ENZYMATIC MODIFICATION OF ANHYDROUS MILKFAT WITH N-3 AND N-6 FATTY ACIDS AND ITS OXIDATIVE STABILITY

by

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(Under the Direction of Casimir C. Akoh)

ABSTRACT

Structured lipids (SLs) can be used in infant formulas as human milkfat analogues to mimic the fatty acid composition of human breast milk. The purpose of this study was to produce a SL with a high content of *sn*-2 palmitic acid from anhydrous milkfat enriched with docosahexaenoic (DHA) and arachidonic (ARA) acids using lipases as biocatalysts. The oxidative stability of the SL was studied in complex oil in water (O/W) infant formula emulsions with added antioxidants. The total percentages of ARA and DHA contained in the SL were 0.63 and 0.50 mol% with 0.55 and 0.46 mol% at the *sn*-2 position, respectively. It also contained 44.97 mol% of *sn*-2 palmitic acid. The oxidative stability of the SL in O/W emulsions was improved using a glucose-cysteine Maillard reaction product and other antioxidants including tocopherol and ascorbyl palmitate. This SL might be totally or partially used in fat blends for infant formula.

INDEX WORDS: Human milkfat analogue, Infant formula, Structured lipids, Anhydrous milkfat, Docosahexaenoic acid, Arachidonic acid, Enzymatic interesterification, Lipid oxidation, Maillard reaction product

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BS, The University of North Georgia, 2014

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2016

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August 2016

DEDICATION

I would like to dedicate my thesis to my mother, father, and family for all of their support throughout this time.

ACKNOWLEDGEMENTS

I would first like to thank my major professor, Dr. Casimir C. Akoh for his thoughtful advice throughout my time at The University of Georgia. Thank you for providing the opportunity to learn and grow as a scientist. I would also like to thank my committee members, Dr. William L. Kerr and Dr. Ronald B. Pegg for their valuable advice. I would also like to thank the professors and technicians in the Department of Food Science and Technology who have contributed to my learning experience and allowing the use of their laboratory equipment.

I would also like to thank our research professional, Vickie Wentzel, for her help, advice, and support in professional and personal matters. I would also like to send a special thank you to my lab co-workers Eben, Carlos, and Maria for their help, encouragement, and support.

I would finally like to thank my family who has always encouraged me to reach my goals in life.

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CHAPTER 1

INTRODUCTION

Human breast milk provides the optimum balanced nutrition for infants and includes a complex mixture of nutrients, antibodies, and hormones that help with the proper growth and function of the infant (Hoddinott, Tappin, & Wright, 2008). Some of the most important macronutrients for infants are lipids, and they provide 50% of the energy in human milk (Bar-Yoseph, Lifshitz, & Cohen, 2013). These lipids include saturated, monounsaturated, and polyunsaturated fatty acids that are vital for proper growth and function during an infant's early stages of life. The lipids in human milkfat (HMF) are mainly triacylglycerols (TAGs) that have three fatty acids (FFAs) attached to a glycerol backbone at the *sn*-1, *sn*-2, and *sn*-3 positions, and some fatty acids are found in specific positions that allow for their proper absorption (Innis, 2011). Specifically, important fatty acids include palmitic, arachidonic (ARA), and docosahexaenoic (DHA) acids. Palmitic acid is a vital source of energy for an infant (Bar-Yoseph, Lifshitz, & Cohen, 2013). ARA and DHA are important for proper development of motor skills, visual acuity, and cognitive function, and they are found in large amounts in the cell membranes of the brain and the retina (Fleith & Clandinin, 2005). Palmitic acid is present in HMF at 15.43-24.46 mol%, and compared to all other fatty acids, comprises 51.17-52.30 mol% at the *sn*-2 position (Lopez-Lopez, Lopez-Sabater, Campoy-Folgoso, Rivero-Urgell, & Castellote-Bargallo, 2002). ARA and DHA are present in HMF at 0.23-0.75 mol% and 0.15-0.56 mol%, respectively, and are located in the *sn*-2 position at 0.67-1.16 mol% and

0.64-0.93 mol%, respectively (Lopez-Lopez, Lopez-Sabater, Campoy-Folgoso, Rivero-Urgell, & Castellote-Bargallo, 2002). Although breastfeeding is the preferred method of supplying the proper nutrition to infants, it cannot always be practiced due to medical reasons or personal preference. This causes an infant to be completely reliant on formula to meet his or her nutritional needs. While infant formula is the next most convenient way to provide nutrition to infants, there have been issues with the lack of stereospecificity of the lipids in the formula causing improper lipid absorption, digestive problems, and inadequate mineral absorption (Bar-Yoseph, Lifshitz, & Cohen, 2013; Mu, 2004). More specifically, palmitic acid is more readily absorbed when located at the *sn*-2 position (Innis, Dyer, Quinlan, & Diersen-Schade, 1995). While the vegetable oils that are used to make infant formulas contain palmitic acid, it is mainly located at the outer *sn*-1 and *sn*-3 positions (Karupaiah & Sundram, 2007; Lopez-Lopez, Lopez-Sabater, Campoy-Folgoso, Rivero-Urgell, & Castellote-Bargallo, 2002). Research has grown over the years to provide HMF analogues that more closely resemble HMF in both total and positional fatty acid compositions. However, this can become a challenge due to the specific positional characteristics of the TAGs in HMF. Enzymatic modification can be a useful technique to produce structured lipids (SLs) in order to achieve the specific positional requirements. SLs are lipids that have been modified to change the native structure of the lipid, including both TAG species and positional composition due to additions or rearrangements of specific desired fatty acids on the glycerol backbone (Akoh & Kim, 2015). The ideal HMF analogue would contain the appropriate amounts of *sn*-2 palmitic acid, ARA, and DHA, and could contain other beneficial fatty acids such as medium chain fatty acids (MCFAs) as a good source of readily available energy (Carnielli, Rossi,

Badon, Gregori, Verlato, Orzali, et al., 1996; Fleith & Clandinin, 2005). Previous studies have been successful in increasing the *sn*-2 palmitic acids and including ARA and DHA, but studies that compare physical blending, interesterification, and acidolysis methods in relation to differences in fatty acid profiles, reaction times, antioxidant contents, oxidative stability, melting and crystallization profiles, and reaction yields are few.

The oxidation of structured lipids in infant formula can also be a major concern because they contain polyunsaturated fatty acids and mineral supplements which increase the rate of oxidation due to unsaturation and mineral prooxidant qualities (Martin, Reglero, & Señoráns, 2010). Oxidation can occur during processing, thermal treatments, and storage, and it leads to rancidity, deterioration, and product quality loss in the infant formula product (Karpińska, Borowski, & Danowska-Oziewicz, 2001). A way to improve the stability is to add antioxidants into the food product, but this usually involves the addition of synthetic antioxidants. Over the years, the demand for natural antioxidants has increased due to the consumer preference for clean labels. The Maillard reaction has been shown to produce natural compounds that have antioxidant characteristics. The Maillard reaction is a natural non-enzymatic browning process that occurs in food during thermal processing and involves the reaction between a reducing sugar, such as glucose or fructose, and the amino group from amino acids, peptides, or proteins to form brown melanoidins (Hwang, Kim, Woo, Lee, & Jeong, 2011). The antioxidant mechanisms of action include scavenging of reactive oxygen species and metal chelation (Maillard, Billaud, Chow, Ordonaud, & Nicolas, 2007; Morales & Babbel, 2002). Many different combinations of amino acids and sugars can produce antioxidant compounds; however, one combination has shown promising results for the inhibition of lipid oxidation and

involves both L-cysteine and D-glucose (Maillard, Billaud, Chow, Ordonaud, & Nicolas, 2007). While the antioxidant capacities have been studied, experiments involving the effect of a glucose-cysteine MRP in complex oil in water (O/W) food emulsions, such as infant formula, are scarce. Also, studies involving the oxidative stability of food products containing structured lipids are few (Martin, Reglero, & Señoráns, 2010).

This thesis includes five chapters. The first chapter is an introduction that includes the research objectives. The second chapter is a literature review of related topics including HMF, infant formulas, structured lipids, enzymatic interesterification, lipases, anhydrous milkfat, arachidonic and docosahexaenoic acids, HMF analogues, lipid oxidation, methods for measuring lipid oxidation, antioxidant mechanisms, and antioxidant properties of Maillard reaction products. The third chapter includes the synthesis of three structured lipid HMF analogues using anhydrous milkfat (AMF), tripalmitin, and single cell algal oils containing ARA and DHA as substrates. Three different methods were compared including physical blending, enzymatic interesterification, and enzymatic acidolysis reactions to determine the best method for synthesis. Products were compared with respect to differences in fatty acid profiles, reaction times, antioxidant contents, oxidative stability, melting and crystallization profiles, and reaction yields. The enzymatic reactions used an *sn*-1,3 specific immobilized lipase as a catalyst. The fourth chapter presents the effects of a glucose-cysteine Maillard reaction product on lipid oxidation of the interesterified HMF analogue produced in chapter three in an infant formula complex food emulsion. The fifth chapter presents the conclusion on the entire research along with possible future work.

The objectives of this research were:

1. To synthesize HMF analogues via three different methods with AMF as the main substrate and ARA and DHA using an immobilized *sn*-1,3 specific lipase, and to compare the products with respect to differences in fatty acid profiles, reaction times, antioxidant contents, oxidative stability, melting and crystallization profiles, and reaction yields.
2. To determine the antioxidant effects of a glucose cysteine-MRP on the lipid oxidation of the HMF analogue in a complex O/W food emulsion and compare the effects to other common food antioxidants such as α-tocopherol (TOC) and ascorbyl palmitate (AP).

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CHAPTER 2

LITERATURE REVIEW

Human Milkfat

The human body experiences rapid growth and development during the first twelve months of life. During this time, the body experiences increases in weight by 115%, body length by 34%, and head circumference 22% (Chumlea & Guo, 2005; Vanderhoof & Berseth, 2004). This rapid growth is possible due to the optimum nutrient contents of human breast milk. Healthy full term infants have a functional digestive system at birth; however, the amounts of digestive enzymes may be lower compared to older infants (Isaacs, 2016). Breast milk is a complex nutritional fluid that provides the optimum nutrition for growing infants and contains antibodies, enzymes, and hormones that have health benefits and protect the infants from illnesses and infections (Hoddinott, Tappin, & Wright, 2008). Breast milk also contains important lipids known collectively as human milkfat (HMF) that account for 50% of the energy in breast milk (Bar-Yoseph, Lifshitz, & Cohen, 2013). HMF contains important fatty acids for the proper growth and function including palmitic acid and long chain polyunsaturated fatty acids (LCPUFAs) for energy and proper growth of tissues (Fleith & Clandinin, 2005). Membrane LCPUFAs are also used for the production of cell messengers such as eicosanoids (prostaglandins, thromboxanes, and leukotrienes) that influence many cell functions (Fleith & Clandinin, 2005; Jensen, 1999). HMF is mainly in the form of triacylglycerol (TAG) molecules (98-99%) that contain three fatty acids attached to the *sn*-1, *sn*-2, and *sn*-3 positions (see

Figure 2.1). The positioning of certain types of fatty acids on the TAG backbone are essential in providing proper nutrition and absorption of the fatty acids in infants (Bar-Yoseph, Lifshitz, & Cohen, 2013). The positioning of the fatty acids on the glycerol backbone are not random in HMF because mammary glands have developed pathways to produce TAGs that are different in structure compared to fats in body tissues and lipids from oil sources (Bar-Yoseph, Lifshitz, & Cohen, 2013). A list of the major fatty acids and their percentages in HMF can be seen in **Table 2.1**. These include oleic (28.30-43.83 mol%), palmitic (15.43-24.46 mol%), linoleic (10.61-25.30 mol%), and stearic (4.60-8.13 mol%) acids (Lopez-Lopez, Lopez-Sabater, Campoy-Folgoso, Rivero-Urgell, & Castellote-Bargallo, 2002). Essential fatty acids are also in HMF including linoleic (10.61-25.30 mol%) and α-linolenic acids (0.41-1.68 mol%) as well as the LCPUFAs arachidonic (ARA, 0.23-0.75 mol%) and docosahexaenoic (DHA, 0.15-0.56 mol%) acids (Lopez-Lopez, Lopez-Sabater, Campoy-Folgoso, Rivero-Urgell, & Castellote-Bargallo, 2002). HMF has a large percentage of saturated fatty acids esterified to the *sn*-2 position with unsaturated fatty acids esterified at the outside *sn*-1 and *sn*-3 positions (Bar-Yoseph, Lifshitz, & Cohen, 2013). This is different compared to vegetable oils which contain more saturated fatty acids at the *sn*-1,3 positions and unsaturated fatty acids at the *sn*-2 position (Bar-Yoseph, Lifshitz, & Cohen, 2013). One of the major fatty acids at the *sn*-2 position is palmitic acid (51.17-52.30 mol%), and numerous studies have shown that palmitic acid is much better absorbed at the *sn*-2 position compared to the outer *sn*-1 and *sn*-3 positions (Bar-Yoseph, Lifshitz, & Cohen, 2013; Carnielli, Luijendijk, van Goudoever, Sulkers, Boerlage, Degenhart, et al., 1995; Innis, Dyer, Quinlan, & Diersen-Schade, 1995; Karupaiah & Sundram, 2007; López-López, Castellote-Bargalló, Campoy-

Folgoso, Rivero-Urgel, Tormo-Carnicé, Infante-Pina, et al., 2001). Therefore, the positioning of the fatty acids within the TAG will affect how they are digested and absorbed. This leads to unique TAG species that include OPO (13.69-28.46%), PLO (12.69-21.25%), and PPO (4.79-8.27%) (see **Table 2.2**) (Zou, Huang, Jin, Guo, Liu, Cheong, et al., 2012). Other properties of HMF include a final melting temperature of 37.3 °C near body temperature and an onset of crystallization temperature of 16.7 °C (Lopez, Briard-Bion, Bourgaux, & Pérez, 2013).

Infant Formula

While breast-feeding is considered the best option for infants during their early stages of life, human milk substitutes have been developed for mothers who choose not to breastfeed for various reasons. Infant formula is intended to substitute, by itself, the nutritional requirements of infants for their optimum growth and function. This includes lipids, carbohydrates, proteins, vitamins, and minerals (CAC, 1981; Innis, 1991). The Food and Drug Administration (FDA) and the Code of Federal Regulations (CFR) specify regulation requirements of infant formulas for the United States. An important goal in producing infant formula is to achieve a fatty acid composition similar to HMF because the lipid portion provides energy and essential fatty acids for the structure and function of cell membranes (Innis, 1991). Similar fatty acid profiles to HMF can be achieved by blending edible vegetable oils mainly from palm, soybean, safflower, and or corn oil (Innis, 1991). However, vegetable oils usually contain low amounts (5-20%) of saturated fatty acids at the *sn*-2 position compared to HMF (Prosser, Svetashev, Vyssotski, & Lowry, 2010). A list of ranges of the total and *sn*-2 positional fatty acid compositions in commercial infant formulas can been seen in **Table 2.3** (Lopez-Lopez,

Lopez-Sabater, Campoy-Folgoso, Rivero-Urgell, & Castellote-Bargallo, 2002). The major fatty acids in the total fatty acid composition are oleic (34.34-44.69 mol%), palmitic (17.96-27.42 mol%), linoleic (8.93-17.02 mol%), and lauric (5.19-12.64 mol%) acids. One of the eleven infant formulas sampled also contained DHA (0.20 mol%) and ARA (0.36 mol%). The major fatty acids in the *sn*-2 position varied widely. Only two of the eleven infant formulas sampled contained higher than 30 mol% palmitic acid at the *sn*-2 position while many contained high amounts of oleic acid at the *sn*-2 position (26.33-49.40 mol%). This shows that the *sn*-2 palmitic acid in the infant formulas was lower than HMF (51.17-52.30 mol%). However, ARA and DHA were detected at the *sn*-2 position in only one of the eleven infant formulas with 0.48 and 0.23 mol%, respectively (Lopez-Lopez, Lopez-Sabater, Campoy-Folgoso, Rivero-Urgell, & Castellote-Bargallo, 2002). The large difference in palmitic acid at the *sn*-2 position could have significant impact on an infant's digestion. Palmitic acid, as mentioned previously, is much better absorbed while at the *sn*-2 position. When located at the *sn*-1 and *sn*-3 positions digestive problems can occur because after lipases cleave the *sn*-1,3 positions, the palmitic free fatty acids (FFAs) readily form insoluble soaps with divalent ions such as calcium and cannot be absorbed (Bar-Yoseph, Lifshitz, & Cohen, 2013). This causes inadequate absorption of palmitic acid and calcium ions, as well as constipation (Bar-Yoseph, Lifshitz, & Cohen, 2013; Bongers, De Lorijn, Reitsma, Groeneweg, Taminiau, & Benninga, 2007). While some infant formulas did contain ARA and DHA, other infant formulas rely on the bioconversion of linoleic and alpha-linoleic acids into LCPUFAs including eicosapentaenoic acid (EPA, C20:5n3), γ-linolenic acid (GLA C18:3n6), DHA, and ARA as shown in **Figure 2.2** (Lee, Gura, Kim, Arsenault,

Bistrian, & Puder, 2006). However, studies have shown that this conversion is low and these fatty acids are not adequately converted to LCPUFAs due to the slow conversion rate of desaturase enzymes (Clandinin, Chappell, Heim, Swyer, & Chance, 1981; Makrides, Neumann, Jeffrey, Lien, & Gibson, 2000). However, supplementation of preformed ARA and DHA to formula fed infants did increase their concentrations in comparable amounts to breastfeed infants (Lapillonne, Groh-Wargo, Gonzalez, & Uauy, 2013). Therefore, addition of preformed ARA and DHA are important for proper nutrition. However, this supplementation does have challenges due to the unsaturation of these fatty acids and their susceptibility to oxidation.

Structured Lipids

Structured lipids (SLs) are lipids that have been modified from their natural form to have specific food and nutraceutical applications (Akoh & Kim, 2015). The term modification means that a change in the native structure of the lipid, including both triacylglycerol species and positional composition, occurred due to additions or rearrangements of specific desired fatty acids on the glycerol backbone (Akoh & Kim, 2015). These modifications can be achieved through interesterification reactions, and the modified lipids could include monoacylglycerols (MAGs), diacylglycerols (DAGs), phospholipids (PLs), and more commonly triacylglycerols (TAGs). The modification of lipids can yield desirable biological, physical, and chemical properties that can affect their melting behavior, absorption, and nutritional value (Akoh & Kim, 2008). SLs have various applications in the food and nutraceutical industries. In the food industry, SL products include HMF analogues, cocoa butter substitutes, reduced calorie and *trans*-free fats, MAGs and DAGs as emulsifiers, as well as modified phospholipids as emulsifiers (Akoh

& Kim, 2015). Medium and long chain TAGs (MLCTs) are TAGs that contain essential long chain fatty acids (LCFAs) at the *sn-2* position with medium chain fatty acids (C6:0-C12:0, MCFA) at the *sn*-1,3 positions. It has been suggested that during digestion the MCFAs and short chain fatty acids (C2:0-C6:0, SCFA) at the *sn*-1,3 positions are hydrolyzed by digestive lipases and absorbed more rapidly than LCFAs (Jandacek, Whiteside, Holcombe, Volpenhein, & Taulbee, 1987). This is because SCFAs and MCFAs are transported directly through the portal vein to the liver due to their smaller size and greater solubility (Jandacek, Whiteside, Holcombe, Volpenhein, & Taulbee, 1987; Akoh and Kim, 2015). The essential LCFAs are more efficiently absorbed from the *sn*-2 position in the form of a 2-monoacylglycerol (2-MAG) after lipolysis. Modified phospholipids are also examples of SLs that are more easily digested because they are in a more bioavailable form and may serve as more efficient carriers of fatty acids for incorporation into membranes and tissues compared to TAGs (Akoh & Kim, 2015; Høy & Straarup, 2000; Lee, Akoh, Flatt, & Lee, 2000; Lemaitre-Delaunay, Pachiaudi, Laville, Pousin, Armstrong, & Lagarde, 1999; Ochoa, Hernández-Becerra, Cavazos-Garduño, García, & Vernon-Carter, 2013). Therefore, chemical and enzymatic interesterification are promising techniques to provide beneficial SLs for various applications in the food and nutraceutical industries. The uses of SLs are summarized in **Table 2.4**.

Enzymatic Interesterification

Modifications to form SLs can be completed by chemical or enzymatic interesterification reactions. Chemical interesterification is more commonly used in industry due to the high cost of the enzymes used as biocatalysts. However, only randomized products can be produced with chemical methods. Enzymatic modification has the advantage of using

milder reaction conditions with higher yields, and it can be used to make specific non-random products for certain applications (Weete, Lai, & Akoh, 2008). There has been growing interest in enzymatic modifications due to the products having specific structures that have nutritional and functional benefits. The high cost of enzymes can also be overcome because they can be used more than once if immobilized onto a support (Weete, Lai, & Akoh, 2008). For both chemical and enzymatic methods, there are different types of reactions based on the nature of the starting substrates. Acidolysis reactions involve TAG and FFA substrates in which the FFAs are incorporated into the TAG molecules. Interesterification reactions involve two or more separate TAG molecules or esters. Alcoholysis is a reaction in which the substrates are FFA or TAG molecules and an alcohol, and examples include using glycerol (glycerolysis) and ethanol (ethanolysis) as the alcohol (Rousseau & Marangoni, 2008). The reaction schematics can be seen in **Figure 2.3**. Both chemical and enzymatic interesterification reactions can take place in a batch reactor where all substrates and catalyst are added and stirred over time as well as in continuous packed bed reactors where catalysts are continuously added to substrates to reduce mass transfer limitations (Rousseau & Marangoni, 2008; Willis & Marangoni, 2008). For enzymatic interesterification, lipases and phospholipases are the preferred enzymes to use as biocatalysts to modify fats and oils. They hydrolyze the fatty acids on TAGs or phospholipids to esterify with a new fatty acid while in a hydrophobic environment. Lipases can be used for acidolysis, interesterification, and alcoholysis reactions using organic solvent or solvent-free methods. Different enzymes can be used to achieve certain TAG structures. For example, there are nonspecific enzymes where random interesterification occurs and *sn*-1,3-specific enzymes where the *sn*-1 and *sn*-3

fatty acids of the TAG molecules are exchanged. *Candida rugosa* and *Candida antarctica* (Novozyme 435®) lipases are examples of nonspecific enzymes, while *Rhizomucor miehei* (Lipozyme RM IM®) and *Thermomyces lanuginosus* (Lipozyme TL IM®) lipases are examples of *sn*-1,3-specific enzymes where the names in parenthesis represents immobilized versions from Novozyme® (Weber & Mukherjee, 2008).

Lipases

A very promising sustainable alternative to the conventional industrial chemistry involves using enzymes. Lipases are the most frequently used enzymes in the fats and oils industry due to their specificity that allows them to be used in many different reactions with many different substrates (Fernandez-Lafuente, 2010). Lipases all have a common property in that they have and active center that is secluded from the reaction medium by a lid comprised of a polypeptide chain. This lid can vary is size; however, in the presence of a hydrophobic medium, the lid moves to allow for interaction between the substrates and the enzyme active site and the reaction medium. This is referred to as the interfacial activation of the lipases and is very important for the use of these enzymes as biocatalysts (Fernandez-Lafuente, 2010). When lipases are in hydrophobic medium (such as oils and fats), the enzyme will first hydrolyze the triacylglycerol causing the liberation of native fatty acids forming a less substituted acylglycerol containing at least one hydroxyl group. This hydrolysis is followed by the esterification of a new fatty acid to the newly created hydroxyl group (Willis & Marangoni, 2008)

Thermomyces lanuginosus (TL) lipase is a thermostable enzyme that is commercially available in immobilized (Lipozyme TL IM®) and soluble (Lipolase®) forms (Fernandez-Lafuente, 2010). Immobilization of the lipases is done to increase cost

effectiveness and reusability (Willis & Marangoni, 2008). Immobilization involves the attachment of the enzyme to a solid support material to form a water insoluble immobile state (Mustranta, Forssell, & Poutanen, 1993). The advantages of lipase immobilization include higher catalyst productivity, reuse and recovery of enzymes, prevention of product protein contamination, enhanced storage and stability from denaturation caused by heat, and convenient handling of the enzyme (Weete, Lai, & Akoh, 2008). The solid supports used in immobilization include, polyethylene, styrene, acrylic polymers, Duolite, Celite, silica gel, activated carbon, clay, and Sepharose (Malcata, Reyes, Garcia, Hill, & Amundson, 1990). The immobilization to produce Lipozyme TL IM® is done using a silica support (Peng, Xu, Mu, Høy, & Adler-Nissen, 2002). The enzyme is comprised of 269 amino acids and has an alpha-helical surface loop consisting of a lid that covers the active site containing the catalytic triad of Ser-His-Asp (Fernandez-Lafuente, 2010; Rousseau & Marangoni, 2008). This enzyme is also *sn*-1,3 specific as previously mentioned indicating that the enzyme can selectively cleave the *sn*-1, and *sn*-3 positions. This *sn*-1,3 specificity is due to steric hindrance that prevents the fatty acid located at the *sn*-2 position from binding to the active site (Weete, Lai, & Akoh, 2008). This is an advantage compared to chemical catalysts, which do not contain this specificity as previously stated. One of the main uses of Lipozyme TL IM® is for the modification of fats and oils to produce healthier lipids (Álvarez & Akoh, 2015; Nagachinta & Akoh, 2013a; Teichert & Akoh, 2011a, 2011b). This lipase has been used to successfully produce human milkfat analogues in many studies due to its selectivity for the *sn*-1,3 positions (Álvarez & Akoh, 2015; Teichert & Akoh, 2011a, 2011b). This allows for the addition of important fatty acids such as ARA, DHA, γ-linolenic acid, and medium chain

fatty acids (MCFAs) while conserving the important palmitic acid at the *sn*-2 position (Álvarez & Akoh, 2015; Nagachinta & Akoh, 2013a; Teichert & Akoh, 2011b). Although, this lipase is frequently used by the food industry, it also has applications in other fields including the production of biodiesel, fine chemicals, and pharmaceuticals (Fernandez-Lafuente, 2010).

Anhydrous Milkfat

Anhydrous milkfat (AMF) is a processed product obtained from milk, cream, or butter in which almost all traces of moisture and nonfat milk solids have been removed (Illingworth & Bissell, 1994). AMF is also sometimes called anhydrous butteroil, anhydrous butterfat, butterfat, or butteroil. The definition given by the International Dairy Federation (IDF) states that AMF must have a minimum milkfat percentage of 99.8%, a maximum water percentage of 0.1%, a maximum FFA percent as oleic acid of 0.3%, and a peroxide value of 0.2 milliequivalents of O_2/kg (Federation, 1977). They also specify that the product is from prime quality milk, cream or butter, the physical structure should be smooth, and the taste and odor must be clean and bland at 20-25 °C (Federation, 1977). AMF is normally recombined with dairy products such as whole milk, ice creams, cheese, and butter, specifically in countries that do not have an adequate local milk production industry to satisfy the demand of the population such as the Middle East and parts of South-East Asia (Kieseker, 1982). The process of removing the nonfat milk solids differs depending on which raw material the milkfat is derived from (cream, butter, milk), and also varies between countries. The final step in processing involves the removal of water (dehydration) and allows for a low possibility of microbial and enzymatic deterioration. The removal of moisture also removes dissolved oxygen and

reduces oxidation and prevents flavor deterioration of the oil (Illingworth & Bissell, 1994). AMF represents one of the most complex natural fats due to the variety in fatty acids (Månsson, 2008). The fatty acid composition of milkfat can vary depending on many factors including the breed of cow, stage of lactation, ruminal fermentation, season, and feed related factors (Månsson, 2008). The saturated and unsaturated fatty acids account for approximately 70% and 30% by weight of fatty acids, respectively. The total fatty acid profile of the AMF substrate used in this study can be seen in **Table 2.5**. Based on data provided by Mansson (2008), the most abundant fatty acids include palmitic (C16:0, 28.7-34.1 wt%), stearic (C18:0, 10.3-13.3 wt%), and myristic (C14:0, 10.0-12.1 wt%). Of the saturated fatty acids, approximately 10.9% are short-chain fatty acids (C4:0-C10:0). This includes butyric (C4:0, 4.0-5.1 wt%) and caproic (C6:0, 2.1-2.9 wt%) acids. Of the fatty acids in milkfat, 25% represent monounsaturated fatty acids with oleic acid (C18:1) accounting for 19.7-24.7 wt%. The polyunsaturated fatty acids account for 2.3 wt% and include linoleic (C18:2, 1.4-1.8 wt%) and α-linolenic (C18:3; 0.6-0.9 wt%) acids. AMF also contains natural *trans*-fatty acids with total ranges between 0.6-3.9 wt%. The *trans*-fatty acids are mainly comprised of C18:1-*trans* and C18:2-*trans* with percentages of 2.0-3.3 and 0.1-0.5 wt%, respectively (Månsson, 2008). *trans*-Fatty acids are formed in cows due to bacterial bioconversion of fatty acids in ruminant animals (Precht & Molkentin, 1995). The main fatty acids at the *sn*-2 position of AMF are palmitic (C16:0), myristic (C14:0), and oleic (C18:1) acids with approximately 37.8, 18.0, and 15.3 wt%, respectively (Straarup, Laurtitzen, Faerk, Høy, & Michaelsen, 2006). This is similar to HMF (see **Table 2.1**). However, the palmitic acid percentage in AMF is still lower in comparison to HMF.

Arachidonic and Docosahexaenoic Acids

The LCPUFAs, ARA and DHA, are important for growth and development and for the structure and function of cell membranes. ARA is an omega-6 (n-6) fatty acid that is found mainly in animal tissues such as beef, poultry, and fish as well as marine algae. DHA is a part of the omega-3 series (n-3) and is mainly found in fish and fish oil (Watkins & German, 2008). Other more sustainable sources of ARA and DHA are from single cell oils derived from fungi and algae in which ARA and DHA can be produced in large quantities. Commercial examples include ARASCO® (40% ARA) and DHASCO® (40% DHA) which are produced from the unicellular fungus *Mortierella alpina* and unicellular algae *Crypthecodinium conhii,* respectively (Weber and Mukherjee, 2008). Total and positional fatty acid profile of ARASCO® and DHASCO® oils are presented in **Table 2.6**. These LCPUFAs are useful in the development of the infant retina and brain, and are present in large concentrations in the cell membranes of these tissues (Fleith & Clandinin, 2005). Studies have shown that an increase in DHA and ARA consumption by infants leads to enhanced visual acuity and cognitive function compared to infants who did not consume these LCPUFAs (Fleith & Clandinin, 2005; Makrides, Neumann, Simmer, Gibson, & Pater, 1995; Willatts, Forsyth, DiModugno, Varma, & Colvin, 1998). Providing infants with the appropriate amounts of DHA and ARA is important due to the rapid growth of the neuronal and retinal tissues after birth (Hoffman, Boettcher, & Diersen-Schade, 2009). Infants normally obtain ARA and DHA through breast milk. However, if a mother cannot breast-feed due to medical or personal reasons, the infant in completely reliant on formula for his or her nutritional needs. Studies have shown that formula fed infants show lower amounts of ARA and DHA in their plasma, brain cortex,

and red blood cells if the formula is not supplemented with adequate amounts of the LCPUFAs (Clandinin, Van Aerde, Parrott, Field, Euler, & Lien, 1997). However, supplementation of infant formula with preformed ARA and DHA did adequately increase the concentration to comparable amounts in the formula fed infants compared to breastfed infants (Clandinin, Van Aerde, Parrott, Field, Euler, & Lien, 1997). It is important to note that ARA and DHA can both be synthesized in the body from the essential fatty acid precursors linoleic acid and α-linolenic acids, respectively (see **Figure 2.2**). These precursors are converted into ARA and DHA by a series of steps that involve desaturase and elongase enzymes. However, studies have shown that the rate of this conversion can be quite slow. One study found that infant formulas with the essential fatty acid precursors did not adequately increase DHA and ARA levels possibly due to the slow conversion rate by desaturase enzymes (Makrides, Neumann, Jeffrey, Lien, & Gibson, 2000). This again illustrates the importance of the addition of preformed ARA and DHA. As mentioned previously, the total amount of DHA in HMF varies widely across regions depending on diet with mean values from studies in the United States ranging from 0.15 to 0.37 mol%, and the amount of ARA has less variability with ranges from 0.40 to 0.67 mol% (Hoffman, Boettcher, & Diersen-Schade, 2009). Reccomendations for supplementation state that the total fatty acid percentage should be between 0.3-0.7 and 0.2-0.5 mol% for ARA and DHA, respectively, with a ratio of 1-2:1 ARA to DHA (Lapillonne, Groh-Wargo, Gonzalez, & Uauy, 2013). Previous studies have successfully enriched infant formula fat with ARA and DHA with the commercial single cell oils ARASCO® and DHASCO® (Álvarez & Akoh, 2015; Teichert & Akoh, 2011b). However supplementation of infant formula with ARA and DHA does have

challenges including the high susceptibility of these fatty acids to oxidation due to their degree of unsaturation, especially in oil in water (O/W) infant formula emulsions.

Human Milkfat Analogues

As stated earlier, lipids are one of the most important macronutrients for an infant. For proper growth and function, important fatty acids need to be metabolized efficiently, and this is achieved in HMF due to the specific positional location of the fatty acids. One of the most important fatty acids is palmitic acid because it is an important source of energy for the infant, and it is mainly located at the *sn*-2 position of HMF (Bar-Yoseph, Lifshitz, & Cohen, 2013; Innis, Dyer, Quinlan, & Diersen-Schade, 1995; Zou, Pande, & Akoh, 2016). As mentioned previously, this differs from many vegetable oils used in the production of infant formula which usually contain palmitic acid at the *sn*-1 and *sn*-3 positions. In order to make an efficient HMF analogue, the positional composition of the fatty acids must be similar to HMF. Therefore, the use of enzymes can generate the unique positional composition comprised of mainly *sn*-OPO TAGs, where O and P represent oleic and palmitic acids, respectively (Zou, Pande, & Akoh, 2016). Specifically, high oleic oils and fats and oils with palmitic acid located at the *sn*-2 position, such as tripalmitin undergo an interesterification reaction with *sn*-1,3-specific lipases to generate OPO TAGs (Akoh & Kim, 2015). The benefits of using a SL HMF analogue in infant formula include improved fat absorption, softer stools, and a decrease in constipation (Bar-Yoseph, Lifshitz, & Cohen, 2013). Many studies have been able to utilize specific enzymes to produce HMF analogues with similar TAG structures (Álvarez & Akoh, 2015; Rønne, Yang, Mu, Jacobsen, & Xu, 2005; Zou, Pande, & Akoh, 2016). A commercial example of SL based infant formula includes Betapol® from Loders Croklaan

(Unilever). They used an acidolysis reaction catalyzed by an *sn*-1,3-specific lipase with tripalmitin-rich fats from palm stearin fractions and oleic FFAs from high oleic sunflower oil (Akoh & Kim, 2015). Recently, HMF analogues containing LCPUFAs such as DHA and ARA have been produced using enzymes due to their role in membrane structure and function in the brain and the retina (Álvarez & Akoh, 2015; Li, Pande, Sabir, Baeshen, & Akoh, 2014; Nagachinta and Akoh, 2013a; Nagachinta and Akoh 2013b; Teichert and Akoh, 2011b). MCFAs have also been added to the *sn*-1,3 positions of TAGs using enzymes to further improve nutrition and provide rapid energy to infants (Álvarez & Akoh, 2015).

Lipid Oxidation in Bulk Oil and O/W Emulsions

Lipid oxidation is one of the major causes of quality loss, nutritional loss, and undesirable flavors, colors and toxic compounds in many types of food systems (Kim & Min, 2008). There is an increased use of polyunsaturated fatty acids (PUFAs) and mineral supplements in lipid products to add nutritional function, and this can lead to increased rate of oxidation due to the degree of lipid unsaturation and mineral prooxidant qualities. Oxidation can occur in food products during processing, thermal treatments, and storage (Karpińska, Borowski, & Danowska-Oziewicz, 2001). SLs also tend to have lower oxidative stability compared to original oil sources due to the processing steps involved that contribute to antioxidant loss. Lipid oxidation refers to the complex sequence of chemical changes that result from the interaction of lipids with oxygen species (McClements & Decker, 2000). The oxidation of lipids occurs through a free radical chain reaction mechanism involving three stages that include initiation, propagation, and termination as shown in **Figure 2.4.** Initiation involves the reaction with singlet or triplet

oxygen or unsaturated lipids in which a hydrogen atom is lost and a lipid free radical is formed. This can be catalyzed by heat, metals, and light (Kim & Min, 2008). Once the lipid radical (L•, alkyl radical) is formed, it reacts very rapidly with molecular oxygen to form a peroxyl radical (LOO•) (Frankel, 2014). Then, hydrogen is transferred from a lipid molecule to the peroxyl radical and forms hydroperoxides (LOOH) known as primary oxidation products (Frankel, 2014). Propagation also involves more chain radical reactions in which peroxyl and alkoxyl (LO•) radicals react with lipids in close proximity. Termination, the final step of lipid oxidation, involves two radical species coming together to form nonradical products (Frankel, 2014). Alkoxyl radicals can undergo β-scission of C-C bonds that result in the generation of a large variety of products known as secondary oxidation products and include aldehydes, ketones, alcohols, and hydrocarbons. Secondary oxidation products lead to the undesired physicochemical and sensory properties of oxidized oils (McClements & Decker, 2000). Oxidation occurs at the air-oil interface for oils and the oil-water interface for O/W emulsions (McClements & Decker, 2000). Lipids in O/W emulsions tend to have lower oxidative stability compared to bulk oils because they have a larger surface area to come into contact with hydrophilic antioxidants (Martin, Reglero, & Señoráns, 2010). A diagram of a lipid droplet in aqueous medium is shown in **Figure 2.5**. Studies on lipid oxidation in O/W emulsions suggest that the interaction between lipid hydroperoxides and transition metals located at the droplet surface and hydrophilic medium, respectively, is the most common cause of lipid oxidation in emulsion systems (Mei, Decker, & McClements, 1998; Mei, McClements, Wu, & Decker, 1998; Yoshida & Niki, 1992).

The mechanism is illustrated in **Equations 1-7** using ferric iron as the transition metal (McClements & Decker, 2000).

$$Fe^{3+} + LOOH \rightarrow Fe^{2+} + LOO\bullet + H^{+} \quad (1)$$

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO\bullet + OH^{-} \quad (2)$$

$$LOO\bullet + LH \rightarrow LOOH + L\bullet \quad (3)$$

$$LO\bullet + LH \rightarrow LOH + L\bullet \quad (4)$$

$$L\bullet + O_2 \rightarrow LOO\bullet \quad (5)$$

$$LOO\bullet + LH \rightarrow LOOH + L\bullet \quad (6)$$

$$LOO\bullet + LOO\bullet \rightarrow \text{nonradical products} \quad (7)$$

Hydroperoxides in emulsion droplets are surface-active and accumulate at the droplet surface and then come into contact with hydrophilic molecules that initiate further oxidation. Therefore, the rate of lipid oxidation in emulsions could be influenced by the rate the free radicals, hydroperoxides, or lipids can diffuse from one region to another (McClements & Decker, 2000). The droplet size of the oil droplets in O/W emulsion can also affect oxidation rates. A study found that larger droplet sizes tend to increase the rate of oxidation compared to smaller droplet sizes in O/W emulsions despite having a smaller surface area (Nakaya, Ushio, Matsukawa, Shimizu, & Ohshima, 2005). Other factors that affect oxidation rates involve the chemical structure of lipids. For example, saturated fatty acids are much more stable to oxidation compared to unsaturated fatty acids. This is because the double bonds in the unsaturated molecules allow for α-hydrogen abstraction. Increasing the saturated fat content in an emulsion could slow lipid oxidation; however, this is not a feasible strategy because unsaturated fats have physical and sensory characteristics that cannot be matched by saturated fats.

Methods for Measuring Lipid Oxidation

There are a variety of analytical techniques for measuring lipid oxidation in foods. The methods used to measure oxidation in bulk oils can often be used for monitoring the oxidation of O/W emulsions; however, extraction of the oil phase before analysis is often necessary (McClements & Decker, 2000). The methods of analysis are broken down into how lipid oxidation is measured. These techniques include monitoring the loss of initial reactants such as oxygen, lipid species, and antioxidants, as well as the formation of intermediate products of lipid oxidation such as hydroperoxides and conjugated dienes, and secondary oxidation products such as alcohols, aldehydes, hydrocarbons, and ketones (McClements & Decker, 2000). Substrate loss can be evaluated by both chemical tests, including changes in the fatty acid profiles, and physical tests such as weight gain (Shahidi & Wanasundara, 2008). Measuring peroxide value (PV) and conjugated dienes is a common way to monitor the primary changes in oxidation, while techniques such as determining *p*-anisidine value (*p*-AnV), total oxidation value (TOTOX), 2-thiobarbituric acid reactive substance value (TBARS), carbonyls, and volatile compounds can measure the secondary changes using spectroscopic or chromatographic methods (McClements & Decker, 2000). More specifically, the PV is a measurement of the hydroperoxides in the oil, and this value increases and then decreases due to the decay of the hydroperoxides into secondary oxidation products (Frankel, 1996). Also, since propagation in lipid oxidation involves the induction period in which hydroperoxides have not formed and antioxidants are being consumed, the measurement of the induction point at which hydroperoxides form can be used to evaluate the effectiveness of added antioxidants in a food system (Shahidi & Wanasundara, 2008). The *p*-AnV measures the amount of

aldehydes (primarily 2-alkenals and 2,4-alkadienals) that are formed from the deterioration of the hydroperoxides (Frankel, 1996; Shahidi & Wanasundara, 2008). Also, differential scanning calorimetry (DSC) can be used to measure oxidative stability by measuring oxidation induction time (OIT_{ime}) and temperature (OIT_{emp}) (Schmid, Ritter, & Affolter, 2006). Active oxygen method (AOM) and oil stability index (OSI) can be used to determine induction point of the oil from a graph of PVs over time (Shahidi & Wanasundara, 2008). Sensory tests can be used to determine the presence of off flavors and odors that develop from lipid deterioration from oxidation. While there are many methods that can be used to measure lipid oxidation there is no uniform method for detecting the oxidative changes in food systems (Shahidi & Wanasundara, 2008). However, it is recommended to use at least two methods to determine primary and secondary oxidation products (Shahidi & Wanasundara, 2008).

Antioxidants and Antioxidant Mechanisms

Incorporating antioxidants has been shown to be effective in slowing lipid oxidation in foods (Reische, Lillard, & Eitenmiller, 2008). Antioxidants work in a variety of ways and can be classified according to the mechanism of action as primary or secondary antioxidants. Primary antioxidants are known as chain breaking antioxidant, and they act as free radical acceptors and hydrogen atom donors that inhibit or delay the initiation or propagation steps of lipid oxidation (Decker, 2008). Primary antioxidants (A) react with lipid and peroxyl radicals to form more stable antioxidant radical products, and this can be seen **in Equations 8-10** (McClements & Decker, 2000).

$$LOO\bullet + AH \rightarrow LOOH + A\bullet \quad (8)$$

$$LO\bullet + AH \rightarrow LOH + A\bullet \quad (9)$$

$$L\bullet + AH \rightarrow LH + A\bullet \quad (10)$$

The antioxidant radicals produced are less reactive because they are stabilized by resonance. Primary antioxidant radicals can also terminate lipid oxidation by reacting with peroxyl and alkoxyl radicals to form nonradical products as shown in **Equations 11-13** (McClements & Decker, 2000).

$$LOO\bullet + A\bullet \rightarrow LOOA \quad (11)$$

$$LO\bullet + A\bullet \rightarrow LOA \quad (12)$$

$$A\bullet + A\bullet \rightarrow AA \quad (13)$$

α-Tocopherol is a common lipophilic primary antioxidant that donates a phenolic hydrogen atom to free radicals and prevents further oxidation from occurring because the tocopherol radical is stabilized by resonance delocalization as shown in **Figure 2.6** (Decker, 2008). However, an antioxidant that is effective in retarding lipid oxidation in bulk oils may not be effective in emulsions (Frankel, 2014; McClements & Decker, 2000; Porter, 1980). This is known as polar paradox theory in which hydrophilic antioxidants are less effective in O/W emulsions than lipophilic antioxidants and lipophilic antioxidants are less effective in bulk oils than hydrophilic antioxidants (Decker, 2008; McClements & Decker, 2000). This is because in O/W emulsions, lipophilic antioxidants can accumulate at the water-oil interface depending on their affinity for the water-oil interface. This is also true for hydrophilic antioxidants in bulk oils because of their affinity for the air-oil interface (Decker, 2008; Huang, Hopia, Schwarz, Frankel, & German, 1996; McClements & Decker, 2000). This also suggests that amphiphilic antioxidants should be effective in slowing lipid oxidation in emulsions because they can localize at the water-oil interface (McClements & Decker, 2000).

Secondary antioxidants slow lipid oxidation by various mechanisms including chelating prooxidant metals, replenishing hydrogen atoms to primary antioxidants, and quenching of singlet oxygen (Decker, 2008; McClements & Decker, 2000). Ascorbyl palmitate is an example of a secondary antioxidant with multifunctional properties including free radical inhibition, hydrogen atom donation, and metal chelation. It has also been found to replenish hydrogen to tocopherols causing synergistic properties between the two antioxidants (Decker, 2008). The chelation of transition metals has been found to be an important antioxidant mechanism for O/W emulsions (McClements & Decker, 2000). Transition metals such as iron or copper in the aqueous phase are major factors in the promotion of lipid oxidation (McClements & Decker, 2000). Chelators are molecules that can bind metals, and therefore, remove the prooxidant metals from the water-oil interface. This can occur in a variety of different mechanisms including formation of insoluble metal complexes, occupation of metal coordination sites, and steric hindrance of interactions between metals and lipid substrates (Decker, 2008; Reische, Lillard, & Eitenmiller, 2008). Some examples of common synthetic chelators in foods are EDTA, phosphoric acid, and polyphosphates (Reische, Lillard, & Eitenmiller, 2008). There is some concern about using these chelators because the strong binding of minerals could make the minerals less bioavailable. Also, synthetic antioxidant chelators are not viewed as being less label friendly compared to more natural options (McClements & Decker, 2000). Citric acid is an example of a natural secondary antioxidant that can chelate metals; however, citric acid tends to be less effective at chelating metals (McClements & Decker, 2000; Sousa & Silva, 2005). Over the years, the demand for natural antioxidants has increased due to consumer preference. Synthetic antioxidants such as

butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT) have shown some health concerns (Ito, Fukushima, & Tsuda, 1985; McClements & Decker, 2000). Therefore, research on natural antioxidants has grown to meet the consumer demand.

Maillard Reaction Products with Antioxidant Properties

The Maillard reaction is a natural non-enzymatic browning process that occurs in food during thermal processing and involves the reaction between a reducing sugar, such as glucose or fructose, and the amino group from amino acids, peptides, or proteins to form brown melanoidins (Hwang, Kim, Woo, Lee, & Jeong, 2011). The Maillard reaction is complex and involves a series of reactions that form numerous products. For example, a reaction with glucose and ammonia results in more than 15 compounds, a reaction with glucose and glycine can yield 24 products, and a reaction with xylose and glycine can yield up to 100 products (Nursten, 2005). As described by Hodge, the Maillard reaction can be divided into the initial, intermediate, and final stages (Hodge, 1953; Nursten, 2005). This is briefly illustrated in **Figure 2.7**. In the initial stage, the products are colorless with absorption around 280 nm and involve a sugar and amine condensation reaction to form an *n*-substituted glycosylamine. This then undergoes Amadori rearrangement to form an Amadori product (Hodge, 1953; Nursten, 2005). The intermediate stage involves colorless or yellow products with strong absorption in UV light. The reactions that occur in this stage involve sugar dehydration, sugar fragmentation, and amino acid degradation by Strecker degradation. The final stage yields products that are highly colored and the reactions involve aldol condensation, aldehyde-amine condensation, and formation of heterocyclic nitrogen compounds such as melanoidins (Hodge, 1953; Nursten, 2005). These melanoidins contribute to the brown

coloration of cooked foods such as toast and meats, and they absorb light in the visible region around 360 to 420 nm (Nursten, 2005; Wu, Hu, Wei, Du, Shi, & Zhang, 2014). Other factors such as pH and pressure can also contribute to the types of products formed. The pH has a crucial effect on the Maillard reaction after the Amadori compound is formed. In alkaline conditions fission products and reductones are formed, and in acidic conditions furfuraldehydes are formed (Nursten, 2005). The Maillard reaction also produces compounds that contribute to the flavor and aroma of foods. For example, compounds like furfural and 5-hydroxymethylfurfual can lead to meaty and caramel-like flavors while alkylpyrazines give more of a nutty flavor (Mottram, 2007). Many studies have also found that Maillard reaction products (MRPs) have antioxidant capacity with mechanisms that include scavenging of reactive oxygen species and metal chelation (Maillard, Billaud, Chow, Ordonaud, & Nicolas, 2007; Morales & Babbel, 2002). Since, the Maillard reaction is a natural process, the antioxidant compounds formed can be viewed as natural products. A preivous study found that a gelatin and gum arabic MRP had a higher antioxidant activity compared to gelatin and gum arabic alone and a mixture of gelatin and gum arabic. This MRP was used to encapsulate a polyunsaturated oil and resulted in a lower TOTOX value in comparision to a control over 28 days at 4 °C (Ifeduba and Akoh, 2015). Nagachinta and Akoh (2013b) used a MRP produced from corn syrup solids and whey protein isolates as an encapsulant for a SL HMF analogue that had low PVs and thiobarbituric acid reactive substances (TBARs). While there are many possibilities for amino acid and sugar combinations for a MRP, studies have shown that a glucose-cysteine MRP was effective in scavenging lipophilic free radicals and had chelating ability (Elias, McClements, & Decker, 2005; Hernández-Ledesma, Dávalos,

Bartolomé, & Amigo, 2005; Maillard, Billaud, Chow, Ordonaud, & Nicolas, 2007). Also, MRPs derived from glucose have been shown to have chelating ability with chelating sites derived from the hydroxyl groups from glucose (Yoshimura, Iijima, Watanabe, & Nakazawa, 1997). Another study found that the glucose-cysteine MRP had the highest scavenging activity for the DPPH and ABTS radicals in comparison to all of the amino acids formed from glucose and fructose (Hwang, Kim, Woo, Lee, & Jeong, 2011). The mechanism of D-glucose and L-cysteine reacting to form the Amadori compound can be seen in **Figure 2.8.** While the antioxidant capacities have been studied, experiments involving the effect of a glucose-cysteine MRP in complex O/W food emulsions are scarce, and this was addressed in this study.

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Table 2.1 Total and *sn*-2 positional fatty acid composition (mol%) of human milkfat (HMF)

Fatty acid	HMF[a]	
	Total (n=40)	*sn*-2 (n=11)
C4:0	ND	ND
C6:0	ND	ND
C8:0	0.11-0.36	ND
C10:0	0.85-3.08	0.21-0.36
C12:0	4.05-9.35	2.41-4.81
C14:0	3.60-9.13	6.69-10.74
C15:0	0.11-0.48	0.46-0.53
C16:0	15.43-24.46	51.17-52.30
C16:1n7	1.10-2.18	1.72-1.97
C18:0	4.60-8.13	1.68-1.80
C18:1t	ND	ND
C18:1n9	28.30-43.83	13.97-17.43
C18:2t	ND	ND
C18:2n6	10.61-25.30	10.32-11.58
C18:3n3	0.41-1.68	0.28-0.59
C20:4n6	0.23-0.75	0.67-1.16
C20:5n3	0.00-0.24	0.27-0.33
C22:6n3	0.15-0.56	0.64-0.93

ND-not detected

[a] Data was obtained from Lopez-Lopez et al. (2002) and represents a range of averages.

Table 2.2 TAG species percentage of human milkfat (HMF) (%)

TAG Species[a,b]	HMF[c]
MCaLa	0.06-0.45
OLaCa	0.14-0.84
MLaLa	0.12-0.67
OLaLa	0.59-3.31
MMLa	0.56-5.56
LLaO	1.21-2.65
LaMO	2.27-8.44
LaMP	1.70-5.27
LaOO	2.19-9.01
LaPO	5.80-13.52
MPL	0.73-3.97
SMM	1.17-4.64
PLO	12.69-21.25
PPL	5.80-8.87
MPP	0.6902.44
OOO	1.14-3.75
OPO	13.69-28.46
PPO	4.79-8.27
PPP	0.19-0.91
OOS	0.21-0.89
OPS	1.65-3.08
PPS	0.05-0.24

[a] Abbreviations include Ca, capric acid (C10:0); La, lauric acid (C12:0); M, myristic acid; (C14:0) P, palmitic acid (C16:0); S, stearic acid (C18:0); O, oleic acid (C18:1n9); L, linoleic acid (C18:2n6)

[b] Triacylglycerol (TAG) species do no reflect stereochemical configuration

[c] Data obtained from Zou, Huang, Jin, Guo, Liu, Cheong, et al. (2012).

Table 2.3 Total and *sn*-2 positional fatty acid composition (mol%) of commercial infant formulas

Fatty acid	Infant Formula[a]	
	Total (n=12)	*sn*-2 (n=11)
C4:0	0.00-0.16	ND
C6:0	0.03-0.24	ND
C8:0	0.55-1.20	0.03-0.09
C10:0	0.74-1.24	0.16-1.54
C12:0	5.19-12.64	4.68-15.32
C14:0	4.06-5.91	2.23-7.10
C15:0	0.05-0.35	0.02-0.31
C16:0	17.96-27.42	5.88-43.01
C16:1n7	0.14-0.78	0.08-0.52
C18:0	3.05-6.72	0.56-2.38
C18:1t	ND	ND
C18:1n9	34.34-44.69	26.33-49.40
C18:2t	ND	ND
C18:2n6	8.93-17.02	8.14-25.95
C20:4n6	0.00-0.36	0.00-0.48
C20:5n3	0.00-0.07	ND
C22:6n3	0.00-0.20	0.00-0.28

ND-not detected

[a] Data for commercial infant formulas was obtained from Lopez-Lopez et al. (2002) and represents a range of averages.

Table 2.4 Some uses, effects, and desired compositions of various structured lipids[a,b]

Product Type	Uses and Effects	Desired Composition
Medium and Long Chain Triacylglycerols (MLCTs)	• Parenteral and enteral feeding • Rapid energy source • Treatments of lipid malabsorption and metabolic syndromes	*sn*-MLM TAGs
Human Milkfat (HMF) Analogues	• Infant formula • Infant formula enriched with ARA and DHA • Increased lipid absorption for infants	*sn*-OPO TAGs and other TAGs containing ARA,DHA, and MCFAs
Cocoa Butter Alternatives	• Chocolate • Confectionaries • Bakery products	*sn*-POP TAGs *sn*-StOSt TAGs *sn*-POSt TAGs
Reduced Calorie Fats and Low *trans/trans*-Free Fats	• Snack foods • *trans-F*ree margarines • Shortenings • Sauces • Baked chips • Baked goods • Reduces health implications of *trans*-fatty acids	TAGs containing SCFAs TAGs with intermediate properties of substrates
Monoacylglycerols (MAGs)	• Emulsifiers • Emulsifiers with essential fatty acids	2-MAG 2-MAG with essential fatty acids
Diacylglycerols (DAGs)	• Emulsifiers • DAG oils • Hypotriglyceridemic effects • Anti-obesity effects	*sn*-1,3 DAG
Structured Phospholipids (PLs)	• Emulsifiers • Phospholipids enriched with n-3 and MCFAs • More bioavailable carrier of fatty acids	PLs with 1 acyl group attached (Lyscopholipids) PLs enriched with various fatty acids

[a] Data obtained from Akoh and Kim (2008, 2015).

[b] Abbreviations are as described; TAG, triacylglycerol; FFA, free fatty acids; M, medium chain fatty acid; L, long chain fatty acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; P, palmitic acid; O, oleic acid; St, stearic acid; SCFA, short chain fatty acid; LCFA,

long chain fatty acid; MCFA, medium chain fatty acid; MAGs, monoacylglycerols; DAGs, diacylglycerols; PL, phosopholipid

Table 2.5 Total and *sn*-2 positional fatty acid composition (mol%) of anhydrous milkfat (AMF)

Fatty acid	AMF[a]	
	Total	*sn*-2
C4:0	3.41 ± 0.05	ND
C6:0	1.90 ± 0.03	ND
C8:0	1.26 ± 0.02	1.61 ± 0.03
C10:0	2.93 ± 0.04	4.19 ± 0.03
C12:0	3.39 ± 0.04	5.27 ± 0.12
C14:0	10.62 ± 0.08	18.34 ± 1.20
C15:0	1.14 ± 0.01	1.82 ± 0.03
C16:0	29.83 ± 0.15	32.60 ± 1.55
C16:1n7	1.37 ± 0.01	2.06 ± 1.93
C18:0	9.97 ± 0.89	7.14 ± 0.76
C18:1t	3.75 ± 0.87	5.18 ± 0.51
C18:1n9	22.69 ± 0.16	13.58 ± 0.50
C18:2t	0.83 ± 0.04	ND
C18:2n6	3.30 ± 0.56	2.67 ± 0.12

Values are mean ± SD (n=3)

ND-not detected

[a] Data obtained from Sproston and Akoh (2015).

Table 2.6 Total and *sn*-2 positional fatty acid composition (mol%) of ARASCO and DHASCO

Fatty acid	ARASCO[a,b]		DHASCO	
	Total	*sn*-2	Total	*sn*-2
C4:0	ND	ND	ND	ND
C6:0	ND	ND	ND	ND
C8:0	ND	ND	0.44 ± 0.00	ND
C10:0	ND	ND	1.48 ± 0.01	ND
C12:0	ND	ND	5.65 ± 0.04	3.21 ± 0.02
C14:0	0.33 ± 0.06	ND	11.82 ± 0.07	6.18 ± 0.46
C15:0	ND	ND	ND	ND
C16:0	10.45 ± 0.20	3.18 ± 1.49	10.33 ± 0.04	3.97 ± 1.07
C16:1n7	0.14 ± 0.01	ND	2.90 ± 0.02	2.16 ± 0.05
C18:0	8.56 ± 0.15	ND	0.41 ± 0.05	ND
C18:1t	ND	ND	ND	ND
C18:1n9	21.51 ± 0.39	58.06 ± 1.33	16.79 ± 0.06	33.02 ± 1.73
C18:2t	0.20 ± 0.06	3.07 ± 0.78	ND	ND
C18:2n6	7.96 ± 0.02	15.21 ± 0.78	0.55 ± 0.06	5.31 ± 0.83
C18:3n6	1.62 ± 0.06	1.80 ± 0.11	0.03 ± 0.00	ND
C20:3n6	2.61 ± 0.09	1.28 ± 0.22	ND	ND
C20:4n6	40.90 ± 0.20	16.53 ± 1.10	0.39 ± 0.19	ND
C24:0	3.95 ± 0.31	ND	ND	ND
C22:6n3	ND	ND	48.54 ± 0.30	45.04 ± 1.33

Values are mean ± SD (n=3)

ND-not detected

[a] Abbreviations are as described; ARASCO, arachidonic acid single cell oil; DHASCO, docosahexaenoic acid single cell oil.

[b] Data obtained from Sproston and Akoh (2015).

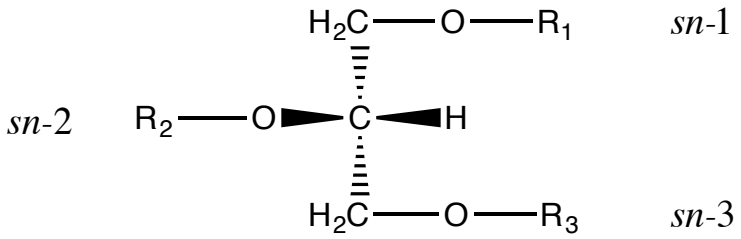

Figure 2.1 Stereospecific numbering (sn) of triacylglycerol structure with R groups as fatty acids. Figure recreated from O'Keefe (2008).

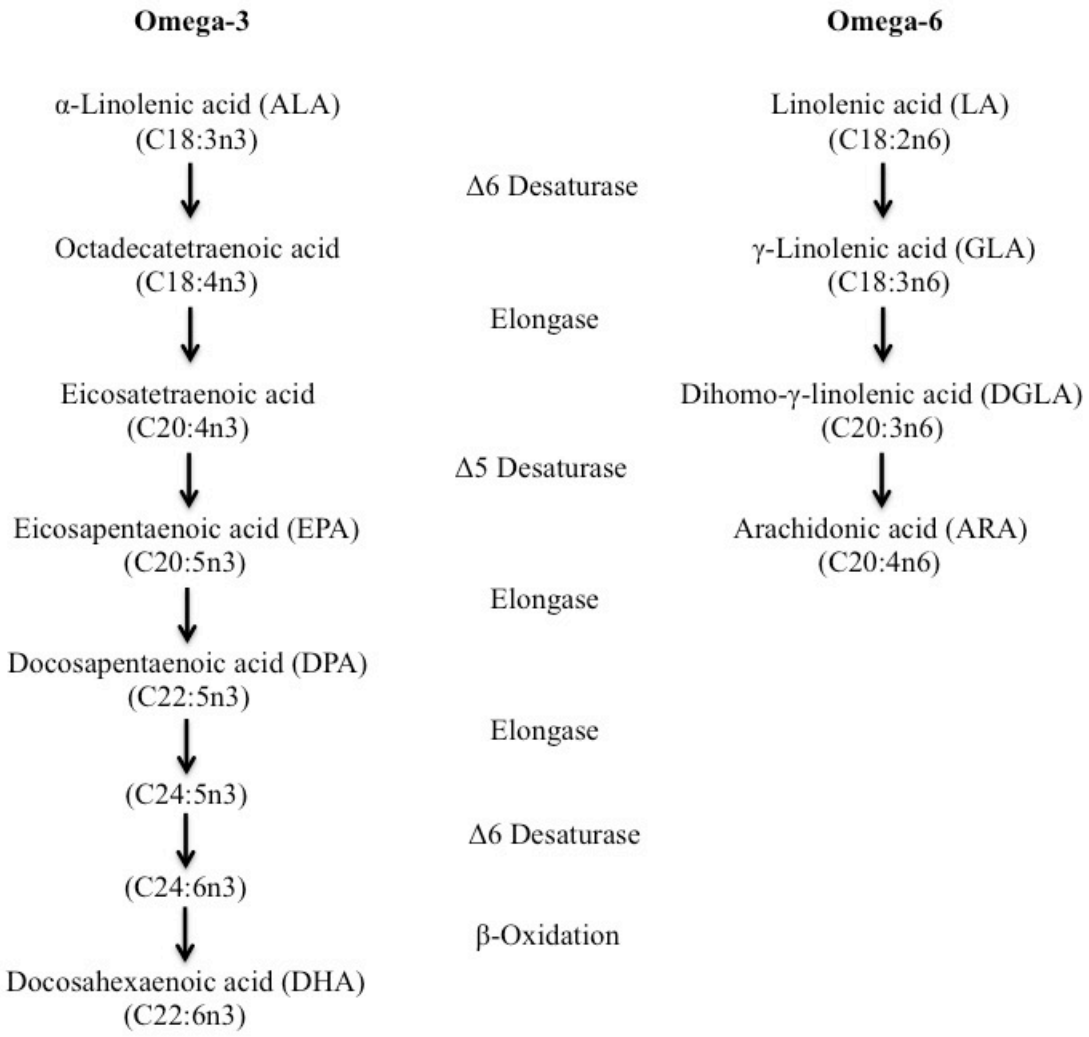

Figure 2.2 Synthesis of omega-3 (n-3) and omega-6 (n-6) lon chain polyunsaturated fatty acids (LCPUFAs). Figure recreated and adapted from Lee, Gura, Kim, Arsenault, Bistrian, & Puder (2006).

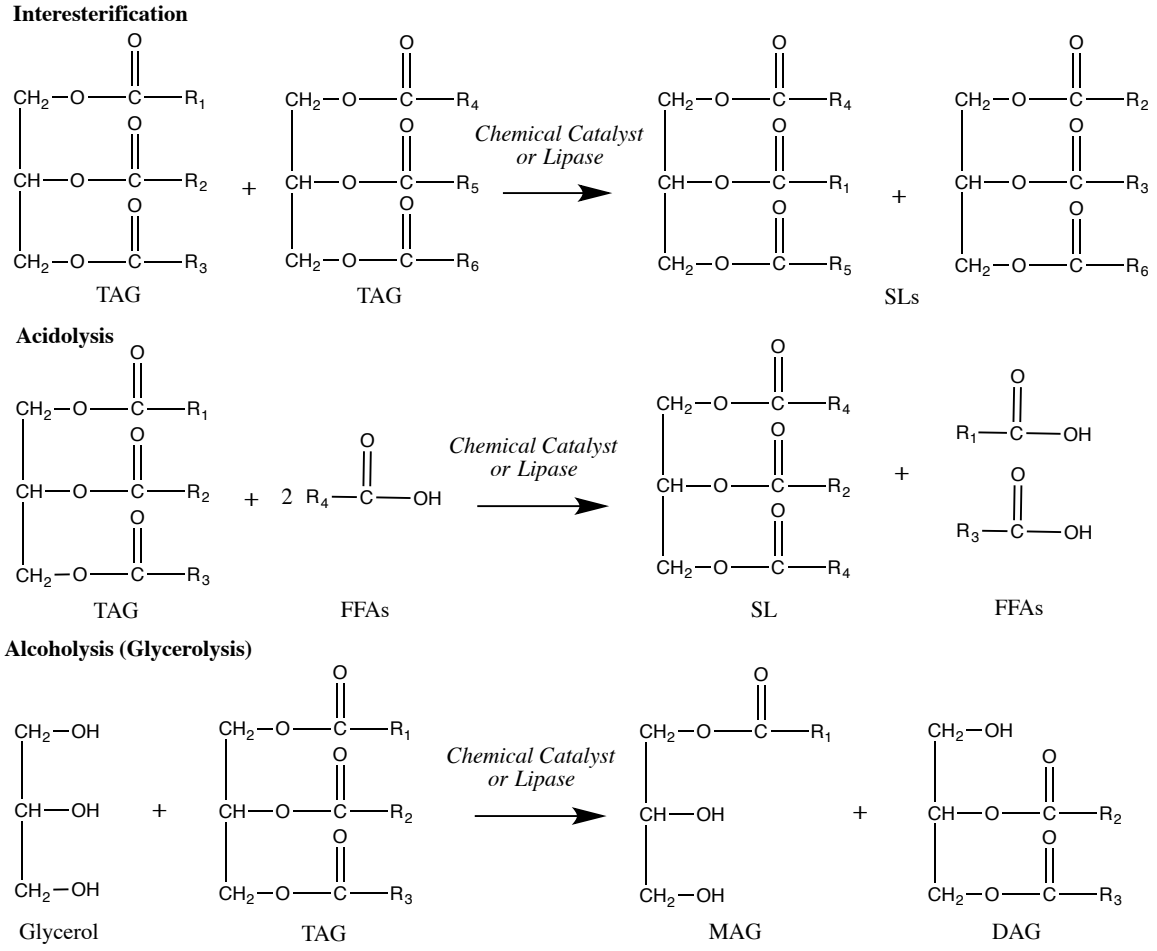

Figure 2.3 Reaction schematic for structured lipid synthesis showing interesterification, acidolysis, and alcoholysis (glycerolysis) reactions. Other reaction products can be formed due to reactions being random in this example.

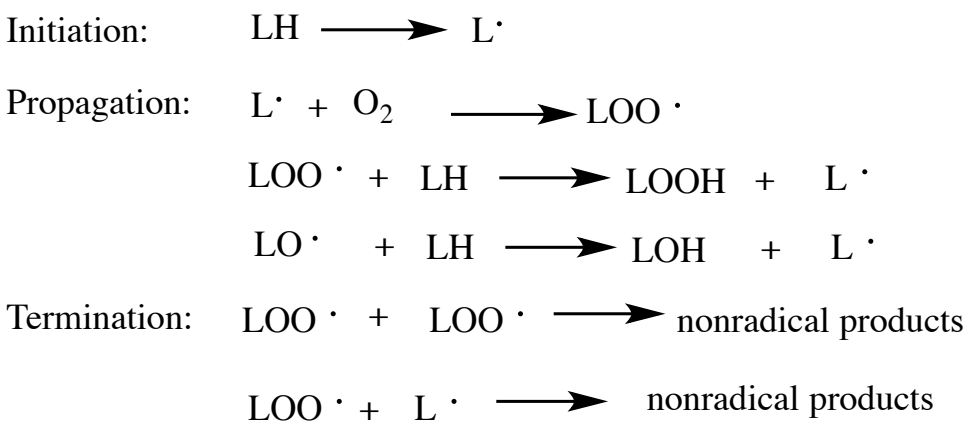

Figure 2.4 Steps in lipid oxidation. Abbreviations are LH, lipid; LOO•, peroxyl radical; L•, alkyl radical; LOOH, hydroperoxide. Adapted from McClements and Decker (2000).

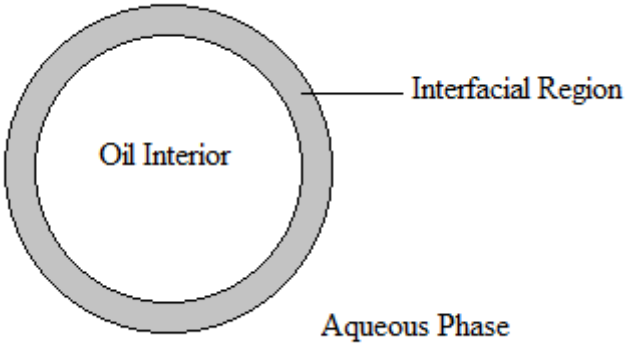

Figure 2.5 Lipid droplet in aqueous media. Image was recreated and adapted from McClements & Decker (2000).

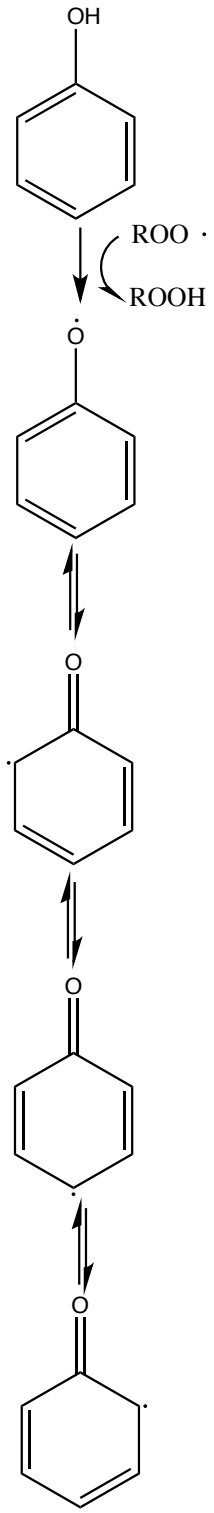

Figure 2.6 Resonance delocalization of a phenolic. Image was recreated from Decker (2008).

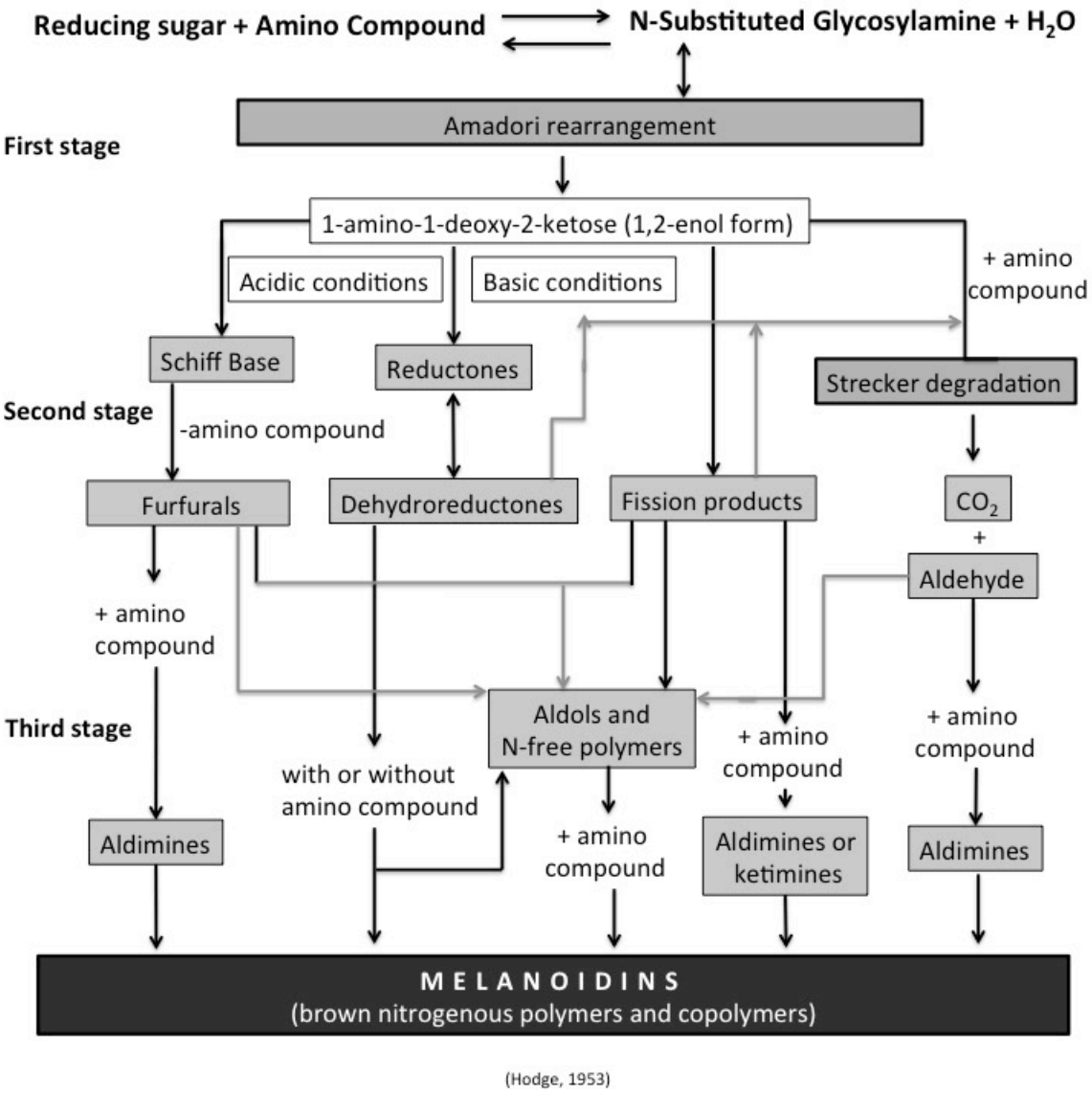

Figure 2.7 Maillard reaction scheme. Image was recreated and adapted from Hodge (1953).

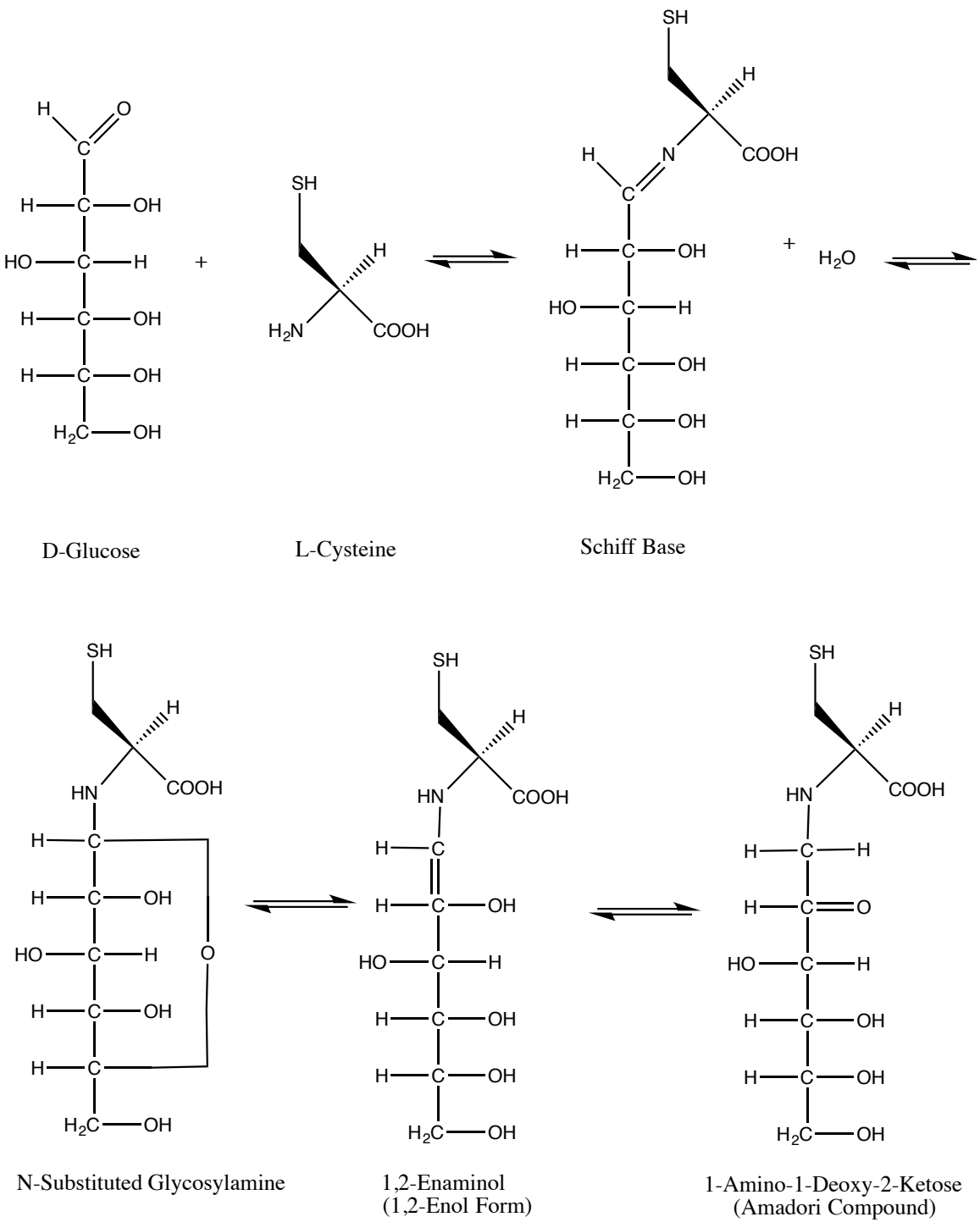

Figure 2.8 Reaction mechanism of D-glucose and L-cysteine to form an Amadori compound during the Maillard reaction. Image was recreated and adapted from Hodge (1953).

CHAPTER 3

ENZYMATIC MODIFICATION OF ANHYDROUS MILKFAT WITH N-3 AND N-6 FATTY ACIDS FOR POTENTIAL USE IN INFANT FORMULA: COMPARISION OF METHODS[1]

Sproston, M. J., & Akoh, C. C. (2015). *Journal of the American Oil Chemists' Society, 93*, 251-265. Reprinted here with the permission from Springer.

Abstract

A structured lipid (SL) with a high amount of *sn*-2 palmitic acid was synthesized from anhydrous milkfat and was then enriched with docosahexaenoic (DHA) and arachidonic (ARA) acids using an immobilized lipase. Three different methods were compared including physical blending, enzymatic interesterification, and enzymatic acidolysis. Products were compared with respect to differences in fatty acid profiles, reaction times, antioxidant contents, oxidative stability, melting and crystallization profiles, and reaction yields. The acidolysis method was the least suitable for the synthesis of desired product because of a low reaction yield, low incorporation of DHA, low oxidative stability, and the extra processing steps required. The physical blending and interesterification methods were suitable, but the interesterification product (IE-SL) had higher amounts of ARA at the *sn*-2 position. The IE-SL contained total ARA and DHA of 0.63 and 0.50 mol%, and 0.55 and 0.46 mol% at the *sn*-2 position, respectively. The IE-SL also contained 44.97 mol% *sn*-2 palmitic acid. The reaction yield for the IE-SL was 91.84%, and its melting completion and crystallization onset temperatures were 43.1 and 27.1 °C, respectively. This SL might be totally or partially used in commercial fat blends for infant formula.

Introduction

Human breast milk is a complex mixture of nutrients that provides the optimum balanced nutrition for growing infants after birth and supplies antibodies and hormones to help protect infants from certain illnesses and infections (Hoddinott, Tappin, & Wright, 2008). One of the most important macronutrients for infants during early stages of life is lipids, which account for about 50% of the energy in human breast milk (Bar-Yoseph, Lifshitz, & Cohen, 2013). Breast milk also provides important fatty acids for the growth and

development of infants including saturated fatty acids and long chain polyunsaturated fatty acids (LCPUFAs) (Fleith & Clandinin, 2005). Although, breast milk is the preferred method of supplying nutrition to infants, breastfeeding cannot always be practiced due to medical or personal reasons. When this occurs, commercial infant formulas are used causing the infant to be completely reliant on infant formula to meet his or her lipid and nutritional needs. Research has grown over the years in formulating lipid analogues that mimic human milkfat (HMF) to provide proper nutrition for infants who are not breastfed. Modification of TAG molecules to form structured lipids (SLs) can be achieved through interesterification reactions using *sn*-1,3 specific lipases as biocatalysts (Akoh & Moussata, 2001). The position of certain fatty acids on the TAG molecule has been shown to affect absorption (Bar-Yoseph, Lifshitz, & Cohen, 2013). Many experiments have shown that palmitic acid is better absorbed when it is at the *sn*-2 position in comparison to the *sn*-1 and *sn*-3 positions, and in HMF, palmitic acid is present at the *sn*-2 position in amounts between 50-60 mol% (Innis, Dyer, Quinlan, & Diersen-Schade, 1995; Karupaiah & Sundram, 2007). The high amount of palmitic acid at the *sn*-2 position, rather than at the *sn*-1,3 positions, differs from many edible oil sources including the vegetable oils used for the commercial production of infant formula (Bar-Yoseph, Lifshitz, & Cohen, 2013). This affects digestion because lipases cleave the *sn*-1,3 positions forming free fatty acids (FFAs) and a 2-monoacylglycerol (2-MAG) before being absorbed (Mu & Høy, 2004). Because of the pH of the intestine, palmitic acid as a FFA has a high affinity to bind to calcium ions forming insoluble soaps which cause digestive problems for infants including harder stools, a decrease in calcium absorption, and a loss in energy (Bar-Yoseph, Lifshitz, & Cohen, 2013). However, when

the palmitic acid is located at the *sn*-2 position, the 2-MAG formed is readily absorbed (Karupaiah & Sundram, 2007). Lipid sources from animal fats, such as anhydrous milkfat (AMF), have higher amounts of *sn*-2 palmitic acid compared to vegetable oils with approximately 30.0 mol% in animal fats and up to 20 mol%, in vegetable oils, but the amounts are still lower than HMF (Straarup, Lauritzen, Faerk, Høy, & Michaelsen, 2006).

Other important fatty acids for infant growth and development are LCPUFAs because of their role in membrane structure and function. Specifically, docosahexaenoic acid (C22:6n3, DHA) and arachidonic acid (C20:4n6, ARA) are present in large amounts in the membranes of the brain and retina (Fleith & Clandinin, 2005). Providing infants with the appropriate amounts of DHA and ARA is important due to the rapid growth of the neuronal and retinal tissues after birth (Hoffman, Boettcher, & Diersen-Schade, 2009). Studies have also shown that formulas supplemented with the essential fatty acid precursors, such as linoleic and alpha linolenic acids, do not adequately increase DHA and ARA levels possibly due to the slow conversion rate by desaturase enzymes (Makrides, Neumann, Jeffrey, Lien, & Gibson, 2000). However, supplementation of infant formula with preformed ARA and DHA did adequately increase the concentration to comparable amounts in the red blood cells, plasma, and the brain cortex of formula fed infants compared to breastfed infants (Clandinin, Van Aerde, Parrott, Field, Euler, & Lien, 1997). Current recommendations state that the total fatty acid percent of ARA and DHA in infant formula should be between 0.3 to 0.7 mol% and 0.2 to 0.5 mol%, respectively, with a ratio of 1-2:1 ARA to DHA (Lapillonne, Groh-Wargo, Gonzalez, & Uauy, 2013). Supplementation of DHA and ARA in infant formula does have challenges

including the susceptibility of these fatty acids to oxidation due to their degree of unsaturation.

The objective of this work was to synthesize a HMF analogue with anhydrous milkfat (AMF) as the main substrate with ARA and DHA using Lipozyme TL IM® lipase derived from *Thermomyces lanuginosus* for potential use in infant formula. AMF was used as the main substrate due to its high *sn*-2 palmitic acid content and a fatty acid profile with medium and long chain fatty acids similar to HMF. The commercial immobilized *sn*-1,3 specific lipase, Lipozyme TL IM®, was used in order to conserve the palmitic acid at the *sn*-2 position of AMF TAGs. An intermediate structure lipid (IM-SL) was synthesized using tripalmitin and AMF to incorporate more *sn*-2 palmitic acid. The incorporation of ARA and DHA into the IM-SL was made from ARASCO® and DHASCO® oils in amounts within the limits stated above. Also, three common methods of synthesis were compared including physical blending and enzymatic interesterification of the IM-SL with ARASCO® and DHASCO® TAGs and enzymatic acidolysis between the IM-SL and ARASCO® and DHASCO® FFAs. Previous studies involving the production of HMF analogues have used an acidolysis, interesterification, or physical blending method; however, there is very little published on comparisons between the methods. In order to determine the best method of synthesis, comparisons of the final products include the differences in fatty acid profiles, reaction times, antioxidant content, oxidative stability, melting and crystallization profiles, and reaction yields.

Materials and Methods

Materials

Anhydrous milkfat (AMF) was kindly donated by the Dairy Farmers of America (Winthrop, MN), and tripalmitin was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). ARASCO® (40% ARA) and DHASCO® (40% DHA) single cell algal oils were purchased from DSM Nutritional Products (Columbia, MD). The immobilized lipase, Lipozyme TL IM® (*sn*-1,3 specific *Thermomyces lanuginosus* lipase), was obtained from Novozymes North America, Inc. (Franklinton, NC). The lipid standards, Supelco 37 component FAME mix, 2-oleoylglycerol, Supelco tocopherol standards, and the all *trans*-*β*-carotene were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), while heptadecanoic acid (C17:0) was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). The TAG standard mixes (GLC reference standard 437 and 570) were purchased from Nu-Chek Prep, Inc. (Elysian, MN). Other chemicals and solvents were purchased from Fisher Scientific (Norcross, GA), Sigma Aldrich Chemical Company (St. Louis, MO), and J. T. Baker Chemicals (Center Valley, PA).

Determination of Appropriate Weight Ratios for Reactions

In order to determine the weight ratio needed for the IM-SL in order to synthesize a product with the appropriate percentages of palmitic acid, calibration curves were made using the weight ratio versus the mole percentage of both the *sn*-2 and total palmitic acid. The weight ratios analyzed were physical blends of between 0.0 and 0.5 (w/w) tripalmitin to AMF. In order to determine the appropriate weight ratios for the final products, the same process was repeated with weight ratios of physicals blends between 0.0 and 0.1 (w/w) ratio of a 2:1 (w/w) ARASCO/DHASCO TAG blend to the IM-SL for the physical

blend product (PB-SL) and the interesterification product (IE-SL), and a 2:1 (w/w) ARASCO/DHASCO FFA blend to the IM-SL for the acidolysis product (ACID-SL). The ARASCO/DHASCO FFA blend was made by mixing a 2:1 (w/w) ratio of ARASCO® to DHASCO® TAGs, and the TAGs were converted to FFAs following a saponification procedure (Teichert & Akoh, 2011). The calibration curves were the weight ratio (w/w) versus the mole percentage of ARA and DHA in the physical blends, and the linearity of each curve was analyzed using linear regression analysis. The physical blends were analyzed by determining their fatty acid profile including total fatty acid analysis and positional analysis as described below.

Determination of Fatty Acid Profiles

For the analysis of the total fatty acid percentages, 0.1 g of sample was weighed into separate Teflon-lined screw-capped test tubes, and 0.5 mL of 20 mg/mL C17:0 in hexane internal standard was added to each in order to determine the mole percentages. The lipid samples were then converted into fatty acid methyl esters (FAME) following the AOAC Official Method 996.01, Section E with minor modifications (Satchithanandam, Fritsche, & Rader, 2001). The modifications were as previously described (Álvarez & Akoh, 2015). Supelco 37 component FAME mix was used as an external standard for identification of fatty acids (FAs) and run parallel with the samples on the GC.

Positional Analysis

For *sn*-2 analysis, a 1 mL sample of lipid was pipetted into separate Teflon-lined screw-capped test tubes. A modified version of Luddy et al. (1964) was used to perform pancreatic lipase-catalyzed *sn*-2 analysis. The modifications were as previously described (Álvarez & Akoh, 2015). The 2-MAG band was scraped off of the TLC plates into test

tubes, and the samples were converted into FAME as described above. One hundred microliters of 20 mg/mL C17:0 in hexane was added to the samples to determine the percentages. The *sn*-2 mole percentage represents the percentage of that fatty acid at the *sn*-2 position compared to all other fatty acids at the *sn*-2 position.

GC Analysis

The FAMEs were analyzed using an Agilent Technology 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) with a flame ionization detector (FID). A Supelco SP-2560 column, 100 m x 250 μm, 0.20 μm film was used for analysis. The injection volume was 1 μL with a split ratio of 5:1 for *sn*-2 samples and 50:1 for total fatty acid analysis. Helium was the carrier gas at a flow rate of 1.1 mL/min. The injection and detector temperature was 250 °C. The oven was held at 140 °C for 5 min and then the temperature was increased to 240 °C at 4 °C/min. Once the temperature reached 240 °C, it was held at 240 °C for 17 min. The relative FAME content was calculated as mol%. Samples were analyzed in triplicate, and the average and standard deviations were reported.

TAG Molecular Species Analysis

The TAG molecular species analysis was completed using a RP-HPLC (Agilent Technologies 1260 Infinity, Santa Clara, CA) with a Sedex 85 evaporative light scanning detector (ELSD) on a Beckman Ultrasphere® C18 4.6 mm x 250 mm, 5 μm nominal particle size column. Samples were prepared and analyzed as previously described (Álvarez & Akoh, 2015).

In order to find the appropriate time for the interesterification reactions for the IM-SL and the IE-SL product with ARASCO® and DHASCO® TAGs, TAG molecular

species analysis was used to monitor the decrease in the tripalmitin peak over time using RP-HPLC. Since tripalmitin was a starting substrate, the reaction would be complete when the tripalmitin peak disappeared or was at its lowest percentage compared to other TAGs in the lipid sample. A reference standard was used to determine retention time of tripalmitin. In order to determine significant differences in the tripalmitin peak area percentages over time, statistical analysis was used as described later.

Gram-scale Interesterification

The reaction schematics for the SLs are shown in **Figure 3.1**. For the solvent-free interesterification reaction, 40 g of oil in the appropriate weight ratio determined using the calibration curves were placed in an Erlenmeyer flask. For the IM-SL, a weight ratio of 0.2 (w/w) tripalmitin to AMF was used, and for the interesterification reaction a 0.02 (w/w) ratio of a 2:1 (w/w) ARASCO/DHASCO TAG blend to the IM-SL was used. One milliliter of sample was removed to represent the starting oil composition and was stored in an amber vial in a -20 °C freezer until RP-HPLC analysis. Then, molecular sieve and lipase (Lipozyme TL IM®) were added both at a 5% (w/w) weight of substrates. The flask was closed with a rubber stopper to prevent water from getting into the reaction medium, and the reaction took place in a shaking water bath at 65 °C for 9 h with shaking at 200 rpm. From the reaction, 1 mL of oil was sampled every 30 min, and the samples were immediately filtered through an anhydrous sodium sulfate column to remove any trace of water and the biocatalyst. The samples were then placed in amber capped vials and stored in a -20 °C freezer until RP-HPLC analysis to monitor the decrease in tripalmitin and to determine the reaction time.

For the solvent-free acidolysis reaction, the time of the reaction was determined by the time needed to incorporate the desired mole percentage of ARA and DHA during the reaction. The weight ratio of 0.03 (w/w) of a 2:1 (w/w) ARASCO/DHASCO FFA blend to IM-SL was used as determined by the calibration curve for the acidolysis reaction. The reaction parameters were the same as described for the interesterification reaction. From the acidolysis reaction solution, 1 mL was sampled every 30 min for 6 h. Immediately after being removed, the samples were filtered through an anhydrous sodium sulfate column to remove any trace of water and biocatalyst. In order to remove the FFAs, samples were deacidified by using a modified method of alkaline extraction (Lee & Akoh, 1998). Each 1 mL sample was dissolved in 4 mL of hexane in an Erlenmeyer flask. Then, 4 mL of neutralized 95% ethanol were added, and phenolphthalein solution was added as an indicator. The sample was then titrated with 0.032N NaOH until a pink color appeared showing the endpoint was reached. After titration, the samples were transferred to screw-capped test tubes and were centrifuged at 200 rpm for 5 min. The top hexane layer containing the TAGs was transferred to another test tube and concentrated under nitrogen to remove the hexane. Then, the samples were placed in capped vials and stored in a -20 °C freezer until the samples were analyzed to determine the fatty acid profile as described above.

Large Scale Synthesis of Intermediate Structured Lipid, Interesterification, and Acidolysis Products

Eight hundred grams of the IM-SL were made by mixing a 0.2 (w/w) ratio of tripalmitin to AMF. Two hundred grams of both the IE-SL and ACID-SL products were synthesized. For the interesterification reaction, a weight ratio of 0.02 (w/w) of a 2:1 (w/w)

ARASCO/DHASCO TAG blend to IM-SL was used, and for the acidolysis reaction a weight ratio of 0.03 (w/w) of a 2:1 (w/w) ARASCO/DHASCO FFA blend to IM-SL was used as determined by their corresponding calibration curves. All of the reactions were solvent-free, and they occurred in a 1 L stirred-batch reactor under vacuum with a circulating water bath at 65 °C and mixing at 200 rpm for 5 h. During the reaction, the reactor was covered in foil to reduce exposure to light. Each reaction used Lipozyme TL IM® at 5% (w/w) weight of substrates and molecular sieve at 5% (w/w) weight of substrates. At the end of the reaction, the enzyme and molecular sieve were removed by vacuum filtration with a Buchner funnel. The enzyme was washed with hexane to recover any remaining product, and the hexane solution was pooled with the structured lipid that was the filtrate. The hexane was then removed using a rotary evaporator at 60 °C. Each product was then filtered through a large anhydrous sodium sulfate column under vacuum. Two hundred grams of the (PB-SL) product were made by mixing a weight ratio of 0.02 (w/w) of a 2:1 (w/w) ARASCO/DHASCO TAG blend to IM-SL. After filtration, the SLs were placed in amber Nalgene bottles and were flushed with nitrogen before being stored in a -80 °C freezer. Approximately 5 g of each SL were kept for analysis of FFA percent, *β*-carotene, and tocopherol content before short-path distillation.

Short-Path Distillation

Short-path distillation was used to remove any remaining FFAs from the large-scale synthesis of the SLs excluding the PB-SL. Short-path distillation was performed as previously reported (Teichert & Akoh, 2011a). A KDL-4 (UIC, Inc., Joliet, IL) short-path unit was used. After short-path distillation, the percentage of FFAs as oleic was determined according to the AOCS Official Method Ca 5a-40 (AOCS, 2011a). Reaction

yields were calculated with Eq. (1) with the initial and final values representing the weight and FFA amount before and after short-path distillation. The structured lipids were analyzed for their fatty acid profile and positional analysis as previously stated.

$$Percent\ Yield = \frac{(Final\ weight\ (g))(1-final\ FFA)}{(Initial\ weight\ (g))(1-Initial\ FFA)}\ x\ 100 \quad (1)$$

Oxidative Stability Analysis

Two grams of the PB-SL, IE-SL, and ACID-SL were placed in separate Teflon-lined screw-capped test tubes, and a separate test tube with sample was made for each 12 h sampling period over five days. The samples were oxidized in accelerated conditions at 65 °C in the dark in a water bath. The samples were removed every 12 h and were tested for both peroxide value (PV) and *p*-anisidine value (*p*-AnV*).* The PV was determined according to the IDF method (Shantha & Decker, 1994). The *p*-AnV was determined according to the AOCS Official Method Cd 18-90 (AOCS, 2011b). The total oxidation (TOTOX) value was calculated as 2(PV) + (*p*-AnV) (Shahidi & Wanasundara, 2002). The absorbance of each sample was determined using an UV-1601 visible spectrophotometer (Shimadzu Corp., Columbia, MD).

The oxidative induction temperature (OIT_{emp}) was determined using a differential scanning calorimeter (DSC) 204 F1 Phoenix (NETZSCH Instruments North America, Burlington, MA). Eight to twelve milligrams of lipid sample were weighed into aluminum pans, and the pierced lids were hermetically sealed. The initial temperature was 40 °C, and then the temperature was increased to 300°C at a rate of 10 °C/min in the presence of oxygen with a flow rate of 50 mL/min. Once the temperature reached 300 °C, the DSC was cooled to 20 °C at a rate of 20 °C/min. The oxidative induction temperature

was determined by calculating the onset of oxidation using NETZSCH Proteus Thermal Analysis software.

Tocopherol and *β*-Carotene Analysis

Tocopherol Analysis

Tocopherols were analyzed using a normal phase HPLC with a Shimadzu LC-6A pump equipped with an RF-10AXL fluorescence detector (Shimadzu Corp., Columbia, MD), a Spectra Series As100 autosampler (Thermo Separation Products, Inc., San Jose, CA), and Agilent Chemstation software. The method, samples, and standards were completed and prepared as previously described (Teichert & Akoh, 2011b). The concentrations in ppm were determined by dividing the detected micrograms of each tocopherol by the grams of injected lipid.

β-Carotene Analysis

To analyze *β*-carotene, a modified version of Li (2013) method was used, and analysis was completed in subdued light. The stock solution was prepared by preparing a 3 μg/mL solution in hexane. The purity of the solution was determined and the concentration was adjusted appropriately. The solution was then evaporated under nitrogen until the flasks were completely dry to remove hexane. The volumetric flask was then filled to the mark with HPLC grade chloroform. A standard curve was made by diluting the stock solution to the corrected purity concentrations of between 0.002 to 2.55 μg/mL. The lipid samples were prepared by dissolving approximately 0.3 g of lipid in 1 mL of HPLC grade chloroform. The *β*-carotene concentration was determined by using a RP-HPLC, and peak areas were compared to the standard curve to determine the amount of *β*-carotene detected. The amount detected (μg) was divided by the grams of lipid injected to

determine the *β*-carotene content in ppm. The mobile phase was 50:50 methanol to methyl-tert-butyl-ether (MTBE), and the column used was a Beckman Ultrasphere® C18, 4.6 mm x 250 mm, 5 µm nominal particle size column. A diode array detector (DAD) was used to determine the *β*-carotene peak at 450 nm. The flow rate was 2 mL/min, and the injection volumes for standards and samples were 20 µL. The column was held at 25 °C.

Melting and Crystallization Profiles

The melting and crystallization profiles were determined using a differential scanning calorimeter (DSC) 204 F1 Phoenix (NETZSCH Instruments North America, Burlington, MA) following AOCS Official Method Cj 1-94 (AOCS, 2011c). Nitrogen was used as the protective gas (purge).

Statistical Analysis

Analyses were completed in triplicate or duplicate. All statistical analyses were conducted with SAS software package (SAS Institute Inc., Cary, NC). Duncan's Multiple Range test was performed to determine the significance of difference at $p \leq 0.05$.

Results and Discussion

Total and Positional Fatty Acid Profiles and Determination of Reaction Parameters

AMF, ARASCO®, and DHASCO® oils were characterized because they were starting substrates in this study. The fatty acid profiles are shown in **Table 3.1**. The total and positional analyses of the fatty acid composition of AMF were in agreement with previous studies (Straarup, Lauritzen, Faerk, Høy, & Michaelsen, 2006). The fatty acid profiles of ARASCO® and DHASCO® oils were also in compliance with the manufacture's certificate of analysis. The major fatty acids in AMF were myristic (10.62

± 0.08 mol%), palmitic (29.83 ± 0.15 mol%), stearic (9.97 ± 0.89 mol%), and oleic (22.69 ± 0.16 mol%) acids. The *sn*-2 position of AMF had high amounts of myristic (18.34 ± 1.20 mol%), palmitic (32.60 ± 1.55 mol%), stearic (7.14 ± 0.76 mol%), and oleic (13.58 ± 0.50 mol%) acids. Although the palmitic acid at the *sn*-2 position was high, it was still lower than HMF (Straarup, Lauritzen, Faerk, Høy, & Michaelsen, 2006). Because of this, more *sn*-2 palmitic acid needed to be incorporated into the AMF TAGs. The major fatty acids in ARASCO® were palmitic (10.45 ± 0.20 mol%), stearic (8.56 ± 0.15 mol%), oleic (21.51 ± 0.39 mol%), and ARA (40.90 ± 0.20 mol%) acids. The major fatty acids at the *sn*-2 position of ARASCO® were oleic (58.06 ± 1.33 mol%), linoleic (15.21 ± 0.78 mol%), and ARA (16.53 ± 1.10 mol%) acids. DHASCO® had similar major fatty acids including myristic (11.82 ± 0.07 mol%), palmitic (10.33 ± 0.04 mol%), oleic (16.79 ± 0.06 mol%), and DHA (48.54 ± 0.30 mol%) acids. The *sn*-2 position of DHASCO® had large amounts of oleic acid (33.02 ± 1.73 mol%) and DHA (45.04 ± 1.33 mol%).

The calibration curves to determine the mole percentages of the total and *sn*-2 palmitic acid in physical blends for the intermediate SL (IM-SL) were used to find appropriate weight ratios to achieve *sn*-2 percentages closer to HMF (50 mol%). Also, since tripalmitin has palmitic acid in all three positions, the addition of tripalmitin would increase the total amount of palmitic acid in all positions. This could cause the total palmitic acid mole percentage to be too high in comparison to HMF. For this reason, a calibration curve for the total palmitic acid was also used in order to see how the ideal ratio for the desired percentage of *sn*-2 palmitic acid affects the total palmitic acid percentage. The linear regression models for the mole percentage of *sn*-2 and total

palmitic acid had R^2 values of 0.996 and 0.980, respectively. The ideal weight ratio predicted for a mean percentage of 50 mol% palmitic acid at the *sn*-2 position was found to be 0.45 (w/w) tripalmitin to AMF; however, when this ratio was used to predict the total palmitic acid, the total mean percentage was calculated to be 52.95 mol%. This value was too high in comparison to HMF, which has a total palmitic acid percentage of around 30 mol% (Straarup, Lauritzen, Faerk, Høy, & Michaelsen, 2006). Therefore, a lower ratio was needed in order to decrease the percentage of total palmitic acid. The ratio chosen was 0.2 (w/w) tripalmitin to AMF, and the linear model predicted that the mean percentage of *sn*-2 palmitic acid would be approximately 40.24 mol% and the mean total percentage would be approximately 41.00 mol%. The predicted value of *sn*-2 palmitic acid was lower than ideal, but acyl migration was predicted to increase the palmitic acid at the *sn*-2 position during the reaction. Even though the enzyme used was an *sn*-1,3 specific lipase, a side reaction known as acyl migration can occur in which fatty acids at the *sn*-1,3 positions migrate to the *sn*-2 position and vise versa (Xu, Skands, Høy, Mu, Balchen, & Adler-Nissen, 1998). This usually occurs due to the presence of diacylglycerols that are formed as intermediates in the reaction (Xu, 2000). The change in palmitic acid was followed in order to determine if the interesterification reaction between tripalmitin and AMF using Lipozyme TL IM® would increase the palmitic acid at the *sn*-2 position. The results shown in **Table 3.2** indicated that the means for the *sn*-2 palmitic acid were 39.67 ± 0.53 mol% for the physical blend before the reaction and 43.51 ± 0.46 mol% for the IM-SL after the reaction. According to the results, the percentages were significantly different, and therefore, the *sn*-2 palmitic acid increased after the reaction by about 3.80 mol%. The total palmitic acid percentage was also

slightly higher than ideal, but the acidolysis reaction was tested to see if the reaction would decrease the total mole percentage due to substitution of fatty acids on the glycerol backbone, and this will be discussed later. Other changes in the *sn*-2 composition include a decrease in capric (C10:0), myristic (C14:0), palmitoleic (C16:1n7), and *cis*-oleic (C18:1n9) acids and an increase in stearic acid (C18:0).

Calibration curves were also completed to determine the appropriate weight ratios of a 2:1 (w/w) ARASCO/DHASCO TAG blend for the interesterification reaction and a 2:1 (w/w) ARASCO/DHASCO FFA blend for the acidolysis reaction to the IM-SL in order to incorporate the target mole percentage of ARA and DHA in the TAGs. The R^2 for both calibration curves of ARA and DHA were greater than 0.990. From the curves, the weight ratio of 0.02 (w/w) 2:1 (w/w) ARASCO/DHASCO TAG blend to the IM-SL was found to provide the desired mole percentages of ARA and DHA for the interesterification reaction. Also, a weight ratio of 0.03 (w/w) 2:1 (w/w) ARASCO/DHASCO FFA blend to the IM-SL provided the desired mole percentage of DHA and ARA for the acidolysis reaction.

The time course of interesterification was studied for the IM-SL and the IE-SL by determining the time at which the relative percentage of the tripalmitin peak was the lowest because this would represent when the reaction substrate was used. **Figure 3.2A** shows that tripalmitin was not completely used up during the interesterification reaction and an equilibrium percentage of tripalmitin was reached. The peak percentages for the time periods between 240 and 510 min were not significantly different with the exception of 420 min, which had a peak percentage that was significantly higher. The time of around 5 h (300 min) was chosen as the reaction time for the IM-SL and the IE-SL to

ensure equilibrium was established. The IE-SL had similar curves due to the addition of small amounts of ARASCO® and DHASCO® TAGs. For the acidolysis reaction, the time chosen for the reaction was determined by the time at which the incorporation of ARA and DHA in the TAGs were both within the recommended percentage limits. The incorporation of ARA and DHA into the TAGs over time is shown in **Figure 3.2B**. The ARA incorporation increased rapidly possibly due to steric hindrance or discrimination by the enzyme. However, the DHA incorporation took more time and did not reach the incorporation limit of 0.20 mol% until 5 h (300 min). A time of 5 h was chosen for the acidolysis reaction because both the ARA and DHA incorporation percentages were within the recommended limits of 0.3 to 0.7 mol% and 0.2 to 0.5 mol%, respectively. Also, this shows that the acidolysis reaction was more challenging in comparison to the interesterification reaction, especially for DHA incorporation. This is most likely because the interesterification reaction rearranges the fatty acids between TAG molecules and does not involve FFA being incorporated into the TAG molecule as with the acidolysis reaction.

After the reaction parameters were determined, large-scale preparation and synthesis were completed for the PB-SL, IE-SL, and ACID-SL. The fatty acid compositions and statistical differences are shown in **Table 3.3**. Ranges of fatty acid percentages in HMF and infant formulas were also included for comparison. The total palmitic acid percentages for all three products were 42.56 ± 0.16, 43.14 ± 0.87, and 42.35 ± 0.14 mol% for the PB-SL, IE-SL, and ACID-SL, respectively. The results show that the total palmitic acid for all three products did not differ indicating that the acidolysis reaction did not reduce the total palmitic acid. This could be due to the fact

that small amounts of the FFA blend were incorporated into the TAG, which did not have a large effect on the fatty acid profile. The results show that the three products contained total mole percentages of ARA and DHA that were within the limits recommended for supplementation and had similar percentages to HMF. The ACID-SL did however have significantly lower amounts of total DHA with 0.23 ± 0.09 mol% compared to the PB-SL and IE-SL with 0.44 ± 0.06 and 0.50 ± 0.12 mol%, respectively. This was most likely due to the fact that the incorporation of DHA, as previously mentioned, occurred at a slower rate in comparison to the incorporation of ARA possibly due to the steric hindrance or discrimination by the enzyme. The percentages of total DHA for the final products were also higher than the range of percentages found in 12 commercial infant formulas (0.00-0.20 mol%, see **Table 3.3**). The total mole percentage of ARA did not statistically differ for all three products with amounts of 0.66 ± 0.06, 0.63 ± 0.05, and 0.68 ± 0.04 mol% for the PB-SL, IE-SL, and the ACID-SL, respectively. The percentages of total ARA were also higher than the range of percentages found in infant formulas (0.00-0.36 mol%). Other total fatty acid percentages in all of the products that showed similar amounts in a study of the composition of HMF include capric (C10:0), myristic (C14:0), palmitoleic (C16:1n7), and stearic (C18:0) acids (Lopez-Lopez, Lopez-Sabater, Campoy-Folgoso, Rivero-Urgell, & Castellote-Bargallo, 2002). In comparison to percentages in infant formulas, the final products were more similar to HMF for capric (C10:0) and palmitoleic (C16:1n7).

Comparisons of the *sn*-2 composition of the final products were also conducted and data is shown in **Table 3.3**. The results for the *sn*-2 fatty acid composition show that the *sn*-2 palmitic acid did not significantly differ between methods with 45.17 ± 0.76,

44.97 ± 1.02, and 46.01 ± 2.46 mol% for the PB-SL, IE-SL, and ACID-SL, respectively. Also, the amount of lauric (C12:0), myristic (C14:0), palmitoleic (C16:1n7), and linoleic (C18:2n6) acids significantly decreased after both interesterification and acidolysis reactions, but they still provide percentages similar to HMF (Lopez-Lopez, Lopez-Sabater, Campoy-Folgoso, Rivero-Urgell, & Castellote-Bargallo, 2002, see **Table 3.3**). The percentage of oleic acid (C18:1n9) significantly increased from 14.37 ± 0.10 mol% in the PB-SL to 17.00 ± 0.25 and 16.80 ± 0.87 mol% in the IE-SL and ACID-SL, respectively, showing percentages closer to HMF and lower than commercial infant formulas (26.33-49.40%). LCPUFAs in HMF are mainly found in TAGs esterified to the *sn*-2 and *sn*-3 positions. It has been suggested that due to the weak positional specificity of single cell algal oils, absorption of the LCPUFAs could be affected. Therefore, the percentage of the *sn*-2 ARA and DHA levels are important (Lapillonne, Groh-Wargo, Gonzalez, & Uauy, 2013). The percentage of *sn*-2 DHA was similar for all three products with percentages of 0.25 ± 0.07, 0.46 ± 0.12, and 0.37 ± 0.14 mol% for the PB-SL, IE-SL, and ACID-SL, respectively, and these are similar percentages present in HMF (Straarup, Lauritzen, Faerk, Høy, & Michaelsen, 2006). The PB-SL did have statistically lower amounts of *sn*-2 ARA with 0.15 ± 0.05 mol% in comparison to the IE-SL and ACID-SL with 0.55 ± 0.13 and 0.52 ± 0.12 mol%, respectively. This is because no enzyme was used. Also, the percentage of *sn*-2 ARA in ARASCO® was lower (16.53 ± 1.10 mol%) in comparison to the *sn*-2 DHA in DHASCO® (45.04 ± 1.33 mol%, see **Table 3.1**), yet the amount of ARA incorporated with the enzyme was high. Also, this indicates that the enzymatic reactions to form the SL products increased the *sn*-2 mole percentage of ARA possibly due to acyl migration. This could be due to a fast migration

rate of polyunsaturated fatty acids (Xu, Skands, Høy, Mu, Balchen, & Adler-Nissen, 1998). This would be beneficial to infant nutrition as *sn*-2 ARA and DHA amounts are important for incorporation into membrane lipids (Lapillonne, Groh-Wargo, Gonzalez, & Uauy, 2013). In comparison to infant formulas, the *sn*-2 percentages of ARA and DHA in the IE-SL and ACID-SL were higher than the range found in infant formulas; however, the upper range percentages of 0.48 mol% ARA and 0.28 mol% DHA were only found in 1 out of 11 infant formulas sampled (Lopez-Lopez, Lopez-Sabater, Campoy-Folgoso, Rivero-Urgell, & Castellote-Bargallo, 2002, see **Table 3.3**). The *sn*-2 palmitic acid was also higher in the final products in comparison to the infant formulas sampled, with the exception of 1 infant formula out of 11 containing 43.01 mol% *sn*-2 palmitic acid (Lopez-Lopez, Lopez-Sabater, Campoy-Folgoso, Rivero-Urgell, & Castellote-Bargallo, 2002, see **Table 3.3**).

The reaction yields for the IE-SL and ACID-SL were calculated as 91.84 and 89.29%, respectively. One reason for the lower reaction yield for the acidolysis reaction could be because more FFAs (5.77 ± 0.27%) were formed compared to the interesterification reaction (3.84 ± 0.07%, see **Table 3.4**). Since the parameters such as time and temperature for the short-path distillation were the same for both products, the amount of FFAs remaining in the ACID-SL after distillation was slightly higher with 0.32 ± 0.06% as oleic compared to 0.15 ± 0.03% as oleic for the IE-SL. This shows that the ACID-SL would require more time and have more product loss because there is a higher percentage of FFAs formed that must be removed, and this is represented by the lower reaction yield.

Oxidation and Antioxidant Content

To test the oxidative stability of the PB-SL, IE-SL, and ACID-SL under accelerated conditions, the PV and *p*-AnV were determined in order to calculate the TOTOX value over a period of 120 h. The PV is a measure of the hydroperoxides, which are primary oxidization products that are formed during oxidation. This value increases and then decreases due to the decay of the hydroperoxides into secondary oxidation products (Frankel, 1996). The *p*-AnV is a measure of secondary oxidation products, which are mainly comprised of aldehydes that are formed from the deterioration of the hydroperoxides (Frankel, 1996). The TOTOX value takes into consideration both the PV and *p*-AnV, and the value increases with increasing lipid oxidation. The TOTOX, PV, and *p*-AnV values of the SLs over time are shown in **Figure 3.3**. The TOTOX value for PB-SL stayed relatively constant over the 120 h oxidation period. However, both the SLs experienced different levels of oxidation. The ACID-SL experienced the greatest amount of oxidation, which began around 12 h, and was the least stable to oxidation. The IE-SL had TOTOX values in between the ACID-SL and the PB-SL showing intermediate stability. The same trend was observed for the PV and *p*-AnV values where the ACID-SL had higher PV and *p*-AnV values compared to the IE-SL and PB-SL over time. The decrease in PV and TOTOX value of the ACID-SL after 96 h was most likely because of the deterioration of the primary oxidation products to the secondary oxidation products.

The oxidative induction temperature (OIT_{emp}) was also determined for the final products to measure the thermal oxidative stability. The OIT_{emp} is determined as the onset of decomposition during a dynamic increase in temperature in an oxidative environment (Schmid, Ritter, & Affolter, 2006). The OIT_{emp} values are shown in **Table 3.5**. The PB-

SL had a significantly higher OIT_{emp} of 215.9 ± 4.30 °C compared to the ACID-SL which had an OIT_{emp} of 205.3 ± 1.80 °C. However, the OIT_{emp} for the IE-SL was 209.9 ± 1.30 °C and did not significantly differ from either the PB-SL or ACID-SL showing it had intermediate oxidative stability, and this trend was also observed in the oxidation experiment.

The rapid oxidation of the ACID-SL could be for many reasons. One reason could be because of the difference in the percentage of FFAs in the final products as previously mentioned. Since FFAs are more susceptible to oxidation, a higher percentage of FFAs could cause lipid oxidation to occur at a faster rate (Shahidi & Wanasundara, 2002). It is important to note that the FFA percentages for the products could be lower with further processing, such as deodorization. Another explanation could be from the degradation of antioxidants due to many factors including exposure to light, oxygen, and heat during processing. Therefore, the tocopherol and *β*-carotene amounts were determined for the starting substrates, final products, and between processing steps.

Tocopherols are a part of the vitamin E family, and are comprised of a hydrophilic chromanol ring containing a hydrophobic isoprenoid side chain. They are characterized as *α-, β-, γ-*, and *δ*-tocopherols depending on the placement of methyl groups on the chromanol ring (Sabliov, Fronczek, Astete, Khachaturyan, Khachatryan, & Leonardi, 2009). The antioxidant property is due to the redox properties of the chromanol ring (Sies & Stahl, 1995). Tocopherols help prevent oxidation by reacting with radicals formed from lipid oxidation to inhibit radical chain reactions that produce primary and secondary oxidation products (Frankel, 1996). Another antioxidant present in milkfat is *β*-carotene, which gives it a characteristic yellow color. The antioxidant activity of *β*-

carotene is due to the extended system of conjugated double bonds that aid in inhibiting free radicals (Sies & Stahl, 1995). The antioxidant contents in the starting substrates and final products are shown in **Table 3.5**. In AMF, α-tocopherol was the most abundant antioxidant (23.93 ± 3.23 ppm) followed by β-carotene (4.14 ± 0.05 ppm), and γ-tocopherol (2.92 ± 0.48 ppm), and there were lower amounts of β-tocopherol (0.65 ± 0 .20 ppm) and δ-tocopherol (0.85 ± 0.14 ppm). ARASCO® and DHASCO® oils both had high amounts of tocopherols. The most predominant tocopherols in ARASCO® were γ-tocopherols (192.31 ± 11.55 ppm), α-tocopherol (93.19 ± 6.11 ppm), and δ-tocopherol (70.96 ± 4.23 ppm). DHASCO® had similar predominant tocopherols including γ-tocopherol (221.60 ±11.95 ppm), α-tocopherol (113.70 ± 6.49 ppm), and δ-tocopherol (96.23 ± 5.10 ppm). DHASCO® also contained 190.0 ± 1.91 ppm of β-carotene, while β-carotene was not detected in ARASCO®. The 2:1 (w/w) blend of ARASCO/DHASCO FFAs showed lower amounts of tocopherols and β-carotene with the predominant tocopherols being γ-tocopherol (123.48 ± 6.78 ppm), and δ-tocopherol (61.72 ± 3.65 ppm). The lower amount could be due to the reaction to convert the TAGs into FFAs, which includes exposure to heat.

The comparison of antioxidant contents in the final products is shown in **Table 3.5**. The PB-SL contained α-tocopherol (6.51 ± 0.67 ppm), β-tocopherol (0.42 ± 0.15 ppm), γ-tocopherol (4.32 ± 0.30 ppm), δ-tocopherol (1.42 ± 0.27 ppm), and β-carotene (4.09 ± 0.03 ppm). The IE-SL contained α-tocopherol (2.98 ± 0.10 ppm), γ-tocopherol (2.19 ± 0.21 ppm), δ-tocopherol (0.62 ± 0.10 ppm), and β-carotene (3.67 ± 0.02 ppm), while β-tocopherol was not detected. The ACID-SL contained α-tocopherol (1.00 ± 0.16 ppm), δ-tocopherol (0.57 ± 0.02 ppm), and β-carotene (1.75 ± 0.04 ppm), but β-

tocopherol and γ-tocopherol were not detected. The PB-SL had significantly higher amounts of all antioxidants in comparison to the IE-SL and ACID-SL products. This is because the PB-SL did not go through short–path distillation. The IE-SL and ACID-SL products differed in α-tocopherol, γ-tocopherol, and β-carotene content with the ACID-SL having significantly lower amounts. Also, β-tocopherol was not detected in both SL products, and γ-tocopherol was not detected in the ACID-SL. This could explain why the PB-SL was the most stable to oxidation and the ACID-SL was the least stable to oxidation because of low antioxidant content. This also suggests that addition of antioxidants to SLs would lead to an increase in oxidative stability since the PB-SL contained the IM-SL, a structured lipid, and ARASCO® and DHASCO® TAGs with high amounts of tocopherols and β-carotene.

To determine if there was a loss in antioxidants during processing steps, the antioxidant content of the SLs was determined for each processing step shown in **Table 3.4.** Nearly all of the antioxidants did not show a significant difference in antioxidant content before and after the interesterification or acidolysis reaction with the exception of α-tocopherol for the ACID-SL and β-carotene for the IM-SL. A significant difference in antioxidant content before and after short-path distillation was seen in nearly all of the products, and this is consistent with previous studies (Akoh & Moussata, 2001). This could be due to exposure to heat, light, and also removal of antioxidants with the FFAs during short-path distillation. Also, the IE-SL and ACID-SL had an increase in antioxidant content compared to the IM-SL, a starting substrate, due to the addition of ARASCO® and DHASCO® which contained tocopherols and β-carotene as previously stated.

TAG Molecular Species and Melting and Crystallization Profiles

The predicted TAG molecular species in AMF, IM-SL, PB-SL, IE-SL, and ACID-SL were determined in order to show the change in TAG species due to enzymatic reactions, and this is shown in **Table 3.6**. The TAGs shown are not representative of stereochemical configuration. The peak determinations were made according to elution time of TAG standards, equivalent carbon number (ECN), and published works of milkfat TAGs (Gastaldi, Medana, Giancotti, Aigotti, Dal Bello, & Baiocchi, 2011). The major TAG species in AMF were BMP or BPO (10.66 ± 0.46%), BPP (20.98 ± 0.37%), BPS or CoPP (7.30 ± 0.35%), CyPP or CoPS (6.24 ± 0.49%), and PPO (5.58 ± 0.22%). Major differences in AMF and IM-SL were observed and include decreases in the percentage of BMP or BPO (10.66 ± 0.46 to 7.91 ± 0.36%), BPP (20.98 ± 0.37 to 11.77 ± 0.10%), BPS or CoPP (7.30 ± 0.35 to 3.86 ± 0.36%), and CyPP or CoPS (6.24 ± 0.49 to 3.96 ± 0.02%). There were increases in MPP (3.06 ± 0.06 to 6.28 ± 0.34%), PPO (5.58 ± 0.22 to 9.59 ± 0.46%), PPP (3.50 ± 0.36 to 13.68 ± 0.59%), and OSS (1.52 ± 0.11 to 4.56 ± 0.41%). The high increase in tripalmitin (PPP) was due to the use of tripalmitin as a substrate and because it was not completely used up in the reaction possibly due to a chemical equilibrium as previously stated. The percentage of tripalmitin TAG did not significantly differ between methods and was 13.75 ± 0.54%, 13.22 ± 0.39%, and 12.83 ± 0.02% for the PB-SL, IE-SL, and ACID-SL, respectively. While there are reports of tripalmitin in HMF (0.19-0.91%, see **Table 3.6**), the percentage is much lower than what is present in the final products (Zou, Huang, Jin, Guo, Liu, Cheong, et al., 2012). The relative peak percentages of most of the TAG species of the final products were similar due to the small amounts of ARASCO® and DHASCO® added. A study comparing the TAG

molecule species of HMF and other fat sources found 23 different TAGs with concentrations higher than 1% in HMF. Of those, 13 TAG molecular species were found in the final products including CPO, LaOO, LaPO, PLO, MOO, MPO, OOO, OOP, PPO, PSM, OOS, OPS, PPS. While most TAGs detected in the products had similar percentages to what was found in the HMF there were still TAGs in the final products that were not present in HMF including many containing butyric acid (Gastaldi, Medana, Giancotti, Aigotti, Dal Bello, & Baiocchi, 2011; Zou, Huang, Jin, Guo, Liu, Cheong, et al., 2012). Also, the percentages of PLO and OOP in the final products were much lower than HMF, which contains 12.69-21.94 PLO and 13.69-28.46 OOP (Zou, Huang, Jin, Guo, Liu, Cheong, et al., 2012 see **Table 3.6**). The identification of ARA and DHA in TAG molecular species analysis was not observed most likely due to their small incorporation into the final products.

The melting and crystallization profiles of AMF, IM-SL, PB-SL, IE-SL, and ACID-SL were determined using DSC. The melting completion temperature (T_{mc}) of AMF was 36.3 °C with an onset of crystallization temperature (T_{co}) of 17.7 °C. The T_{mc} increased for the IM-SL to 43.5 °C, and the T_{co} increased to 26.0 °C. This is due to the addition of tripalmitin, which has a high melting point (64-68 °C). The T_{mc} for the PB-SL, IE-SL, and ACID-SL were similar to the IM-SL and were 43.2, 43.1, and 43.0 °C, respectively. The T_{co} for the final products were also similar to the IM-SL and were 26.3, 27.1, and 27.1°C for the PB-SL, IE-SL, and ACID-SL, respectively. The small change in the melting and crystallization profiles of the final products containing algal oils is most likely due to the relatively low addition of the algal oils to the IM-SL. The high melting point of the final products could be a problem because HMF analogues with high melting

points are more difficult to handle, transport, and combine with other hydrophilic ingredients in a formulation (Álvarez & Akoh, 2015).

Conclusion

Three human milkfat analogues were produced that may more closely fit the nutritional needs of infants and help improve metabolic functions. Of the three SLs synthesized, the IE-SL would be a suitable HMF analogue for potential use in infant formula due to the increased amounts of ARA at the *sn*-2 position compared to the PB-SL. Although, the PB-SL was the most stable to oxidation, antioxidants could be added to the IE-SL to improve stability, and this will be studied in the future. The ACID-SL product would be the least suitable for use in commercial infant formula due to a lower reaction yield, lower incorporation of DHA, additional processing steps, greater loss of antioxidants, and the high susceptibility to oxidation compared to the other products. A possible drawback to the products includes a high melting completion and crystallization onset temperatures due to the high percentage of the tripalmitin TAG. Blending with other oils or HMF analogues can alleviate this problem. However, the IE-SL would provide TAGs with higher amounts of *sn*-2 palmitic acid and the recommended levels of DHA and ARA to help the growth and development of infants.

Acknowledgements

We thank the Dairy Farmers of America for providing the anhydrous milkfat. This research was supported in part by Food Science Research, University of Georgia.

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Table 3.1 Total and positional fatty acid composition (mol%) of starting substrates

Fatty acid	AMF[a]		ARASCO[b]		DHASCO[c]	
	Total	*sn*-2	Total	*sn*-2	Total	*sn*-2
C4:0	3.41 ± 0.05	ND	ND	ND	ND	ND
C6:0	1.90 ± 0.03	ND	ND	ND	ND	ND
C8:0	1.26 ± 0.02	1.61 ± 0.03	ND	ND	0.44 ± 0.00	ND
C10:0	2.93 ± 0.04	4.19 ± 0.03	ND	ND	1.48 ± 0.01	ND
C12:0	3.39 ± 0.04	5.27 ± 0.12	ND	ND	5.65 ± 0.04	3.21 ± 0.02
C14:0	10.62 ± 0.08	18.34 ± 1.20	0.33 ± 0.06	ND	11.82 ± 0.07	6.18 ± 0.46
C15:0	1.14 ± 0.01	1.82 ± 0.03	ND	ND	ND	ND
C16:0	29.83 ± 0.15	32.60 ± 1.55	10.45 ± 0.20	3.18 ± 1.49	10.33 ± 0.04	3.97 ± 1.07
C16:1n7	1.37 ± 0.01	2.06 ± 1.93	0.14 ± 0.01	ND	2.90 ± 0.02	2.16 ± 0.05
C18:0	9.97 ± 0.89	7.14 ± 0.76	8.56 ± 0.15	ND	0.41 ± 0.05	ND
C18:1t	3.75 ± 0.87	5.18 ± 0.51	ND	ND	ND	ND
C18:1n9	22.69 ± 0.16	13.58 ± 0.50	21.51 ± 0.39	58.06 ± 1.33	16.79 ± 0.06	33.02 ± 1.73
C18:2t	0.83 ± 0.04	ND	0.20 ± 0.06	3.07 ± 0.78	ND	ND
C18:2n6	3.30 ± 0.56	2.67 ± 0.12	7.96 ± 0.02	15.21 ± 0.78	0.55 ± 0.06	5.31 ± 0.83
C18:3n6	ND	ND	1.62 ± 0.06	1.80 ± 0.11	0.03 ± 0.00	ND
C20:3n6	ND	ND	2.61 ± 0.09	1.28 ± 0.22	ND	ND
C20:4n6	ND	ND	40.90 ± 0.20	16.53 ± 1.10	0.39 ± 0.19	ND
C24:0	ND	ND	3.95 ± 0.31	ND	ND	ND
C22:6n3	ND	ND	ND	ND	48.54 ± 0.30	45.04 ± 1.33

Values are mean ± SD (n=3)

ND-not detected

[a] Other fatty acids found in trace amounts were C11:0, C14:1, C15:1, and C18:3n3

[b] Other fatty acids found in trace amounts were C20:1, C18:3n3, C20:2, C22:0, and C24:1

[c] Other fatty acids found in trace amounts were C14:1, C20:1

Table 3.2 Fatty acid composition (mol%) of the small scale physical blend (PB) and intermediate structured lipid (IM-SL)[a]

Fatty Acid	Total[b]	*sn*-2	
		PB[c]	IM-SL
C4:0	2.78 ± 0.06	ND	ND
C6:0	1.59 ± 0.02	ND	ND
C8:0	1.07 ± 0.01	1.59 ± 0.02a	1.47 ± 0.09a
C10:0	2.46 ± 0.02	4.68 ± 0.02a	3.84 ± 0.18b
C12:0	2.83 ± 0.04	5.66 ± 0.05a	4.78 ± 0.99a
C14:0	8.86 ± 0.14	18.44 ± 0.19a	12.74 ± 0.96b
C15:0	0.97 ± 0.02	1.63 ± 0.04a	2.47 ± 0.69a
C16:0	41.98 ± 0.67	39.67 ± 0.53a	43.51 ± 0.46b
C16:1n7	1.54 ± 0.04	2.15 ± 0.09a	1.64 ± 0.08b
C18:0	9.53 ± 0.11	3.79 ± 0.09a	6.58 ± 0.93b
C18:1t	3.13 ± 0.08	1.15 ± 0.50a	0.78 ± 0.35a
C18:1n9	17.13 ± 0.18	17.48 ± 0.65a	16.16 ± 0.41b
C18:2t	0.40 ± 0.02	ND	1.84 ± 0.39b
C18:2n6	2.59 ± 0.13	2.58 ± 0.31a	3.35 ± 0.81a

Values are mean ± SD (n=3)

ND-not detected

Different letters in each row of *sn*-2 show significant difference ($p \leq 0.05$)

[a] IM-SL synthesized with 0.2 (w/w) ratio of tripalmitin to AMF with 5 h reaction time

[b] Other fatty acids found in trace amounts were C11:0, C14:1, C15:1, and C18:3n3

[c] Represents the physical blend of the IM-SL before the enzymatic reaction

Table 3.3 Total and positional fatty acid composition (mol%) of the final structured lipid products, HMF, and commercial infant formulas

Fatty acid	Total					*sn*-2				
	PB-SL[a]	IE-SL[b]	ACID-SL[c]	HMF[d] (n=40)	Infant Formula (n=12)	PB-SL	IE-SL	ACID-SL	HMF (n=12)	Infant Formula (n=11)
C4:0	2.26 ± 0.09a[e]	1.68 ± 0.16b	1.88 ± 0.12b	ND	0.00-0.16	ND	ND	ND	ND	ND
C6:0	1.43 ± 0.04a	1.25 ± 0.05b	1.31 ± 0.03b	ND	0.03-0.24	0.51 ± 0.45a	0.55 ± 0.50a	1.00 ± 0.90a	ND	ND
C8:0	0.98 ± 0.02a	0.91 ± 0.03b	0.93 ± 0.01b	0.11-0.36	0.55-1.20	1.62 ± 0.22a	1.16 ± 0.45a	1.24 ± 0.48a	ND	0.03-0.09
C10:0	2.36 ± 0.03a	2.31 ± 0.03a	2.30 ± 0.01a	0.85-3.08	0.74-1.24	3.52 ± 0.34a	3.40 ± 0.12a	2.40 ± 1.58a	0.21-0.36	0.16-1.54
C12:0	2.79 ± 0.02a	2.79 ± 0.04a	2.78 ± 0.01a	4.05-9.35	5.19-12.64	4.25 ± 0.21a	3.91 ± 0.10ab	3.76 ± 0.30b	2.41-4.81	4.68-15.32
C14:0	8.82 ± 0.04a	9.07 ± 0.24a	8.93 ± 0.03a	3.60-9.13	4.06-5.91	13.57 ± 0.41a	12.00 ± 0.25b	12.34 ± 0.55b	6.69-10.74	2.23-7.10
C15:0	0.98 ± 0.01a	0.99 ± 0.00a	0.98 ± 0.01a	0.11-0.48	0.05-0.35	1.55 ± 0.10a	1.18 ± 0.03b	1.24 ± 0.02b	0.46-0.53	0.02-0.31
C16:0	42.56 ± 0.16a	43.14 ± 0.87a	42.35 ± 0.14a	15.43-24.46	17.96-27.42	45.17 ± 0.76a	44.97 ± 1.02a	46.01 ± 2.46a	51.17-52.30	5.88-43.01
C16:1n7	1.53 ± 0.01a	1.54 ± 0.03a	1.54 ± 0.02a	1.10-2.18	0.14-0.78	1.81 ± 0.18a	1.49 ± 0.04b	1.52 ± 0.07b	1.72-1.97	0.08-0.52
C18:0	8.97 ± 0.24a	8.23 ± 0.15b	7.69 ± 0.11c	4.60-8.13	3.05-6.72	6.65 ± 0.25a	7.74 ± 0.80a	6.68 ± 0.35a	1.68-1.80	0.56-2.38
C18:1t	3.16 ± 0.19a	3.76 ± 0.10b	3.66 ± 0.34b	---	---	1.51 ± 0.05a	2.70 ± 0.04b	3.02 ± 0.70b	---	---
C18:1n9	17.83 ± 0.22a	17.92 ± 0.51a	18.79 ± 0.38b	28.30-43.83	34.34-44.69	14.37 ± 0.10a	17.00 ± 0.25b	16.80 ± 0.87b	13.97-17.43	26.33-49.40
C18:2t	0.34 ± 0.04a	0.35 ± 0.20a	0.63 ± 0.14b	---	---	0.96 ± 0.30a	ND	ND	---	---
C18:2n6	2.49 ± 0.12a	2.60 ± 0.43a	2.95 ± 0.15a	10.61-25.30	8.93-17.02	2.28 ± 0.14a	0.74 ± 0.64b	1.33 ± 0.04b	10.32-11.58	8.14-25.95
C20:4n6	0.66 ± 0.06a	0.63 ± 0.05a	0.68 ± 0.04a	0.23-0.75	0.00-0.36	0.15 ± 0.05a	0.55 ± 0.13b	0.52 ± 0.12b	0.67-1.16	0.00-0.48
C22:6n3	0.44 ± 0.06a	0.50 ± 0.12a	0.23 ± 0.09b	0.15-0.56	0.00-0.20	0.25 ± 0.07a	0.46 ± 0.12a	0.37 ± 0.14a	0.64-0.93	0.00-0.28

Values are mean ± SD (n=3)

ND-not detected

Different letters within each row for total and *sn*-2 separately show significant difference ($p \leq 0.05$)

Other fatty acids found in trace amounts were C11:0, C14:1, C15:1, C18:3n3, and C20:1

[a] PB-SL physical blend of 0.02 (w/w) ratio of 2:1 ARASCO:DHASCO TAGs to IM-SL

[b] IE-SL synthesized with 0.02 (w/w) ratio of 2:1 (w/w) ARASCO:DHASCO TAGs to IM-SL with 5 h reaction time

[c] ACID-SL synthesized with 0.03 (w/w) ratio of 2:1 (w/w) ARASCO:DHASCO FFAs to IM-SL with 5 h reaction time

[d] Data for HMF and infant formulas was obtained from Lopez-Lopez *et al.* (2002) and represents a range of averages

Table 3.4 Comparison of antioxidant content (ppm) throughout processing steps for structured lipids

Structured Lipid	Antioxidant	Before Reaction	After Reaction[a]	After Short-path Distillation
IM-SL[b]	α-Tocopherol	20.81 ± 4.03a	16.86 ± 0.03a	6.91 ± 0.04b
	β-Tocopherol	0.55 ± 0.02a	0.38 ± 0.05a	ND
	γ-Tocopherol	2.42 ± 0.50a	2.06 ± 0.09a	0.97 ± 0.03b
	δ-Tocopherol	0.41 ± 0.25a	0.21 ± 0.01a	ND
	β-Carotene	3.74 ± 0.01a	3.11 ± 0.01b	2.64 ± 0.01c
	FFA %[c]	--	4.21 ± 0.12a	0.25 ± 0.01b
PB-SL[d,e]	α-Tocopherol	6.51 ± 0.67	--	--
	β-Tocopherol	0.42 ± 0.15	--	--
	γ-Tocopherol	4.32 ± 0.30	--	--
	δ-Tocopherol	1.42 ± 0.27	--	--
	β-Carotene	4.09 ± 0.03	--	--
	FFA %	0.23 ± 0.09	--	--
IE-SL[f]	α-Tocopherol	6.51 ± 0.67a	5.79 ± 1.23a	2.98 ± 0.10b
	β-Tocopherol	0.42 ± 0.15a	0.44 ± 0.25a	ND
	γ-Tocopherol	4.32 ± 0.30a	4.03 ± 1.11a	2.19 ± 0.21a
	δ-Tocopherol	1.42 ± 0.27a	1.56 ± 0.56a	0.62 ± 0.10a
	β-Carotene	4.09 ± 0.03a	4.01 ± 0.02a	3.67 ± 0.02b
	FFA %	--	3.84 ± 0.07a	0.15 ±0.03b
ACID-SL[g]	α-Tocopherol	5.29 ± 0.27a	3.62 ± 0.03b	1.00 ± 0.16c
	β-Tocopherol	0.36 ± 0.07a	0.26 ± 0.02a	ND
	γ-Tocopherol	4.46 ± 0.15a	4.41 ± 0.24a	ND
	δ-Tocopherol	1.94 ± 0.15a	2.07 ± 0.13a	0.57 ± 0.02b
	β-Carotene	3.31 ± 0.05a	3.32 ± 0.06a	1.75 ± 0.03b
	FFA %	--	5.77 ± 0.27a	0.32 ± 0.06b

Values are mean ± SD (n=2)

Different letters in each row show significant difference ($p \leq 0.05$)

ND-not detected

[a] Represents the product after the enzymatic reaction but before short-path distillation

[b] IM-SL synthesized with 0.2 (w/w) ratio of tripalmitin to AMF with 5 h reaction time

[c] FFA % as oleic acid

[d] PB-SL physical blend of 0.02 (w/w) ratio of 2:1 (w/w) ARASCO:DHASCO TAGs

[e] After reaction and after short-path distillation values were not listed because the physical blend did not undergo any reaction or distillation

[f] IE-SL synthesized with 0.02 (w/w) ratio of 2:1 (w/w) ARASCO:DHASCO TAGs to IM-SL with 5 h reaction time

[g] ACID-SL synthesized with 0.03 (w/w) ratio of 2:1 (w/w) ARASCO:DHASCO FFAs to IM-SL with 5 h reaction time

Table 3.5 Antioxidant content (ppm) and OIT_{emp} (°C) of starting substrates and final products

	α-T[a]	β-T	γ-T	δ-T	β-Carotene	OIT_{emp}
Substrates						
AMF	23.93 ± 3.23	0.65 ± 0.20	2.92 ± 0.48	0.85 ± 0.14	4.14 ± 0.05	--
ARASCO	93.19 ± 6.11	7.16 ± 0.05	192.31 ± 11.55	70.96 ± 4.23	ND	--
DHASCO	113.70 ± 6.49	10.63 ± 0.76	221.60 ± 11.95	96.23 ± 5.10	190.0 ± 1.91	--
ARA/DHA FFA Blend[b]	24.20 ± 1.18	6.54 ± 0.49	123.48 ± 6.78	61.72 ± 3.65	16.02 ± 0.27	--
Products						
PB-SL[c]	6.51 ± 0.67a	0.42 ± 0.15a	4.32 ± 0.30a	1.42 ± 0.27a	4.09 ± 0.03a	215.9 ± 4.30a
IE-SL[d]	2.98 ± 0.10b	ND	2.19 ± 0.21b	0.62 ± 0.10b	3.67 ± 0.02b	209.9 ± 1.30ab
ACID-SL[e]	1.00 ± 0.16c	ND	ND	0.57 ± 0.02b	1.75 ± 0.04c	205.3 ± 1.80b

Values are mean ± SD (n=2)

ND-not detected

Letters in each column for the final products show significant difference at ($p \leq 0.05$)

[a] α-Tocopherol

[b] 2:1 (w/w) ARASCO:DHASCO FFA blend

[c] PB-SL physical blend of 0.02 (w/w) ratio of 2:1 (w/w) ARASCO:DHASCO TAGs to IM-SL

[d] IE-SL synthesized with 0.02 (w/w) ratio of 2:1 (w/w) ARASCO:DHASCO TAGs to IM-SL with 5 h reaction time

[e] ACID-SL synthesized with 0.03 (w/w) ratio of 2:1 (w/w) ARASCO:DHASCO FFAs to IM-SL with 5 h reaction time

Table 3.6 Relative percent (%) of peak areas of triacylglycerol (TAG) molecular species based on equivalent carbon number (ECN)

TAG Species[a]	ECN[b] (DB)	AMF	IM-SL[c]	PB-SL[d]	IE-SL[e]	ACID-SL[f]	HMF[g] (n=45)
BMM	32 (0)	2.27 ± 0.54a	1.89 ± 0.11a	2.19 ± 0.26a	3.43 ± 0.08b	3.66 ± 0.05b	ND
BMO	34 (1)	4.08 ±0.44a	2.94 ± 0.23bc	2.71 ± 0.06c	3.64 ± 0.21a	3.46 ± 0.04ab	ND
BMP or BPO	34 (0), 36 (1)	10.66 ± 0.46a	7.91 ± 0.36b	7.67 ± 0.16b	9.70 ± 0.26c	10.10 ± 0.22ac	ND, ND
BPP	36 (0)	20.98 ± 0.37a	11.77 ± 0.10b	11.60 ± 0.23b	10.39 ± 0.34c	10.50 ± 0.094c	ND
BOS or CoPO	38 (1)	3.78 ± 0.19a	2.45 ± 0.14bc	2.65 ± 0.09b	1.99 ± 0.03c	2.28 ± 0.25bc	ND, ND
BPS or CoPP	38 (0)	7.30 ± 0.35a	3.86 ± 0.36b	3.83 ± 0.27b	2.21 ± 0.03c	2.37 ± 0.95c	ND, ND
CyPP or CoPS	40 (0)	6.24 ± 0.49a	3.96 ± 0.02b	4.02 ± 0.04b	4.86 ± 0.03b	4.42 ± 0.76b	ND, ND
LaMO or CPO	42 (1)	3.69 ± 0.18a	2.24 ± 0.08b	2.44 ± 0.08b	2.37 ± 0.11b	2.29 ± 0.28b	2.27-8.44, ND
LaMP	42 (0)	3.59 ± 0.38a	3.13 ± 0.09a	3.18 ± 0.10a	3.16 ± 0.26a	2.98 ± 0.18a	1.70-5.27
CPP	42 (0)	4.00 ± 0.03a	3.56 ± 0.05b	3.84 ± 0.22ab	3.70 ± 0.24ab	3.87 ± 0.08ab	ND
LaOO or LaPO	44 (2), 44 (1)	3.43 ± 0.10a	4.01 ± 0.29bc	3.72 ± 0.19ab	4.30 ± 0.04c	4.26 ± 0.08c	2.19-9.01, 5.80-13.52
PLO	46 (3)	2.84 ± 0.32a	1.74 ± 0.03b	1.91 ± 0.36b	1.98 ± 0.43b	2.00 ± 0.32b	12.69-21.25
MOO or MPO	46 (2), 46 (1)	4.86 ± 0.42a	6.81 ± 1.65a	5.65 ± 0.34a	5.60 ± 0.06a	5.83 ± 0.02a	ND, ND
MPP	46 (0)	3.06 ± 0.06a	6.28 ± 0.34b	6.39 ± 0.13b	6.32 ± 0.06b	6.27 ± 0.59b	0.69-2.44
OOO	48 (3)	1.48 ± 0.01a	1.52 ± 0.32a	1.77 ± 0.29a	1.70 ± 0.15a	1.44 ± 0.13a	1.14-3.75
OOP	48 (2)	3.58 ± 0.12a	3.45 ± 0.13a	3.37 ± 0.20a	3.20 ± 0.12a	3.25 ± 0.05a	13.69-28.46
PPO or PSM	48 (1), 48 (0)	5.58 ± 0.22a	9.59 ± 0.46b	9.57 ± 0.76b	9.63 ± 0.31b	9.72 ± 0.32b	4.79-8.27, ND
PPP	48 (0)	3.50 ± 0.36a	13.68 ± 0.59b	13.75 ± 0.54b	13.22 ± 0.39b	12.83 ± 0.02b	0.19-0.91
OOS	50 (2)	0.76 ± 0.10a	0.62 ± 0.09a	0.60 ± 0.08a	0.63 ± 0.03a	0.67 ± 0.09a	0.21-0.89
OPS or PPS	50 (1), 50 (0)	2.46 ± 0.12a	2.66 ± 0.37a	2.58 ± 0.03a	2.88 ± 0.12a	2.78 ± 0.10a	1.65-3.08, 0.05-0.24
OSS	52 (1)	1.52 ± 0.11a	4.56 ± 0.41b	4.82 ± 0.20b	4.81 ± 0.29b	4.72 ± 0.06b	ND

Values are mean ± SD (n=2)

Different letters in each row show significant difference ($p \leq 0.05$)

ND-not detected

B, butyric acid (C4:0); Co, caproic acid (C6:0); Cy, caprylic acid (C8:0); C, capric acid (C10:0); La, lauric acid (C12:0); M, myristic acid; (C14:0) P, palmitic acid (C16:0); S, stearic acid (C18:0); O, oleic acid (C18:1n9); L, linoleic acid (C18:2n6)

[a] TAG Species do not reflect stereochemical configuration

[b] Equivalent carbon number (ECN)=TC-(2 x DB); TC is total carbon number of acyl group and DB is total number of double bonds in TAG shown in parenthesis

[c] IM-SL synthesized with 0.2 (w/w) ratio of tripalmitin to AMF with 5 h reaction time

[d] PB-SL physical blend of 0.02 (w/w) ratio of 2:1 (w/w) ARASCO:DHASCO TAGs to IM-SL

[e] IE-SL synthesized with 0.02 (w/w) ratio of 2:1 (w/w) ARASCO:DHASCO TAGs to IM-SL with 5 h reaction time

[f] ACID-SL synthesized with 0.03 (w/w) ratio of 2:1 (w/w) ARASCO:DHASCO FFAs to IM-SL with 5 h reaction time

[g] Data obtained from Zou et al. (2012)

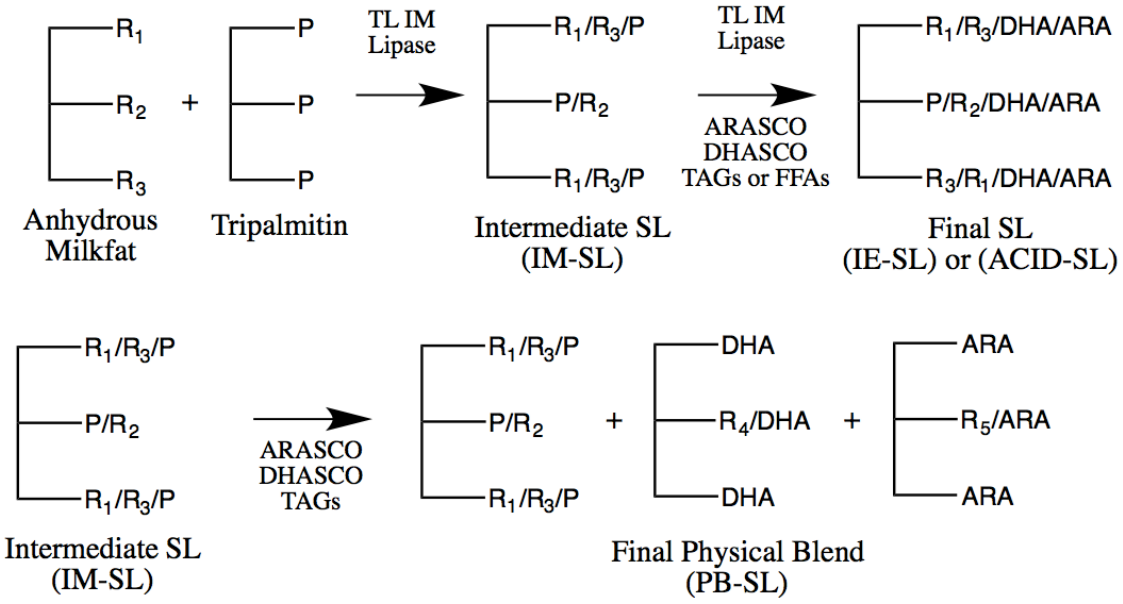


Figure 3.1 Reaction scheme for SL synthesis. IM-SL synthesized by enzymatic interesterification of AMF and tripalmitin by using a *sn*-1,3 specific lipase, Lipozyme TL IM®. Final products synthesized by three methods. The IE-SL synthesized with purified IM-SL and ARASCO® and DHASCO® TAGs using Lipozyme TL IM®. The ACID-SL synthesized with purified IM-SL and ARASCO® and DHASCO® FFAs using Lipozyme TL IM®. The PB-SL produced by blending purified IM-SL with ARASCO® and DHASCO® TAGs. P, palmitic acid (C16:0); ARA, arachidonic acid (C20:4n6); DHA, docosahexaenoic acid (C22:6n3); R_1, R_2, R_3 are other fatty acids in AMF. R_4 and R_5 represent other fatty acids in DHASCO® and ARASCO® oils, respectively.

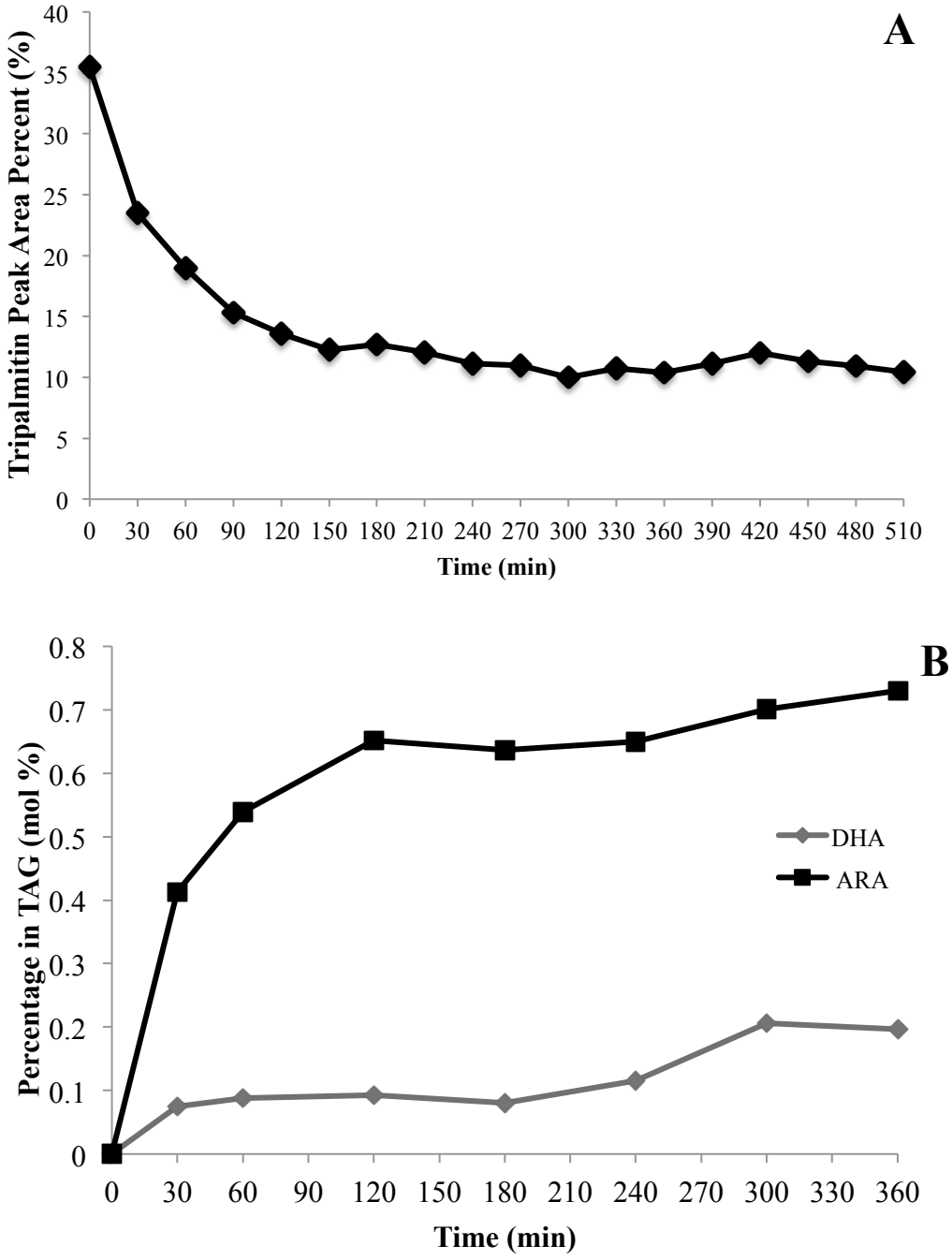
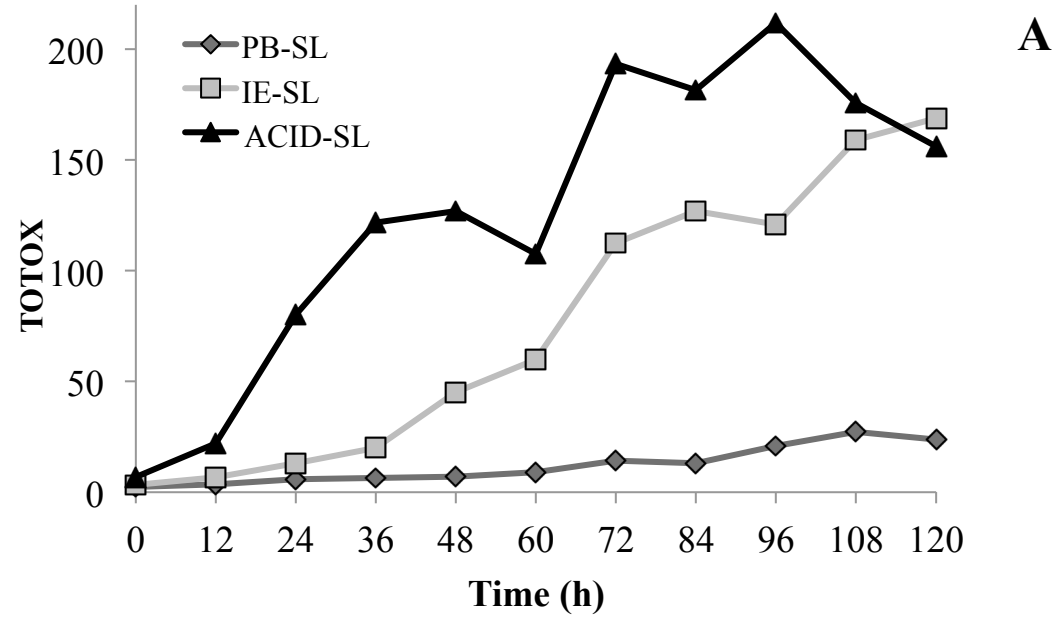
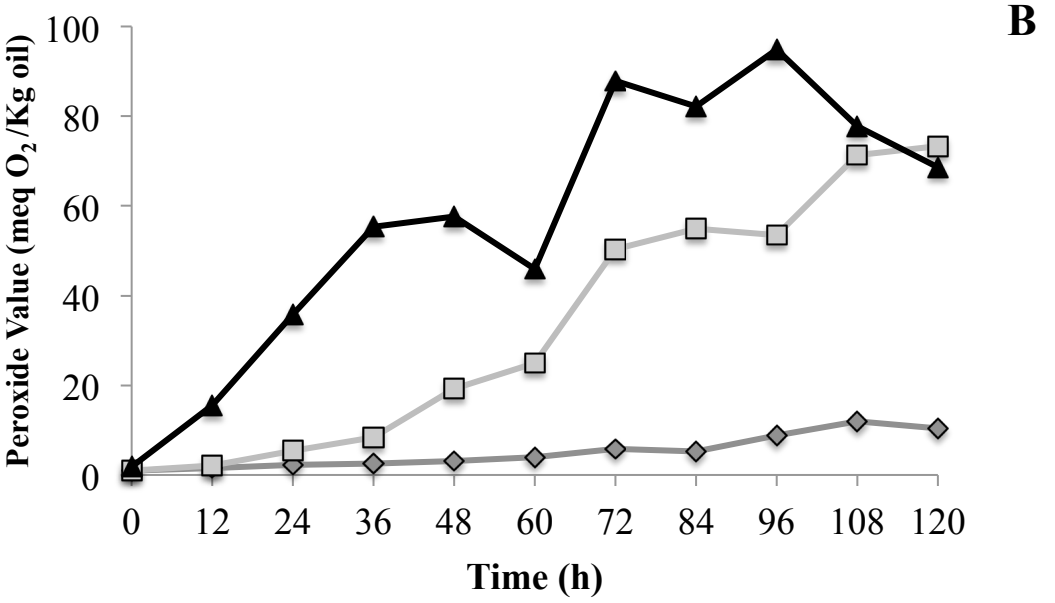


Figure 3.2 (A) Relative peak area percent of tripalmitin versus reaction time of an interesterification reaction of 0.2 (w/w) ratio of tripalmitin to AMF monitored by RP-HPLC. **(B)** Incorporation of DHA and ARA in the acidolysis reaction of 0.03 (w/w) ratio of 2:1 ARASCO:DHASCO FFAs to IM-SL over time.


A
PB-SL
IE-SL
ACID-SL
TOTOX
200
150
100
50
0
0
12
24
36
48
60
72
84
96
108
120
Time (h)


B
Peroxide Value (meq O_2/Kg oil)
100
80
60
40
20
0
0
12
24
36
48
60
72
84
96
108
120
Time (h)

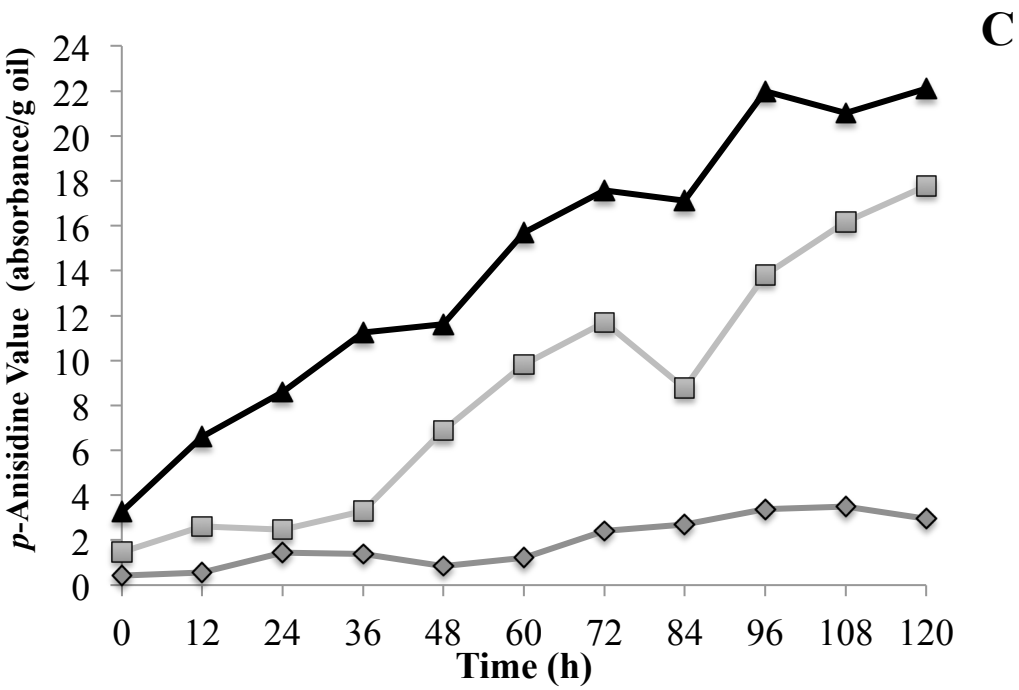


Figure 3.3 Oxidative stability measured as TOTOX (**A**), PV (**B**), and *p*-AnV (**C**) of the PB-SL, IE-SL, and ACID-SL products under accelerated conditions at 65 °C for 120 h. TOTOX was calculated as 2(PV) + (*p*-AnV). PB-SL produced by blending a 0.02 (w/w) ratio of 2:1 (w/w) ARASCO:DHASCO TAGs to IM-SL. IE-SL synthesized with 0.02 (w/w) ratio of 2:1 (w/w) ARASCO:DHASCO TAGs to IM-SL with 5 h reaction time. ACID-SL synthesized by 0.03 (w/w) ratio of 2:1 (w/w) ARASCO:DHASCO FFAs to IM-SL with 5 h reaction time.

CHAPTER 4

EFFECTS OF A GLUCOSE-CYSTEINE MAILLARD REACTION PRODUCT ON LIPID OXIDATION OF A STRUCTURED-LIPID IN A COMPLEX FOOD EMULSION[2]

[2] Sproston, M. J., & Akoh, C. C. Submitted to *The Journal of Food Science,* 06/06/2016

Abstract

A glucose-cysteine Maillard reaction product (MRP) was produced and its antioxidant effects on lipid oxidation were determined for a structured-lipid enriched with polyunsaturated fatty acids in a complex emulsion. Trolox equivalent antioxidant capacities (TEAC) were determined for MRP heating intervals of 2, 4, and 6 h and were compared to *α*-tocopherol (TOC), MRP with TOC (TOC-MRP), and TOC with ascorbyl palmitate (TOC-AP). Emulsions were produced with total antioxidant additions of 0.02% of the oil, and lipid oxidation was monitored by peroxide and *p*-anisidine values over 56 days. Positive correlations between browning and heating time as well as TEAC were observed. Total TEAC values for the MRP at 6h, TOC, TOC with the MRP at 6h, and TOC-AP were 2.51, 3.87, 2.68, and 2.76 mg trolox eq/g, respectively. Oxidation results indicated a possible antioxidant effect for the MRP at 6h on secondary oxidation for days 14-28. These results suggest that the MRP at 6h could be useful in inhibiting secondary oxidation in complex emulsions.

Introduction

The oxidation of lipids can be a major concern in food emulsion products especially because of the increased use of polyunsaturated fatty acids and mineral supplements to increase nutritional function and to benefit health. These can lead to an increased rate of oxidation due to the degree of lipid unsaturation and mineral prooxidant qualities. Oxidation can occur in products during processing, heat treatments, and storage, and can lead to rancidity, deterioration, and quality loss in food products (Karpińska, Borowski, & Danowska-Oziewicz, 2001). More specifically, structured lipids tend to have lower oxidative stability compared to the original oil source due to the processing steps

involved that contribute to antioxidant loss. The lipids in oil-in-water (O/W) emulsions also have lower oxidative stability compared to the bulk oil due to the larger surface area for contact with hydrophilic prooxidants (Martin, Reglero, & Señoráns, 2010). A way to improve the stability is to add antioxidants into the food product, but this usually involves the addition of synthetic antioxidants. Over the years, the demand for natural antioxidants has increased due to consumer preference. An example of a natural antioxidant includes the products from the Maillard reaction. The Maillard reaction is a natural non-enzymatic browning process that occurs in food during thermal processing and involves the reaction between a reducing sugar, such as glucose or fructose, and the amino group from amino acids, peptides, or proteins to form brown melanoidins (Hwang, Kim, Woo, Lee, & Jeong, 2011). Many studies have found that Maillard reaction products (MRPs) have antioxidant capacity with mechanisms that include scavenging of reactive oxygen species and metal chelation (Maillard, Billaud, Chow, Ordonaud, & Nicolas, 2007; Morales & Babbel, 2002). A preivous study found that a gelatin and gum arabic MRP had a higher antioxidant activity compared to gelatin and gum arabic alone and a mixture of gelatin and gum arabic. This MRP was used to encapsulate a polyunsaturated oil and resulted in a lower TOTOX value in comparision to a control over 28 days at 4 °C (Ifeduba & Akoh, 2015). Nagachinta and Akoh (2013) used a MRP produced from corn syrup solids and whey protein isolates as an encapsulant for a structured lipid human milkfat analogue that had low PVs and thiobarbituric acid reactive substances (TBARs). While there are many amino acid and sugar combinations possible to obtain a natural antioxidant, studies have shown that a glucose-cysteine MRP was effective in scavenging lipophilic free radicals and had chelating ability (Maillard, Billaud, Chow, Ordonaud, & Nicolas, 2007). Another

study found that the glucose-cysteine MRP had the highest scavenging activity for the DPPH and ABTS radicals in comparison to all of the amino acids formed from glucose and fructose (Hwang, Kim, Woo, Lee, & Jeong, 2011). While the antioxidant capacities have been studied, experiments involving the effect of a glucose-cysteine MRP in complex O/W food emulsions are scarce. Also, studies involving the oxidative stability of food products containing structured lipids are few (Martin, Reglero, & Señoráns, 2010).

The objective of this study was to determine the antioxidant effects of a glucose cysteine-MRP on lipid oxidation of a structured lipid in a complex O/W food emulsion and compare the effects to other common food antioxidants including α-tocopherol (TOC) and ascorbyl palmitate (AP). This study wanted to determine if there was a synergistic effect between the MRP and TOC and also to compare the antioxidant effect to combinations of TOC and AP, which has been reported to show a synergistic effect (Decker, 2008). Also, correlations between extent of browning and heating time as well as antioxidant capacity for the MRP were studied. An infant formula emulsion was used as the food matrix. A structured lipid enriched with *sn*-2 palmitic acid and polyunsaturated fatty acids specifically produced for infant nutrition was used to prepare the infant formula that contained vitamins and minerals required in the formulation.

Materials and Methods

Materials

Anhydrous milkfat (AMF) was kindly donated by the Dairy Farmers of America (Winthrop, MN), and tripalmitin was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). ARASCO® (40% arachidonic acid, ARA) and DHASCO® (40% docosahexaenoic acid, DHA) single cell oils were purchased from DSM Nutritional

Products (Columbia, MD). The immobilized lipase, Lipozyme TL IM® (*sn*-1,3 specific *Thermomyces lanuginosus* lipase), was obtained from Novozymes North America, Inc. (Franklinton, NC). The structured lipid used was previously synthesized as described by Sproston and Akoh (2015). L-Cysteine was purchased from AMRESCO (Solon, Ohio) and anhydrous dextrose was purchased from VWR International (Randor, PA). Trolox and (+/-)-*α*-tocopherol were purchased from Arcos Organics (Geel, Belgium). Ascorbyl palmitate, horseradish peroxidase (HRP), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), and 3-(2-pyridyl)-5-6-diphenyl-1-triazine-4',4"-disulfonic acid salt (Ferrozine) were purchased from Sigma Aldrich Chemical Company (St. Louis, MO). 5-(Hydroxymethyl)furfural (5-HMF) was purchased from Oakwood Chemical (N. Estill, SC). High heat nonfat dry milk was kindly provided by O-AT-KA Milk Products Cooperative, Inc. (Batavia, NY), and SI-100 carrageenan and locust bean gum were generously provided by Ingredients Solutions, Inc. (Waldo, ME). Hilmar Ingredients (Hilmar, CA) kindly donated α-lactalbumin enriched whey protein concentrate and 5120 IF lactose. A micronutrient premix mainly consisting of vitamins, minerals, and nucleotides was formulated by Fortitech, Inc. (Schenectady, NY). Liquid lecithin was purchased from Modern Products Inc. (Mequon, WI). Dimodan® Distilled monoacylglycerol (MAG) was a product of Danisco USA Inc. (New Century, KS). Other chemicals and solvents were purchased from Fisher Scientific (Norcross, GA), Sigma Aldrich Chemical Company (St. Louis, MO), or J. T. Baker Chemicals (Center Valley, PA).

Production of MRP and Extent of Browning

In order to prepare the dry blended MRP, a solution containing 1.0 M L-cysteine and 1.0

M anhydrous dextrose (glucose) was made in deionized water. The solution was stirred and then freeze-dried with a Unitop 600L, Freezemobile 25SL unit (The Virtis Company, Gardiner, NY). The solution was prefrozen at -40 °C and dried for 48 h at 13 Pa to a final temperature of 25 °C. The freeze dried samples were pulverized using a Kitchen Aid Coffee Grinder (Kitchen Aid, St. Joseph, MI). This was done to allow thorough mixing of the substrates. Then, the sample was incubated at 85 °C for 2, 4, and 6 h under 77% relative humidity in a closed desiccator containing saturated potassium iodide solution at the bottom. This yielded the products referred to as MRP-2h, MRP-4h, and MRP-6h, respectively. The extent of browning was measured by dissolving MRP (3 mg/mL) in water and measuring the absorbance using a UV-1601 UV–visible spectrophotometer (Shimadzu Corp., Columbia, MD) at 294 nm to determine the formation of intermediate products and at 420 nm to determine the extent of browning (Wu, Hu, Wei, Du, Shi, & Zhang, 2014).

Determination of 5-(Hydroxymethyl)furfural (5-HMF)

The concentration of HMF in the samples was determined using RP-HPLC-DAD (Agilent Technologies 1260 Infinity, Santa Clara, CA) using a modified version of the method described by Truzzi, Annibaldi, Illuminati, Finale, Rossetti, and Scarponi (2012) with an Ultrasphere® C18 5 µm x 4 mm x 250 mm, 5 µm nominal particle size column. The column temperature was held at 30 °C, and the samples were analyzed at 285 nm with a 10 µL injection volume. The mobile phase was an isocratic mixture of 10% (v/v) methanol in ultra pure water with a flow rate of 2 mL/min. MRP samples were dissolved in mobile phase to achieve a 1 mg/mL solution and were vortexed for 2 min. Then, samples were centrifuged for 5 min at 2000 RPM (670.8 g) and filtered through a waters

HA 0.45 μm pore size membrane before being injected into the RP-HPLC. The concentration of HMF in the samples was determined by comparing the HMF peak area to an external standard curve and values were reported as μg HMF per mg of MRP sample.

Determination of Antioxidant Capacity

Total antioxidant capacity was determined by the ABTS/H_2O_2/HRP decolorization method as described by Arnao, Cano, and Acosta (2001). The hydrophilic and lipophilic contributions of the MRP products (MRP-2h, MRP-4h, MRP-6h), and equal combinations of each MRP and TOC (TOC-MRP) as well as TOC and AP (TOC-AP) were used to calculate the total antioxidant capacity. Briefly, the MRP products were first dissolved in 50 mM sodium phosphate buffer (pH=7.5) and individual solutions of α-tocopherol and ascorbyl palmitate were made by dissolving in ethyl acetate to achieve a 1 mg/mL solution. Then, aliquots of the solutions were pipetted into separate test tubes to achieve a final concentration of 2 mg total sample in 5 mL buffer and 5 ml ethyl acetate. Samples were vortexed for 2 min and then centrifuged for 5 min at 1000 RPM (167.7 g). The top layer was separated from the bottom layer representing the lipophilic and hydrophilic extracts, respectively. The hydrophilic assay solution contained 2 mM ABTS, 30 μM H_2O_2, and 0.25 μM HRP in 50 mM sodium phosphate buffer (pH=7.5) in a total volume of 3 mL. The lipophilic assay solution contained 1 mM ABTS, 30 μM H_2O_2, and 0.15 μM HRP in pure ethanol for a total volume of 3 mL. Once the colored reaction mixtures gave a stable absorbance value at 730 nm, 10 μL of antioxidant extract solution were added. For the blanks, 10 μL of buffer or ethyl acetate were added to the test tubes containing assay solution. The absorbance values of the samples were determined at 730

nm after 10 min incubation at 25 °C. The loss of color was determined by the difference in absorbance between the blank and the sample. The change in absorbance represents the amount of ABTS radical cation scavenged by the antioxidant samples tested. The antioxidant capacity was expressed as mg trolox equivalent per 1 g dry weight of sample. The calculation of trolox equivalent was determined using a dose response curve of loss of absorbance from varying amounts (0-30 nmol) of trolox. A synergistic effect between the antioxidants was found if equation 1 was true.

$$TEAC_{TOC-MRP} > (0.5 * TEAC_{TOC}) + (0.5 * TEAC_{MRP}) \quad (1)$$

Where $TEAC_{TOC\text{-}MRP}$ represents the TEAC of the TOC-MRP combination and $TEAC_{TOC}$ and $TEAC_{MRP}$ represent the individual TEAC of TOC and MRP, respectively. The 0.5 represents the equal contribution of each individual antioxidant.

Emulsion Formation

The interesterified structured lipid used in emulsion formulation containing anhydrous milkfat, ARA, and DHA was synthesized previously (Sproston and Akoh 2015). This structured lipid contained oleic acid, linoleic acid, ARA, and DHA with 17.92, 2.60, 0.63, and 0.50 mol%, respectively. It also contained palmitic, myristic, stearic, lauric, and capric acids with 43.14, 9.07, 8.23, 2.79, 2.31 mol%, respectively. Emulsions were produced using a modified version of Zou and Akoh (2013). The MRP-6h was first dissolved in water, and then was added to the polar phase. Emulsions contained total antioxidant additions of 0.02% of the oil with MRP-6h, TOC, TOC with MRP-6h (TOC-MRP-6h), and TOC with AP (TOC-AP) and were compared to a control containing no added antioxidant. Modifications to the procedure include the absence of sodium azide in the formulation as well as a modified sterilization procedure. Briefly, after emulsion

formation, 50 mL aliquots of sample were placed in glass bottles for each sampling day. All bottles were autoclaved during the same treatment at 110 °C for 15 min. Sterility was ensured by the absence of growth of colonies on aerobic plates after being incubated at 35 °C for 48 h. After sterilization, the bottles were stored in the dark at 25 °C for 56 days.

Particle Size Distribution

The particle sizes of the emulsion samples were analyzed using an LS 13, 320 MW particle size analyzer (Beckman Coulter, Inc. Miami, FL) to ensure each emulsion sample had comparable particle sizes. Samples were added dropwise to deionized water in the dispersion cell until an obscuration value of 8-10% was achieved. Particle size distribution was analyzed with low sonication and the mean particle sizes and standard deviations were reported. The particle sizes were also determined on day 56 to ensure that emulsion samples had comparable particles sizes after storage.

Extraction of Oil from Emulsion Samples and Oxidation Analysis

Oil was extracted following a modified version of the IDF method 172:1995 (Federation, 1995). Modifications were as described by (Ifeduba & Akoh, 2015). Thirty milliliters of emulsion samples were used as the initial volume for extraction. Immediately after extraction, the peroxide values (PV) and *p*-anisidine values (*p*-AnV) were determined in triplicate following the IDF method (Shantha & Decker, 1994) and the AOCS Official Method Cd18-90 (AOCS, 2011), respectively. Oil was extracted every 7 days until day 28, and then extracted every 14 days until day 56. Induction time was determined by the intersection of tangent lines near the curve. The tangents lines were determined by creating linear models at points near the curve. An antioxidant effect was defined as if the PV or *p*-AnV of a sample was significantly lower than the control for the same sampling

day using statistics as described below. A synergistic effect was defined using an equation similar to equation 1. However, the greater than sign was changed to the less than sign since the PV and *p*-AnV would be lower for the combinations compared to the individual contributions if a synergistic effect was observed. This is shown in equation 2.

$$PV\ or\ pAnV_{TOC-MRP} < (0.5 * PV\ or\ pAnV_{TOC}) + (0.5 * PV\ or\ pAnV_{MRP}) \quad (2)$$

Determination of Metal Chelation %

The ferrous iron chelating ability of the antioxidants was determined by the method described by Dinis, Madeira, and Almeida, (1994). Briefly, stock antioxidant solutions were prepared in deionized water for MRP and ethanol for TOC and AP. Then, appropriate equal combinations were made, and the total antioxidant concentrations were adjusted to 200 µg/mL (0.02%). One milliliter of antioxidant solution was added to a test tube followed by 0.1 mL of 2 mM iron(II) chloride solution. Samples were vortexed, and 0.2 mL of 5 mM ferrozine solution was added to develop a pink ferrozine iron(II) complex. Blank solutions were prepared containing the iron(II) chloride solution, ferrozine solution, and either deionized water or ethanol depending on the antioxidant tested. Absorbance of the solutions were analyzed at 562 nm and chelation percent was determined with equation 3 where A_c and A_s represent the control and sample absorbance, respectively.

$$Chelation\ Percent = \left(\frac{A_c - A_s}{A_c}\right)\ x\ 100 \quad (3)$$

Statistical Analysis

All samples were performed in triplicate or duplicate with results expressed as mean ± SD. All statistical analyses were conducted with Microsoft Excel using the DSAASTAT

version 1.101 macro (DSAA, Perugia, Italy). Duncan's Multiple Range test was performed to determine significance of difference at $p \leq 0.05$.

Results and Discussion

Production of MRP and Determination of Browning

Over time, the heated mixture of D-glucose and L-cysteine powder had a color change from white to yellow to brown. The extent of browning of a 3 mg/mL solution was measured using a UV–visible spectrophotometer at wavelengths of 294 nm and 420 nm to determine the increase in both intermediate and final products, respectively, with increased heating time. The intermediate products represent low molecular weight compounds while the final products represent the colored compounds from the final stages of the Maillard (Ruiz-Roca, Navarro, & Seiquer, 2008). **Figure 4.1A** shows that as heating time increased, the absorbance at 294 and 420 nm both increased continuously showing a positive curvilinear correlation with r-values of 0.998 and 0.997, respectively. This indicates that as heating time increased, the extent of browning and intermediate product formation increased for the glucose-cysteine model. This is consistent with Ruiz-Roca, Navarro, and Seiquer (2008) where a positive correlation between browning and heating time was also observed, but for a glucose-lysine system. The figure also indicates that there was a greater production of intermediate products compared to the colored melanoidin products possibly due to the relatively short heating time that was not sufficient for the colored products of the Maillard reaction to form. It is important to note that the color change could be due to the formation of MRPs as well as the caramelization of glucose. However, studies have shown that the browning observed from sugar and amino acid mixtures is mainly due to the Maillard reaction and only small amounts of

browning are observed from caramelization of glucose at 100 °C (Morales & Jiménez-Pérez, 2001).

Antioxidant Capacity

Correlation between extent of browning and TEAC

The TEAC assay was chosen in order to determine the hydrophilic, hydrophobic, and total antioxidant capacities of the MRPs and combinations of MRP and TOC. There was a positive correlation observed for extent of browning for the intermediate and browned MRPs and total TEAC values with r-values of 0.988 and 0.986, respectively (see **Figure 4.1B**). This indicates that as browning increased, the total antioxidant capacity also increased for the time intervals tested. Phisut and Jiraporn (2013) also found that as browning intensity increased, DPPH radical scavenging activity, ferric reducing antioxidant power, hydroxyl scavenging activity, and inhibition of lipid peroxidation also increased.

TEAC of antioxidants

The antioxidant capacity for the hydrophilic extract shown in **Table 4.1** indicates the MRP-6h had significantly higher antioxidant capacity (1.05 ± 0.06 mg trolox eq/g) compared to all hydrophilic samples followed by TOC-MRP-6h (0.53 ± 0.02 mg trolox eq/g), TOC-MRP-2h (0.39 ± 0.01 mg trolox eq/g), and MRP-2h (0.25 ± 0.01 mg trolox eq/g). The hydrophilic TEAC value of TOC-MRP-4h was 0.09 ± 0.01 mg trolox eq/g. The MRP-4h had a prooxidant effect with -0.11 ± 0.05 mg trolox eq/g. This data also indicates the MRP-6h had a significantly higher hydrophilic TEAC compared to all MRP samples. This could be due to the greater concentration of final products formed due to an increased heating time (Phisut & Jiraporn, 2013). However, it is unknown why the MRP-

4h showed a prooxidant effect compared to the other heating times. There was no synergistic effect observed between these MRPs and TOC combinations, and this was expected due to TOC being hydrophobic. Previous studies have either found a synergistic effect between MRPs and α-tocopherol or no effect at all (Chiu, Tanaka, Nagashima, & Taguchi, 1991; Wijewickreme & Kitts, 1998). This could be due to the different products formed depending on the heating time and the substrates used to produce the MRPs.

The hydrophobic antioxidant capacity was also determined for the antioxidant samples, and they are shown in **Table 4.1**. The hydrophobic antioxidant capacity of TOC was significantly higher compared to all the samples with 3.87 ± 0.06 mg trolox eq/g. This was followed by TOC-AP (2.76 ± 0.08 mg trolox eq/g), TOC-MRP-6h (2.16 ± 0.13 mg trolox eq/g), TOC-MRP-2h (2.08 ± 0.10 mg trolox eq/g), MRP-4h (1.78 ± 0.01 mg trolox eq/g), TOC- MRP-4h (1.72 ± 0.09 mg trolox eq/g), MRP-6h (1.45 ± 0.07 mg trolox eq/g), and MRP-2h (0.79 ± 0.07 mg trolox eq/g). The MRP-2h had significantly lower antioxidant capacity compared to all hydrophobic extracts, and this is possibly due to the shorter reaction time and slow development of browning compounds. However, the MRP samples had higher hydrophobic TEAC values indicating that the products formed were more hydrophobic. This is consistent with a previous study that found that the majority of low molecular weight intermediate products formed from the Maillard reaction had a nonpolar behavior (Bekedam, Roos, Schols, Van Boekel, & Smit, 2008). Also, since cysteine can have hydrophobic character, the intermediate products and final products could be more nonpolar (Nursten, 2005). However, high molecular weight melanoidins tend to have polar character (Bekedam, Schols, Van Boekel, & Smit, 2006). This could help explain why there was a significant decrease in the hydrophobic TEAC

values between the MRP-4h and MRP-6h but a significant increase in hydrophilic TEAC. This could be because hydrophilic antioxidant compounds were being formed due to increased reaction time.

A total antioxidant capacity of the samples was determined by the addition of both TEAC values for the hydrophilic and hydrophobic extracts, and they are shown in **Table 4.1**. The highest total antioxidant capacity was observed for TOC (3.87 ± 0.06). This was followed by TOC-AP (2.76 ± 0.08) and TOC-MRP-6h (2.68 ± 0.13 mg trolox eq/g) which were not significantly different. The TOC-MRP-6h had a significantly higher total TEAC value compared to the MRP-6h (2.51 ± 0.10 mg trolox eq/g), but there was no synergistic effect observed between TOC and MRP-6h for the hydrophobic and total TEAC. The combination of TOC and MRP-2h was the only sample to show a slight synergistic effect for the hydrophobic and total TEAC. The TOC-MRP-4h (1.81 ± 0.09 mg trolox eq/g) was significantly lower compared to all TOC-MRP and TOC-AP samples, and this could be due to MRP-4h having a prooxidant effect in a hydrophilic environment. The total TEAC value of MRP-2h of 1.04 ± 0.07 mg trolox eq/g was significantly lower compared to all samples. This is possibly due to the short reaction time and slow development of browning compounds as mentioned previously. Because the total antioxidant capacity was the highest for the MRP-6h and TOC-MRP-6h, as a function of time, compared to the other MRP products and TOC-MRP combinations, these were studied in the emulsion oxidation experiment to compare with the control, TOC, and TOC-AP.

5-(Hydroxymethyl)furfural (5-HMF) Determination

The formation of 5-HMF with heating time of the glucose-cysteine model was also

studied. HMF was not observed in the initial unheated powder but was observed in the MRP-2h, MRP-4h, and MRP-6h samples in the amounts of 2.30 ± 0.03, 2.64 ± 0.03, and 2.35 ± 0.02 μg/mg MRP, respectively, and were all significantly different from each other. This indicates that a reaction occurred in which HMF was formed. The decrease in HMF from the MRP-4h to MRP-6h could be explained by that fact that HMF is an intermediate compound that gets used up when the final colored products form during the Maillard reaction (Mastrocola & Munari, 2000). Since the MRP-6h contained more colored compounds as described previously (see **Figure 4.1**) this would be likely.

Particle Size Analysis of Emulsions

The initial and final (day 56) particle size of each emulsion was determined to ensure uniform droplet sizes to prevent oxidation because of the differences in particle size and surface area. Nakaya, Ushio, Matsukawa, Shimizu, and Ohshima (2005) found that larger droplet sizes tend to increase the rate of oxidation compared to smaller droplet sizes in O/W emulsions. The results of the initial and final droplet sizes are shown in **Table 4.2**. The initial droplet sizes of the control, MRP-6h, TOC, TOC-MRP-6h, and TOC-AP emulsions were 1.12 ± 0.56, 1.49 ± 1.11, 1.63 ± 1.14, 1.69 ± 1.15, and 1.56 ± 1.23 μm, respectively, and the initial droplet sizes were similar to each other. After 56 days of storage, the droplet sizes were statistically similar with 1.26 ± 0.73, 1.60 ± 1.17, 1.71 ± 1.25, 1.78 ± 1.22, and 1.76 ± 1.20 for the control, MRP-6h, TOC, TOC-MRP-6h, and TOC-AP, respectively. These values show that the droplet sizes remained similar throughout storage, and it was unlikely that oxidation from differing droplet sizes occurred.

Effect of antioxidants on oxidation in a complex food emulsion

Primary Oxidation

Antioxidants can be classified into two broad categories known as primary and secondary antioxidants based on their mechanisms of action. Primary antioxidants include antioxidants that act as free radical acceptors and hydrogen atom donators that inhibit or delay the initiation or propagation steps of lipid oxidation (Decker, 2008). α-Tocopherol is a common lipophilic primary antioxidant that donates a phenolic hydrogen atom to free radicals and prevents further oxidation from occurring because the tocopherol radical is stabilized by resonance (Decker, 2008). Propagation in lipid oxidation also involves the induction period in which hydroperoxides have not formed and antioxidants are being consumed. By measuring the induction period before hydroperoxides form, the effectiveness of added antioxidants in a food system can be determined (Shahidi & Wanasundara, 2002). Secondary antioxidants have mechanisms that involve chelating prooxidant metals, replenishing hydrogen atoms to primary antioxidants, and quenching of singlet oxygen (Decker, 2008). Ascorbyl palmitate is an example of a secondary antioxidant with multifunctional properties including free radical inhibition, hydrogen atom donation, and metal chelation. It has also been found to replenish hydrogen atoms to tocopherols causing synergistic properties between the two antioxidants (Decker, 2008). The influence of the antioxidants on primary oxidation was monitored by peroxide value (PV) of the extracted oil over time. The PV is a measure of the hydroperoxides, and this value increases and then decreases due to the decay of the hydroperoxides into secondary oxidation products (Frankel, 1996). **Figure 4.2A** shows the PV over time of the oil extracted from the O/W emulsions. The control, which contained no added antioxidant,

remained relatively stable over time with a slight increase in PV from days 7 to 21 following a decrease that is most likely due to primary oxidation products reacting to form secondary oxidation products. The PV for the MRP-6h was statistically higher compared to the control from days 7 to 14, but it was statistically lower from days 21 to 28. After day 28, the control and MRP-6h were statistically similar, and there were no distinct induction points of oxidation for both. This indicates that the MRP-6h could have either no effect or a slight observed antioxidant effect for primary oxidation. Since the MRP-6h did show a slight antioxidant effect from days 21 to 28, it could be possible that the MRP had free radical scavenging activity for lipophilic radicals, and this could be explained by the hydrophobic TEAC results mentioned previously. The MRP having a slight antioxidant effect would be consistent with Maillard, Billaud, Chow, Ordonaud, and Nicolas (2007) where a glucose-cysteine MRP was effective in scavenging lipophilic free radicals. Studies have shown that the free radical scavenging activities of the glucose-cysteine MRPs are attributed to the sulfhydryl group of cysteine due to hydrogen atom donation (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005; Maillard, Billaud, Chow, Ordonaud, & Nicolas, 2007). Elias, McClements, and Decker (2005) also found that cysteine residues from β-lactoglobulin oxidized before lipid oxidation was detected indicating antioxidant behavior. However, Maillard, Billaud, Chow, Ordonaud, & Nicolas (2007) found that the sulfhydryl group of cysteine was less involved in scavenging lipophilic free radicals because the heated MRP was more efficient than the physical unheated mixture of glucose and cysteine. In comparison, TOC, TOC-MRP-6h, and TOC-AP all had distinct induction times of 21.6, 41.8, and 41.6 days, respectively (see **Figure 4.3**). This indicates that primary oxidation did occur in these samples over

time. The lower induction time for TOC could be due to α-tocopherol having a possible prooxidant effect at the concentration tested. This prooxidant effect could be because high concentrations of the tocopheroxyl radicals could initiate chain reactions or increase the rate of oxidation in combination with other prooxidants in the food matrix including transition metal ions, hydroperoxides, and reactive oxygen species (Kim & Min, 2008). This was also observed in previous studies in which α-tocopherol at a concentration of 0.02% of the oil showed a prooxidant effect in an O/W emulsion (Osborn-Barnes & Akoh, 2003; Zou & Akoh, 2015). These studies concluded that the antioxidative or prooxidative effect of α-tocopherol depended on concentration, test system, and oxidation time. This could explain why the high TEAC value for TOC did not correlate with a low induction time and PVs of the oil. The increase in PV over time for TOC-MRP-6h and TOC-AP is most likely due to the addition of TOC in the oil because the MRP-6h showed no point of induction. The lower PV of TOC-MRP-6h and TOC-AP compared to TOC could be because TOC was added at a lower concentration for the combinations (0.01%).

Secondary Oxidation

Secondary oxidation was monitored by determining the *p*-AnV of the extracted oil over time, and the results are shown in **Figure 4.2B**. The *p*-AnV is a measure of secondary oxidation products, which are mainly comprised of aldehydes that are formed from the deterioration of the hydroperoxides (Frankel, 1996). The *p*-AnV of the control oil sample increased over time after day 7 showing that secondary oxidation was occurring. The control also had an induction time of 6.3 days in which secondary oxidation continuously increased (see **Figure 4.2B** and **Figure 4.3**). The MRP-6h showed an antioxidant effect, in which the *p*-AnV was statistically lower than the control, for days 14-28 and was

statistically similar to the control on days 42 and 56. This shows the MRP-6h at 0.02% of the oil was effective in reducing secondary oxidation of the structured lipid in an O/W emulsion for approximately 28 days. TOC showed an antioxidant effect from days 14 to 21 and was statistically similar to the control from days 28 to 42. The induction time of 19.8 days for TOC was lower than the MRP-6h (23.6 days) and the other antioxidant combinations tested (see **Figure 4.3**). This could be due to the higher concentration of *α*-tocopherol used as mentioned above. Also, TOC is a primary antioxidant preventing primary oxidation. Therefore, TOC is not expected to have an antioxidant effect for secondary oxidation when primary oxidation is occurring. The TOC-MRP-6h showed an antioxidant effect on days 14, 21, and 42, and the induction time for the TOC-MRP-6h was 41.7 days. While there was an increase in *p*-AnV from days 14 to 28, the antioxidant combination seemed to remain stable from days 28 to 42 followed by a large increase in *p*-AnV. Therefore, the induction time was determined for the curve closer to day 42. While the *p*-AnV for TOC-AP was similar to the control initially from days 0 to 14, there were antioxidant effects observed on days 21 and 42. The induction point for TOC-AP of 39.8 days was similar to the TOC-MRP-6h (41.7 days). The effectiveness of the MRP-6h could be due to many reasons including chelation of prooxidant metals and scavenging of oxygen (Decker, 2008). Other studies found that lipid oxidation-derived aldehydes such as hexanal can react with amino acids in a Maillard-type reaction forming browning products of low and high molecular weight that have antioxidant activities (Adams, Kitrytė, Venskutonis, & De Kimpe, 2009; Kitrytė, Adams, Venskutonis, & De Kimpe, 2012). Kitrytré, Adams, Venskutonis, and De Kimpe (2012) found that the high molecular weight reaction products resulting from a heated model system containing

hexanal, lysine, and glucose had a high radical scavenging activity and was higher than standard glycine/glucose melanoidins. This shows that the products from the interaction of lipid oxidation-derived aldehydes, glucose, and amino acids can produce compounds with antioxidant capacities. The ability for the glucose-cysteine MRP to chelate metals was further studied and will be discussed below.

Chelation Percentage of Antioxidants

MRPs can have strong chelating ability that can prevent lipid oxidation by chelating transition metal ions that initiate the oxidative chain reactions (Maillard, Billaud, Chow, Ordonaud, & Nicolas, 2007). The chelating ability depends on which sugars and amino acids were used to produce the MRP. Specifically, MRPs derived from glucose have been shown to have chelating ability with chelating sites derived from the hydroxyl groups from glucose (Yoshimura, Iijima, Watanabe, & Nakazawa, 1997). Other compounds such as AP have strong chelating properties due to hydroxyl groups. Also, thiol derived MRPs have been found to have chelation properties because of the presence of the thiol group (Maillard, Billaud, Chow, Ordonaud, & Nicolas, 2007). The chelating ability of MRP-6h, TOC, TOC-MRP-6h, and TOC-AP was determined at a concentration of 0.02% in appropriate solvents and is shown in **Figure 4.4**. The MRP-6h and TOC-AP had the highest chelation percentage of 5.75 ± 0.20 and 4.57 ± 0.51%, respectively, and they were not significantly different. This was followed by TOC-MRP-6h and TOC with 3.51 ± 0.16 and 1.93 ± 0.54%, respectively, in which TOC was significantly lower. In comparison, one study found that a 0.096% (960 μg/mL) solution of the commonly used metal chelator ethlyenediaminetetraacetic acid (EDTA) had an iron(II) chelation percent of 92.5% using the same method (Yu, Liu, Xing, Liu, Guo, Wang, et al., 2006). While

MRP-6h had one of the highest chelation percentages compared to the antioxidants tested, the percentage was still low. This could be because of the 6 h reaction time in which the free sulfhydryl group could decrease while other products were formed. This was seen in a study where the copper chelating ability of glucose-cysteine mixture was higher compared to the glucose-cysteine MRP (Maillard, Billaud, Chow, Ordonaud, & Nicolas, 2007). These results could suggest that the chelating ability of the MRP-6h was helpful in reducing secondary oxidation.

Conclusion

A glucose-cysteine MRP was produced and compared to other antioxidants at a total concentration of 0.02% of the oil to determine its effects on lipid oxidation in complex O/W food emulsions of a structured-lipid enriched with polyunsaturated fatty acids. Positive correlations were found between the extent of browning of the MRP and heating time as well as extent of browning and total TEAC values. The results showed that as heating time increased the product was darker in color and displayed higher antioxidant capacity. The effects of the antioxidants on oxidation was determined by PV, *p*-AnV, induction time, and chelation %. The MRP-6h showed little to no observed effect on primary oxidation, but the MRP-6h had an antioxidant effect for days 14-28 on secondary oxidation. The mechanism of inhibitory effect on secondary oxidation is unknown; although, chelation percentages showed the MRP-6h had similar percentages to TOC-AP and were the highest compared to the other antioxidants tested. The MRP-6h and TOC-MRP-6h could be useful in inhibiting secondary oxidation in complex O/W emulsion matrices; however, more studies are needed to determine appropriate concentrations and the mechanisms of action.

Acknowledgements

We thank the Dairy Farmers of America for providing the anhydrous milkfat. This research was supported in part by Food Science Research, University of Georgia.

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Table 4.1 Trolox equivalent antioxidant capacity (mg trolox eq/g of antioxidant) of antioxidants and antioxidant combinations.

Antioxidant	Hydrophilic Antioxidant Capacity	Hydrophobic Antioxidant Capacity	Total Antioxidant Capacity
MRP-2h	0.25 ± 0.01d	0.79 ± 0.07f	1.04 ± 0.07e
MRP-4h	-(0.11) ± 0.05f	1.78 ± 0.01d	1.67 ± 0.04d
MRP-6h	1.05 ± 0.06a	1.45 ± 0.07e	2.51 ± 0.10c
TOC	ND	3.87 ± 0.06a	3.87 ± 0.06a
TOC-MRP-2h	0.39 ± 0.01c	2.08 ± 0.10c	2.47 ± 0.10c
TOC-MRP-4h	0.09 ± 0.01e	1.72 ± 0.08d	1.81 ± 0.09d
TOC-MRP-6h	0.53 ± 0.02b	2.16 ± 0.13c	2.68 ± 0.13b
TOC-AP	ND	2.76 ± 0.08b	2.76 ± 0.08b

Different letters in each column show significant differences ($p \leq 0.05$)

Values are mean ± SD (n=3)

ND- not determined due to being lipophilic

Table 4.2 Particle size analysis of oil in water emulsions on days 0 and 56.

Emulsion	Day 0 (μm)	Day 56 (μm)
Control	1.12 ± 0.56a	1.26 ± 0.73a
MRP-6h	1.49 ± 1.11a	1.60 ± 1.17a
TOC	1.63 ± 1.14a	1.71 ± 1.25a
TOC-MRP-6h	1.69 ± 1.15a	1.78 ± 1.22a
TOC-AP	1.56 ± 1.23a	1.76 ± 1.20a

Different letters in each column show significant differences ($p \leq 0.05$)

Values are mean ± SD (n=2)

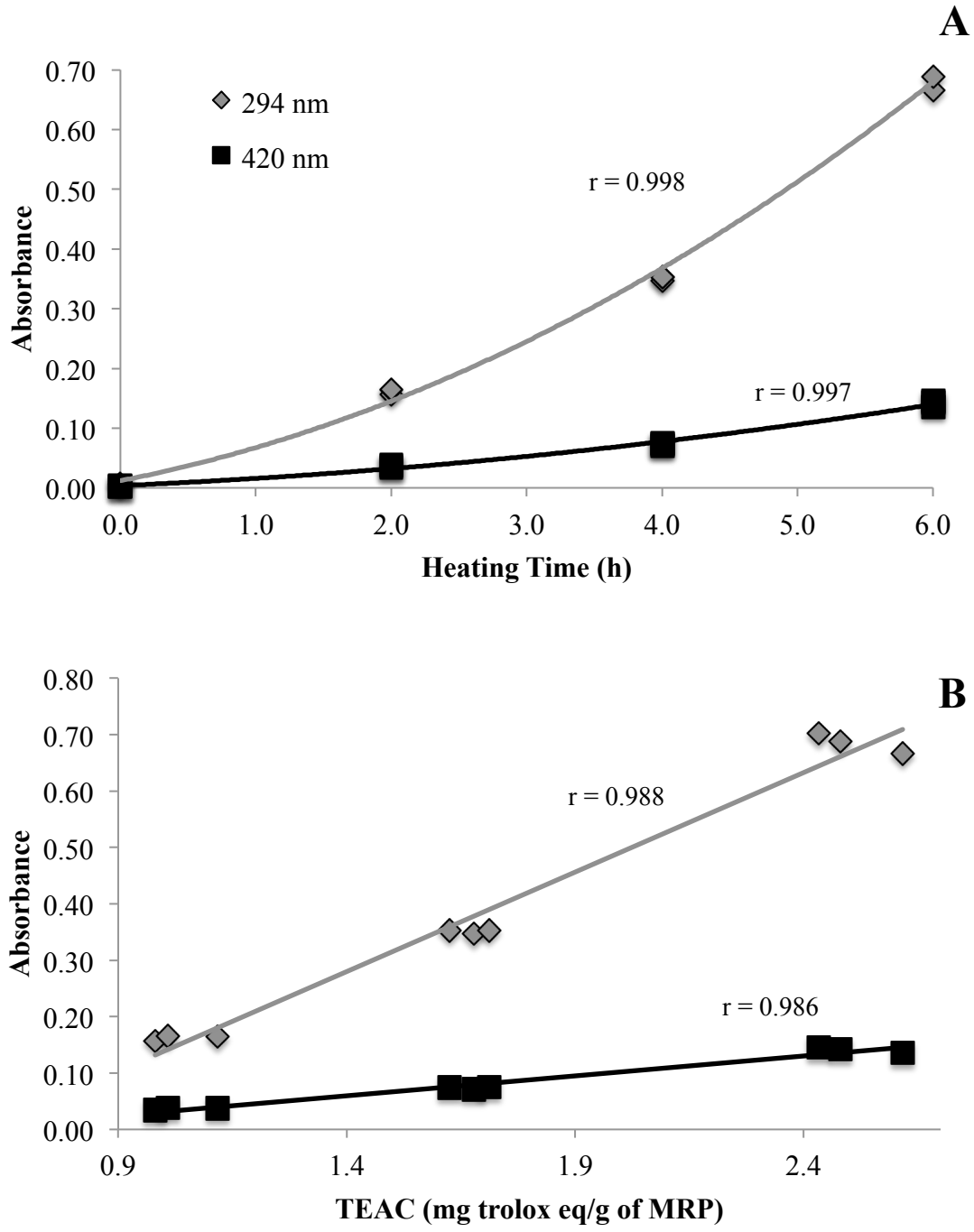

Figure 4.1 (A) The extent of browning versus heating time of the glucose-cysteine MRP; **(B)** The extent of browning versus total TEAC of the glucose-cysteine MRP.

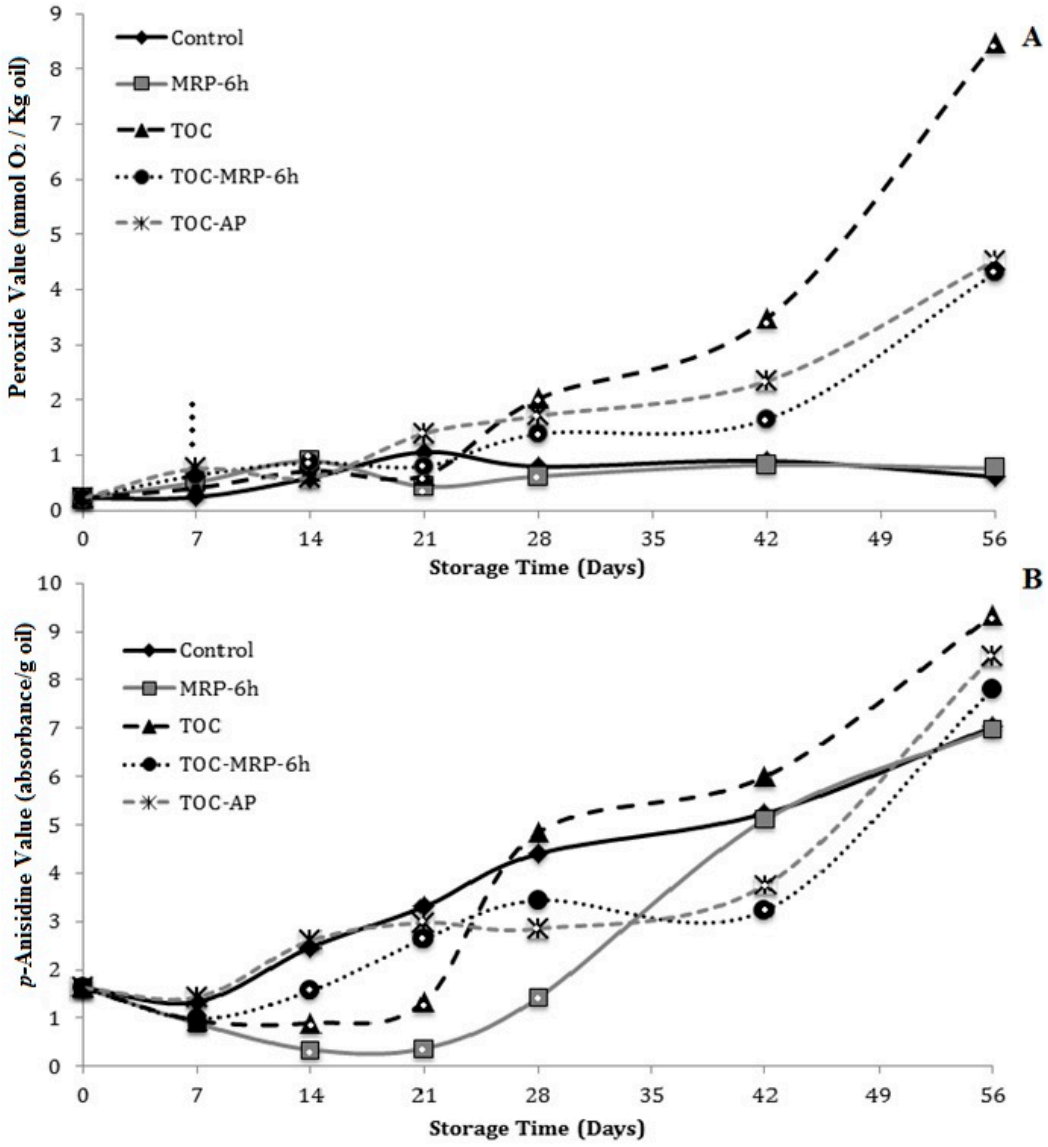

Figure 4.2 Effect of antioxidants (0.02% of the oil) on oxidative stability of a structured lipid in an infant formula emulsion over time at 25 °C: **(A)** peroxide values (PV) over time where white and black dots on the shapes indicate a significant difference ($p \leq 0.05$) from the control on the same day. The black dots above day 7 indicate that all samples were statistically higher than the control; **(B)** *p*-Anisidine values (*p*-AnV) over time where white dots indicate a significant difference ($p \leq 0.05$) from the control on the same day.

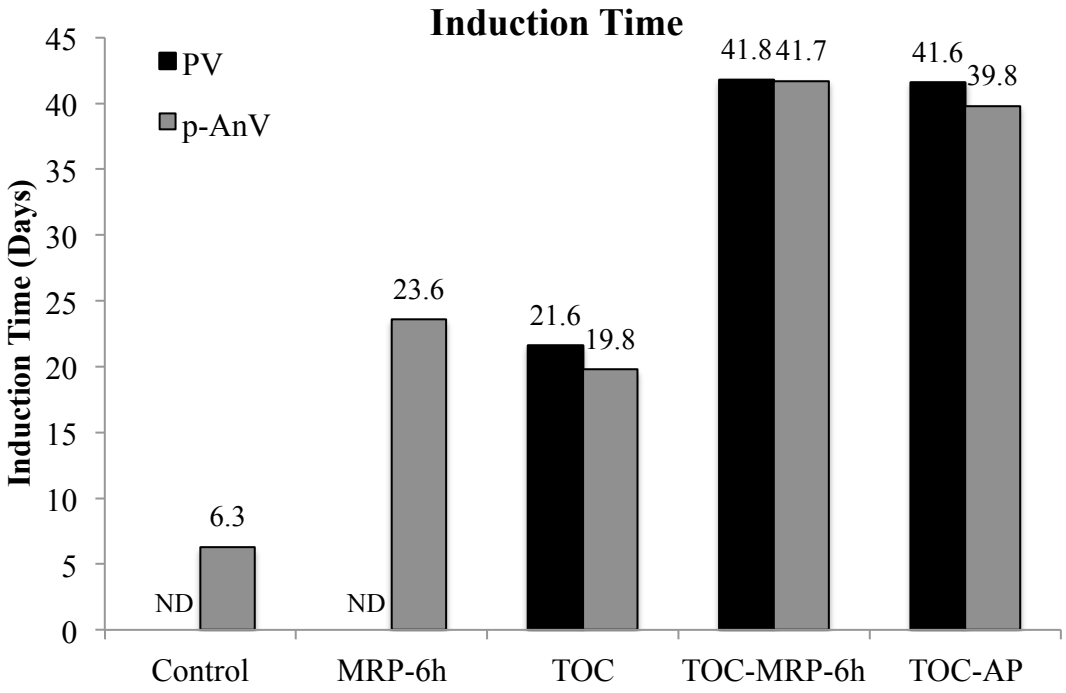

Figure 4.3 Induction times of oil determined by PV and *p*-AnV over time. The abbreviation ND represents that the value was not detected.

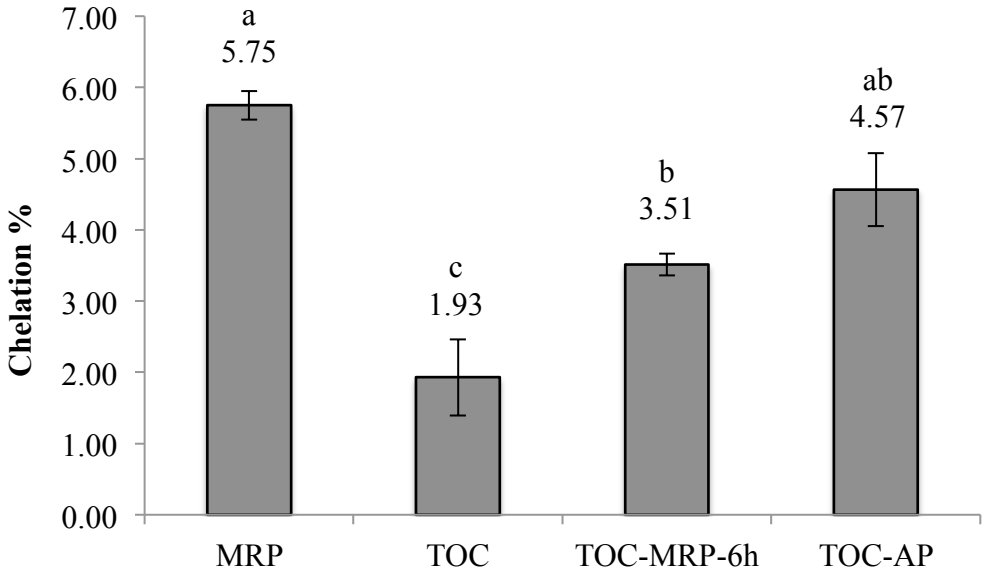

Figure 4.4 Chelation % of antioxidants (0.02% solutions). Different letters for each antioxidant represent a significant difference ($p \leq 0.05$).

CHAPTER 5

CONCLUSIONS

When infants cannot be breastfed, infant formulas are the most convenient alternative to supply proper nutrition. However, the triacylglycerols (TAGs) in many infant formulas differ in stereospecific structure compared to TAGs in human milkfat (HMF). These differences can lead to complications associated with their absorption and utilization. The differences can also cause inadequate absorption of palmitic acid, calcium ions, and can cause digestive problems. HMF analogues that contain high amounts of palmitic acid at the *sn*-2 position which are also enriched with arachidonic (ARA) and docosahexaenoic (DHA) acids can be produced to more closely meet the nutritional needs of infants.

In the first part, anhydrous milkfat was enriched with ARA and DHA by lipase catalyzed reactions in two stages. First, an intermediate structured lipid (IM-SL) was produced to increase the content of palmitic acid at the *sn*-2 position using a ratio of 0.2 (w/w) tripalmitin to anhydrous milkfat and an *sn*-1,3 specific lipase (5 wt % of substrates) at 65°C. Three different methods used to enrich the IM-SL with ARA and DHA were compared with respect to differences in fatty acid profiles, reaction times, antioxidant contents, oxidative stability, melting and crystallization profiles, and reaction yields. The three methods included physical blending, enzymatic interesterification (IE), and enzymatic acidolysis (ACID). All three products had adequate amounts of *sn*-2 palmitic acid, DHA, and ARA. However, out of the three products, the interesterifed structured lipid (IE-SL) produced using a 0.02 (w/w) ratio of 2:1 ARASCO/DHASCO

TAGs to the IM-SL, was the most suitable HMF analogue due to the increased amount of ARA at the *sn*-2 position. The IE-SL contained total ARA and DHA of 0.63 and 0.50 mol%, and 0.55 and 0.46 mol% at the *sn*-2 position, respectively. The IE-SL also contained 44.97 mol% *sn*-2 palmitic acid. The reaction yield for the IE-SL was 91.84% and its melting completion and crystallization onset temperatures were 43.1 and 27.1 °C, respectively. The ACID-SL product was the least suitable for possible use in commercial infant formula due to a lower reaction yield, lower incorporation of DHA, additional processing steps required, greater loss of antioxidants, and the high susceptibility to oxidation compared to the other products. This study was able to compare three methods for the synthesis of HMF analogous that could be totally or partially used in commercial fat blends for infant formula.

In the second part, the oxidative stability of the IE-SL was studied in complex oil in water infant formula emulsions, and the effects of antioxidants were compared. A glucose-cysteine Maillard reaction product (MRP) was produced and its antioxidant effects on lipid oxidation was compared to a control containing no added antioxidant, *α*-tocopherol (TOC), combinations of TOC and MRP (TOC-MRP), and combinations of TOC and ascorbyl palmitate (TOC-AP). Antioxidants were added at a concentration of 0.02% of the oil. The effects of the antioxidants were determined by trolox equivalent antioxidant capacity (TEAC), peroxide value (PV), *p*-anisidine value (*p*-AnV), induction time, and chelation %. For the production of the MRP, there were positive correlations between heating time for the intermediate (r=0.998) and browned (r=0.997) products as well as heating time and TEAC for the intermediate (r=0.988) and browned (r=0.986) products. This indicates that as heating time increased the product was darker in color

and displayed higher antioxidant capacity. The MRP heated for 6 h (MRP-6h) had the highest total antioxidant capacity (2.51 ± 0.10 mg trolox eq/g), compared to the other MRP samples; however this value was lower than TOC (3.87± 0.06 mg trolox eq/g). Oxidation results indicated a possible antioxidant effect for the MRP-6h on secondary oxidation for days 14-28 with an induction time of 23.6 days, while there was little to no observed antioxidant effect for primary oxidation. TOC had lower induction times of 21.6 and 19.8 days for primary and secondary oxidations, respectively. TOC-MRP-6h and TOC-AP had similar induction times of 41.8 and 41.6, respectively, for PV and 41.7 and 39.8 for *p*-AnV, respectively. The mechanism of inhibitory effect on secondary oxidation is unknown; although, chelation percentages showed that MRP-6h (5.75%) had a similar percentage to TOC-AP (4.57%) and were the highest compared to the other antioxidants tested. The MRP-6h and TOC-MRP-6h were useful in inhibiting secondary oxidation of the IE-SL in a complex O/W emulsion; however, more studies are needed to determine appropriate concentrations and the mechanisms of action.

Future studies could evolve from this research. One possibility would be to reduce the use of pure products such as tripalmitin that was used in the first part of the study. This would reduce the total palmitic acid, cost, and the melting completion and onset of crystallization temperatures to better mimic human milkfat. Since there was evidence of the *sn*-1,3 specific lipase increasing the palmitic acid at the *sn*-2 position, the optimum reaction conditions could be found to determine if acyl migration could increase the *sn*-2 palmitic acid in anhydrous milkfat to higher amounts. Another project could involve determining the mechanism of action of the MRP and the appropriate concentrations at which the MRP could inhibit lipid oxidation in emulsions. This HMF analogue could also

be compared to other HMF analogues used by the industry today in terms of taste, palatability, handling, and transport to further enhance the product. Another study could involve the metabolism and absorption of the IE-SL compared to infant formulas and HMF. This would determine if the metabolism and absorption of the IE-SL would be statistically similar or different to HMF and current commercial infant formulas. These studies would help determine the suitability of the IE-SL for possible use in future commercial applications.