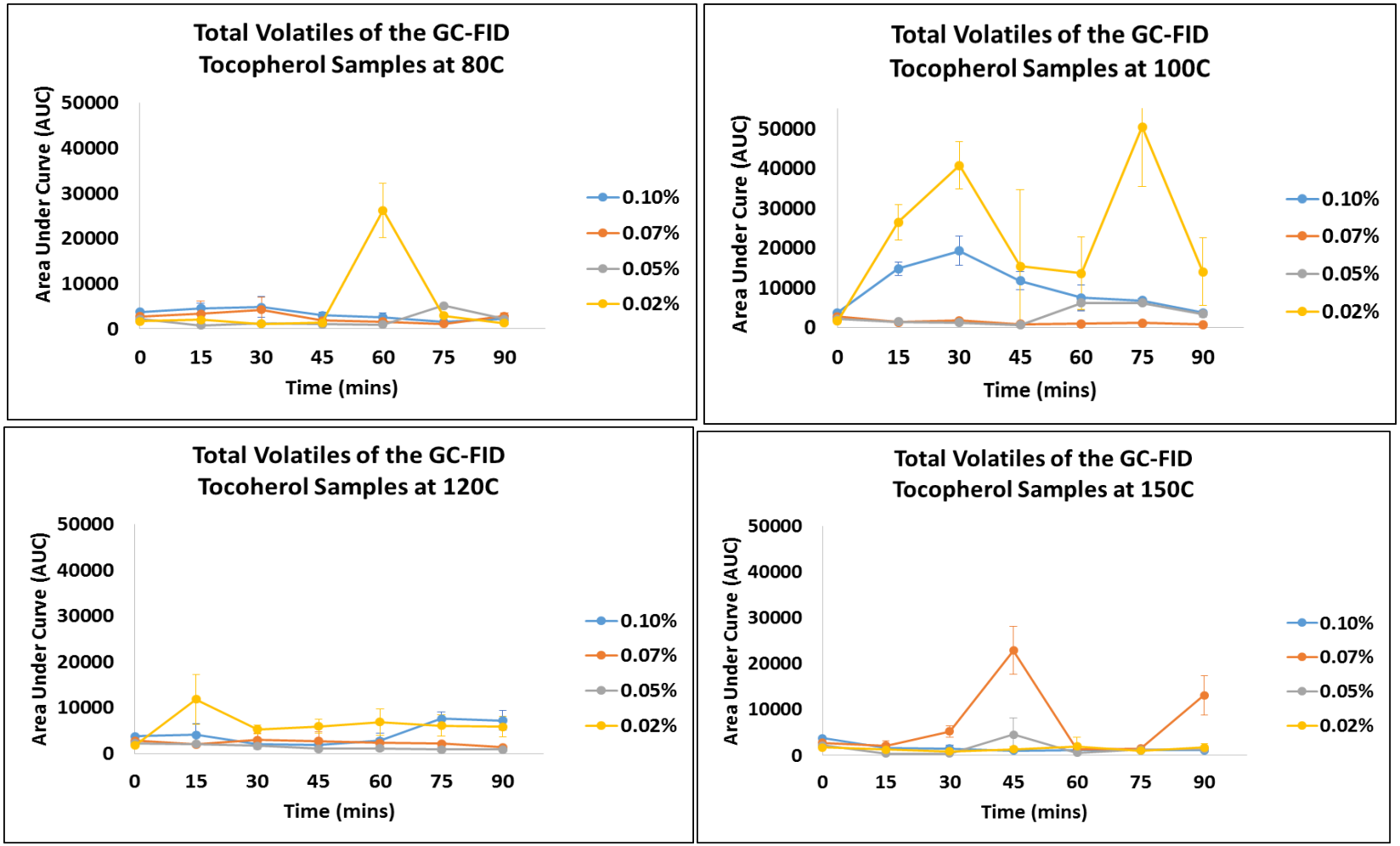


Figure 29: Area under the curve of the GC-FID analysis for the tocopherol samples at 80°C, 100°C, 120°C, and 150°C over time.

Table 16. GC-FID peak areas of volatile compounds in Blends formulated with water from different sources. Starting materials.

	Milli-Q	Spring	Packer
Nonanal	273.15	152.86	3828.42
2-Decenal	69.73	81.93	2862.97
Hexanal	218.98	102.99	2088.9
Pentanal	179.71	103.84	1510.64
Octanal	185.4	109.7	1477.07
Pentane	171.03	152.92	1433.73
Heptanal	146.86	96.41	1273.8
Hexanoic acid	31.42	30.34	926.39
2,4-Decadienal	57	3.71	833.49
Butanal	56.67	85.74	700.02
2-Pentenal	40.72	18.53	570.85
Decanol	40.72	9.98	517.05
2-Octenal	49	21.32	361.91
2-Nonenal	85.45	24.24	348.8
Decanal	36.97	25.04	279.84
2,4-Nonadienal	19.61	6.38	264.48
1-Pentene		24.65	198.02
Undecanal	6.6	13.03	191.14
2-Hexenal	11.65	8.64	159.55
2,4,6-Octatrienal	11.04	5.28	141.27
Octanoic Acid	8.92	20.17	121.12
4-Heptenal	12.45	5.59	92.13
2,4-Heptadienal	1.85	5.11	71.36
2-Pentyl furan	9.76	6.27	55.15

Table 17. GC-FID peak areas of volatile compounds found in the Milli-Q water Blend as a function of heating time at 80 °C. Peak areas detected are in the micromolar range for concentrations of products.

		Milli-Q Water - 80 °C					
Heating time, min.	**0**	**15**	**30**	**45**	**60**	**75**	**90**
Butanal	56.67	46.57	10.93	24.95	33.97	17.07	47.34
Nonanal	273.15	152.65	89.87	66.91	81.34	51.63	25.64
Octanal	185.4	84.3	53.04	58.38	38.31	32.4	17.29
2-Decenal	69.73	120.04	70.07	43.88	37.72	28.45	14.44
4-Heptenal	12.45	61.32	36.42	28.37	26.41	21.06	11.95
Hexanal	218.98	44.2	20.91	16.2	21.83	18.66	8.27
Pentanal	179.71	37.52	48.97	13.19	15.04	10.4	6.99
Decanal	36.97	19.43	12.46	10.03	7.99	6.55	3.82
Undecanal	6.6	17.23	11.66	8.48	6.24	5.11	3.7
Octanoic Acid	8.92	13.77	9.07	7.86	9.69	7.14	3.66
2,4,6-Octatrienal	11.04	ND	8.2	9.12	5.06	6.41	2.49
2-Nonenal	85.45	17.07	10.96	8.11	6.16	3.92	1.52
2-Octenal	49	14.14	7.2	5.35	5.07	3.52	1.46
2,4-Decadienal	57	27.01	10.88	5.91	5.55	2.79	1.37
Decanol	40.72	12.46	7.19	4.26	3.66	2.45	0.98
2-Hexenal	11.65	5.84	3.04	2.28	2.26	1.61	0.8
Pentane	171.03	ND	ND	ND	ND	ND	ND
2-Pentenal	40.72	ND	ND	ND	ND	ND	ND
Heptanal	146.86	ND	ND	ND	ND	ND	ND
Hexanoic acid	31.42	25.26	ND	12.12	ND	ND	ND
2-pentyl furan	9.76	ND	ND	ND	ND	ND	ND
2,4-Heptadienal	1.85	1.85	ND	ND	ND	ND	ND
2,4-Nonadienal	19.61	7.11	1.13	1.86	2.04	1.35	ND
1-Pentene	0	ND	ND	ND	ND	ND	ND

Table 18: GC-FID peak areas of volatile products found in the Packer water Blend as a function of heating time at 80 °C. Peak areas detected are in the micromolar range for concentrations of products.

		Packer Water - 80 °C					
Heating time, min.	0	15	30	45	60	75	90
Nonanal	3828.42	18.56	12.19	22.24	17.38	26.36	401.46
2-Decenal	2862.97	7.05	4.56	7.54	9.56	13.31	320.76
Pentane	1433.73	21.12	14.48	30.69	15.11	19.85	279.44
Hexanal	2088.9	7.87	5.38	6.02	9.97	13.99	274.89
2,4-Decadienal	833.49	1.62	1.1	1.28	9.65	4.26	210.95
Butanal	700.02	20.32	10.14	10.23	10.61	15.96	150.84
Pentanal	1510.64	4.8	7.28	4.42	6.53	8.06	146.39
Octanal	1477.07	11.7	7.65	13.05	10.45	13.72	144.61
Heptanal	1273.8	7.67	5.06	8.99	7.37	10.23	129.51
2,4,6-Octatrienal	141.27	1.76	0.57	2.39	2.91	2.15	91.5
2-Nonenal	348.8	4.41	1.04	1.95	1.3	1.94	41.85
1-Pentene	198.02	7.9	6.69	9.77	7.07	6.31	38.94
Decanol	517.05	ND	ND	ND	1.29	0.97	34.66
Undecanal	191.14	2.88	2.22	2.81	4.63	6.7	32.26
2-Hexenal	159.55	ND	ND	ND	1.33	1.28	25.27
2-Pentenal	570.85	ND	ND	ND	ND	ND	ND
4-Heptenal	92.13	ND	ND	ND	ND	ND	ND
Hexanoic acid	926.39	2.25	1.49	ND	ND	ND	ND
2-Pentyl furan	55.15	ND	ND	ND	ND	ND	ND
2,4-Heptadienal	71.36	ND	ND	ND	ND	ND	ND
2-Octenal	361.91	ND	ND	ND	ND	ND	ND
Octanoic Acid	121.12	ND	ND	ND	ND	ND	ND
2,4-Nonadienal	264.48	ND	ND	ND	ND	ND	ND
Decanal	279.84	ND	ND	ND	ND	ND	ND

Table 19: GC-FID peak areas of volatile products found in the 0.02% tocopherol/Spring water Blend as a function of heating time at 80 °C. Peak areas detected are in the micromolar range for concentrations of products.

		0.02% Tocopherol/Spring Water 80 °C					
Heating time, min.	0	15	30	45	60	75	90
Hexanal	102.99	217.96	144.9	21.55	1425.69	219.27	146.98
pentanal	103.84	102.75	63.73	12.8	1050.6	133.64	84.65
butanal	85.74	86.41	68.58	47.52	951.87	124.44	76.25
Nonanal	152.86	138.99	79.81	7.68	2267.93	181.21	73.69
Octanal	109.7	101.35	67.63	5.51	1345.41	135.68	66.08
Heptanal	96.41	87.66	58.46	11.08	1108.46	112.38	59.43
2-decenal	81.93	101.67	45.98	2.47	1678.11	108.74	36.44
2-pentenal	18.53	37.47	22.77	90.94	282.39	36.57	18.94
decanol	9.98	19.03	10.69	43.81	362.03	57.2	16.6
Decanal	25.04	21.02	11.79	13.68	328.64	32.52	15.58
hexanoic acid	30.34	29.33	19.39	2.27	617.24	33.06	15.51
2,4-Decadienal	3.71	15.46	12.74	4.95	358.57	28.13	13.28
2-nonenal	24.24	17.46	9.35	2.56	313.43	27.18	11.95
Undecanal	13.03	8.5	11.79	13.68	254.2	23.43	10.78
2-octenal	21.32	14.24	9.66	4.81	331.61	36.86	9.43
Octanoic Acid	20.17	21.07	15.84	3.16	541.28	86.4	8.95
2-hexenal	8.64	7.19	5.39	11.2	189.95	10.06	6.34
2,4,6-Octatrienal	5.28	4.69	3.19	2.31	219.64	6.44	3.74
2,4-Nonadienal	6.38	5.87	2.6	11.49	125.98	6.86	2.29
4-heptenal	5.59	1.92	1.56	14.23	113.06	4.48	1.77
2-pentyl furan	6.27	0.97	0.71	2.78	124.18	7.73	1.28
1-pentene	24.65	ND	ND	ND	ND	ND	ND
pentane	152.92	ND	ND	ND	ND	ND	ND
2,4-Heptadienal	5.11	ND	ND	ND	ND	ND	ND

Table 20: Peak areas from GC-FID of the compounds found in the 0.05% tocopherol Blend as a function of heating time at 80 °C. Peak areas detected are in the micromolar range for concentrations of products.

	0.05% Tocopherol 80C						
Compound	**0**	15	30	45	60	75	90
Hexanal	125.58	127.71	251.08	190.03	186.16	410.71	254.19
Nonanal	200.26	46.69	69.35	47.78	47.69	362.03	135.55
pentanal	121.02	58.16	65.31	53.32	40.89	194.7	96.93
pentane	177.35	50.71	82.17	74.38	60.86	232.02	93.64
butanal	119.61	48.27	70.2	56.71	54.96	160.9	88.59
Octanal	136.08	41.06	2.64	42.08	40.08	191.14	83.04
2-decenal	111.16	29.5	35.29	26.72	26.62	326.78	80.2
Heptanal	117.72	36.36	46.41	35.88	35.04	167.55	71.49
2-pentenal	23.64	15.17	65.31	23.04	18	80.22	38.38
hexanoic acid	39.36	9.83	13.9	10.63	10.05	72.39	25.83
Octanoic Acid	25.6	7.61	7.57	7.32	6.71	46.47	22.99
Decanal	30.74	8.49	9.37	7.28	6.69	43.2	17.08
2,4-Decadienal	9.04	4.15	3.79	2.93	3.41	89.87	17.03
decanol	14	5.97	6.88	5.6	4.8	43.99	16.81
2-nonenal	30.97	6.12	6.91	5.53	5.34	56.48	16.31
2-octenal	26.13	9.22	12.86	10.1	9.31	40.73	15.3
Undecanal	17.13	5.47	5.87	4.33	4.26	35.91	11.68
2-hexenal	9.82	5.05	5.55	4.77	3.91	20.97	7.67
2,4-Nonadienal	7.86	2.07	2.59	ND	ND	24.7	6.34
2,4,6-Octatrienal	6.2	3.52	4.98	3.92	3.51	17.4	5.76
4-heptenal	6.5	1.19	1.02	1.07	1.08	10.09	0.94
1-pentene	29.55	ND	ND	ND	ND	ND	ND
2-pentyl furan	7.46	ND	52.32	ND	ND	ND	ND
2,4-Heptadienal	6.63	ND	ND	ND	ND	4.84	ND

Table 21: Peak areas from GC-FID of the compounds found in the 0.07% tocopherol Blend as a function of heating time at 80 °C. Peak areas detected are in the micromolar range for concentrations of products.

	0.07% Tocopherol 80C						
Compound	**0**	15	30	45	60	75	90
Nonanal	284.67	270.75	253.34	122.37	121.21	65.68	176.45
butanal	122.06	49.25	751.03	241.6	181.56	98.4	147.81
Hexanal	157.21	239.43	172.86	100.06	137.19	79.91	146.63
2-decenal	156.96	207.75	212.96	79.42	69.22	37.83	142.06
pentane	197.21	209.68	121.96	95.35	75.01	53.19	110.12
Octanal	178.16	148.39	137.37	86.34	77.66	53.86	102.03
pentanal	136.11	179.81	114.39	89.72	85.8	57.77	101.58
Heptanal	150.92	132.66	118.99	76.01	70.2	50.26	91.04
2,4-Decadienal	14.92	122.38	70.07	33.33	24.56	7.8	49.26
hexanoic acid	38.22	49.76	49.52	26.34	15.47	13.15	33.34
decanol	19.3	32.08	35.12	14.59	14.38	8.62	32.26
Decanal	39.05	35.52	35.05	21.56	35.05	12.06	27.62
2-nonenal	33.2	28.43	43.26	19.91	16.21	9.24	26.04
2-octenal	33.97	33.3	22.19	10.41	11.62	9.94	19.3
Undecanal	20.7	24.47	22.58	13.54	11.15	8.14	18.37
2-hexenal	11.83	15.95	25.36	10.62	14.59	9.95	12.22
2,4,6-Octatrienal	ND	11.94	6.31	3.71	3.43	1.28	6.45
1-pentene	26.17	ND	ND	ND	ND	ND	ND
2-pentenal	31.56	ND	ND	ND	ND	ND	ND
4-heptenal	8.13	ND	ND	ND	ND	ND	ND
2-pentyl furan	3.63	ND	ND	ND	ND	ND	ND
2,4-Heptadienal	8.92	ND	ND	ND	ND	ND	ND
Octanoic Acid	5.73	ND	ND	ND	ND	ND	ND
2,4-Nonadienal	7.02	ND	ND	ND	ND	ND	ND

Table 22: Peak areas from GC-FID of the compounds found in the 0.10% tocopherol sample as a function of heating time at 80 °C. Peak areas detected are in the micromolar range for concentrations of products.

	0.10% Tocopherol 80C						
Compound	**0**	**15**	**30**	**45**	**60**	**75**	**90**
Nonanal	435.29	356.29	350.67	207.42	139.78	98.89	128.75
Hexanal	244.72	263.64	276.01	177.48	119.1	79.9	113.83
Octanal	223.5	239.12	244.2	153.99	109.68	72.88	100.01
Pentanal	160.6	226.58	239.54	154.27	103.65	65.87	98.43
Heptanal	182.89	208.85	211.97	137.75	99.03	63.13	89.86
2-Decenal	3.16	232.14	269.25	148.39	92.98	71.96	79.05
Butanal	137.83	115.97	190.53	112.46	87.73	46.25	77.18
Pentane	225.93	172.91	183.5	125.66	109.76	57.42	73.89
2-Nonenal	43.97	66.67	69.93	44.53	27.86	19.84	28.93
2-Octenal	ND	63.62	61.99	39.73	27.99	19.72	26.71
Hexanoic acid	95.02	85.48	79.08	49.42	30.29	20.65	26.43
2,4-Decadienal	0.19	49.5	59.47	45.23	30.02	24.75	26.27
Decanal	48.15	60.13	61.56	38.83	27.54	17.59	24.14
Decanol	44.68	47.53	48.32	26.03	17.42	14.78	16.59
Undecanal	3	36.2	16.26	24.94	17.57	6.74	15.25
2-Hexenal	14.05	17.76	18.78	11.5	8.14	ND	8.1
1-Pentene	38.3	26.33	19.37	22.89	11.9	ND	7.84
4-Heptenal	10.24	14.36	20.21	9.48	6.68	4.1	5.72
2,4-Nonadienal	13.09	20.99	22.05	12.96	8.22	5.75	4.78
2,4,6-Octatrienal	49.83	10.08	12.09	6.96	4.75	3.05	4.02
2-Pentyl furan	4.86	7.09	8.2	5.19	2.22	1.85	2.11
2-Pentenal	ND	ND	ND	ND	ND	ND	ND
2,4-Heptadienal	14.56	15.7	17.56	10.4	ND	ND	ND
Octanoic acid	12.87	9.94	11.94	ND	ND	ND	ND

Table 23. Starting GC-FID peak areas of volatile compounds found in Blend 3G formulated with different levels of mixed tocopherols.

Toc level (%)	**0.02**	**0.05**	**0.07**	**0.10**
Nonanal	152.86	200.26	284.67	435.29
Hexanal	102.99	125.58	157.21	244.72
Pentane	152.92	177.35	197.21	225.93
Octanal	109.7	136.08	178.16	223.5
Heptanal	96.41	117.72	150.92	182.89
Pentanal	103.84	121.02	136.11	160.6
Butanal	85.74	119.61	122.06	137.83
Hexanoic acid	30.34	39.36	38.22	95.02
2,4,6-Octatrienal	5.28	6.2	ND	49.83
Decanal	25.04	30.74	39.05	48.15
Decanol	9.98	14	19.3	44.68
2-Nonenal	24.24	30.97	33.2	43.97
1-Pentene	24.65	29.55	26.17	38.30
2,4-Heptadienal	5.11	6.63	8.92	14.56
2-Hexenal	8.64	9.82	11.83	14.05
2,4-Nonadienal	6.38	7.86	7.02	13.09
Octanoic Acid	20.17	25.6	5.73	12.87
4-Heptenal	5.59	6.5	8.13	10.24
2-Pentyl furan	6.27	7.46	3.63	4.86
2-Decenal	81.93	111.16	156.96	3.16
Undecanal	13.03	17.13	20.7	3.00
2,4-Decadienal	3.71	9.04	14.92	0.19
2-Octenal	21.32	26.13	33.97	ND
2-Pentenal	18.53	23.64	31.56	ND

Starting volatile levels in water samples showed clearly the importance of water quality and handling on sample oxidation (Table 16). Blends prepared with Packer water had markedly higher levels of volatiles than the other two Blends. This data supports not only chemical indications of higher oxidation with this Blend (marginally higher conjugated dienes and hydroperoxides), but also our assertions that standard oxidation analyses inaccurately portray extent of oxidation in Blends based on tropical fats.

The water samples showed interesting behavior in heating studies. The more highly contaminated waters (Packer and Spring). with presumably high levels of metals and organic contamination (Azoulay et al, 2001; Diduch et al, 2011), reached higher volatile levels at only 100 °C, whereas the more pure water (Milli-Q) reached highest volatiles at 120 °C (Figure 28). At 80 °C, volatiles generally started low and maintained low levels during heating of all formulations, supporting CD and PV assays that indicated low degradation. Volatiles reached the highest levels at 100 °C, with levels in Packer and spring water Blends exceeding those in Milli-Q water Blends. Recall that at 100 °C, hydroperoxide decomposition and formation appeared to be in balance (Figure 23) so in these samples there was a continual generation of volatiles even after heating. However, the volatiles data indicates that the "balance" in hydroperoxides at 100 °C and the similarities between the three waters resulted from greater LOOH decomposition in the Packer and Spring water samples, making oxidation levels appear erroneously comparable in all water formulations when measured by CD and PV. Above 100 °C, volatiles continually decreased as temperature increased because higher levels of volatiles had already been released during direct heating. This also paralleled increased production of CD and PV at 120 and 150 °C (Figure 23). Overall, total volatiles supported Milli-Q water

Blends as the most stable. We will return to discussion of the strange volatile release patterns in the next section on non-volatile carbonyls.

Pursuing reaction differences further, we examined individual volatiles generated in the Blends. The major volatile compounds found in the water samples during the GC study were aldehydes (Tables 17-19). Shorter chain aldehydes have more pungent and fruity scents and while longer chain aldehydes have oilier or fried scents; the latter are likely major contributors to off odors that were detected during heating. In all water samples, from 80°C to 120°C, the dominant products were nonanal, octanal, hexanal, pentanal, butanal, and 2-decenal, although the proportions of each changed between water formulations as well as before and after heating. Standard LO$^{\bullet}$ scissions of oleic acid can explain nonanal, octanal, and 2-decenal while LO$^{\bullet}$ scissions of linoleic acid can explain hexanal, pentanal, and butanal. Butanal may also be generated from linolenic acid. Interestingly, Blends made with Packer and Spring water showed a dominance of linoleic acid products both before and after heating, while Milli-q water formulations showed more oleic acid products after heating, demonstrating that reactions were not the same in the three Blends.

At 150°C, the samples began to deviate and different products dominated in each. The Milli-Q samples had the same high levels of the previous products but pentane and heptanal emerged in higher levels. In Packer samples, heptanal, 1-pentene and pentane reached higher levels while in spring water Blends, decanal and 4-heptenal emerged in higher levels. These new compounds resulted from a mixture of increased attack at different positions on oleic acid (decanal and heptanal), increased attack at C13 of linoleic

acid (pentane), and increased secondary degradation of initial LO• scission products at the higher temperature.

Another important observation from the volatiles data was increased attack at C9 of linoleic acid, with greater generation particularly of 2,4-decadienal and nonadienal in the Packer water samples. Other studies in this laboratory have found this shift to be characteristic of metal catalysis in oxidation (unpublished data), and is consistent with our contention that the Packer waters, in particular, must have contained high levels of metals. The volatiles show once again that the quality of the water used to prepared the Blends is critical to its stability and to the off-flavors and odors the Blends develop during heating and oxidation.

The tocopherol samples (Figure 29) seem to show the same patterns as the water samples with total volatiles low at 80°C, reaching the highest levels at 100 °C, then decreasing dramatically after heating at higher temperatures. Under most conditions, and especially at 100 °C, highest levels of volatiles were observed with the lowest levels of tocopherols (0.02%), consistent with stabilization of hydroperoxides (and inhibition of decomposition to volatile products) by tocopherols. As with the water samples, decreased volatile levels at higher temperatures were an artifact of the analytical method which determined products still being generated in the oils after heating. Information about non-volatile products in the oil throughout the heating period would help clarify reaction processes.

Consistent with the water study, the major volatile compounds found by GC in the tocopherol samples after heating at 80 °C were aldehydes, predominantly nonanal, hexanal, octanal, butanal, heptanal, and 2-decenal (Tables 19-22). Interestingly, although total

volatiles showed generally unremarkable changes, the levels of primary products and the complexity of products increased with tocopherol levels. It is difficult to determine optimum concentration of tocopherols from this data because there are some inconsistencies in the picture given by total and individual volatiles. It appears that 0.02% and 0.10% tocopherols are at opposite ends of the spectrum with respect to protective effects. 0.02% Tocopherol seems to have little effect in limiting production of volatiles while 0.10% tocopherol appears to be exerting a pro-oxidant effect. There was no tocopherol level that distinctly protective at all temperatures or under all conditions.

Generally, in both water and tocopherol samples, the major aldehydes found in the samples started at higher levels at time zero and decreased over time. This could result from excessive volatilization, as discussed above, from extensive decomposition of primary products into secondary compounds. Or from shifts in scissions so that a greater proportion of products remain attached to the glycerol backbone. We used HPLC analysis of carbonyl products to shed further light on what may be happening.

5.3.2.4 HPLC

Non-volatile compounds, mainly aldehydes, needed to be identified in order to complete the picture of what chemical changes were occurring in the blends during heating. As mentioned previously, lipid hydroperoxides and CDs cannot be tasted at the levels detected in these experiments, but informal tasting of the blends confirmed distinct off-flavors. Off-odors were also detected. GC analysis provided valuable information about possible sources of odors, but gave only one part of the story. This initiated studies to

analyze non-volatile secondary products that may contribute to off-flavors and may help explain reaction pathways in the various Blend formulations.

Immediately after heating, samples of the heated blends were placed in a 5 mL clear glass vial, flushed with argon, and sealed with a Teflon-lined screw cap and Parafilm^{TM}. The vials were wrapped in aluminum foil to prevent light penetration, and stored in a -80°C freezer until analysis was performed. Before analysis, the vials were removed from the freezer and incubated at 40 °C until liquefied (less than 10 mins). Approximately 50 µL of the blend was pipetted into a test tube and reacted with the DNPH reaction mixture; carbonyl products (as hydrazones) were then separated by HPLC and identified by comparison to standards.

Total non-volatile carbonyls are presented for the water samples in Figure 30 and for the tocopherol samples in

Figure *31*1. These results show more strongly than any other tests that 1) the Packer water contained oxidation catalysts and generated much higher levels of carbonyl products than either of the other two waters, 2) oxidation increased with the addition of mixed tocopherols, with 0.07% and 0.10% tochoperols markedly increasing levels of carbonyl products, and 3) conjugated dienes and peroxide values are inadequate for determining extent of oxidation and distinguishing flavor and odor impacts in blends based on predominantly saturated tropical fats.

In the interest of space, the individual compound breakdown of only the samples tested at 80°C are shown below; all other data is available upon request. The patterns of breakdown are similar at the different temperatures, but amounts generated increased with temperature.

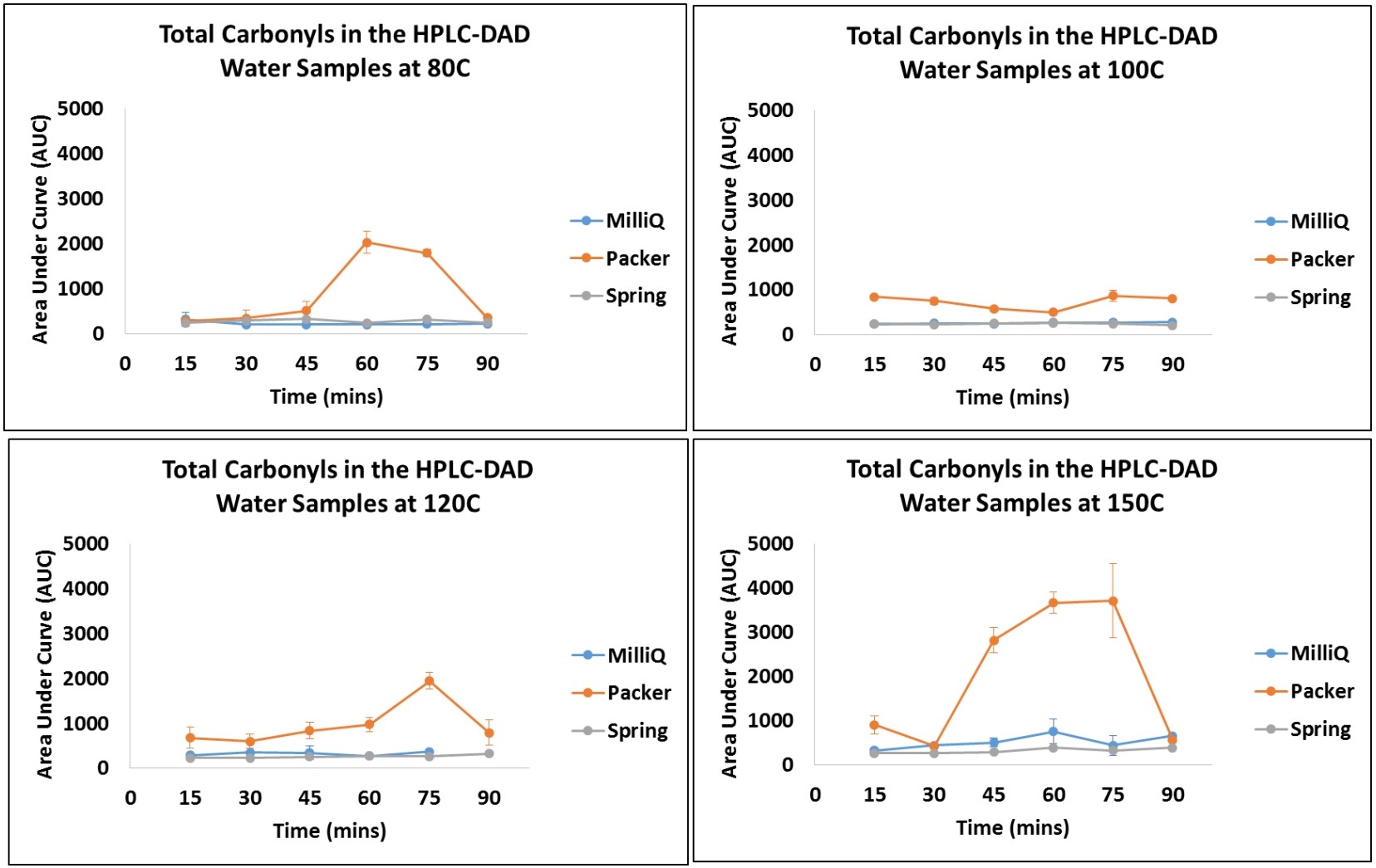


Figure 30: Effect of water source and heating temperature on total non-volatile carbonyls detected in Blends by DNPH.

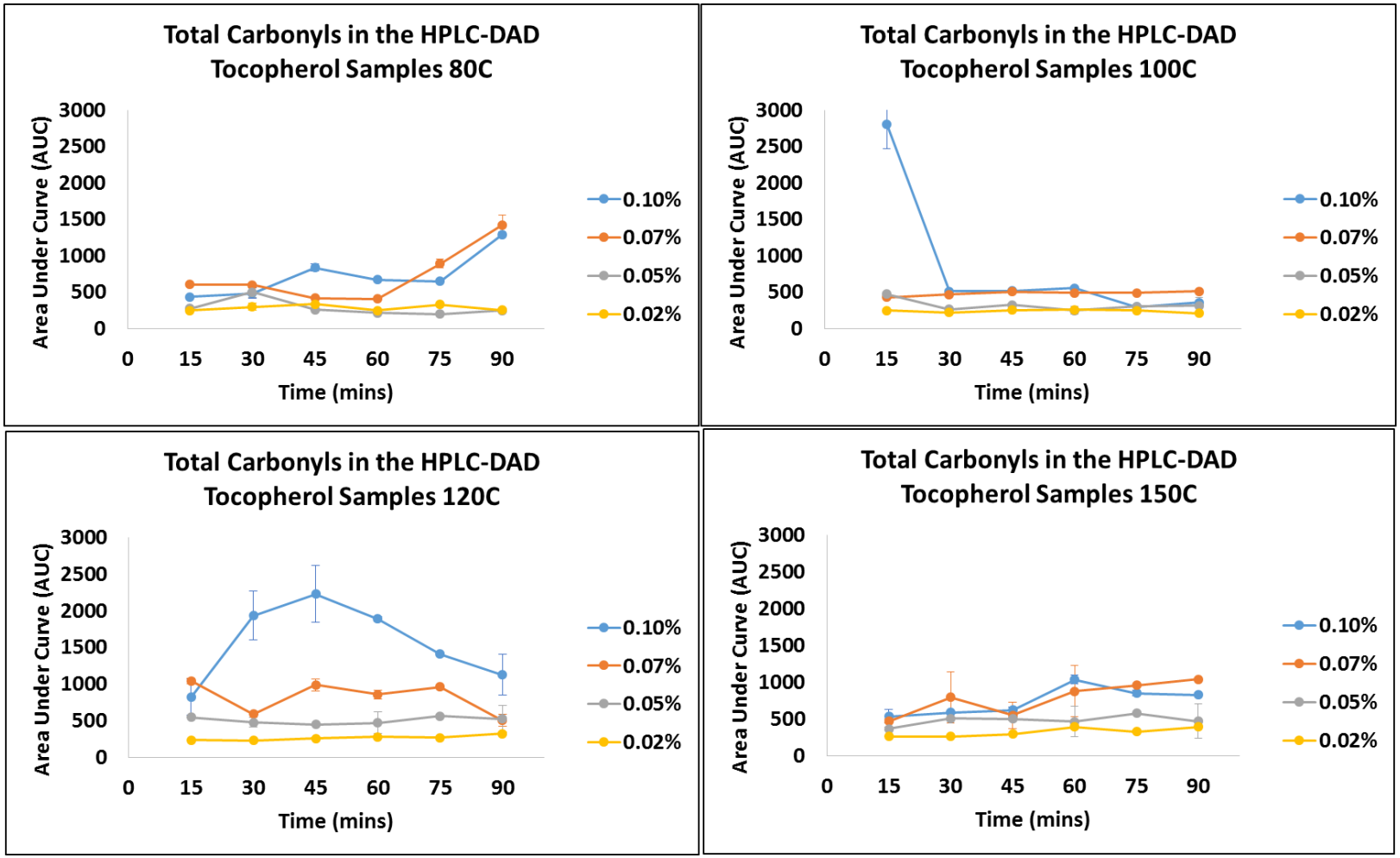


Figure 31: Effect of tocopherol levels and heating temperature on total non-volatile carbonyls detected in Blends by DNPH.

Water effects: The major non-volatile aldehydes detected and identified in the water Blends are listed in Table 24. The volatile distribution was very interesting in containing no nonanal or hexanal, which were the volatile carbonyls in highest concentrations, but showing instead butanal and pentanal as the dominant products, particularly in the Blends with Packer water. Butanal was present at levels well above the taste threshold of 5 ppm (Burdock, 2010) at all temperatures in Packer Blends and at the two highest temperatures in the other Blends. Butanal has a pungent fermented flavor and pentanal has a sharp, bitter almond flavor (Table 25). While the off-flavor does not match these descriptors exactly, it is likely that butanal is a major contributor to the off-flavors. The total flavor impact could well result from a combination of the aldehydes present, all of which were present above threshold levels (Table 25).

Levels of non-volatile aldehydes, particularly butanal, were consistently and markedly higher in the Packer water samples at all temperatures, and the differences were significant ($p < 0.05$). This is in contrast to volatile products and hydroperoxides where levels detected in Packer water Blends were comparable to or sometimes even lower than the other two formulations. This apparent disparity can be explained by rapid metal-catalyzed decomposition of hydroperoxides into secondary products (Schaich, 2005).

$$RCH_2CH_2OOH \longrightarrow RCH_2CH_2O^{\bullet} + {}^{\bullet}OH \longrightarrow RCH_2CHO \qquad (8)$$

Levels of volatile aldehydes did not reveal this difference probably because aldehydes continued to react and transform to different products during the 20 minutes of headspace equilibration at elevated temperatures. Altogether, this data shows once again that a single assay, particularly hydroperoxides, does not adequately reflect level and extent of oxidation in more saturated Blends.

Table 24: Peak areas (AUC) of non-volatile aldehydes detected by DNPH reaction with Blend 3G formulated with different waters and heated for up to 90 minutes at different temperatures. Peak areas cited correspond to micromolar concentrations of the aldehydes (data not shown).

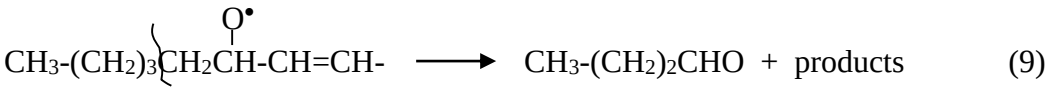
	Peak areas					
Heating time (min)	**15**	**30**	**45**	**60**	**75**	**90**
MILLI-Q WATER			**80 °C**			
Butanal	26.81	19.63	20.62	20.59	21.89	24.1
			100 °C			
Butanal	21.84	23.95	25.36	31.43	78.69	27.28
2,4-Nonadienal	18.21	20.79	21.78	30.85	ND	ND
			120 °C			
Butanal	125.92	135.5	81.32	31.43	153.47	ND
Pentanal	28.55	26.29	34.48	ND	21.86	ND
2-Hexen-1-al	11.42	10.61	10.88	ND	ND	ND
2,4-Nonadienal	ND	ND	21.48	30.85	ND	ND
			150 °C			
Butanal	130.24	85.62	139.61	170.31	150.25	149.23
Pentanal	28.38	ND	34.48	11.41	11.05	11.12
Decanal	ND	ND	ND	14.37	15.68	18.13
PACKER WATER			**80 °C**			
Butanal	19.94	32.02	61.67	690.76	304.06	22.74
Pentanal	4.62	10.42	24.28	215.03	187.03	ND
Hexanal	2.70	10.66	8.49	60.75	75.50	ND
2-Hexen-1-al	1.39	ND	4.00	1.36	23.84	ND
4-Heptanal	ND	ND	ND	ND	4.52	5.56
2,4-Nonadienal	6.71	9.13	10.26	10.63	9.61	12.05
2,4-Decadienal	2.63	10.88	3.29	13.59	2.74	3.03
			100 °C			
Butanal	25.71	138.98	138.66	136.06	139.22	135.81
Pentanal	ND	ND	ND	11.82	20.34	31.49
			120 °C			
Butanal	45.67	54.21	122.91	109.37	115.47	66.04
Pentanal	14.10	ND	ND	ND	14.81	14.69

Hexanal	10.38	ND	ND	ND	12.2	ND
2-Hexen-1-al	ND	ND	ND	ND	ND	10.23
2,4-Heptadienal	ND	ND	5.5	ND	ND	14.80
Heptanal	ND	1.58	4.36	2.32	7.12	6.78
Octanal	ND	ND	ND	ND	5.84	5.11
2,4-Nonadienal	ND	ND	ND	ND	11.44	11.21
2,4-Decadienal	ND	7.76	8.61	8.52	11.83	16.49
			150 °C			
Butanal	91.23	34.24	461.95	502.63	777.61	66.59
Pentanal	ND	ND	ND	117.04	166.76	10.3
2-Hexen-1-al	ND	ND	394.05	2.69	5.38	4.57
2,4-Heptadienal	ND	ND	84.06	5.75	13.14	14.66
Heptanal	7.59	6.62	90.89	5.16	4.93	4.16
Octanal	8.99	4.06	50.76	ND	ND	ND
4-Heptanal	ND	ND	81.57	ND	ND	ND
2,4-Nonadienal	ND	ND	80.4	9.31	13.15	12
2,4-Decadienal	16.39	19.18	54.96	ND	ND	ND
Undecanal	1.47	1.53	1.81	ND	2.04	2.21
SPRING WATER			**80 °C**			
Butanal	10.19	13.58	14.40	16.38	14.15	31.23
2,4-Nonadienal	18.67	13.22	16.33	17.93	19.10	20.72
			100 °C			
Butanal	22.79	24.87	28.45	30.67	32.89	10.51
2,4-Nonadienal	18.87	ND	22.75	6.93	16.92	ND
			120 °C			
Butanal	28.72	24.73	27.96	29.41	31.64	39.05
Nonanal	ND	11.38	20.62	22.15	14.85	20
			150 °C			
Butanal	34.54	22.95	27.68	14.12	11.4	11.99
Pentanal	ND	ND	ND	13.67	13.22	19.96
2,4-Nonadienal	19.66	20.05	30.74	ND	ND	ND
2,4-Decadienal	ND	ND	ND	22.05	21.28	34.14

Table 25: Peak areas (AUC) and concentrations (mM) of aldehydes found in HPLC-DAD analysis of 0.10% tocopherol sample at 80 °C over time.

Compound	Off-Flavor	Threshold Value (mM) In Oil	
		Odor	Taste
Butanal	Cocoa, green, grainy, Fermented, pungent		
Pentanal	Sharp, bitter almond	0.0028	0.0017
Hexanal	Green-fruity, bitter almond	0.0032	0.0008
Octanal	Fatty, soapy-fruity	0.0025	0.0003
Nonanal	Tallowy, starch-glue	0.0928	0.0014
Decanal	Orange peels	0.0429	0.0045
2-Nonenal	Tallowy, starch-glue	0.025	0.0003
2,4-Nonadienal	Fatty, oily	0.0181	0.0033
2,4-Decadienal	Deep-fried	0.0141	0.0007

Formation of butanal seemed to be related most to temperature and water quality, presumably metals. Small amounts of butanal can be formed directly from scission of C13-O• of linoleic acid two carbons away toward the distal end of the chain (Reaction 9):



(9)

At the present time we can only speculate that thermal energy and metals facilitate this scission.

That high levels of non-volatile aldehydes were detected above human threshold levels for perception after heating even at 80°C in the Packer water Blends emphasizes the low stability and low quality of these blends. Low levels of aldehydes suggest that Milli-Q water preserved the quality and stability of the blend. Together these results argue that

these Blends are not appropriate for use in heating applications, except perhaps at temperatures lower than 80 °C, and that high purity water (18 MΩ resistivity) is required for stability of these blends.

Tocopherol effects. In the tocopherol samples, aldehyde levels generally increased with tocopherol level up to 0.07% then decreased somewhat at 0.10% tocopherols (Figure 31). At the same time, diversity of different aldehydes continually increased with tocopherol concentrations and temperature (Table 26), with the net result of higher total aldehydes at 0.10% tocopherols as heating time increased. Again butanal was the major product by far, followed by pentanal. At higher tocopherol levels and temperatures, butanal, pentanal, hexanal, 2-hexen-1-al, 2,4-heptadienal, octanal, heptanal, 2,4-nonadienal, and decanal were also detected.

The appearance of increased aldehydes explains the apparent lack of protection shown by tocopherols in chemical assays (conjugated dienes and peroxide values) in the shelf life study (Figure 21) as well as the marginal pro-oxidant activity of 0.10% and 0.07% tocopherols in the heating study (Figure 25) -- the presence of higher tocopherols enhances both formation (or stabilization) and decomposition of hydroperoxides. 0.10% and 0.07% tocopherol in the Blends. Tocopherols are well-known to switch from anti- to pro-oxidant at high concentrations (Frankel, 1996; Frankel, 1994; Cillard, 1980; Peers, 1981).

How can this action of tocopherols be explained? One possibility is that tocopherols provide a ready source of hydrogen atoms that can be abstracted by $LOO^{\bullet}$ to create LOOH, hydroperoxides. If alternate reactions are normally more active (Schaich, 2005), this H donation forces a shift between pathways with generation of different

products. For example, we have found LOO• addition to double bonds to be a very fast reaction that produces high levels of epoxides in preference to hydroperoxides. The presence of tocopherols can shift the reaction balance to favor hydroperoxide formation (Figure 32). This leads to a paradoxical increase in hydroperoxides by tocopherols, and also increased levels on LOOH decomposition products, such as those seen in the HPLC and GC analyses.

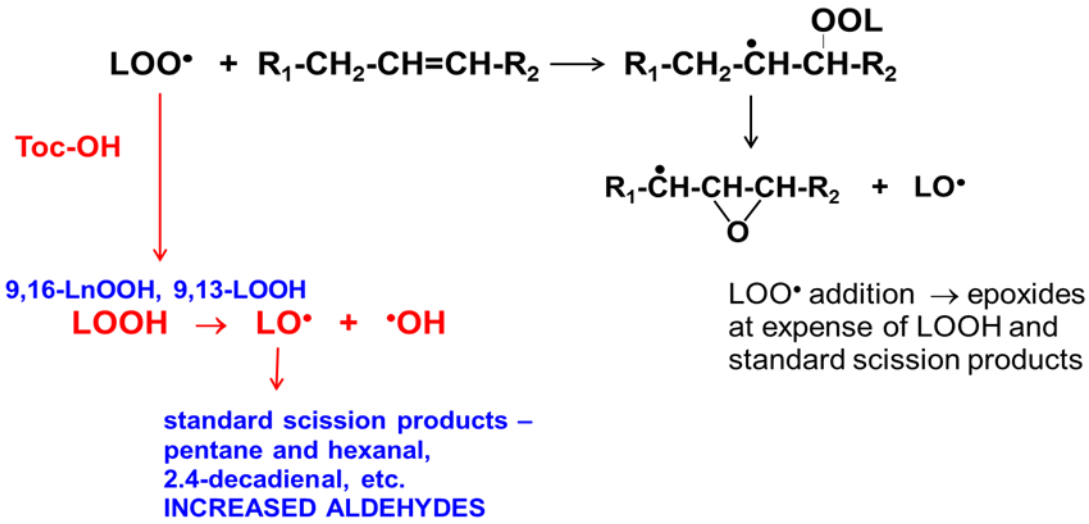


Figure 32. Possible shift in oxidation pathways cause by H donation by tocopherols.

Although 0.02% tocopherol showed low levels of non-volatile aldehydes, these samples also had high hydroperoxide concentrations and high volatiles found in the GC analyses, suggesting that 0.02% tocopherol may be insufficient to compete for propagating radicals in this system. The volatiles were also high for the 0.10% tocopherol sample, again showing that this concentration of tocopherols may be too high and causing pro-oxidant effects. At this point, with low hydroperoxides, volatiles, and non-volatile aldehydes, 0.05% added tocopherols appears to achieve the greatest stabilization of Blend 3G under all tested conditions. However, it is clear that the tocopherols are not affecting initiation

processes, so additional studies with chelators and other antioxidants must be conducted to optimize stability of these Blends.

Surprisingly, the DNPH assay did not detect the full distribution of aldehydes registered in gas chromatography analyses even though the analyses for non-volatile aldehydes were conducted directly on the oil phase without requirement for volatilization. We expected to see more non-volatile aldehydes and perhaps different products that were scission pairs of volatile products. This loss was not due to lack of identification since all peaks seen in the HPLC study were identified. In addition, neither non-volatile aldehydes nor volatile products identified compounds that clearly characterized the overwhelming metallic and acrid off-flavor in the blends. Several factors could contribute to these disparities:

(1) Poor sensitivity of the HPLC diode array detector compared to the GC flame ionization detector; FIDs in this application typically have a detection limit of 0.1 ppm of carbon, whereas the DAD's detection limit is at least 30 ppm of carbonyls (Shimadzu website; Prieto-Blanco, 2011).

\ (2) Lower reactivity of some aldehydes with DNPH, or conversely, higher reactivity of butanal with DNPH, effectively concentrating it in the assay. Indeed, we have observed that extinction coefficients in this DNPH assay are high for short chain aldehydes and decrease as the chain length increases (Yao, 2015).

(3) Further degradation, oxidation, or transformation of initial products in the heated oil during the 20 minutes equilibration before GC analyses.

(4) Non-volatile products remaining in the oil phase are epoxides or other compounds rather than aldehydes, so are undetected and unaccounted for.

(5) The main culprits of the metallic taste were not effectively identified by the study; metallic off-tastes from lipids come from epoxides, furans, and ketones (particularly vinyl ketones) (Swoboda and Peers, 1977a, 1977b; Stark and Forss, 1962; Day et al, 1963), which were products that could not be identified through the methods in this study.

(6) The quality of the starting material was poor and contained some non-lipid compounds that promoted off-flavors or formed them by co-oxidation with the lipids.

(7) The medium chain saturated fatty acids in coconut oil decompose in a different pattern than traditional vegetable and cooking oils so products are unknown and unaccounted for by the assays used.

Table 26: Effect of added tocopherol levels on aldehydes produced in Blend 3G prepared with Spring water and heated for up to 90 min at 80 °C. Peak areas shown for aldehydes found by HPLC-DAD analysis correspond to micromolar concentrations.

Heating time (min)	**15**	**30**	**45**	**60**	**75**	**90**
			0.10% tocopherol			
Butanal	33.59	57.37	103.55	90.04	78.57	183.31
Pentanal	6.21	15.41	ND	30.27	20.52	87.51
Hexanal	3.86	4.04	9.79	ND	ND	ND
2-hexen-1-al	3.41	8.16	10.8	11.94	10.27	43.55
2,4-heptadienal	2.02	2.69	7.26	6.56	7.93	33.37
Heptanal	ND	ND	6.6	10	7.01	26.88
2,4-nonadienal	9.28	9.82	14.85	13.99	15.01	15.51
2-nonenal	3.79	2.79	2.33	2.71	2.25	3.43
Decanal	ND	ND	ND	1.37	1.36	1.36
			0.07% tocopherol			
Butanal	59.86	59.64	15.15	14.88	55.37	211.52
Heptanal	3.92	4.03	10.07	5.06	12.85	19.5
Nonanal	ND	ND	ND	ND	14.11	13.03
2,4-decadienal	6.53	8.37	10.98	13.44	ND	4.18
Decanal	4.04	3.27	3.46	3.19	ND	ND
			0.05% tocopherol			
Butanal	37	29.75	29.11	17.21	19.27	27.1
2,4-nonadienal	25.23	21.41	22.1	ND	ND	10.87
			0.02% Tocopherol			
Butanal	10.19	13.58	14.4	16.38	14.15	31.23
2,4-nonadienal	18.67	13.22	16.33	17.93	19.1	20.72

6. SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Margarine-type blends based on tropical fats with and without canola and flax seed oils were evaluated for thermal and shelf life stability. Not surprisingly, addition of the highly unsaturated oils to the mixtures of coconut oil and palm shortening for nutritional support markedly destabilized the system to oxidation. Replacing these oils with higher proportions of saturates and high oleic sunflower oil (Blend 3G) increased resistance to oxidation but removed the "health" support. Stable addition of polyunsaturated fatty acids for health will probably require special stabilization methods, such as encapsulation

Formulation of the more stable Blend 3G with water from three different sources showed that handling during preparation of the Blends as well as [presumed] metal content of the water used were both critical issues for insuring stability of the blends. During heating and refrigerated storage, samples prepared with high purity Milli-Q water degraded the least, while samples prepared with ground water from the Packer showed the highest levels and most complex patterns of oxidation. Hence, it is imperative that such products be formulated and manufactured with high purity water, e.g. with 18 MΩ resistivity.

Combining conjugated dienes and peroxide values with measures of volatile and non-volatile secondary products revealed that degradation of Blend 3G increases notably at high temperatures (>80 °), releasing volatiles (odors) and off-flavors. Thus, these Blends should definitely not be used for frying applications and have only limited application, if any, for lower temperature baking applications.

Formulation of Blend 3G with four different levels of mixed tocopherols (200, 500, 700, and 1000 ppm) as antioxidants showed that 200 ppm tocopherols was probably inadequate for full protection and 500 ppm appeared to present best inhibition of the four

levels, while 700 and 1000 ppm became pro-oxidant, increasing both hydroperoxides and secondary products. Tocopherols appear to be acting by at least two mechanisms: stabilizing hydroperoxides via hydrogen bonding, and adding H atoms to LOO• to enhance LOOH (hydroperoxide) formation, thereby shifting oxidation pathways away from other mechanisms such as LOO• additions and epoxide formation.

Stabilization strategies for these Blends have been developed largely blind, with little consideration for endogenous antioxidants. In order to optimize stabilization of Blends based on tropical fats, levels of endogenous antioxidants such as tocols and tocotrienols must be determined accurately and counted when calculating requirements for added antioxidants.

In addition, since these Blends are emulsions and biphasic, consideration must be given to including other types of antioxidants, particularly metal chelators, and adding oil-soluble antioxidants into the oil phase and water-soluble antioxidants into the water phase before emulsifying to increase overall efficacy. Emulsions complicate oxidation reactions with the oil-water interface which makes choosing antioxidants somewhat difficult; emulsions differ greatly from bulk oils, because of the oil-water interface (Frankel, 1994). α-tocopherol has been found to be most effective when solubilized in the lecithin membrane surrounding the droplets of an emulsion rather than the interior, because the interfacial membrane is where the free-radicals initiate lipid oxidation (Coupland, 1996).

Determining oxidative degradation in products based on tropical fats with predominantly saturated fatty acids present significant challenges. Standard monitoring of oxidation by conventional conjugated dienes detects changes only in unsaturated

components. Peroxide values can reflect ROOH formed by thermal scissions of saturated fatty acids as well as oxidation of unsaturated fatty acids as well, but do not distinguish ROOH source so interpretation is not straightforward. Furthermore, peroxide values alone do not accurately reflect the extent of oxidation since they decompose in the presence of light, heat, and metals. Thus, it is not clear whether low peroxide values in tropical fat Blends indicate materials that are stable and unoxidized or materials that have already progressed to secondary stages of oxidation.

This paradox was shown clearly with Blend 3G where low CD and PV values suggested low oxidation and stable products, yet off-flavors and odors were notable, indicating that additional reactions were active and contributing to product quality (and potential toxicity). Analysis of volatile and non-volatile carbonyl products showed differences between Blend 3G formulations much more distinctively and revealed some differences in product distributions and reactivity, but perhaps most importantly demonstrated that these Blends were actively oxidizing and undergoing significant secondary degradations even when standard peroxide values suggested they were “stable” and relatively unreactive. In other words, CD and PV alone gave an erroneous picture of oxidation in these systems. Hence, analysis of epoxides, carbonyls, alcohols, and alkanes (monomers and dimers) should be added to standard CD and PV to track degradation more accurately and to identify source of off-flavors, which as yet remain unidentified. This is an important lesson for analytical laboratories.

These coconut oil Blends are indeed chemically very interesting systems, yet surprisingly, relatively little is known about degradation pathways of tropical fats. Considering the intense current interest in replacing hydrogenated fats with natural fats

such as coconut oil and palm shortening, it would seem imperative to more fully elucidate oxidation and thermal degradation reactions of predominantly saturated fats to understand what reactions are occurring, to plan accurate analyses, and to develop most effective stabilization strategies

Results of this study demonstrate clearly that Blends based on tropical fats undergo oxidative and thermal degradation even with scrupulously careful handling and protection in the laboratory. Therefore, practical commercial applications with shelf life of even a few months will require oxygen-impermeable packaging (whether plastic or glass) and would be benefitted by mixing, transferring, and packaging the Blends under inert gas.

Finally, the source of the distinctive off-flavor must be identified, whether as a contaminant, a lipid oxidation product, or a co-oxidation product of some other component, and must be controlled or eliminated if these Blends are to become commercially viable and acceptable to consumers.

7. FUTURE WORK

NMR studies are proving to be a fast method of determining lipid oxidation. NMR can examine for multiple analytes at once; hydroperoxides, aldehydes, epoxides, and other species can all be detected at once and can give a better idea as to the mechanisms that are working to contribute to oxidation and degradation.

Chemical assays for epoxides, alcohols, and aldehydes should become standard procedures in analyzing Blends that are predominantly saturated. Model system studies should be undertaken to track oxidation pathways and products and observe how they change with Blend composition and handling conditions.

Other antioxidant studies should be implemented. There was no metal chelator added into the water phase which allowed potential oxidation at the interface. Adding EDTA or citric acid to the water should be implemented and examined.

In order to better understand the blend's appeal, taste panels need to be employed. The blend should be incorporated into model foods, such as muffins, cakes, cookies, and other foods, and tasted against those foods made with other fats (butter and other vegetable oils). Alongside the foods, the fats should be extracted and analyzed for hydroperoxides and other analytes examined in this study to see how the panelists are responding to the oxidation compounds. Liking studies should also be implemented, to see how the blends match up against the traditional oils in foods. Taste panels should also be conducted to understand the threshold values at which oxidation can be detected in each of the component oils. These panels would give good feedback as to how consumers are responding to the product.

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