ENZYMATIC SYNTHESIS AND APPLICATION OF STRUCTURED LIPIDS FOR INFANT FORMULA

by

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(Under the Direction of Casimir C. Akoh)

ABSTRACT

A human milk fat (HMF) analogue synthesis from tripalmitin, stearidonic acid (SDA) soybean oil, and docosahexaenoic acid (DHA) was scaled up by interesterification catalyzed by Novozym 435 and acidolysis by Lipozyme TL IM, and then purified by short-path distillation (SPD). This structured lipid (SL) contained 5.4 mol% DHA and 8.0 mol% SDA, with 57.0 mol% palmitic acid esterified at *sn*-2 position. Although tocopheryl and tocotrienyl fatty acid esters were formed during interesterification and acidolysis, >50% of vitamin E isomers were lost into distillates (wastes) during SPD, which contributed mostly to the rapid oxidative deterioration of SLs in the recent and past studies. Response surface methodology was applied to investigate the effects of lecithin, monoacylglycerol, locust bean gum (LBG), and carrageenan on the physical and oxidative properties of SL-based infant formula (IF) emulsion. Particle size, optical stability, viscosity, relative content of DHA and SDA, and total oxidation value were assessed during 28-day storage. In addition, the effect of permitted antioxidants (added at 0.005 and 0.02% of oil, respectively), including α-tocopherol, β-carotene, ascorbyl palmitate, ascorbic acid, citric acid, and their combinations, on the lipid oxidation was

evaluated under an accelerated storage. The peroxide value, anisidine value, and hexanal concentration of emulsion samples were measured. It was found that the optimal conditions to achieve the highest physical and oxidative stability of SL-based IF emulsion were 0.2 g/100 mL lecithin, 0.4 g/100 mL monoacylglycerol, 0.045 g/100 mL LBG, 0.015 g/100 mL carrageenan, and 0.005% ascorbyl palmitate. On the basis of measurement of peroxide and anisidine values over a 28-day period, annatto TRF was found to be a more effective antioxidant than palm TRF and α-tocopherol in inhibiting lipid oxidation in menhaden fish oil and SL-based IF emulsion at 0.02% and 0.05%. Addition of α-tocopherol (0-75%) did not interfere with the antioxidant activity of tocopherol-free annatto TRF in the foods tested. Overall, this study has important implications for the successful incorporation of SL into IF products for better infant nutrition and health.

INDEX WORDS: Structured lipid, Lipase, Infant formula, Tocopherol, Tocotrienol, Antioxidant, Lipid oxidation, Oil-in-water emulsion, Annatto, Stearidonic acid soybean oil, Human milk fat

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DEDICATION

To my dear wife, my parents, and my sister

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CHAPTER 1

INTRODUCTION

There is a growing interest among food chemists and nutritionists concerning structured lipids (SLs) due to their desired physiochemical properties and/or health benefits for food or nutritional applications, such as human milk fat (HMF) analogues. Lipase-catalyzed synthesis of SLs, commonly interesterification and acidolysis, is preferred over chemical synthesis because of its specificity and better control over the final products (Xu, 2000). Human milk is the best source of nutrients for infants. However, it is not always possible to feed infants with human milk due to physiological and cultural reasons. The fatty acid composition and distribution of HMF are believed to be a gold standard, and omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFAs) have been shown to play an essential role in the retinal and neurological development of infants (Schuchardt, Huss, Stauss-Grabo, & Hahn, 2010). Therefore, SLs, which mimic the unique structure of HMF as well as containing n-3 LCPUFAs in the triacylglycerol backbone, are needed and may maximize health benefits for infants.

Moreover, it is important to consider whether such tailor-made lipids are oxidatively stable to allow their use as ingredients. Many studies (Maduko, Park, & Akoh, 2008; Martin, Reglero, & Senorans, 2010; Senanayake & Shahidi, 2002; Teichert & Akoh, 2011) have reported that SLs produced by either lipase-catalyzed interesterification or acidolysis are characterized by a lower oxidative stability compared to the initial fat/oil substrates. The most serious concern is the loss of endogenous

antioxidants during production and purification of SLs, especially tocopherols and tocotrienols, when vegetable oils are used as substrates. However, the reason or the basis for the loss of these important antioxidants is poorly understood.

Furthermore, after synthesis and characterization of SLs as HMF analogues, little work has been carried out to further apply these HMF analogues as an ingredient in actual food system, such as ready-to-feed infant formula (IF). The formation and stabilization of an oil-in-water (O/W) emulsion is an integral step in the manufacture of infant nutritional products. Physical instability (e.g., creaming) and lipid oxidation are among the most fundamental problems that can occur in IF emulsions, limiting the shelf-life and acceptance of the final products. Therefore, it is necessary to understand the interactions among different food ingredients and optimize the formulation (e.g., emulsifiers, thickeners, and/or antioxidants) to minimize the physical and oxidative instability.

Additionally, there is a growing interest in food industry to apply natural antioxidant to retard or inhibit lipid oxidation, due to their efficacy, safety, and additional health-promoting benefits. Although the antioxidant and biological properties of tocopherols, especially α-tocopherol, have been investigated extensively, little is known about antioxidant activities of tocotrienols in food systems. An expanding body of evidence has demonstrated that tocotrienols possesses powerful cholesterol-lowering, anticancer, and neuroprotective properties that are often not exhibited by tocopherols (Aggarwal, Sundaram, Prasad, & Kannappan, 2010; Watson & Preedy, 2009). Nevertheless, unexpectedly, α-tocopherol has been shown to attenuate tocotrienol health benefits in vivo (e.g., hypocholestrolemic and anticancer effects) (Qureshi et al., 1996;

Shibata et al., 2010). Little is known whether α-tocopherol can interfere with the antioxidant ability of tocotrienols in foods. The major commercial sources of tocotrienols are palm, rice bran and annatto. Palm or rice tocotrienol is a mixture of tocopherols and tocotrienols, whereas annatto tocotrienol is virtually tocopherol-free and composed of only γ- and δ-tocotrienols (Watson et al., 2009).

To fill these gaps, the research entitled "enzymatic synthesis and application of structured lipids for infant formula" is carried out, and the whole dissertation is divided into seven chapters. The first chapter gives introduction about the rationales, hypotheses, and objectives of the research. The second chapter is a literature review of topics related to infant nutrition, human milk composition, SLs as HMF analogues, infant formula, and lipid oxidation in emulsions. The third chapter discusses the fate of endogenous tocopherols and tocotrienols during production and purification of SLs. The fourth and fifth chapters discuss the effect of emulsifiers, thickeners, and antioxidants on the stability of SL-based IFs. The sixth chapter is on the antioxidant behaviors of tocotrienol-rich fractions (TRFs) from different sources in bulk oils and O/W emulsions. The last chapter covers the highlights of the whole study, along with some suggestions for future work.

The goal of this dissertation research is to improve infant nutrition and health by exploring and delivering functional and physiological SLs in their diets. To achieve this goal, the specific objectives and hypotheses are set as follows.

Objective 1: Investigate the fate of endogenous vitamin E isomers during production and purification (short-path distillation, SPD) of SLs. Specifically, (1) identify tocopheryl and/or tocotrienyl fatty acid esters formed during lipase-catalyzed

interesterification and acidolysis; (2) quantitatively determine tocopherols and tocotrienols present in all of the fractions after SPD. **Hypothesis 1:** The formation of tocopheryl and/or tocotrienyl fatty acid esters, during either lipase-catalyzed interesterification or acidolysis, is responsible for the compromised oxidative stability of SLs (e.g., HMF analogues).

Objective 2: Develop a physically and oxidatively stable SL-based IF emulsion to deliver HMF analogues enriched with beneficial LCPUFAs for infant nutrition and health. Specifically, (1) investigate the effects of two important categories of ingredients, emulsifiers (lecithin and monoacylglycerol) and thickeners (locust bean gum and carrageenan), on the physical and oxidative stability of SL-based IF emulsion, and optimize their levels to achieve the highest stability; (2) examine the effects of permitted antioxidants on the oxidative stability of SL-based IF emulsion, and find the best candidate to minimize lipid oxidation. **Hypothesis 2:** The physical and oxidative stabilities of SL-based IF emulsion is highly influenced by the types and concentrations of emulsifiers, thickeners, and antioxidants.

Objective 3: Determine and compare the antioxidant activities of commercial annatto and palm TRFs in actual food systems, fish oil (bulk oil) and SL-based ready-to-feed IF (O/W emulsion). Additionally, test whether α-tocopherol can interfere with the antioxidant activity of tocopherol-free annatto TRF in the above foods. **Hypothesis 3:** Annatto TRF is an effective natural antioxidant in foods.

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CHAPTER 2

LITERATURE REVIEW

Nutritional needs during infancy

Infancy is a period (0-12 months) marked by the most rapid physical growth and development of a person's life (NRC, 2006; Trahms & McKean, 2008). Infancy can be divided into several stages, with physical growth, behavioral developments, nutrition needs, and feeding patterns varying significantly (Isaacs, 2011). The most rapid changes occur in early infancy (0-6 months). Typically, infants double their birth weight by 4 to 6 months (Pipes & Trahms, 1993). The digestive system of healthy full-term infants is functional at birth, although the levels of digestive enzymes may be lower than older infants (Isaacs, 2011; Pipes et al., 1993). It takes about 6 months for the gastrointestinal tract to mature (Hagan, Shaw, & Duncan, 2008). In late infancy (7-12 months), infants' physical maturation and mastery of purposeful activity allow them to eat a wide variety of foods. World Health Organization (WHO) and American Academy of Pediatrics (AAP) recommend starting feeding complementary foods after exclusive breastfeeding for 6 months (Eidelman et al., 2012; WHO, 2009). Inadequate nutrition in infancy may lead to lifelong consequences. Therefore, to meet the growth demands of infants, adequate intakes of calories, protein, fat, carbohydrate, vitamins, and minerals are required. The dietary reference intakes (DRIs) for infants (NRC, 2006) are extrapolated from the intakes of full-term infants exclusively fed human milk (Table 2.1).

Energy

The caloric needs during infancy are higher than other life stages. The average caloric need of infants in the first 6 months is 108 kcal/kg body weight, and the need is reduced to 98 kcal/kg body weight from 7 to 12 months (Isaacs, 2011; NRC, 2006). About half of infant calories should be obtained from fat to meet the demands of growth and development (Trahms et al., 2008). Factors that account for the caloric needs of infants include weight, growth rate (e.g., catch-up and lag-down periods), sleep/wake cycle, temperature and climate, physical activity, metabolic response to food, and health status (Isaacs, 2011).

Protein

Protein needs of infants vary with the same factors as calorie needs (Isaacs, 2011). However, they are also influenced by body composition due to the requirements for tissue replacement, deposition of lean body mass, and growth (Isaacs, 2011; Trahms et al., 2008). Recommended protein intake from birth to 6 months is 9.1 g of protein per day, and from 7 to 12 months the need is 11 g of protein per day (Table 2.1). Moreover, the amino acids that are essential for adults are also required by infants, including phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine. Additionally, Tyrosine, cystine, and taurine are essential for premature infants (Fomon, 1993).

Fat

Infants need fat, a concentrated source of calories, to support their high demand for calories. The current recommendation for infants younger than 1 year of age is to consume a minimum of 30 g of fat per day (Table 2.1). Cholesterol intake should not be

restricted during infancy because of the needs for gonad and brain development (Isaacs, 2011). Besides total fat, infants also require essential fatty acids, linoleic acid (LA) and α-linolenic acid (ALA), as substrates for the syntheses of hormones, eicosanoids, and long-chain polyunsaturated fatty acids, especially arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). The current recommendation of linoleic acid (a precursor of the n-6 fatty acids) is 4.4 g/day for infants younger than 6 months of age and 4.6 g/day for infants from 7 months to 1 year of age. The need for α-linolenic acid (a precursor of the n-3 fatty acids) is 0.5 g/day during the first year of life. Recently the importance of ARA and DHA for the retinal and neurologic developments in infants has been recognized (Kidd, 2007; Schuchardt, Huss, Stauss-Grabo, & Hahn, 2010). However, their dietary intakes are uncertain.

Carbohydrate

Carbohydrates (e.g., lactose in breast milk) supply 30-60% of the energy intake during infancy (Trahms et al., 2008). However, some infants cannot tolerate lactose and an alternative formula may be required in their diet. Recommended intake from birth to 6 months is 60 g per day, and from 7 to 12 months the need is 95 g per day.

Water

The recommended total water intake for infants is 0.7 L/day for infants up to 6 months and 0.8 L/day for infants from 7 to 12 months of age. Concentrated and excessively diluted milk or formula is inappropriate for infants. In very hot and humid environments, infants may require additional water. In addition, juice is not needed before the age of 6 months, and colas and tea should be avoided (Eidelman et al., 2012).

Micronutrients

For the micronutrients, specific supplements are recommended for breastfed infants under circumstances.

Iron

Although iron in human milk is highly bioavailable, infants who are only fed human milk are at risk at 4 to 6 months of age for developing a negative iron balance and may deplete the iron reserves by 6 to 9 months (Kim, Cheong, Jun, Choi, & Son, 1996). Therefore, the recommended adequate intake (AI) for infants from 0 to 6 months of age is 0.27 mg/day, and for infants from 7 to 12 months, the AI is 11 mg/day. Iron fortified cereals and infant formula are good complementary sources (WHO, 2009).

Fluoride

Fluoride is necessary for the tooth development. Fluoride supplements are recommended if the family lives in a place that does not provide fluoridated water (Eidelman et al., 2012; Isaacs, 2011). The DRI for fluoride is 0.01 and 0.5 mg daily for infants < 6 months and 7-12 months of age, respectively.

Vitamin D

Human milk supplies all the vitamins the infant needs except for vitamin D. It only contains approximately 0.5 μg of vitamin D (Trahms et al., 2008). Thus, breastfed infants are at risk for vitamin D deficiency due to low amounts in breast milk and limited sun exposure. A vitamin D supplement of 5 μg per day for breastfed infants is recommended to prevent vitamin D deficiency and rickets.

Vitamin B_{12}

Vitamin B_{12} supplements may be needed if the mother has followed a vegan diet for a long time before and during the pregnancy. The AI for infants is 0.4 μg/day during the first 6 months and 0.5 μg/day during the second 6 months of life.

Vitamin K

Deficiency of Vitamin K may result in bleeding or hemorrhagic disease of the new born (Trahms et al., 2008). The AI for infants is 2 μg/day during the first 6 months and 2.5 μg/day during the second 6 months of life. To reduce the risk of hemorrhagic disease, an injection of vitamin K is required for the newborns in many states of America (Eidelman et al., 2012).

Human milk composition

Human milk provides a complex of nutrients that an infant needs in the first 12 months of life, including proteins, carbohydrates, fats, vitamins, minerals, water, and other physiological components (e.g., white cells and enzymes) (Riordan, 2005; Table 2.2)**.** Human milk is not a uniform body fluid. It varies not only from individual to individual but also with stages of lactation, time of feeding, and maternal diet (Akers & Groh-Wargo, 1999). On the basis of expression stage, human milk can be divided into colostrum (0-7days), transitional milk (7-14 days), mature milk (14 days-7 months), and extended lactation (7 months-2 years) (Akers et al., 1999). Each stage has its own biochemical components and properties. For example, yellowish colostrum is lower in carbohydrate, fat, and energy than mature milk but higher in protein, fat-soluble vitamins (e.g., vitamin A), minerals, and antibodies (e.g., immunoglobulin A, IgA) (Riordan,

2005). Thus colostrum can provide important immune protections to an infant when he or she is first exposed to the microorganisms in the environment (WHO, 2009).

Fat

Human milk contains 3-5 g/100 mL fat, which provides >50% of the dietary energy and essential fatty acids (LA and ALA) required by the infants (Jenness, 1979). Moreover, human milk fat (HMF) is a structural component of cell membranes, a carrier of fat-soluble vitamins, and a precursor for the synthesis of hormones and eicosanoids (e.g., prostaglandins, thromboxanes, and leukotrienes) (Jensen, 1999). HMF is composed of triacylglycerols (TAGs, 98-99%), phospholipids (0.26-0.80%), sterols (mainly cholesterols, 0.25-0.34%), and other minor components (Jensen, 1999).

The TAGs in HMF have specific compositions (Jensen, 1999; Lopez-Lopez, Sabater, Folgoso, Urgell, & Bargallo, 2002; Straarup, Lauritzen, Faerk, Hoy, & Michaelsen, 2006). The major fatty acids (FAs) are oleic acid (28-44%), palmitic acid (15-25%), linoleic acid (11-25%), myristic acid (4-9%), and stearic acid (5-8%) (Table 2.3). It also contains minor medium-chain FAs (e.g., caprylic acid and capric acid), long-chain polyunsaturated fatty acids (LCPUFAs; e.g., DHA, EPA, and ARA), and conjugated linolenic acid (mainly *cis*-9, *trans*-11 C18:2). The FA composition of HMF is highly influenced by maternal diet (Riordan, 2005). Medium-chain FAs provide rapid source of energy for infants. DHA and ARA are critical components of cellular membranes and essential for the retinal and brain development (Schuchardt, Huss, Stauss-Grabo, & Hahn, 2010). DHA and EPA combinations have been shown to benefit neurological disorders, such as attention deficit hyperactivity disorder (Kidd, 2007). LA

and ALA and their products DHA, EPA, and ARA in proper balance may be required for optimal nutrition of infants (Jensen, 1999; Michalski, 2013).

The TAGs in HMF also have a unique stereospecific structure ("OPO") with palmitic acid primarily esterified at *sn*-2 position (about 60%) and unsaturated FAs mainly at *sn*-1,3 positions (Lopez-Lopez et al., 2002; Straarup et al., 2006). This unique FA distribution greatly affects their digestion, absorption, and metabolism. During fat digestion, pancreatic lipase (*sn*-1,3 specific enzyme) hydrolyzes FAs at *sn*-1,3 positions as free fatty acids, producing 2-monoacylglycerol which is efficiently absorbed. However, the absorption efficiency of the FAs from *sn*-1,3 positions depends on the length of carbon chain and degree of unsaturation. If palmitic acid is predominately located at *sn*-1,3 positions, like physically blended vegetable oils used in traditional infant formula, it is released as free palmitic acid. At the alkaline condition of small intestine, these free palmitic acid interact readily with cations (e.g., calcium and magnesium) to form insoluble soaps which are excreted as hard stools (Lien, 1994; Carnielli et al., 1995; Lopez-Lopez et al., 2001). This results in the unavailability of palmitic acid and minerals to infants. Therefore, the unique TAG structure of HMF improves absorption of palmitic acid (energy) and calcium and has substantial benefits for the growth of infants (Innis, 2011). That is why recent researches in the field of infant formula tend to synthesize lipids to mimic TAG structure of HMF.

In human milk (oil-in-water), lipid molecules are organized in the form of a unique emulsified structure, milk fat globules (MFGs). It contains a core composed of TAGs, surrounded by a biological milk fat globule membrane (MFGM) (Lopez and Menard, 2011; Michalski, Briard, Michel, Tasson, & Poulain, 2005). In mature milk,

MFGs have a mean diameter of 4-5 μm and a surface area of about 2 m^2/g (Lopez et al., 2011). The size of MFGs is larger in colostrum and transitional milk than in mature milk (Michalski et al., 2005). Interestingly, MFG size has been found to be correlated with lipid content, indicating that increasing milk fat content is associated with larger MFGs rather than an increased number of MFGs of similar size (Michalski et al., 2005). Compared to MFGs, homogenized lipid droplets in infant formula have a smaller particle size (~1 μm) and larger surface area (~22 m^2/g) (Michalski et al., 2005). The MFGM with a trilayer structure contains phospholipids, glycolipids, proteins (e.g., membrane transporters, receptors, and enzymes), and lipid rafts rich in cholesterol and sphigomyelin (Lopez et al., 2011). All these together can protect infants against infections (Hamosh et al., 1999) and enhance lipid digestion and absorption (Armand et al., 1996). In addition, the protective structure of MFGM also contributes to the high oxidative stability of expressed human milk despite its richness in LCPUFAs (Michalski, 2013).

Protein

Mature human milk contains 0.8-0.9 g/100 mL protein with balanced amino acids to meet the nutritional needs of infants (Jenness, 1979). It mainly contains casein and whey protein. Whey protein, which is acidified in the stomach, forms a soft and flocculent curd that is easily digested, whereas casein (the primary protein in cow milk) forms a tough and less digestible curd that requires a high expenditure of energy (Riordan, 2005). The level of whey protein and casein changes as lactation proceeds, resulting in a whey protein/casein ratio of about 90:10 in early lactation, 60:40 in mature milk, and 50:50 in late lactation (Kunz and Lonnerdal, 1992). Whey protein in human milk is also composed of five major components, α-lactalbumin, serum albumin,

lactoferrin, immunoglobulins, and lysozyme (Raiha, 1985). α-Lactalbumin is the dominant whey protein in human milk. Compared to cow milk, human milk contains higher content of α-lactalbumin but no β-lactoglobulin (Raiha, 1985). Lactoferrin, immunoglobulins (especially IgA), and lysozyme play important roles in the immunological defense of infants (Riordan, 2005). Lactoferrin also protects infants against iron-deficiency (Raiha, 1985). About 25% of the total nitrogen of human milk represents nonprotein compounds including urea, uric acid, creatine, creatinine, and a large number of amino acids (e.g., glutamic acid and taurine) (Jenness, 1979; Raiha, 1985).

Carbohydrate

The main carbohydrate in human milk is lactose (about 7 g/100 mL), although a small amount of galactose, fructose, and oligosaccharides is also present (Jenness, 1979). Lactose enhances calcium absorption and metabolizes readily to supply energy for the rapid growth of infants (Riordan, 2005). The enzyme lactase is present in the intestinal mucosa after birth (Riordan, 2005). However, its activity gradually diminishes after weaning, resulting in lactose intolerance. Oligosaccharides promote the growth of *Bifidobacteria and Lactobacilli*, balancing intestinal microbes and protecting against infection from pathogenic bacteria (Dai, Nanthkumar, Newburg, & Walker, 2000).

Vitamins and minerals

Breast milk normally contains sufficient vitamins for an infant, except vitamin D and K (Jenness, 1979; WHO, 2009). Therefore, the infant needs exposure to sunlight to generate endogenous vitamin D or a supplement of vitamin D. Only a small amount of vitamin K, which is required for the synthesis of blood-clotting factors, is present in

human milk (Riordan, 2005). After birth, intakes of human milk can promote gastrointestinal bacterial colonization, which increases the levels of vitamin K (Riordan, 2005). The principal mineral constituents of human milk are sodium, potassium, calcium, magnesium, phosphorus, and chloride (Jenness, 1979; WHO, 2009). The minerals iron and zinc are present at a relatively low concentration, but their bioavailability is high (WHO, 2009).

Lipase-catalyzed synthesis of structured lipids (SLs) as HMF analogues (HMFAs)

The fatty acid composition and distribution of HMF are believed to be a gold standard, and many approaches have been tried to produce SLs to mimic HMF (Table 2.4). SLs are generally defined as TAGs that have been chemically or enzymatically modified from their natural biosynthetic state by changing the FA composition and/or the positions of FAs in the glycerol backbone to yield novel lipids (Akoh & Kim, 2008). SLs can be synthesized by direct esterification, acidolysis, alcoholysis, and interesterification reactions.

Direct esterification: Glycerol + FA → TAG + Water

Acidolysis: $TAG_1 + FA_1 \rightarrow TAG_2 + FA_2$

Alcoholysis: $TAG_1 + Alcohol_1 \rightarrow TAG_2 + Alcohol_2$

Interesterification: $TAG_1 + TAG_2 \rightarrow TAG_3 + TAG_4$

Lipase-catalyzed synthesis is preferred over chemical synthesis because of specificity, mild reaction conditions, ease of product recovery, energy efficiency, and minimal waste disposal (Xu, 2000). Factors, including lipase activity and load, substrates, synthesis procedures, temperature, water activity, reaction time, and reactors, could affect the composition, yield, and purity of the products. For example, diacylglycerols (DAGs)

are considered as the intermediates in the reaction, which are produced by hydrolysis from original TAGs and reesterified with FAs to form new TAGs. However, DAGs are also the precursor of side reactions (acyl migration) since they are not thermodynamically stable, which lead to the formation of by-products. Therefore, all these factors should be considered when selecting strategies for enzymatic production of HMFAs.

Lipases

Lipases (triacylglycerol hydrolase, EC 3.1.1.3) can catalyze hydrolytic and synthetic reactions of fats and oils in the presence or absence of organic solvents. A certain amount of water is also needed to maintain the lipase in an active three-dimensional structure, making the active site accessible to the substrate via an interfacial activation mechanism (Willis & Marangoni, 2008). Commercial lipases are available from microbial, plant, and animal sources. Among them, microbial lipases are the most attractive ones as they are thermostable, without co-lipase requirements, and of different specifications that have been extensively described (Xu, 2000). Lipases from different sources display distinct FA and positional specificity. Lipase from *Candida rugosa* exhibits discrimination against DHA, γ-linolenic acid (GLA), and stearidonic acid (SDA) (Weber & Mukherjee, 2008). Recently, pancreatic lipase has been shown to selectively hydrolyze docosapentaenoic acid over EPA and DHA due to the FA specificity rather than regiospecificity (Akanbi, Sinclair, & Barrow, 2014). Lipases can also exhibit specificity to certain FA chain length (Weber et al., 2008; Xu, 2000). *sn*-1,3 Specific lipases (e.g., lipase from *Rhizomucor miehei*) preferentially catalyze reactions on the external (*sn*-1 or *sn*-3) positions of the glycerol backbone, without affecting the internal position (*sn*-2) due to steric hindrance (Stadler, Kovac, Haalck, Spener, & Paltauf, 1995).

In contrast, nonspecific lipases do not possess positional or FA selectivity and produce products that are similar to those obtained through chemical reactions (Macrae, 1983).

The stability of lipases is one of the major concerns for industrial applications due to their high cost. Immobilization is often used to improve their stability and reusability, thus lowering the cost of the reaction. Immobilization also affects the selectivity, chemical, and physical properties of the lipases (Rodrigues, Ortiz, Berenguer-Murcia, Torresd, & Fernandez-Lafuente, 2013). So far, Lipozyme TL IM (*Thermomyces lanuginosus* immobilized on silica gel, *sn*-1,3 specific lipase) , Lipozyme RM IM (*Rhizomucor miehei* immobilized on microporous ion exchange resin, *sn*-1,3 specific lipase), and Novozym 435 (*Candida antarctica* immobilized on macroporous acrylic resin beads, nonspecific lipase) have gained more attention for the production of HMFAs (Table 2.4). Lipozyme TL IM was found to have much lower catalytic activity than Lipozyme RM IM in the acidolysis, while they had similar catalytic activity in interesterification (Yang, Fruekilde, & Xu, 2003).

Lipase content affects the reaction rate. A high enzyme load accelerates the reaction and improves the incorporation of acyl donors. However, an increase in enzyme load also increases acyl migration and production cost (Xu, 2000). Therefore, a suitable enzyme load (usually 10-20% of total substrates) is important for shaping the fatty acid profiles of the products.

Substrates

Substrates in the form of ethyl ester, TAG, or free fatty acid (FFA) are often used as acyl donors to produce HMFAs. The reaction between two TAGs by *sn*-1,3 specific lipases results in similar TAG species to those produced by randomization, even though

their contents may not be totally the same (Xu, 2000). The selection of ethyl esters as acyl donors are mainly due to their easy separation by distillation but more DAG formation may occur (Xu, 2000). FFAs are commonly used as acyl donors due to their easy availability and high reactivity. Table 2.4 summarizes the substrates used in recent studies to synthesize HMFAs. Due to a high proportion of palmitic acid at *sn*-2 position, lard has been selected as a starting material for the production of HMFAs. However, because of religious constraints and potential health concerns, lard is not widely used. Palm stearin, a fraction of palm oil with a high content of tripalmitin, has been successfully applied to produce HMFAs. Vegetable oils, such as olive and amaranth oils, are rich sources of oleic and linoleic acids. Oils containing LCPUFAs (e.g., DHA, EPA, and ARA) are used to benefit the retinal and brain development of infants. SDA soybean oil (SDASO) is also used to increase the intake of n-3 FAs in infants. SDA converts to EPA more efficiently than ALA by skipping Δ6-desaturase rate-limiting step (Lemke et al., 2010), and is considered a "pro-EPA" fatty acid. Also, SDA is more stable than EPA and DHA for commercial use due to less unsaturation. Medium-chain FAs (especially caprylic and capric acids) from Neobee can provide a quick source of energy and a protection to bacterial and viral infections. In addition, a proper balance of LA/ALA or n-6/n-3 PUFAs of final products should also be considered for the selection of acyl donors to meet the nutrition requirements of infants.

The TAG compositions of the products depend on the substrate ratios after reaction equilibrium has been achieved. A high substrate ratio certainly moves the reaction equilibrium to the product side and improves the acyl incorporation. The choice of substrate mole ratio is also related to the cost of downstream processing and the

associated difficulties of separating FFAs and acyl donors by distillation to obtain purified HMFAs. Therefore, a reasonable amount of substrates should be selected.

Synthesis procedure

There are two general reaction routes for HMFA production. The first route is conducted in one step, i.e. acidolysis of TAG with FA, or interesterification between two TAGs or between TAG and ethyl or methyl ester of FA. One-step process is normally simple and easily performed without additional hydrolysis steps. Betapol (Loders Croklaan, Glen Ellyn, IL, USA), an example of commercial HMFAs, is produced by acidolysis of tripalmitin with unsaturated FAs catalyzed by *sn*-1,3 specific lipase (King & Padley, 1989).

The second route is carried out in two steps, i.e., hydrolysis and reesterification with the acyl groups to be incorporated, which can produce products of a high yield and purity. A typical two-step approach involves alcoholysis of TAG by *sn*-1,3 specific lipase to first obtain 2-monoacylglycerol (MAG), followed in the second step by direct esterification of purified 2-MAG with unsaturated FAs (Schmid, Bornscheuer, Soumanou, McNeill, & Schmid, 1999).

Recently, Pande, Sabir, Baeshen, and Akoh (2013) used multiple lipases for HMFA synthesis. They reported that although enzymes had better reusability in two-stage (sequential addition of lipase) synthesis, one-stage (dual lipase) synthesis was faster and also resulted in higher incorporation of acyl groups (e.g., ARA and DHA) than two-stage synthesis.

Temperature

Temperature has a dual effect on the rate of reaction. According to the Arrhenius law, an increase in temperature usually results in an acceleration effect during reactions catalyzed by enzymes. At elevated temperatures, operation is also easy, since a high temperature increases solubility of substrates in organic solvents or decreases the viscosity of substrates in a solvent-free system for food applications. However, higher temperature leads to higher lipase deactivation rates and more acyl migration as well. A compromise among incorporation, acyl migration, and lipase deactivation has to be made to select the right reaction temperature (usually 50-70 °C) (Willis et al., 2008).

Water activity

A minimum amount of water is essential to activate the lipase. However, higher quantities of water result in a higher hydrolysis of TAGs as well as a higher acyl migration that may affect the yield and purity of products (Willis et al., 2008). The optimum water activity for lipase-catalyzed reactions is generally between 0.25 and 0.45, which usually corresponds to moisture contents between 0.5 and 1% (Soumanou, Perignon, & Villeneuve, 2013).

Reactors

The commonly used reactors for the production of HMFAs are stirred batch reactor and fixed bed reactor. Stirred batch reactor is often used in laboratory due to its simplicity and low cost. The substrates and immobilized lipases are fed manually, and the reaction takes place at a specific temperature and time with continuous stirring. The product is collected after separation from lipases by filtration. The reaction rate in a stirred batch reactor was found to be higher than that obtained in a fixed bed reactor

(Jimenez et al., 2010). However, a gradual decline in enzymatic activity with increasing batch numbers was reported (Holm & Cowan, 2008). Therefore, addition of lipases or a longer reaction time without addition is required.

Fixed bed reactors are generally used for a large-scale synthesis because of their efficiency, low cost, and ease of operation. Fixed bed reactors consist of a column packed with immobilized enzymes through which the substrates and products are pumped in and out at the same rate. Increasing residence time in the reactor could increase the product yield (Willis et al., 2008). However, substrate utilization is limited due to the equilibrium nature of the reaction (Soumanou et al., 2013). Therefore, multi-stage processes have been utilized to increase the incorporation rate of FAs into TAGs, such as the production of Betapol in industry (Akoh & Xu, 2002; Soumanou et al., 2013). After acidolysis reaction in the first fixed bed reactor, products were purified by distillation to remove FFAs. The purified product was then reacted again with new acyl donors (e.g., oleic acid) for the acidolysis reaction in the second fixed bed reactor. After a second purification step to remove FFAs, the products were fractionated and refined for edible uses.

Evaluation of HMFAs

Different HMFAs have different FA compositions and positional distributions. Therefore, some criteria are needed to evaluate their quality. Wang et al. (2010) reported a model to evaluate the degree of similarity of HMFAs to HMF on the basis of total and *sn*-2 FA composition. A "deducting score" principle was used in the model to evaluate the degree of similarity. Zou et al. (2011) applied this model to evaluate HMFAs from palm stearin, and a high score of 85.8 was obtained. Recently, Zou et al. (2013) extended the previous evaluation model and used TAG composition as a comparison base.

Stability of HMFAs

HMFSs produced with fats and oils containing unsaturated fatty acids (e.g., LCPUFAs) can deteriorate during storage and produce off-flavors due to oxidation. In addition, the reaction processes for HMFAs increase FFA concentration, which also contributes to the development of unacceptable off-flavors. Therefore, oxidation stability of HMFAs provides insights on the acceptability and nutritional quality of these tailor-made lipids for formulation and consumption. In general, most studies have reported less oxidative stability of HMFAs compared to original oils (Martin, Reglero, & Senorans, 2010). This decrease is mainly attributed to the loss of endogenous antioxidants (e.g., tocopherols) during purification steps (e.g., distillation) (Maduko, Park, & Akoh, 2008; Sørensen, Xu, Zhang, Kristensen, Jacobsen, 2010). Hamam and Shahidi (2006) also proposed that the formation of tocopheryl fatty acid esters in acidolysis may compromise the oxidative stability of modified oils. Therefore, tocopherols, other natural antioxidants (e.g., rosemary extract) or synthetic ones (e.g., ascorbyl palmitate) should be supplemented to stabilize HMFAs.

Infant formula (IF)

Human milk is the best source of nutrients for infants. However, it is not always possible to feed infants with human milk due to physiological and cultural reasons, thus an alternative formula is needed. IF is defined as a food which purports to be or is represented for special dietary use solely as a food for infants by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk (FDA, 1938). Typically, infant nutritional products contain dairy proteins, vegetable oils, lactose, vitamins, minerals, food additives (e.g., thickeners, emulsifiers, antioxidants, and

acidity regulators), and other nutrients. The AAP recommends that iron-fortified cow's milk-based IF is the most appropriate milk feeding from birth to 12 months for infants who are not breastfed or who are partially breastfed (Baker et al., 1999).

Types

Infant nutritional products are commercially available in several forms, including convenient ready-to-feed liquid, concentrated liquid, and powdered products. Ready-to-feed liquid IF does not require addition of water and is convenient for feeding, whereas both concentrated liquid and powdered formulas require dilution prior to use. Thus, the safety of these formulas may be compromised if the water is contaminated. In addition, powdered IFs are not subject to the same sterilization process as liquid formulas, thus they are more susceptible to bacterial contaminations, such as *Enterobacter sakazakii* (WHO, 2007). Therefore, infants who are not breastfed and of high-risk (e.g., preterm babies) should be encouraged to use sterile liquid formula or formula which has undergone an effective decontamination procedure (e.g. use of boiling water to reconstitute or heat reconstituted formula (WHO, 2007).

Based on the ingredients, IFs can also be divided into milk protein-based (whey protein dominant or casein dominant), lactose-free milk protein-based, soy protein-based, hydrolyzed protein-based (extensively or partially), and amino acid-based products (Martinez & Ballew, 2011; Smith, Clinard, & Barnes, 2011). The commonly used protein includes skim milk powder, demineralized whey protein, whey protein concentrate, α-lactalbumin whey protein concentrate, milk protein isolate, soy protein isolate, hydrolyzed protein, sodium caseinate, and calcium caseinate (McSweeney, 2008). Casein is the predominant protein in cow's milk, whereas the primary protein in human milk is

whey protein. To mimic human milk, some cow milk-based infant formulas have been adapted to contain more whey protein by changing the whey protein/casein ratio from about 20:80 to 60:40. In addition, products fortified with α-lactalbumin, which is rich in tryptophan and cysteine (Heine, Klein, & Reeds, 1991), have been a recent innovation to mimic human milk. Lactose-free milk protein-based products are suitable for infants who are lactose-intolerant but who can tolerate milk protein. Soy protein-based products are available for infants who display milk protein or lactose intolerance. Infants with allergies or intolerance to intact milk or soy proteins often consume hypoallergenic IFs, including partially or extensively hydrolyzed protein-based and amino acid-based products.

Based on the infant's growth, they can also be categorized into preterm, term, follow-up (6-12 months), and toddler (1-3 years) formulas. There are also other IFs for special needs, including human milk fortifiers and metabolic formulas. For preterm infants, particularly very low birth weight (VLBW) infants (< 1500 g), human milk alone is inadequate to meet the nutritional needs. Therefore, fortification of human milk with nutrients is recommended. Currently commercial human milk fortifiers contain protein, carbohydrate, fat, and up to 23 vitamins and minerals (Martinez et al., 2011). Metabolic formulas are designed for infants with rare metabolic disorders, including maple syrup urine disease, phenylketonuria, and tyrosinemia (Smith et al., 2011).

Processing

The manufacturing processes of IFs depend on the type. For liquid products, the formation and stabilization of an oil-in-water (O/W) emulsion is an integral step, generally produced by mixing and homogenizing an oil phase in an aqueous phase,

mainly consisting of dairy proteins, carbohydrate, minerals, and vitamins. Specifically, once oil and aqueous phases are blended, the mixture is temporarily stored or transported via pipeline to pasteurization equipment. After pasteurization, the coarse emulsion is further homogenized to form a uniform mixture with a small droplet size (typically ~1 μm). Before packaging, the compositions are standardized to ensure that key parameters (e.g., pH and micronutrient concentrations) are at appropriate levels. Then the packaged products are ready for sterilization, which is achieved by thermal treatments such as ultra high temperature processing (e.g., 135-150 °C for 3-5 s) or in-container retort sterilization (e.g. 120 °C for 5-10 min) or a combination of these processes (McSweeney, 2008).

Generally, powdered IF is manufactured using three types of processes: a dry blending process, a wet mixing/spray drying process, and a combined process (FDA, 2003). In the dry blending process, the ingredients received from suppliers in a dehydrated powdered form are mixed together to achieve a uniform blend. This process does not involve the use of water. However, there is no heat treatment to destroy bacteria in the final product. Thus, if one or more ingredients are contaminated by even low numbers of harmful bacteria, these bacteria are likely to be present in the finished product. In the wet mixing/spray drying process, ingredients are blended together, homogenized, pasteurized, and spray dried to form a powdered product. Due to the use of pasteurization to destroy harmful bacteria, this process is less dependent on the microbiological quality of ingredients. However, this process requires frequent wet cleaning of equipments (e.g., spray dryer), which may be a source of product contamination. In the combined process, a base powder (consisting mainly of protein and

fat components) is produced using the wet mixing/spray drying process, and then the base powder is dry blended with the carbohydrate, mineral, and vitamins.

Regulatory requirements

As a human milk substitute intended to meet the normal nutritional requirements of infants, IF is highly regulated worldwide. Regulations for energy and nutrients are summarized in Table 2.5, and permitted additives are listed in Table 2.6. Requirements for special ingredients are discussed as follows.

All ingredients and food additives should be gluten-free (CAC, 1981).

The use of sesame and cotton seed oils should be prohibited (EC, 2006). Lauric and myristic acids together should not exceed 20% of total FAs (CAC, 1981; EC, 2006; Koletzko et al., 2005). Commercially hydrogenated fats and oils should not be used (CAC, 1981). For endogenous *trans*-FAs, the content should not exceed 3% of total FAs (CAC, 1981; EC, 2006; Koletzko et al., 2005). The erucic acid content should not exceed 1% of total FAs (CAC, 1981; EC, 2006; Koletzko et al., 2005). The total content of phospholipids should not exceed 300 mg/100 kcal (about 2 g/L) (CAC, 1981; EC, 2006; Koletzko et al., 2005). Prior to 2002, ARA and DHA were not ingredients for IFs in the United States. Currently most manufacturers in the United States add ARA and DHA to their IF products. However, Food and Drug Administration (FDA) treats the evaluation of their safety as new ingredients, and AAP takes no official position on their addition (USDA, 2009). Other regulatory authorities (e.g., Codex Alimentarius Commission, CAC) permit the optional addition of n-3 and n-6 LCPUFAs. In general, the content of ARA should be at least the same concentration as DHA, whereas the content of EPA should not exceed the DHA concentration (CAC, 1981; EC, 2006; Koletzko et al., 2005).

In addition, European Commision (EC) recommends that the content of n-3 and n-6 LCPUFAs (C20 -C22) should not exceed 1 and 2% of the total fat content, respectively (EC, 2006). For n-6 LCPUFAs, ARA content should also not exceed 1% of the total fat content (EC, 2006). European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) states that the optional addition of DHA should not exceed 0.5% of total fat intake until its safety and benefits have been adequately demonstrated (Koletzko et al., 2005).

Lactose and glucose polymers including maltose, maltodextrin, and glucose syrup may be used as carbohydrates in IFs (CAC, 1981; EC, 2006; Koletzko et al., 2005). Only precooked and gelatinized starches that are naturally gluten-free may be added to IFs up to 30% of total carbohydrates and up to 2 g/100 mL (CAC, 1981; EC, 2006; Koletzko et al., 2005). Sucrose and glucose may only be added to hydrolyzed protein-based IFs and their content should not exceed 20% of the total carbohydrate content and 2 g/100 kcal, respectively (EC, 2006). Fructose addition should be avoided in IFs due to hereditary fructose intolerance which may cause death in young infants (CAC, 1981; EC, 2006; Koletzko et al., 2005).

Prebiotics (commonly fructo- and galacto-oligosaccharides) are nutrients that support the growth of nonpathogenic bacteria in the intestine, while probiotics, including *Bifidobacteria* and *Lactobacilli*, are good for the gut health and decrease the risk of infections during infancy. In addition, formulas supplemented with probiotics have been reported to reduce the incidence of eczema, necrotizing enterocolitis, and mortality in high-risk infants (e.g., VLBW infants) (Martinez et al., 2011). If prebiotics are added, their content should not exceed 0.8 g/100 ml in a combination of 90% galacto-

oligosaccharide and 10% fructo-oligosaccharide (EC, 2006). The optional addition of taurine and total nucleotides should not exceed 12 mg/100 kcal and 5 mg/100 kcal, respectively (CAC, 1981; EC, 2006; Koletzko et al., 2005). Although manufacturers have started adding nucleotides, prebiotics, and probiotics to IF products, more research is needed to confirm their benefits.

Lipid oxidation in O/W emulsions

An emulsion consists of two immiscible liquids (usually oil and water), with one being dispersed in the other in the form of small spherical droplets (McClements, 2008). A system that consists of oil droplets dispersed in an aqueous phase is referred to as an O/W emulsion, such as milk, soups, infant formula, and mayonnaise, whereas a system that consists of water droplets dispersed in an oil phase is called a water-in-oil (W/O) emulsion, such as margarine, butter, and spread. Emulsions are thermodynamically unstable systems due to the free energy required to increase the surface area between the oil and water phases and different density between two phases (McClements & Decker, 2000). Physical instability of emulsions can occur through a variety of mechanisms, including creaming, flocculation, coalescence, sedimentation, Ostwald ripening, and phase inversion (McClements, 2008). To form kinetically stable emulsions, emulsifiers are needed before homogenization, forming a protective membrane that prevents the droplets from aggregating. The most common emulsifiers used in food industry are amphiphilic proteins (e.g., whey protein, casein, soy protein, and egg protein), phospholipids (e.g., soy lecithin), and small-molecule surfactants (e.g., MAG and DAG). Due to the large surface area that facilitates interactions between oil droplets and water-soluble components, lipid oxidation in O/W emulsions are significantly different from in

W/O emulsions which occur at a rate similar to that in bulk oils (McClements et al., 2000). This section will focus on O/W emulsions, which may be considered to be consisted of three distinct regions: the interior of the droplet (oil and oil-soluble components), the continuous phase (water and water-soluble ingredients), and the interfacial region (surface-active molecules).

Mechanisms of lipid oxidation

Lipid oxidation is a complex process mainly initiated by light, heat, enzymes, and metals, and can be categorized into photooxidation, enzymatic oxidation, and autoxidation (Kiokias, Varzakas, Arvanitoyannis, & Labropoulos, 2010). Photooxidation involves excitation of a photosensitizer (e.g., chlorophyll and riboflavin) and energy transfer to triplet oxygen, producing singlet oxygen which reacts directly with the double bonds of unsaturated lipids by addition rather than through free radical intermediates to initiate lipid oxidation (Shahidi & Zhong, 2010). Certain enzymes (e.g., lipoxygenase in cereals, fruits, and vegetables) can directly catalyze the formation of lipid hydroperoxides from molecular oxygen and lipid substrates (Barham et al., 2010). Although photooxidation and enzymatic oxidation may play a role, the most common and important process is lipid autoxidation.

Lipid autoxidation is an autocatalytic process via a free radical chain mechanism that proceeds through initiation, propagation, and termination stages (Kiokias et al., 2010).

Initiation: $RH \rightarrow R^{\bullet} + H^{\bullet}$

or $RH + X^{\bullet}$ (initiator radical) $\rightarrow R^{\bullet} + XH$

Propagation: $R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$

$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$

$ROOH \rightarrow OH\bullet + RO\bullet$

$RO\bullet \rightarrow$ Secondary oxidation products (e.g., aldehydes)

Termination: $R^{\bullet} + R^{\bullet} \rightarrow R\text{-}R$

$R^{\bullet} + ROO^{\bullet} \rightarrow ROOR$

$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2$

$RO\bullet + RO\bullet \rightarrow ROOR$

$RO\bullet + R^{\bullet} \rightarrow ROR$

In the presence of initiators (e.g., heat, light, irradiation, and metal ions), initiation occurs as hydrogen is abstracted from an unsaturated lipid (RH), resulting in a lipid free radical ($R^{\bullet}$). This lipid radical immediately reacts with molecular oxygen (O_2) to form a lipid peroxyl radical ($ROO^{\bullet}$) and starts to propagate. The propagation stage of oxidation is fostered by lipid-lipid interactions, whereby the lipid peroxyl radical reacts with another unsaturated lipid, generating a new lipid free radical and lipid hydroperoxide (ROOH). The propagation can be continuously repeated until radicals (e.g., $R^{\bullet}$ or $ROO^{\bullet}$) react with each other to terminate the process by forming stable and nonreactive molecules (e.g., ROOR). During propagation, lipid hydroperoxides, which is recognized as the primary products of oxidation, are formed. They are unstable and can decompose to a complex mixture of secondary oxidation products by homolytic cleavage followed by β-scission of the lipid alkoxyl radical (RO•) produced. The formed secondary oxidation products, including aldehydes, ketones, alcohols, acids, and hydrocarbons, are responsible for the sensory properties of oxidized oils and fats. In addition, reactions between radicals and antioxidants can also terminate the propagation step.

Among different initiator, transition metals (e.g., cobalt, iron, magnesium, and copper) are the major prooxidants in O/W emulsions, and they can initiate lipid autoxidation by hydrogen abstraction from an unsaturated lipid as mentioned previously or reaction with oxygen. For the latter, the anion produced can either lose an electron to produce single oxygen or react with a proton to form a hydroxyl radical, which may serve as a chain initiator (Kiokias et al., 2010).

Initiation: $RH + M^{(n+1)+} \rightarrow R^{\bullet} + H^{+} + M^{n+}$

or $M^{n+} + O_2 \rightarrow M^{(n+1)+} + O_2^{\bullet -}$; $O_2^{\bullet -} \rightarrow {}^1O_2$ or $OH^{\bullet}$

More importantly, transition metals can also promote oxidation by catalyzing the decomposition of lipid hydroperoxides located at the droplet surface into free radicals while the metals themselves undergo redox cycling (Kiokias et al., 2010).

Propagation: $M^{n+} + ROOH \rightarrow RO\bullet + OH^{-} + M^{(n+1)+}$

$M^{(n+1)+} + ROOH \rightarrow ROO^{\bullet} + H^{+} + M^{n+}$

For iron, ferrous (Fe^{2+}) was reported to be a stronger prooxidant than ferric (Fe^{3+}) due to its higher solubility and reactivity (Mei, Decker, & McClements, 1998a; Mei, McClements, Wu, & Decker, 1998b), which means that the presence of iron reducing agents in emulsions can promote oxidation, such as ascorbic acid. On the other hand, metal chelators (e.g., EDTA and citric acid) can increase the oxidative stability.

Factors that influence lipid oxidation in O/W emulsions

Ingredient quality

Ingredient quality has a significant influence on the oxidative stability of O/W emulsions. For example, low-quality oils with a high concentration of lipid hydroperoxides already present oxidize faster than oils of good quality (Let, Jacobsen, &

Meyer, 2005). FFAs can promote lipid oxidation in O/W emulsions due to their ability to increase the negative charge of emulsion droplets and thus increase interactions between transition metals and droplets (Waraho, Cardenia, Rodriguez-Estrada, McClements, & Decker, 2009).

Chemical structure of lipids

The chemical structure of lipids, especially the composition (e.g., number and location of double bonds) and positional distribution of FAs, determines their susceptibility to oxidation.

Typically, the rate of lipid oxidation increases as the degree of FA unsaturation increases. However, some researchers have found the opposite trend in O/W emulsion, with oxidative stability increasing as the degree of FA unsaturation increases (Miyashita, Nara, Ota, 1993). It is possible that compared to saturated FAs, unsaturated FAs are buried more deeply within the hydrophobic interior of the droplets and therefore are less susceptible to attack by prooxidants from aqueous phase.

In addition, it has been found that the closer the double bond is to the methyl end of a FA molecule, the greater its stability to oxidation (Miyashita, Azuma, Ota, 1995). This is probably because the hydrocarbon tail (methyl end) is located in the hydrophobic interior while the carboxyl end orients toward the aqueous phase which is prone to attack.

The positional distribution of FAs in a TAG molecule also plays a role in its oxidative stability. Shen and Wijesundera (2009) revealed that DHA was more stable to oxidation when located at the *sn*-2 position of glycerol backbone.

Droplet characteristics

The effect of oil concentration and particle size on the lipid oxidation in O/W emulsion has been investigated. Osborn-Barnes and Akoh (2004) reported that a decrease in the oil concentration led to an increase in total oxidation in caprylic acid/canola oil SL-based emulsions. One possible explanation for this increase is that the number of radicals generated per oil droplet increases as the oil concentration decreases (McClements et al., 2000).

For a fixed oil concentration, the rate of lipid oxidation increases as the droplet size decreases due to the increased droplet surface, which increases the chance to interact with diffused oxygen, water-soluble radicals and prooxidants. However, if there are only a limited amount of reactants scattering at the droplet surface, changing the droplet size may have no effect on the oxidation rate (McClements et al., 2000). This might explain why some studies found no correlation between droplet size and lipid oxidation (Osborn-Barnes & Akoh, 2004).

Interfacial characteristics

The properties of interface, such as droplet charge and thickness, strongly influence the rate of lipid oxidation since it is the region where water-soluble and lipid-soluble components interact and where surface-active materials (e.g., antioxidants and emulsifiers) concentrate.

Emulsion droplet charge is influenced by the type of emulsifiers (e.g., cationic, anionic or neutral) used to stabilize the emulsions and pH of the emulsions. The role of droplet charge can be demonstrated by the measurement of ζ potential of the emulsion droplets. Several studies have shown that emulsions stabilized with anionic surfactants

oxidize quickly due to the electrostatic attraction of cationic transition metals, whereas cationic surfactants decrease oxidation rates by electrostatically repelling metals away from the lipids (Mancuso, McClements, & Decker, 1999; Mei et al., 1998a; Mei, et al., 1998b; Silvestre, Chaiyasit, Brannan, McClements, & Decker, 2000)

The role of droplet charge has also been demonstrated in protein-stabilized emulsions. In menhaden O/W emulsions stabilized by whey protein isolate, the rate of lipid oxidation was fastest when the pH was greater than the pI of the protein, since emulsion droplet was negatively charged to attract transition metal ions (Donnelly, Decker, & McClements, 1998; Mancuso et al., 1999; Mei et al., 1998a; Mei, et al., 1998b). However, the density of the droplet charge does not always correlate with oxidative stability in protein-stabilized emulsions, probably because proteins have additional properties that may impact oxidation, such as scavenging free radicals and creating thick interfaces (Waraho, McClements, & Decker, 2011).

Emulsion droplet interfacial thickness is dependent on the size of the emulsifier head or tail group. It was observed that lipid oxidation was slower in the emulsion stabilized by the emulsifier with a longer polar head group, which suggests that the thicker interfacial layer is able to act as a physical barrier that separated the lipid substrate from prooxidants in the aqueous phase (Silvestre et al., 2000). In addition, some emulsifier molecules consisting of sugar or amino acid moieties (e.g., cysteine and tyrosine) may also act as chemical barriers to retard lipid oxidation due to their radical scavenging abilities (McClements et al., 2000).

Components in the aqueous phase

Components in the aqueous phase, such as proteins, surfactants, polysaccharides transition metals, salts, sugars, acids, bases, and buffers, may impact lipid oxidation either as prooxidants or antioxidants, depending on their chemical properties.

The nonadsorbed proteins in the continuous phase may increase or decrease the oxidative stability of O/W emulsions through enzymatic or nonenzymatic mechanisms, including catalysis of specific reactions, chelation of transition metals, and free radical scavenging (McClements et al., 2000). For example, glucose oxidase has been shown to retard lipid oxidation in mayonnaise containing fish oils, probably due to oxygen consumption by glucose oxidation (Isaksen & Adler-Nissen, 1997). Casein, transferrin, phosvitin, and lactoferrin have been shown to be effective antioxidants because of their ability to chelate iron (McClements et al., 2000; Waraho et al., 2011).

In O/W emulsions, surfactants (e.g., lecithin) adsorb onto the emulsion droplet surface until the interface is saturated, and the excess surfactants partition into the continuous phase. If the surfactant concentration in the continuous phase exceeds its critical micelle concentration, surfactant micelles form. These surfactant micelles can solubilize lipid hydroperoxides, transition metals, and/or antioxidants out of emulsion droplets into continuous phase to alter lipid oxidation (Waraho et al., 2011).

Polysaccharides are often added to O/W emulsions to enhance the viscosity of aqueous phase, which enhances textural attributes and stabilizes the droplets against physical instability (e.g., creaming). Polysaccarides may also inhibit lipid oxidation in O/W emulsions through metal chelation and free radical scavenging (McClements et al., 2000; Waraho et al., 2011).

Transition metals in the continuous phase, especially iron and copper, can promote lipid oxidation by decomposing lipid hydroperoxides on the droplet surface into free radicals and secondary oxidation products. The rate of copper-mediated oxidation was reported to be over 50-fold faster than that with an equal concentration of iron (Osborn-Barnes & Akoh, 2003)

Salts may act as prooxidants or antioxidants depending on the nature of the system involved. Take sodium chloride as an example, the antioxidant effect is probably because of its ability to reduce the tendency for transition metal ions to accumulate at the droplet interface, whereas the prooxidant effect may be due to its ability to increase the catalytic activity of iron (McClements et al., 2000).

Nonreducing sugars, such as sucrose, have been shown to retard lipid oxidation (Ponginebbi, Nawar, & Chinachoti, 1999). The possible mechanisms include decreasing the concentration of oxygen dissolved in the aqueous phase, increasing the viscosity of the aqueous phase, and scavenging free radicals (McClements et al., 2000). Reducing sugars have been shown to promote lipid oxidation in O/W emulsions due to their ability to reduce transition metal ions to their most active state (e.g., Fe^{2+} and Cu^{+}) (Yamauchi, Goto, Kato, & Ueno, 1984)

Acids, bases, and buffers affect the oxidative stability of O/W emulsions by controlling the pH, and thus different factors (e.g., type of emulsifiers and solubility) may be involved, leading to the inconsistent results in the literature (McClements et al., 2000).

Oxygen concentration

Oxygen is involved in lipid autoxidation as mentioned above. At low oxygen concentrations, the rate-limiting step of lipid oxidation is the diffusion of oxygen through

the aqueous phase. Thus, the rate of lipid oxidation increased with mechanical agitation under oxygen-limiting conditions. However, the diffusion of oxygen is no more a rate-limiting step when oxygen concentration is high, and the diffusion rate of oxygen is faster than the oxidation rate (McClements et al., 2000). Therefore, the effective method to retard oxidation is to reduce the concentration of oxygen, such as packing under vacuum or inert gas such as nitrogen and argon.

Methods for measuring lipid oxidation in O/W emulsions

A wide variety of analytical techniques have been developed to study lipid oxidation in bulk oils, and many of them can also be applied to monitor lipid oxidation in O/W emulsions. The available methods to monitor lipid oxidation in emulsions can be divided into five groups based on what they measure: the absorption of oxygen, the loss of initial substrates, the formation of free radicals, and the formation of primary and secondary oxidation products.

Oxygen absorption can be evaluated through measurements of headspace oxygen concentration. Substrate losses can be evaluated by physical (e.g., weight-gain) or chemical tests (e.g., FA composition). Free radicals can be measured by electron spin resonance. The primary oxidative changes can be evaluated by measuring peroxide value and conjugated dienes, while the common techniques used to monitor secondary oxidation products include *p*-anisidine value, total oxidation value, 2-thiobarbituric acid reactive substance value, carbonyls, and volatile compounds by spectroscopic and/or chromatographic methods. Other techniques, such as differential scanning calorimetry and nuclear magnetic resonance, can also be used for measuring lipid oxidation. In addition, sensory tests are often conducted to provide subjective evaluation of oxidative

deterioration. The details for these techniques have been reviewed extensively in the literature (Shahidi & Wanasundara, 2008) and thus will not be discussed here.

There is no uniform and standard method for detecting all oxidative changes in different emulsion systems. Each method has its advantages and disadvantages. Therefore, it is necessary to select a proper and adequate method for a particular application. The use of two or more methods assessing both primary and secondary oxidation products is highly recommended.

Strategies for controlling lipid oxidation in O/W emulsions

On the basis of factors that influence lipid oxidation in O/W emulsions, different strategies for retarding lipid oxidation can be identified, including controlled preparation and storage conditions, removal of oxygen, usage of fresh and high-quality ingredients to prepare emulsions, interfacial engineering to control the thickness and charge of droplets, and addition of antioxidants.

It is necessary to prepare and store O/W emulsions under carefully controlled conditions to retard or minimize lipid oxidation, such as refrigerated temperatures, low light, and preventing contamination from prooxidants.

Packing under vacuum or nitrogen to exclude oxygen from emulsion products has been used commercially to minimize lipid oxidation in mayonnaise and salad dressings during storage (McClements et al., 2000). However, once the product is opened, lipid oxidation accelerates.

The susceptibility of food emulsions to lipid oxidation can also be improved by ensuring that the ingredients used are low in hydroperoxides, transition metals, or other

prooxidants (e.g., FFAs). However, it may not be economically feasible to purchase high-purity ingredients or to use extra processing steps to purify the ingredients before use.

Compared to single layer emulsions, multilayer emulsions, where the droplet interface is coated by multiple layers of biopolymers, possess a thicker interfacial layer that can provide more physical as well as electrical barriers to reduce the interactions between prooxidants in the continuous phase and emulsion droplets. Some studies have shown that multilayer emulsions can inhibit lipid oxidation when the exterior layer of the emulsion droplet is cationic (Djordjevic, Cercaci, Alamed, McClements, & Decker, 2007; Klinkesorn, Sophanodora, Chinachoti, Decker, & McClements, 2005). Katsuda, McClements, Miglioranza, and Decker (2008) also found that anionic emulsions stabilized with β-lactoblobulin-pectin multilayer had similar oxidative stability as the cationic emulsions with β-lactoblobulin alone.

Addition of antioxidant is one of the most effective and convenient strategies to retard or prevent lipid oxidation (Shahidi & Zhong, 2010). According to the mechanisms of action, antioxidants can be broadly classified as primary antioxidants (e.g., tocopherols, butylated hydroxytoluene, and tertiary butylhydroquinone) which scavenge free radicals to break chain-reactions of oxidation, or secondary antioxidants which protect lipids against oxidation mainly by chelating transition metals, quenching singlet oxygen, replenishing hydrogen to primary antioxidants, and/or scavenging oxygen, such as EDTA, citric acid, and carotenoids (Reische, Lillard, & Eitenmiller, 2008). In some cases, some antioxidants exhibit both primary and secondary inhibition mechanisms and are referred as multiple-function antioxidants, such as ascorbic acid (Reische, Lillard, & Eitenmiller, 2008). Moreover, combinations of antioxidants with different mechanisms

of action, such as α-tocopherol/ascorbic acid, have been reported to exhibit synergistic antioxidant effects (Decker, 2008). Due to the safety concerns about potentially toxic effects of synthetic additives, there is a worldwide trend toward the use of natural antioxidants, Moreover, many natural antioxidants possess additional health-promoting benefits in vivo. Some examples include tocopherols, carotenoids, ascorbic acid, and phenolic compounds from tea (e.g., catechin and epigallocatechin gallate), olive oil (e.g., hydroxytyrosol), rosemary (e.g., carnosic acid), and fruits (e.g., caffeic acid and anthocyanin) (Reische, et al., 2008; Brewer, 2011). Oxidative reactions are believed to be mostly prevalent at the oil-water interface (McClements et al., 2000). Therefore, in addition to its innate potency, the effectiveness of an antioxidant is influenced by its polarity and solubility, which subsequently determine its actual location in O/W emulsion. With respect to interfacial phenomenon, "polar paradox theory" was proposed, which states that polar antioxidants are more effective in less polar media, such as bulk oil, whereas nonpolar antioxidants are more effective in more polar media, such as O/W emulsion (Porter, 1993). The high efficacy of nonpolar antioxidants in O/W emulsion is primarily attributed to their high affinity to orient toward the oil-water interface. However, new evidence suggests that this empirical observation seems to be a particular case of a much wider picture, and more complex factors (e.g., critical concentration) in addition to polarity should be considered to explain antioxidant efficacy (Shahidi, & Zhong, 2011). Furthermore, compounds with antioxidant activity may also exhibit prooxidant behavior under certain conditions. Huang, Frankel, and German (1994) reported that whether α-tocopherol acted as an antioxidant or prooxidant depended on the test system, concentration, oxidation time, and the method used to determine oxidation.

Therefore, all relevant factors must be taken into account when selecting antioxidants for a particular food system, and experiments should be performed before adding them as functional ingredients.

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Table 2.1 Dietary reference intakes: recommended dietary allowances and adequate intakes for infants from birth to 1 year[a]

Nutrient (amount/day)	Age	
	Birth-6 months	7 months-1 year
Energy (kcal)[b]	570 (Male); 520 (Female)	743 (Male); 676 (Female)
Protein (g)	9.1	11*
Fat (g)	31	30
Linoleic acid (g)	4.4	4.6
α-Linolenic acid (g)	0.5	0.5
Carbohydrate (g)	60	95
Water (L)	0.7	0.8
Vitamin A (μg)	400	500
Vitamin D (μg)	5	5
Vitamin E (mg)	4	5
Vitamin K (μg)	2	2.5
Vitamin C (mg)	40	50
Thiamin (mg)	0.2	0.3
Riboflavin (mg)	0.3	0.4
Niacin (mg)	2	4
Vitamin B_6 (mg)	0.1	0.3
Folate (μg)	65	80
Vitamin B_{12} (μg)	0.4	0.5
Pantothenic acid (mg)	1.7	1.8
Biotin (μg)	5	6
Choline (mg)	125	150
Calcium (mg)	210	270
Phosphorus (mg)	100	275
Magnesium (mg)	30	75
Iron (mg)	0.27	11*
Zinc (mg)	2	3*
Iodine (μg)	110	130
Selenium (μg)	15	20
Fluoride (mg)	0.01	0.5
Manganese (mg)	0.003	0.6
Molybdenum (μg)	2	3
Chromium (μg)	0.2	5.5
Copper (μg)	200	220
Potassium (g)	0.4	0.7
Sodium (g)	0.12	0.37
Chloride (g)	0.18	0.57

(NRC, 2006)

[a] Recommended dietary allowances (RDAs; marked by an asterisk) and adequate intakes (AIs) may both be used as goals for individual intake. RDAs are set to meet the needs of 97-98% individuals in a group. An AI is set instead of an RDA if there is insufficient data to set an RDA. For healthy breastfed infants, the AI is the mean intake.

[b] Estimated energy requirements

Table 2.2 Composition of mature human milk

Nutrient	Human milk (per 100 mL)
Energy (kcal)	60-75
Protein (g)	0.8-0.9
Fat (g)	3-5
Carbohydrate (g)	6.9-7.3
Vitamin A (IU)	133-177
β-Carotene (μg)	16-23
Vitamin D (IU)	2.5-5.0
Vitamin E (IU)	0.48
Vitamin K (μg)	0.1-0.23
Vitamin C (mg)	4-6
Thiamin (μg)	16-20
Riboflavin (μg)	31-50
Niacin (μg)	0.18
Vitamin B_6 (μg)	10.7-28
Folate (μg)	4.2-5.2
Vitamin B_{12} (μg)	0.02-0.06
Pantothenic acid (μg)	261
Biotin (μg)	0.53
Calcium (mg)	25-35
Phosphorus (mg)	13-16
Magnesium (mg)	3.0
Iron (mg)	0.02-0.04
Zinc (mg)	0.15-0.25
Selenium (μg)	1.6
Fluoride (μg)	0.5-1.0
Manganese (μg)	0.41
Chromium (μg)	0.03
Copper (μg)	31
Potassium (mg)	44.3-58
Sodium (mg)	11.2-15
Chloride (mg)	37.3

(Akers & Groh-Wargo, 1999; Jenness, 1979; Riordan, 2005)

Table 2.3 Major fatty acid composition of mature human milk (%)[a]

Fatty acid	Human milk[b]		Human milk[c]		Human milk[d]	
	Total	*sn*-2	Total	*sn*-2	Total	*sn*-2
C8:0	ND	ND	ND	ND	0.1-0.4	ND
C10:0	ND	ND	2.9	1.6	0.9-3.1	0.3-0.5
C12:0	4.1	2.5	7.3	6.9	4.1-9.4	4-5.6
C14:0	5.5	6.2	9.4	15.4	3.6-9.1	8.1-11.3
C16:0	21	54.2	27	57.1	15.4-24.5	47.9-56.7
C16:1n-7	3.1	3.5	3.6	1.6	1.1-2.8	1.4-2.4
C18:0	7.1	2.9	7.1	4.9	4.6-8.1	1.4-2
C18:1n-9	40.2	17.1	34.2	8.1	28.3-43.8	11.2-16.7
C18:2n-6	13.4	8.1	7.9	3.7	10.6-25.3	8.2-13.7
C18:3n-3	1.5	0.9	Trace	ND	0.4-1.7	0.5-0.7
C20:4n-6	0.5	0.7	ND	ND	0.2-0.8	0.5-0.8
C22:6n-3	0.2	0.4	ND	ND	0.2-0.6	0.5-0.7
LA/ALA	8.9	NA	ND	ND	14.4-39.5	NA

[a] Abbreviations: LA/ALA, linoleic acid/linolenic acid; ND, not detected; NA, not applicable.
[b] Reference: Innis, Dyer, and Nelson (1994).
[c] Reference: Jensen, Ferris, Lammi-Keefe, and Henderson (1990).
[d] Reference: Lopez-Lopez, Sabater, Folgoso, Urgell, and Bargallo (2002).

Table 2.4 Representative studies on lipase-catalyzed synthesis of human milk fat analogues[a]

Reaction type	Substrates (Mole ratio)	Reaction conditions			Products		Reference
		Lipase and load (%)[b]	T (°C)	Time (h)	PA at *sn*-2 (mol%)	Others	
Interesterification/ Acidolysis	Tripalmitin/extra virgin olive oil/ARASCO and DHASCO FAs (1:1:0.5)	Novozym 435 (10) Lipozyme TL IM (10)	60	6	55.34	5.95% ARA 2.60% DHA	Pande, Sabir, Baeshen, and Akoh (2013)
Acidolysis	Palm olein/ARASCO and DHASCO FAs (1:18)	Novozym 435 (10)	60	24	22.11	8.05% ARA 17.20% DHA	Nagachinta and Akoh (2012)
Acidolysis	Tripalmitin/oleic acid (1:2)	*Carica paraya* lipase	60	24	73.9	28.4% O	Tecelao, Rivera, Sandoval, and Ferreira-Dias (2012)
Interesterification	SDASO/tripalmitin (1:2)	Novozym 435 (10) Lipozyme RM IM (10)	65	18	60.84 60.63	8.15% SDA 8.38% SDA	Teichert and Akoh (2011)
Acidolysis	Tripalmitin/hazelnut oil FAs and Neobee FAs[c] (1:3.35)	Lipozyme RM IM (19.78)	57	NA	NA	12.8 % C8:0 10.6% C10:0 30% PA	Ilyasoglu, Gultekin-Ozguven, and Ozcelik (2011)
Acidolysis	Palm stearin/mixed FAs[d] (1:14.6)	Lipozyme RM IM (10.7)	57	3.4	62.8	29.7% PA	Zou et al. (2011)
Acidolysis	Butterfat/rapeseed and soybean oil FAs (1:2)	Lipozyme RM IM (NA)	65	NA	56.12	NA	Sorensen, Xu, Zhang, Kristensen, and Jacobsen (2010)
Interesterification	Amaranth oil/ethyl palmitate (1:4)	Novozym 435 (10)	60	3	20.8	NA	Pina-Rodriguez and Akoh (2009)
Acidolysis	CAO/DHASCO FAs (1:0.04)	Lipozyme RM IM (10)	60	1.6	20.2	1.9% DHA	
Interesterification	Tripalmitin/BFO (1:3)	Lipozyme RM IM (10)	55	14.4	46.9	1.2% EPA 0.2% DHA	Maduko, Park, and Akoh (2008)
Acidolysis	Tripalmitin/hazelnut oil FAs and GLA (1:16)	Lipozyme RM IM (10) Lipozyme TL IM (6)	55	21.6	74.9 73.9	10.1% GLA 43.6% O 10.0% GLA 44.3% O	Sahin, Akoh, and Karaali (2005)
Acidolysis	Lard/soybean oil FAs	Lipozyme RM IM	61	1	71.1	LA/ALA=10.5	Yang, Xu, He, and

	(1:2.4)	(13.7)					Li (2003)
Interesterification	Tripalmitin/ethyl esters of EPA and DHA (1:5)	Lipozyme TL IM (20)	60	20	NA	42% EPA and DHA	Yang, Fruekilde, and Xu (2003)
Alcoholysis/ esterification	Tripalmitin/ethanol (1:10)	*Rhizopus delemar* immobilized on EP 100 (10)	52	24	NA	80% 2-monopalmitin	Schmid, Bornscheuer, Soumanou, McNeill, and Schmid (1999)
	2-monopalmitin/oleic acid (1:3)	*Rhizopus delemar* immobilized on EP 100 (10)	38	16	96	90% O at *sn*-1,3 position	

[a] Abbreviations: T, temperature; PA, palmitic acid; SDASO, stearidonic acid soybean oil; ARA, arachidonic acid; SDA, stearidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; FA, fatty acid; LA/ALA, linoleic acid/α-linolenic acid; O, oleic acid; GLA, γ-linolenic acid; DHASCO, DHA single-cell oil; ARASCO, ARA single-cell oil; CAO, customized amaranth oil, product of amaranth oil and ethyl palmitate; BFO, oil blend containing 2.1:1.1:0.8:0.4 (v/v/v/v) coconut, safflower, soybean, and fish oils; NA, not available.
[b] Weight percentage of total substrates.
[c] Mixture of medium-chain fatty acids (caprylic and capric acids).
[d] Mixed FAs are from rapeseed oil, sunflower oil, palm kernel oil, stearic acid, and myristic acid.

Table 2.5 Compositional regulations of infant formula

Energy or nutrient	FDA[a]		CAC		EC		ESPGHAN	
(per 100 kcal)	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum
Energy (kcal per 100 mL)	–	–	60	70	60	70	60	70
Protein (g)[b]	1.8	4.5	1.8	3	1.8	3.5	1.8	3
Fat (g)	3.3	6	4.4	6	4	6	4.4	6
Linoleic acid (g)	0.3	–	0.3	–	0.3	1.2	0.3	1.2
α-Linolenic acid (g)	–	–	0.05	–	0.05	–	0.05	–
Linoleic/α-linolenic acids	–	–	5:1	15:1	5:1	15:1	5:1	15:1
Carbohydrate (g)	–	–	9	14	9	14	9	14
Vitamin A (μg RE)[c]	250 IU	750 IU	60	180	60	180	60	180
Vitamin D (μg)[d]	40 IU	100 IU	1	2.5	1	3	1	2.5
Vitamin E (mg α-TE)[e]	0.7 IU	–	0.5	–	0.5	5	0.5	5
Vitamin K (μg)	4	–	4	–	4	25	4	25
Vitamin C (mg)	8	–	10	–	10	30	8	30
Thiamin (μg)	40	–	60	–	60	300	60	300
Riboflavin (μg)	60	–	80	–	80	400	80	400
Niacin (μg)	250	–	300	–	300	1500	300	1500
Vitamin B_6 (μg)	35	–	35	–	35	175	35	175
Folic acid (μg)	4	–	10	–	10	50	10	50
Vitamin B_{12} (μg)	0.15	–	0.1	–	0.1	0.5	0.1	0.5
Pantothenic acid (μg)	300	–	400	–	400	2000	400	2000
Biotin (μg)	1.5	–	1.5	–	1.5	7.5	1..5	7.5
Choline (mg)	7	–	7	–	7	50	7	50
Inositol (mg)	4	–	4	–	4	40	4	40
L-Carnitine (mg)	–	–	1.2	–	1.2	–	1.2	–
Calcium (mg)	60	–	50	–	50	140	50	140
Phosphorus (mg)[f]	30	–	25	–	25	90	25	90
Calcium/ phosphorus	1.1:1	2:1	1:1	2:1	1:1	2:1	1:1	2:1
Magnesium (mg)	6	–	5	–	5	15	5	15
Iron (mg)[g]	0.15	3	0.45	–	0.6	2	0.3	1.3
Zinc (mg)	0.5	–	0.5	–	0.5	1.5	0.5	1.5

Iodine (μg)	5	75	10	−	10	50	10	50
Selenium (μg)	−	−	1	−	1	9	1	9
Fluoride (μg)	−	−	−	100	−	100	−	60
Manganese (μg)	5	−	1	−	1	100	1	50
Copper (μg)	60	−	35	−	35	100	35	80
Potassium (mg)	80	200	60	180	60	160	60	160
Sodium (mg)	20	60	20	60	20	60	20	60
Chloride (mg)	55	150	50	160	50	160	50	160

(CAC, 1981; EC, 2006; FDA, 1985; Koletzko et al., 2005)

[a] Abbreviations: FDA, U. S. Food and Drug Administration; CAC, Codex Alimentarius Commission; EC, European Commision; ESPGHAN, European Society for Paediatric Gastroenterology, Hepatology and Nutrition; −, not specified.

[b] For soy protein isolates, requirements are 2.25-3 g/100 kcal (CAC, ESPGHAN, and EC).

[c] 1 μg RE (retinol equivalent) = 3.33 IU Vitamin A = 1 μg all-*trans* retinol. Retinol contents shall be provided by preformed retinol, while any contents of carotenoids should not be included in the calculation and declaration of vitamin A activity.

[d] 1 μg calciferol = 40 IU vitamin D.

[e] 1 mg α-TE (alpha-tocopherol equivalent) = 1 mg d-α-tocopherol. Vitamin E content shall be at least 0.5 mg α-TE per g PUFA, using the following factors of equivalence to adapt the minimal vitamin E content to the number of fatty acid double bonds in the formula: 0.5 mg α-TE/g linoleic acid (18:2n-6); 0.75 α-TE/g α-linolenic acid (18:3n-3); 1.0 mg α-TE/g arachidonic acid (20:4n-6); 1.25
mg α-TE/g eicosapentaenoic acid (20:5n-3); 1.5 mg α-TE/g docosahexaenoic acid (22: n-3) (CAC, ESPGHAN, and EC); 0.7 IU/g linoleic acid (18:2n-6) (FDA).

[g] For soy protein isolates, requirements are 0.45-2 mg/100 kcal (ESPGHAN and EC).

[h] For soy protein isolates, requirements are 30-100 mg/100 kcal (ESPGHAN and EC).

Table 2.6 Additives permitted in infant formula products[a]

Additive	CAC	EC
Thickeners		
Guar gum	1 g/L in liquid formulas containing hydrolyzed protein	1 g/L in liquid formulas containing hydrolyzed protein
Locust bean gum	1 g/L in all types of IF	1 g/L in follow-on formulas only
Distarch phosphate	5 g/L singly or in combination in soy-based IF only	–
Acetylated distarch phosphate		–
Phosphated distarch phosphate	25 g/L singly or in combination in hydrolyzed protein- and amino acid-based IF only	–
Hydroxypropyl starch		–
Carrageenan[b]	0.3 g/L in regular milk-and soy-based liquid IF only; 1 g/L in hydrolyzed protein- and amino acid-based liquid IF only	0.3 g/L in follow-on formulas only
Pectin	–	5 g/L in acidified follow-on formulas only
Emulsifiers		
Lecithins[c]	5 g/L in all types of IF	1 g/L
Mono- and diacylglycerols[c]	4 g/L in all types of IF	4 g/L
Citric acid esters of mono- and diglycerides of fatty acids[c]		9 g/L in products containing hydrolyzed proteins, peptides or amino acids
Sucrose esters of fatty acids[c]		120 mg/L in products containing hydrolyzed proteins, peptides or amino acids
Starch sodium octenyl succinate		20 g/L in IF for special medical purposes
Acidity Regulators		
Sodium hydroxide	2 g/L singly or in combination and within the limits for sodium, potassium, and calcium in all types of IF	–
Sodium hydrogen carbonate	2 g/L singly or in combination and within the limits for sodium, potassium and calcium in all types of IF	–
Sodium carbonate		
Potassium hydroxide		
Potassium hydrogen carbonate		
Potassium carbonate		
Calcium hydroxide		
L(+) lactic acid	Limited by GMP in all types of IF	*quantum satis*
Citric acid		*quantum satis*
Sodium dihydrogen citrate		2 g/L
Trisodium citrate		
Potassium citrate		–
Antioxidants		
Mixed tocopherol concentrate	10 mg/L in all types of IF singly or in combination	10 mg/L individually or in combination

Ascorbyl palmitate	10 mg/L in all types of IF singly or in combination	10 mg/L
Packaging gases		
Carbon dioxide	GMP	−
Nitrogen	GMP	−

(CAC, 1981; EC, 2006)

[a] Abbreviations: CAC, Codex Alimentarius Commission; EC, European Commision; IF, infant formula; GMP, good manufacturing practice; −, not specified.

[b] Its use is restricted until evaluation is completed.

[c] If more than one of the substances is added, the maximum level for each of those substances is lowered with the relative part as present of the other substances.

CHAPTER 3

IDENTIFICATION OF TOCOPHEROLS, TOCOTRIENOLS, AND THEIR FATTY ACID ESTERS IN RESIDUES AND DISTILLATES OF STRUCTURED LIPIDS PURIFIED BY SHORT-PATH DISTILLATION[1]

[1] Zou, L., and Akoh, C. C. 2013. *Journal of Agricultural and Food Chemistry*, 61, 238-246. Reprinted here with permission of the publisher.

Abstract

The fate of endogenous vitamin E isomers during production and purification of structured lipids (SLs) was investigated. Two SLs involving tripalmitin, stearidonic acid soybean oil, and docosahexaenoic acid were synthesized by transesterification catalyzed by Novozym 435 (NSL) and acidolysis by Lipozyme TL IM (LDHA) and purified by short-path distillation (SPD). The electron impact and chemical ionization mass spectra of tocopheryl and tocotrienyl fatty acid esters in the distillates measured by GC-MS in synchronous scan/SIM mode demonstrated that these esters were formed during acidolysis as well as transesterification. The predominant esters were tocopheryl palmitate, tocopheryl oleate, and tocopheryl linoleate homologues, and no tocopheryl or tocotrienyl linolenate, stearidonate, or docosahexaenoate was found. Meanwhile, none of these esters were detected in the residues for either NSL or LDHA. Less than 50% of vitamin E isomers were present in residues after SPD. This loss played a major role in the rapid oxidative deterioration of SLs from previous studies with less contribution from the formation of tocopheryl and tocotrienyl esters. The lost tocopherols and tocotrienols present at high concentration in the distillates may be recovered and used to improve the oxidative stability of SLs.

Keywords: tocopherol; tocotrienol; tocopheryl/tocotrienyl fatty acid ester; short-path distillation; oxidative stability; structured lipid; stearidonic acid soybean oil

Introduction

Structured lipids (SLs) are generally defined as triacylglycerols (TAGs) that have been chemically or enzymatically modified from their natural biosynthetic state by changing the fatty acids (FA) composition and/or the positions of FAs in the glycerol backbone to yield novel lipids. Lipase-catalyzed synthesis of SLs, commonly transesterification and acidolysis, is preferred over chemical synthesis because of specificity and better control over the final products (Xu, 2000). The current popularity of SLs is well-known because of their desired physical characteristics, chemical properties, and/or health benefits, for food or nutritional applications (Osborn & Akoh, 2002), such as human milk fat analogues, reduced-calorie fats, enteral and parenteral nutrition, *trans*-free margarines, and cocoa butter substitutes. Furthermore, it is important to consider whether such tailor-made lipids are oxidatively stable to allow their use as ingredients. The stability of fats and oils depends on a number of intrinsic and extrinsic factors, mainly the unsaturation degree of FAs, minor components, environmental conditions (e.g., oxygen, light, and temperature), and use of antioxidants (Shahidi & Zhong, 2010).

Tocopherols and tocotrienols, naturally occurring minor components present in vegetable oil, are known as important endogenous antioxidants that protect oil against oxidation. It is widely accepted that the antioxidant activities of the tocopherols and tocotrienols are mainly due to their abilities to donate their phenolic hydrogens to lipid free radicals and retard lipid peroxidation process. The relative antioxidant activities of the tocopherols in vivo are α- > β- > γ- > δ-tocopherol (Eitenmiller & Lee, 2004). However, relative antioxidant activities in model and food systems are variable. α-Tocopherol generally was a more effective antioxidant at low concentration but a less

effective antioxidant at high levels than γ-tocopherol in fats and oils (Seppanen, Song, & Csallany, 2010). γ-Tocotrienol was found to have higher antioxidant activity than α-tocotrienol, and tocotrienols may be better antioxidants than their corresponding tocopherols in certain fat and oil systems (Seppanen et al., 2010).

Modified TAGs, free fatty acids (FFAs) or fatty acid esters are the main components of products after lipase-catalyzed reactions. FFAs and fatty acid esters should be removed from the products before they are used for edible purposes. Modified TAGs, especially those containing polyunsaturated fatty acids, are heat sensitive and have high boiling points. Therefore, thermal decomposition and oxidation easily take place during purification at high temperatures under atmospheric pressure. Furthermore, severe acyl migration may occur under harsh purifying conditions due to the presence of diacylglycerols in the reaction mixture, which act as intermediates for the lipase-catalyzed reactions (Xu, Skands, & Adler-Nissen, 2001). These limitations can be minimized by use of short-path distillation (SPD). SPD, also called molecular distillation, is a thermal separation technique based on an apparatus with a gap between the evaporator and condenser of equal or less dimension than the mean free path of the molecules to be evaporated (Oterhals, Kvamme, & Berntssen, 2010). It is characterized by the combination of very short residence time in the evaporator (1−10 s), high vacuum level (0.1−100 Pa), short distance between the evaporator and condenser (10−50 mm), and approximately collision-free mass transfer of molecules in the distillation space (Lutisan, & Cvengros, 1995; Lutisan, Cvengros, & Micov, 2002). Samples are fractionated into a heavy fraction (residue) and a light fraction (distillate or waste) on the basis of volatility. SPD lowers the boiling temperature and is an excellent method for the

separation, purification, and concentration of thermolabile substances with low vapor pressure. There are many applications of SPD in lipids, such as purification of SLs (Xu, Jacobsen, Nielsen, Heinrich, & Zhou, 2002), fractionation of fatty acid ethyl esters (Vazquez & Akoh, 2010), and concentration of squalene from shark liver oil (Pietsch & Jaeger, 2007).

Several authors (Maduko, Park, & Akoh, 2008; Martin, Reglero, & Senorans, 2010; Senanayake & Shahidi, 2002; Teichert & Akoh, 2011a) have reported that SLs produced by both lipase-catalyzed transesterification and acidolysis are characterized by a lower oxidative stability compared to the initial fat/oil substrates. This decrease may be due to exposure to light, oxygen, and high temperature during reaction and purification steps (e.g., distillation, washing, or filtration). The most serious concern is the loss of endogenous antioxidants during production and purification, especially tocopherols and tocotrienols, when vegetable oils are used as substrates for the production of SLs. Hamam and Shahidi (2006) reported that tocopherols in the oils were esterified with free fatty acids present at high level in the reaction mixture during production of SLs by enzymatic acidolysis, thus leading to the formation of tocopheryl esters that do not provide any stability to the resultant modified oils as they lack any free hydroxyl groups on the phenolic ring of the molecule. These tocopheryl esters may be removed from the reaction product during SPD. However, the mechanism of tocopherol loss associated with transesterification, another important reaction for the production of SLs with much lower content of FFAs, is unknown. For the purification step, many studies (Maduko et al., 2008; Martin et al., 2010; Teichert & Akoh, 2011b; Xu et al., 2002) reported that substantial amounts of tocopherols and tocotrienols were lost during SPD of SLs while

decreasing the FFA content. However, few of them indicated where these tocopherols and tocotrienols were after SPD. These vitamin E isomers may be recovered and added back to SLs to compensate for their oxidative stability. Therefore, the first objective of this study was to investigate the removal or loss of endogenous antioxidants during lipase-catalyzed transesterification by identifying tocopheryl and/or tocotrienyl fatty acid esters present in residues and distillates of SLs purified by SPD. Lipase-catalyzed acidolysis was also examined to verify previous findings (Hamam et al., 2006). Our second objective was to quantitatively determine tocopherols and tocotrienols present in all of the fractions after SPD and to find a possible source of reusable tocopherols and tocotrienols.

Materials and Methods

Materials

Stearidonic acid soybean oil (SDASO) was kindly donated by Monsanto Co. (St. Louis, MO, USA). Docosahexaenoic acid single-cell oil (DHASCO) containing 40% DHA was purchased from Martek Bioscience Corp., now DSM Nutritional Products Ltd. (Columbia, MD, USA). *sn*-1,3 specific lipase Lipozyme TL IM and nonspecific lipase Novozym 435 were obtained from Novozymes A/S (Bagsvaerd, Denmark). Supelco 37 component FAME mix, 2,5-dihydroxybenzoic acid, and α-, β-, γ-, and δ-tocopherols were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Methyl nonadecanoate was obtained from Nu-Chek Prep, Inc. (Elysian, MN, USA), and stearidonic acid methyl ester was purchased from Cayman Chemical (Ann Arbor, MI, USA). Tripalmitin and nonadecanoic acid were purchased from TCI America (Portland, OR, USA). Vitamin E linoleate mixture, consisting of 50−65% tocopheryl linoleate,

25−40% tocopheryl oleate, and no more than 1% α-tocopherol, was generously provided by TRI-K Industries, Inc. (Denville, NJ, USA). All solvents were of analytical grade and purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ, USA) or Fisher Scientific (Norcross, GA, USA).

Scaled-up Synthesis of SLs

Two SLs were respectively scaled up in a 1 L stir-batch reactor with constant stirring at 200 rpm using optimal conditions previously reported (Teichert et al., 2011a; Teichert et al., 2011b). Briefly, structured lipid of SDASO and tripalmitin catalyzed by 10% Novozym 435 (NSL) was produced through transesterification at 65 °C for 18 h with a substrate mole ratio of 1:2. Structured lipid of NSL and DHASCO-FFAs catalyzed by 10% Lipozyme TL IM (LDHA) was prepared through acidolysis at 65 °C for 24 h with a substrate mole ratio of 1:1. DHASCO-FFAs were prepared from DHASCO by saponification as described by Teichert and Akoh (2011b). The reactor was sealed and wrapped with foil to reduce exposure to light and oxygen. After reaction, the resulting products were vacuum filtered through a Whatman no. 1 paper filter containing anhydrous sodium sulfate to dry and separate the SLs from the biocatalyst. If the products needed to be stored before SPD, they were flushed with nitrogen and kept in an airtight amber container at −80 °C.

Short-Path Distillation

SPD was used to remove FFAs from the reaction mixture. SPD was performed using a KDL-4 (UIC Inc., Joliet, IL, USA) system under the following conditions: feed holding temperature of 65 °C for NSL and LDHA, feeding rate of approximately 100 mL/h, evaporator heating temperature of 185 °C, condenser cooling temperature of

20−25 °C, roller speed of 200 rpm, and vacuum of <13.33 Pa. SLs were passed through SPD once (NSL) or twice (LDHA) to obtain FFA concentrations of <1%. FFA content expressed as oleic acid percentage was determined according to AOCS Official Method Ca 5a-40 (AOCS, 2009). The average and standard deviation of triplicate analyses were reported (Table 3.2).

Fatty Acid Composition

Lipid samples were converted to FAMEs following AOAC Official Method 996.01 (AOAC, 2005) with minor modifications. Briefly, 100 mg of sample was weighed into a Teflon-lined test tube, and 100 μL of 20 mg/mL C19:0 in hexane was added as an internal standard and dried with nitrogen to remove the solvent. Two milliliters of 0.5 mol/L NaOH in methanol was added, followed by vortex and incubation at 100 °C for 5 min to saponify the lipid. After cooling under the tap water, 2 mL of 14% boron trifluoride in methanol was added. Then the sample was vortexed for 1 min, incubated at 100 °C for 5 min for methylation, and cooled under tap water. To stop the reaction and extract the FAMEs, 2 mL of hexane and 2 mL of saturated NaCl solution were added. The sample was vortexed for exactly 2 min and then centrifuged at 2000 rpm for 5 min to separate the organic and aqueous layers. The upper organic layer was filtered through an anhydrous sodium sulfate cartridge and recovered into a GC vial for analysis. FAME external standard mixture containing Supelco 37 component FAME mix, methyl nonadecanoate, and stearidonic acid methyl ester was run parallel with the sample.

FAME samples were analyzed with an Agilent 6890N GC (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an autosampler and a flame ionization detector. Separation was achieved on a 100 m × 0.25 mm i.d., 0.20 μm film thickness,

Supelco SP-2560 fused silica capillary column (Sigma-Aldrich Chemical Co.). The temperature program was 140 °C initially for 5 min, ramped to 240 °C at 4 °C/min and held for 20 min. Helium was the carrier gas at a constant flow rate of 1.1 mL/min. Split injection at a ratio of 5:1 was employed, and the injection volume was 1 μL. The injector and detector were maintained at 260 °C. Relative fatty acid content as mole percent was calculated on the basis of response factors and conversion factors (AOAC, 2005; DeVries et al., 1999), using C19:0 as internal standard. The average and standard deviation of triplicate analyses were reported (Table 3.1).

Determination of Tocopherols and Tocotrienols by HPLC

Lipid samples (0.1±0.001 g) were weighed into a test tube, and 5 mL of HPLC-grade hexane was added. The samples were then vortexed for 30 s and centrifuged at 1000 rpm for 10 min. A portion of the hexane layer was transferred into an HPLC vial for analysis. Tocopherol standards were prepared according to the procedure of previous studies (Lee, Landen, Phillips, & Eitenmiller, 1998; Shin, Huang, Pegg, Phillips, & Eitenmiller, 2009; Teichert et al., 2011a). Twenty microliters of the sample extract or tocopherol standard solution was injected into a HPLC system consisting of a Shimadzu LC-6A pump equipped with an RF-10A_{XL} fluorescence detector (Shimadzu Corp., Columbia, MD, USA), a SpectraSeries AS 3000 autosampler (Thermo Separation Products Inc., San Jose, CA, USA), a 250 mm × 4 mm i.d., 5 μm particle size, normal phase LiChrosorb Si 60 Hibar RT column (Merck KGaA, Darmstadt, Germany), and an Agilent Chemstation software (Agilent Technologies Inc.). The isocratic mobile phase contained 0.85% (v/v) isopropanol in hexane, and the flow rate was 1.0 mL/min. The excitation and emission wavelengths of the detector were 290 and 330 nm, respectively.

Quantitation of tocopherols was performed using an external standard calibration curve method. Palm olein was used to identify α-, γ-, and δ-tocotrienols (AOCS, 2009). The fluorescence intensity of tocotrienols is the same as the corresponding tocopherols, so the tocotrienol content may be calculated using the same calibration curve of the corresponding tocopherols according to AOCS Official Method Ce 8-89 (AOCS, 2009). All samples were analyzed in triplicate, and average values and standard deviation were reported (Table 3.2).

Without considering the decomposition by light, oxygen, and heat, the loss percentage of tocopherol or tocotrienol after reaction was calculated on the basis of the law of conservation of mass, using the equation

loss (%) after reaction =

$$\frac{MW_a \times mol_a \times C_a + MW_b \times mol_b \times C_b - (MW_a \times mol_a + MW_b \times mol_b) \times C_{SL}}{MW_a \times mol_a \times C_a + MW_b \times mol_b \times C_b} \times 100 \qquad (1)$$

where MW_a and MW_b are the molecular weights of substrates a and b, respectively; mol_a and mol_b are the mole units of substrates a and b, respectively; C_a, C_b, and C_{SL} are the tocopherol or tocotrienol contents (μg/g) of substrates a, b, and structured lipid after reaction, respectively.

Similarly, the loss percentage of tocopherol or tocotrienol after SPD was calculated as

$$\text{loss (\%) after SPD} = \frac{(C_{SL} - C_R) \times C_W}{(C_W - C_R) \times C_{SL}} \times 100 \qquad (2)$$

where C_W, C_R, and C_{SL} are the tocopherol or tocotrienol contents (μg/g) of distillate or waste after SPD, residue after SPD, and structured lipid after reaction, respectively.

Tocopheryl and Tocotrienyl Esters Analysis by MALDI-TOF-MS

Lipid samples were dissolved in isopropanol at 1 mg/mL. A 20 mg/mL mixture of 2,5-dihydroxybenzoic acid in 50% methanol was used as matrix. For MALDI analysis, 0.8 μL of sample and matrix, respectively, was mixed together in a ratio of 1:1 (v/v) on a stainless steel MALDI target and then air-dried at room temperature. Samples were analyzed on a TOF/TOF 5800 system (AB Sciex, Framingham, MA, USA) equipped with a nitrogen laser (λ = 337 nm) and a reflector detector. An acceleration voltage of 20 kV was used. Mass spectra within an m/z range of 300−900 were acquired in positive ion mode. A magnified image of the MALDI target was used to visually select regions of sample. Spectra were acquired with adjustment of the sample position to produce intense ions for tocopheryl and tocotrienyl esters.

Tocopheryl and Tocotrienyl Esters Analysis by GC-MS

Two ionization modes, EI and PCI, were employed to identify tocopheryl and tocotrienyl esters. GC-MS measurements operated in the EI mode was performed on an Agilent 7890 GC coupled to a 5975C MSD and an autoinjector (Agilent Technologies Inc.). A 30 m × 0.25 mm i.d., 0.25 μm film thickness, Alltech Econo-Cap EC-1 fused silica capillary column (Alltech Associates Inc., Deerfield, IL, USA) was used for separation. The column temperature was maintained at 80 °C for 2 min, then increased at 30 °C/min to 140 °C, and held for 2 min, finally programmed to 325 °C at 20 °C/min, and held isothermally at 325 °C for 70 min (total 85.25 min). Carrier gas was helium with purity above 99.999%, and the flow rate was set at 1 mL/min. The injector temperature was maintained at 290 °C. Splitless injection was employed, and the injection volume was 2 μL. MS detection was performed on a single-quadrupole mass spectrometer under

synchronous scan/SIM mode, and conditions were as follows: ion source, 230 °C; electron energy, 70 eV; transfer line, 300 °C; quadrupole,150 °C; *m/ z* range, 30−780. Characteristic ions in the SIM mode were *m/ z* 430, 416, 416, and 402 for *α*-, *β*-, *γ*-, and *δ*-tocopheryl esters, respectively, and *m/ z* 424, 410, 410, and 396 for *α*-, *β*-, *γ*-, and *δ*-tocotrienyl esters, respectively. Dwell time was 100 ms each. The PCI mass spectra in synchronous scan/SIM mode were acquired on a Shimadzu GCMS QP2010 Ultra system, using ammonia as the reagent gas. Other chromatographic conditions were identical to those described for the EI mode.

Statistical Analysis

One-way ANOVA was conducted using SAS software, version 9.2 (SAS Institute Inc., Cary, NC, USA). Duncan's multiple-range test was performed to determine significant differences of variables ($\alpha = 0.05$).

Results and Discussion

SLs were selected on the basis of enzymatic reaction types and lipase specificity. NSL was synthesized through enzymatic transesterification by enriching SDA soybean oil with about 60% palmitic acid esterified at the *sn*-2 position and about 8% of total SDA. NSL was also a starting substrate for LDHA, which further incorporated DHA by lipase-catalyzed acidolysis. These two SLs may be useful as human milk fat analogues with health benefits of omega-3 fatty acids. The FA profiles of substrates and resulting SLs after SPD are shown in Table 3.1. The results from the FA profile were used to estimate the average molecular weight of the substrates and products. More importantly, the fatty acid composition of the substrates determines the range of FA species that may be available to form tocopheryl and tocotrienyl fatty acid esters. The major FAs of

SDASO were SDA (25.74 mol %), linoleic acid (24.22 mol %), palmitic acid (13.93 mol %), oleic acid (13.21 mol %), α-linolenic acid (11.01 mol %), γ-linolenic acid (7.22 mol %) and stearic acid (3.87 mol %). In the case of NSL, palmitic acid (67.49 mol %), linoleic acid (8.51 mol %), SDA (8.39 mol %), oleic acid (5.04 mol %), and stearic acid (2.54 mol %) were the dominant FAs, which were in agreement to our previous study.[16] The major FAs saponified from DHASCO were DHA (45.51 mol %), followed by oleic acid (23.07 mol %), myristic acid (10.94 mol %), and palmitic acid (7.45 mol %).

During lipase-catalyzed transesterification, FAs are cleaved off the glycerol backbone and not all of these FAs reattach to the TAG, resulting in FFAs. FFA contents of substrates and products are shown in Table 3.2. FFA percentage of NSL after reaction was higher than that of the original SDASO with a significant difference at $P < 0.05$. SPD removed most of the liberated FFAs and restored FFA content similar to fresh oil status (below 0.1%). For LDHA produced by acidolysis, a much higher FFA content (26.95%) was present in the product after reaction because DHASCO-FFAs were one of the starting substrates. Repetitive SPD was required to remove most of the FFA, but FFA percentage (0.35%) was not as low as substrate NSL ($P < 0.05$).

Tocopherol and tocotrienol concentrations of substrates, SLs before and after SPD, and distillates after SPD were measured by normal phase HPLC and are shown in Table 3.2. The elution order of tocopherols and tocotrienols was α-tocopherol, α-tocotrienol, β-tocopherol, γ-tocopherol, γ-tocotrienol, δ-tocopherol, and δ-tocotrienol. Due to the instability of these compounds, all of the test samples were protected from light and oxygen, and analyzed on the day of preparation. According to the law of conservation of mass, loss percentages for different reactions (transesterification and

acidolysis) as well as SPD were calculated, and average values were reported (Table 3.2). For both NSL and LDHA, more vitamin E isomers were lost after SPD than during enzymatic production. Specifically, there were 59 and 73% total losses of tocopherols and tocotrienols during purification by SPD compared to 3 and 21% losses during enzymatic production of NSL and LDHA, respectively. These lost tocopherols and tocotrienols after SPD were found to be present in the distillate at high concentration, which suggests that they should not be discarded as wastes. Instead, they should be recovered and added back to SLs to maintain or protect the oxidative stability of final products. As shown in Table 3.2, losses of α-, β-, γ-, and δ-tocopherols after SPD for LDHA were 61, 100, 86, and 88%, respectively, with 52, 51, 57, and 68% losses for NSL. A possible explanation for the higher loss after SPD in LDHA than NSL may be due to repetitive passes to remove FFAs after acidolysis and therefore longer exposure to heat and light. Another observation was a higher loss of vitamin E isomers during acidolysis of LDHA (27, 20, 27, and 20% for α-, β-, γ-, and δ-tocopherols, respectively) compared to transesterification of NSL (5, 8, 1, and 7%). There are two possible explanations. First, longer reaction time under heat for LDHA (24 h) may contribute in part to the difference. Second, higher amounts of tocopheryl and tocotrienyl esters were estimated to have been formed by acidolysis than transesterification due to the availability of carboxylic acids. As can be seen in Table 3.2, for NSL and LDHA, there was a significant difference in loss percentage ($P < 0.05$) among tocopherol and tocotrienol species during either enzymatic reaction or purification. This may be possibly due to the preference of tocopherols or tocotrienols to form esters during transesterification and acidolysis. As mentioned above, these esters were removed into

the distillate by SPD and contributed to the loss of tocopherols and tocotrienols. This may be partly attributed to relative sensitivity of these isomers in response to environmental stresses (e.g., oxygen, heat, light, and exposure time). For tocotrienols in LDHA, it was expected that tocotrienols will degrade more than corresponding tocopherols during both acidolysis and SPD because of the presence of three unsaturated double bonds in the isoprenoid side chain, like γ-tocotrienol in this study. Surprisingly, α-tocotrienol had a much lower loss than α-tocopherol and other vitamin E isomers. The reason is unclear, and it is unlikely that the structural difference had any effect. Lee and Park (2008) studied the stability of vitamin E isomers extracted from rice bran oil under various temperature and oxygen conditions. They found that α-tocotrienol degraded more rapidly than other isomers, whereas γ-tocopherol was the most stable isomer. Oxygen level also had significant impact on the stability of each isomer in their studies, where severe reductions in γ-tocopherol (by 20%) and γ-tocotrienol (29%) were observed under 2% oxygen conditions, whereas under 0% no degradation was observed even after exposure to 95 °C for 4 h.

Two mass spectrometric techniques, MALDI-TOF-MS and GC-MS, were employed to confirm the hypothesis that tocopheryl and tocotrienyl fatty acid esters were formed during both acidolysis and transesterification. A number of matrices were investigated for the MALDI analysis including 2,5-dihydroxybenzoic acid, α-cyano-4-hydroxycinnamic acid, and sinapinic acid. 2,5-Dihydroxybenzoic acid proved to work best in producing a mass profile of vitamin E linoleate mixture standard in a very clear and systematic manner (Figure 3.1). It can be seen that the spectra of α-tocopheryl oleate, α-tocopheryl linoleate, and α-tocopherol were dominated by sodiated molecules ([M +

Na]$^+$) and showed no protonated molecules ([M + H]$^+$). α-Tocopheryl oleate and linoleate also yielded potassium adduct ions ([M + K]$^+$). After subtraction of the background and correction for isotope contributions, the data produced from distillates and residues of NSL and LDHA were identified on the basis of molecular masses, [M + Na]$^+$ and [M + K]$^+$, which were calculated according to the fatty acid and vitamin E isomer profiles of substrates. Unexpectedly, none of the ester ions were found in the spectra. This may be due to a strong "matrix effect" from abundant endogenous compounds, such as TAGs, diacylglycerols, monoacylglycerols, and FFAs, which interfere with tocopheryl and tocotrienyl fatty acid esters present at much lower levels.

However, this challenge was overcome by selection of GC-MS in synchronous scan/SIM mode to achieve column baseline separation and mass spectrometric separation as well. Synchronous scan/SIM is an acquisition technique by rapid alternation between a full scan and SIM modes, which enables simultaneous collection of common full scan data (qualitative) and SIM data (quantitative) in a single run without sacrificing performance (Meng, 2005). The SIM mode offers significant improvement in sensitivity over full scan mode; thus, it can be utilized to track signals of trace level compounds by eliminating the matrix noise. Because the data were acquired simultaneously, the uncertainty in compound identification was minimized and sample analysis time was reduced. Due to high molecular weight and boiling points of tocopheryl and tocotrienyl esters, high column temperature was used to separate these compounds. Total ion chromatograms of identified tocopheryl and tocotrienyl esters in the distillates of NSL and LDHA in the SIM mode and some typical full scan mass spectra are presented in Figures 3.2 and 3.3, respectively. The peak order of esters was δ-, β(or γ)-, and α-moiety,

separately. Tocopheryl/tocotrienyl fatty acid esters have their own unique EI fragmentation patterns. Taking tocopheryl linoleate as an example (Figure 3.4), after ionization, the molecular ion of tocopheryl linoleate undergoes a rearrangement involving hydrogen transfer from α-carbon to ester oxygen to remove the linoleic acid moiety. The resulting fragments include a neutral ketene molecule and a radical cation of the phenyl alcohol at m/z 430, 416, 416, and 402, respectively (α-, β-, γ-, and δ-tocopheryl linoleate, respectively). The tocopherol ion (base peak) is further fragmented by retro-Diels−Alder reaction with hydrogen transfer into an intense ion at m/z 165, 151, 151, and 137, respectively, and also by α-cleavage into a weaker ion at m/z 205, 191, 191, and 177, respectively. The β- and γ-isomers may not be discriminated because they have the same number of methyl groups at different positions in the chroman ring (Figure 3.4). The fragmentation mechanism was supported by direct analysis of vitamin E linoleate mixture standard (Figure 3.3) and previous studies (Klink, Buchs, & Gulacar, 1994; Pereira et al., 2002). Similarly, tocotrienyl esters undergo the same fragmentation routes as tocopheryl esters, creating different tocotrienyl ions (base peak) but the same secondary fragmented ions (Figure 3.3). As a result, these characteristic fragment ions were chosen in synchronous scan/SIM mode to track and distinguish different types (α, β, γ, or δ) of tocopheryl/tocotrienyl moieties. Finally, α-, β-, γ-, and δ-tocopheryl/tocotrienyl fatty acid esters, although very low in the samples, could be identified on the basis of additional information from observed molecular ions and theoretical molecular mass calculation. Meanwhile, none of these esters were found in the residues of either NSL or LDHA, which indicates that all of the tocopheryl and tocotrienyl esters formed during acidolysis and transesterification were fractionated into distillates after SPD.

Furthermore, under the same analytical conditions, GC-MS/PCI was employed to verify the identification of the tocopheryl and tocotrienyl esters in the distillates, the molecular ions of which were produced at extremely weak abundance in EI mode (Figure 3.3). PCI is a soft ionization technique, and pseudomolecular ion, $[M + NH_4]^+$, is yielded at relatively high intensity when using ammonia as the reagent gas. Tocopheryl and tocotrienyl fatty acid esters identified by GC-MS in EI and PCI modes are summarized in Table 3.3. For NSL by transesterification, δ- and β(or γ)-tocopheryl palmitate, β(or γ)-tocopheryl oleate, and β(or γ)-tocopheryl linoleate were confirmed in both EI and PCI modes, whereas δ- and β(or γ)-tocopheryl myristate, δ- and α-tocopheryl oleate, δ- and α-tocopheryl linoleate, α-tocopheryl palmitate, and δ- and β(or γ)-tocopheryl stearate were confirmed only in EI mode. In the case of LDHA by acidolysis, δ- and β(or γ)-tocopheryl palmitate, α-tocopheryl palmitate, α-tocopheryl oleate, and α-tocopheryl linoleate were verified in PCI and EI modes, whereas β(or γ)-tocopheryl oleate, β(or γ)- tocopheryl linoleate and α-tocotrienyl palmitate were verified only in EI mode. The dominant esters formed were tocopheryl palmitate, tocopheryl oleate, and tocopheryl linoleate homologues, which were found in both NSL and LDHA. However, tocopheryl myristate and stearate and δ-tocopheryl linoleate and oleate were identified only in NSL even though a higher content of corresponding fatty acids was present in the reaction medium of LDHA (Table 3.1), and α-tocotrienyl palmitate was solely formed in LDHA. It is likely that vitamin E isomers in the reaction mixture, not FFA, primarily play a role in the formation of tocopheryl and tocotrienyl fatty acid esters. It is also important to note that none of the tocopheryl/tocotrienyl linolenate, stearidonate, and docosahexaenoate were identified in either NSL catalyzed by nonspecific lipase or LDHA catalyzed by *sn*-1,3

specific lipase. This may be due to the preference of lipases, Lipozyme TL IM and Novozym 435, for a specific fatty acid or fatty acids with a certain chain length range and unsaturation (Weete, Lai, & Akoh, 2008). Peng, Xu, Mu, Hoy, and Adler-Nissen (2002) reported similarly that Lipozyme TL IM showed a slight discrimination over very long chain polyunsaturated fatty acids, eicosapentaenoic acid and DHA, compared to conjugated linoleic acid and caprylic acid.

The assumption that tocopheryl and tocotrienyl fatty acid esters are formed during transesterification in addition to acidolysis was successfully confirmed. All of the esters were fractionated into distillates after SPD. This is the first report that determined these trace-level esters in distillates by GC-MS in EI and PCI modes on the basis of synchronous scan/SIM acquisition technique. Furthermore, >50% of vitamin E isomers were lost into distillates during SPD, which contributed mostly to the rapid oxidative deterioration of SLs in the past studies and to a lesser extent from the formation of tocopheryl and tocotrienyl esters. This implies that these lost tocopherols and tocotrienols in the distillates, previously discarded as wastes, may be recovered and even added back to the final oil products (SLs) to improve their oxidative stability.

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Table 3.1 Total Fatty Acid Profiles (Mole Percent) and Molecular Weight of Substrates and Scaled-up SLs at Optimal Conditions[a]

fatty acid	SDASO (866.74)[b]	DHASCO-FFAs (287.46)	NSL (827.76)	LDHA (835.83)
C8:0	ND	0.46±0.00	ND	ND
C10:0	ND	1.74±0.02a	ND	0.38±0.01b
C12:0	ND	5.48±0.05a	ND	1.31±0.01b
C14:0	0.10±0.00d	10.94±0.10a	1.42±0.00c	3.60±0.01b
C14:1	ND	0.25±0.00a	ND	0.06±0.00b
C16:0	13.93±0.03c	7.45±0.08d	67.49±0.12a	55.94±0.45b
C16:1n-7	0.11±0.00c	3.21±0.01a	0.05±0.01d	0.78±0.01b
C17:0	0.08±0.07a	ND	0.07±0.01b	0.07±0.00b
C18:0	3.87±0.04a	0.72±0.02d	2.54±0.01b	2.14±0.00c
C18:1n-9	13.21±0.15b	23.07±0.12a	5.04±0.01d	9.40±0.16c
C18:2n-6	24.22±0.07a	1.17±0.00d	8.51±0.02b	7.26±0.09c
C20:0	0.21±0.00a	ND	0.08±0.00b	0.07±0.00c
C18:3n-6	7.22±0.03a	ND	2.44±0.01b	2.29±0.03c
C20:1n-9	0.17±0.00a	ND	0.07±0.00b	0.07±0.00b
C18:3n-3	11.01±0.07a	ND	3.71±0.02b	3.06±0.04c
C18:4n-3	25.74±0.14a	ND	8.39±0.03b	7.97±0.07c
C22:0	0.11±0.00a	ND	0.04±0.00b	0.05±0.01b
C24:0	0.03±0.00a	ND	0.01±0.00b	0.01±0.00b
C22:6n-3	ND	45.51±0.38a	ND	5.43±0.06b

[a]Mean ±SD, $n = 3$. Values with different letter in the same row are significantly different by Duncan's multiple-range test ($P < 0.05$). Abbreviations: SDASO, stearidonic acid soybean oil; DHA, docosahexaenoic acid; DHASCO-FFAs, free fatty acids hydrolyzed from DHA single-cell oil; NSL, structured lipid of SDASO/tripalmitin (1:2, mol/mol) at 65 °C for 18 h catalyzed by 10% Novozym 435; LDHA, structured lipid of DHA/NSL (1:1, mol/mol) at 65 °C for 24 h catalyzed by 10% Lipozyme TL IM; ND, not detected.
[b]Average molecular weight (in parentheses), $n = 3$.

Table 3.2 Tocopherol and Tocotrienol Concentration and Free Fatty Acid Percentage of Substrates and Scaled-up SLs[a]

	tocopherol and tocotrienol contents (μg/g)							
	α-T	α-T_3	β-T	γ-T	γ-T_3	δ-T	total	FFA (%)
NSL								
SDASO (Sr)	63.01±1.82b	ND	10.82±0.93b	565.83±4.83b	ND	192.20±5.52b	831.87±13.09b	0.02±0.00b
NSL before SPD	20.88±1.35c (5%C)	ND	3.48±0.08c (8%A)	195.11±8.28c (1%D)	ND	62.57±2.69c (7%B)	282.04±12.41c (3%)	2.17±0.03a
NSL after SPD	10.20±0.28d (52%C)	ND	1.73±0.18d (51%D)	87.14±0.20d (57%B)	ND	20.73±0.16d (68%A)	119.81±0.82d (59%)	0.03±0.01b
W_{NSL} after SPD	694.03±26.35a	ND	155.94±5.49a	4170.62±66.92a	ND	1890.31±12.70a	6910.9±47.78a	NA
LDHA								
DHASCO-FFAs (Sr)	26.76±0.28b	174.60±2.22a	6.09±0.17a	72.64±1.73c	4.74±0.12a	38.04±3.89b	322.87±8.42b	NA
NSL after SPD (Sr)	10.20±0.28c	ND	1.73±0.18d	87.14±0.20b	ND	20.73±0.16c	119.81±0.82d	0.03±0.01c
LDHA before SPD	10.57±0.22c (27%B)	40.46±0.48c (10%D)	2.28±0.01c (20%C)	61.05±0.53d (27%B)	0.63±0.05b (48%A)	20.24±0.16c (20%C)	135.23±1.44c (21%)	26.95±0.02a
LDHA after SPD	5.23±0.09d (61%D)	28.93±0.53d (39%E)	ND (100%A)	13.92±0.20e (86%C)	ND (100%A)	3.75±0.02d (88%B)	51.82±0.84e (73%)	0.35±0.01b
W_{LDHA} after SPD	29.30±0.30a	110.72±1.42b	5.08±0.16b	133.11±1.51a	ND	52.80±2.36a	331.00±5.75a	NA

[a]Mean ±SD, n = 3. Values with different lower case letters in the same column for each SL are significantly different by Duncan's multiple-range test ($P < 0.05$). Average loss percentage of the tocopherol or tocotrienol is given in parentheses, n = 3. Values with different upper case letters in the same row are significantly different by Duncan's multiple-range test ($P < 0.05$). Abbreviations: T, tocopherol; T_3, tocotrienol; FFA, free fatty acid; SPD, short-path distillation; Sr, substrate; SDASO, NSL, LDHA, and DHASCO-FFAs: see Table 3.1 for explanation of abbreviations; W_{NSL} and W_{LDHA}, distillate by short-path distillation from NSL and LDHA, respectively; ND, not detected; NA, not applicable.

Table 3.3 Tocopheryl and Tocotrienyl Esters Identified by GC-MS in Distillates after Short-Path Distillation[a]

			GC-MS/EI[b]			GC-MS/PCI	W_{NSL}		W_{LDHA}	
no.[c]	tocopheryl/tocotrienyl ester	M_r	base peak	M^+	other ions	$[M+NH_4]^+$	EI	PCI	EI	PCI
1	δ-tocopheryl myristate	612	402 (100)	612 (2)	137 (18), 177 (9)	630	+	–	–	–
2	β (or γ)-tocopheryl myristate	626	416 (100)	626 (2)	151(27), 191(5)	644	+	–	–	–
3	δ-tocopheryl palmitate	640	402 (100)	640 (2)	137 (16), 177 (7)	658	+	+	+	+
4	β (or γ)-tocopheryl palmitate	654	416 (100)	654 (1.5)	151(25), 191(4)	672	+	+	+	+
5	δ-tocopheryl oleate	666	402 (100)	666 (1.5)	137 (17), 177 (8)	684	+	–	–	–
6	δ-tocopheryl linoleate	664	402 (100)	664 (1.5)	137 (19), 177 (8)	682	+	–	–	–
7	α-tocopheryl palmitate	668	430 (100)	668 (1.5)	165 (23), 205 (3)	686	+	–	+	+
8	δ-tocopheryl stearate	668	402 (100)	668 (1.2)	137 (17), 177 (6)	686	+	–	–	–
9	β (or γ)-tocopheryl oleate	680	416 (100)	680 (1.5)	151(27), 191(4)	698	+	+	+	–
10	β (or γ)-tocopheryl linoleate	678	416 (100)	678 (1.8)	151(28), 191(4)	696	+	+	+	–
11	β (or γ)-tocopheryl stearate	682	416 (100)	682 (1)	151(26), 191(3)	700	+	–	–	–
12	α-tocotrienyl palmitate	662	424 (100)	662 (3)	165 (28), 205 (12)	680	–	–	+	–
13	α-tocopheryl oleate	694	402 (100)	694 (1)	165 (27), 205 (4)	712	+	–	+	+
14	α-tocopheryl linoleate	692	402 (100)	692 (1)	165 (28), 205 (4)	710	+	–	+	+

[a] Abbreviations: M_r, molecular weight; W_{NSL} and W_{LDHA}: see Table 3.2 for explanation of abbreviations; M^+ and $[M+NH_4]^+$, molecular ion formed by EI and PCI, respectively; +, detected; −, not detected. [b]Characteristic ions formed by EI and their relative abundance (%) (in parentheses). [c]Number corresponds to the numbers in Figure 3.2.

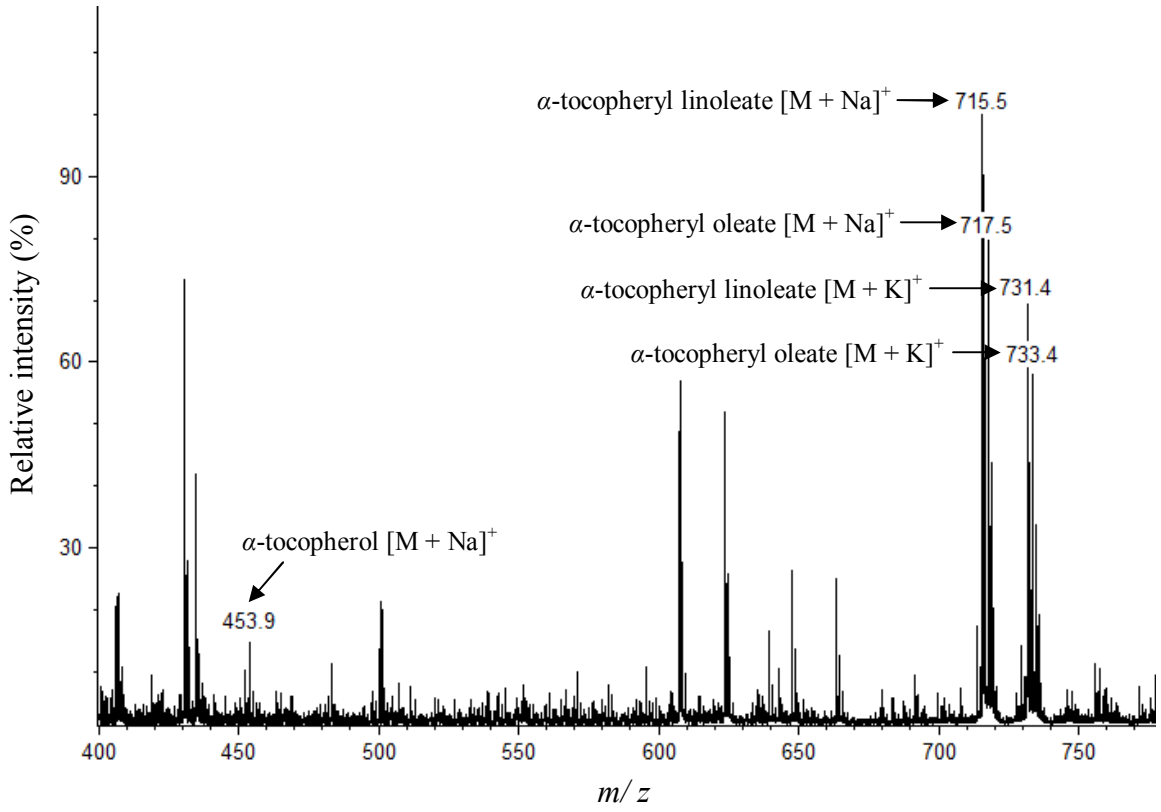

Figure 3.1 MALDI-TOF mass spectra of α-tocopherol, α-tocopheryl oleate and α-tocopheryl linoleate from vitamin E linoleate mixture in positive ion mode.

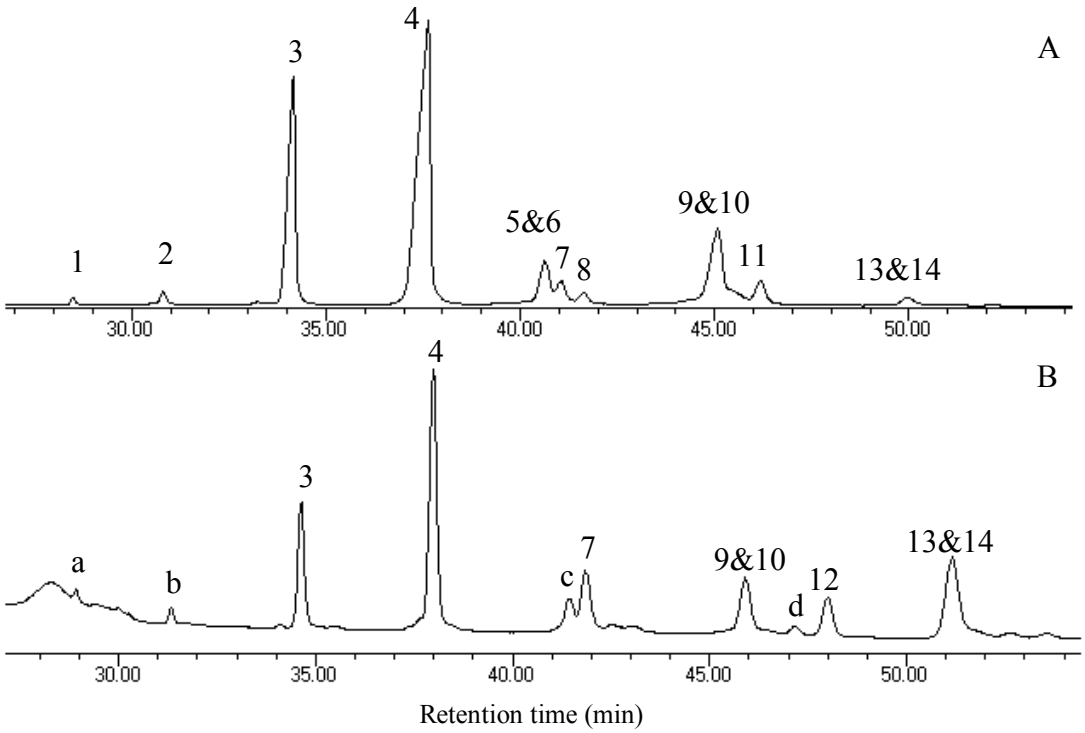

Figure 3.2 Total ion chromatograms of tocopheryl and tocotrienyl esters identified in the (A) W_{NSL} and (B) W_{LDHA} in the SIM mode. Peak numbers correspond to the esters listed in Table 3.3; a, b, c and d are unknown compounds. (W_{NSL} and W_{LDHA}, distillate by short-path distillation from NSL and LDHA, respectively.)

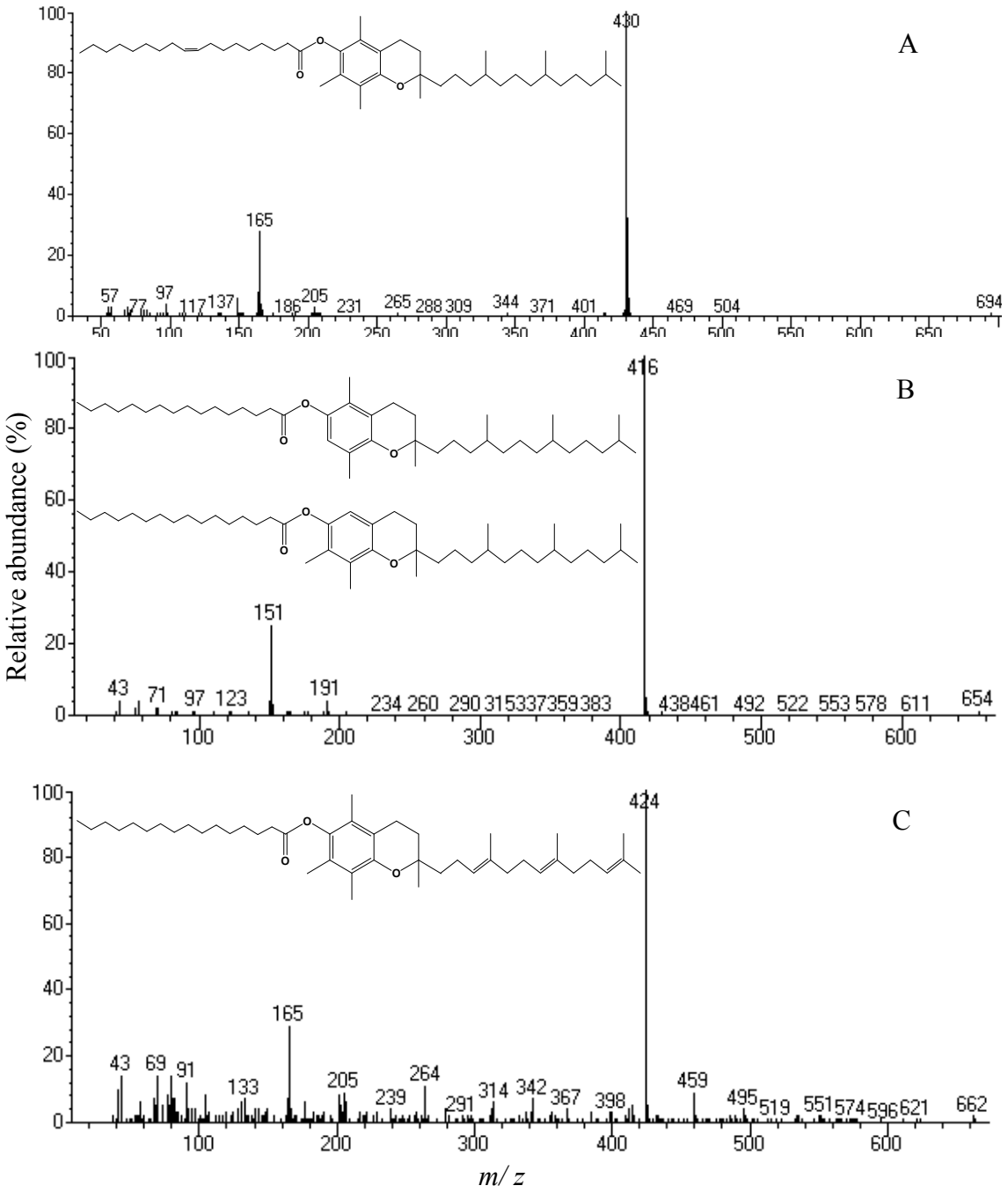

Figure 3.3 Representative structures and EI full scan mass spectra of tocopheryl and tocotrienyl esters: (A) *α*-tocopheryl oleate from vitamin E linoleate mixture, (B) *β*(or *γ*)-tocopheryl palmitate from W_{NSL}, and (C) *α*-tocotrienyl palmitate from W_{LDHA}. (W_{NSL} and W_{LDHA}, distillate by short-path distillation from NSL and LDHA, respectively.)

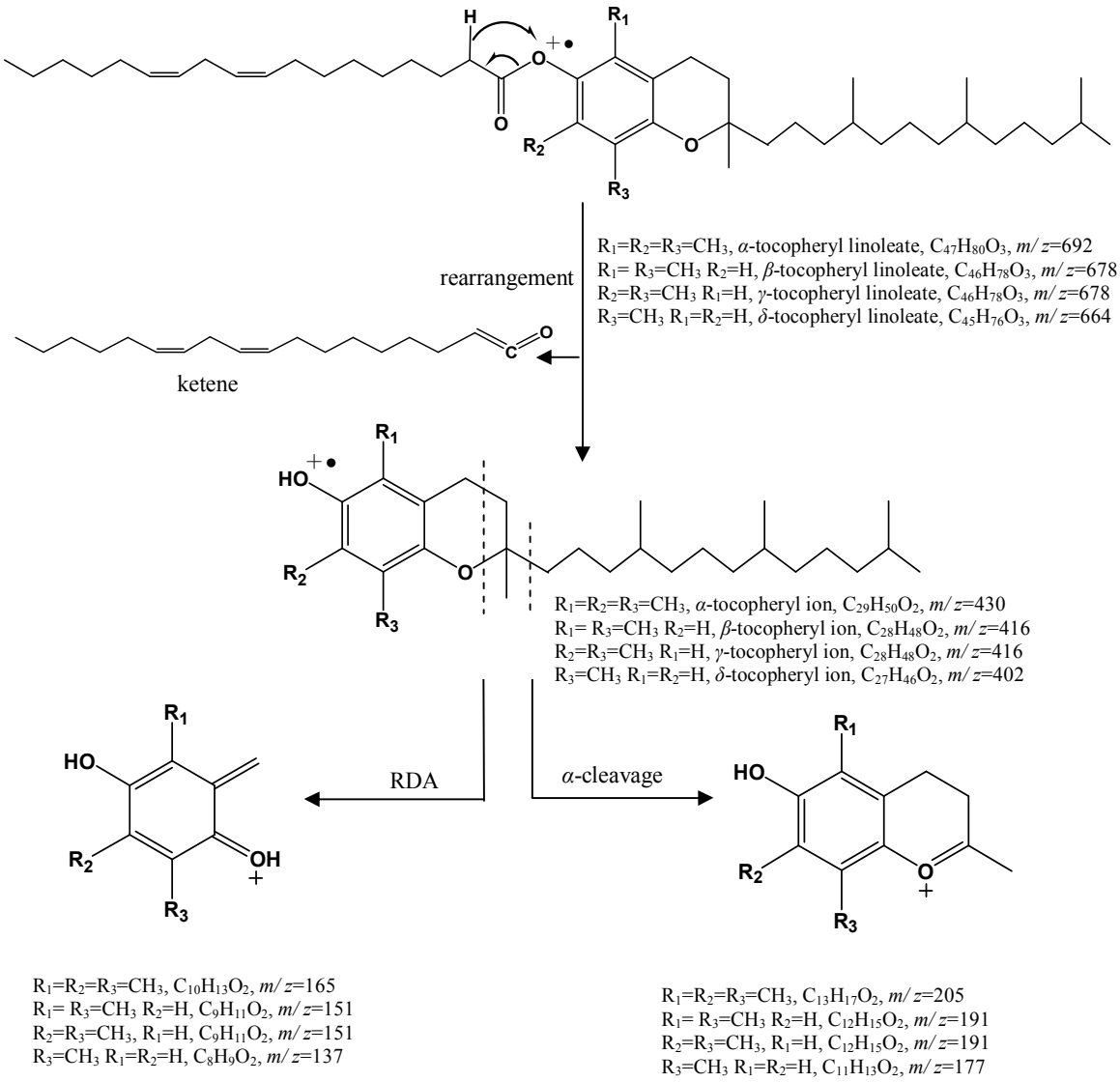

Figure 3.4 Mass spectrometric fragmentation mechanism of α-, β-, γ-, and δ- tocopheryl linoleate in the EI mode. (RDA, retro-Diels−Alder reaction.)

CHAPTER 4

CHARACTERIZATION AND OPTIMIZATION OF PHYSICAL AND OXIDATIVE STABILITY OF STRUCTURED LIPID-BASED INFANT FORMULA EMULSION: EFFECTS OF EMULSIFIERS AND BIOPOLYMER THICKENERS[1]

[1] Zou, L., and Akoh, C. C. 2013. *Food Chemistry*, 141, 2486-2494. Reprinted here with permission of the publisher.

Abstract

Response surface methodology (RSM) was applied to investigate the effects of lecithin (0-0.4 g/100 mL), monoacylglycerol (0-0.4 g/100 mL), locust bean gum (LBG; 0-0.1 g/100 mL), and carrageenan (0-0.02 g/100 mL) on the physical and oxidative properties of structured lipid-based infant formula emulsion containing dairy proteins, lactose, vitamins, minerals, and other micronutrients. Particle size, optical stability, viscosity, relative content of docosahexaenoic acid and stearidonic acid, and total oxidation value were assessed during 28-day storage. ANOVA results showed that the experimental data were satisfactorily fitted to second-order polynomial models by multiple linear regression. The contour plots illustrated that lecithin and monoacylglycerol played a dominant role in controlling the emulsion stability compared to LBG and carrageenan. Lecithin content significantly affected all the responses measured, particularly lipid oxidation. Increasing monoacylglycerol concentration led to an increase in particle size and emulsion viscosity. The optimal condition to achieve the highest stability was predicted to be 0.2, 0.4, 0.045, and 0.015 g/100 mL lecithin, monoacylglycerol, LBG, and carrageenan, respectively. The verification data further demonstrated the suitability of the models explored by RSM. Overall, the findings obtained in this study have important implications for the successful incorporation of structured lipid into infant formula emulsion for better infant nutrition and health.

Keywords: infant formula; structured lipid; emulsion stability; lipid oxidation; response surface methodology; stearidonic acid soybean oil

Introduction

Human milk contains 3−5% fat with 98−99% in the form of triacylglycerols (Jensen, 1996). Human milk fat (HMF) provides essential fatty acids (FAs) and >50% of the dietary energy required by the infants (Wells, 1996). The structure of HMF with 60−70% palmitic acids esterified at *sn*-2 position and unsaturated FAs mainly at *sn*-1,3 positions (Jensen, 1996), is metabolically different from that of physically blended vegetable oils used in infant formula (IF). It improves absorption of palmitic acid (energy) and calcium, and has substantial benefits for the growth of infants (Lien, 1994; Sheila, 2011). Numerous studies have shown that omega-3 long chain polyunsaturated fatty acids (omega-3 LCPUFAs), mainly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), provide extensive health benefits associated with cardiovascular diseases, cognitive disorders, immune dysfunction, and inflammatory diseases (Connor, 2000; Simopoulos, 1991). DHA is also essential for the normal functional development of retina and brain in infants (Connor, 2000; Simopoulos, 1991). Stearidonic acid (SDA), an omega-3 LCPUFA in stearidonic acid soybean oil (SDASO), converts to EPA more efficiently than α-linolenic acid by skipping Δ6-desaturase rate-limiting step (Lemke et al., 2010). Therefore, a structured lipid (SL), which mimics the unique structure of HMF as well as containing DHA and SDA in the triacylglycerol backbone, may maximize health benefits for infants.

Human milk is the best source of nutrients for infants. However, it is not always possible to feed infants with human milk due to physiological and cultural reasons, thus an alternative formula is needed. Ready-to-feed IF is an oil-in-water (O/W) emulsion system, generally produced by homogenizing an oil phase in an aqueous phase mainly

consisting of dairy proteins, carbohydrate, minerals and vitamins. The major physical instability of IF emulsion is creaming, in combination with droplet aggregation (Fligner, Fligner, & Mangino, 1990; McSweeney, 2008), reducing the shelf life of the final products. Lipid oxidation is another unfavorable process in most foods containing omega-3 LCPUFAs. It produces toxic compounds, and alters flavor, texture, appearance, and nutritional value of foods (McClements & Decker, 2000), which are unacceptable to consumers. Therefore, stability of IF emulsion to creaming and oxidation is of practical significance to food scientists and manufacturers.

Governed by Codex Alimentarius regulations (CAC, 1981), lecithin and monoacylglycerol, and locust bean gum (LBG) and carrageenan, are the only non-protein based emulsifiers and thickeners permitted in regular milk-based liquid IF, respectively. Being low-molecular weight oil-soluble surfactants, lecithin (zwitterionic) and monoacylglycerol (non-ionic), may influence the stability of dairy emulsion through several mechanisms: 1) reduce surface tension more rapidly than milk proteins, facilitating the formation of smaller droplets; 2) displace adsorbed milk proteins from O/W interface; 3) interact with interfacial proteins, leading to a thicker and stronger adsorbed layer; 4) increase the viscosity of aqueous phase through the formation of self-bodying mesophase structures (Dickinson, Mauffret, Rolfe, & Woskett, 1989; McSweeney, 2008). Lecithin was also found to be able to increase heat stability of model IF emulsion through the formation of protein-phospholipid complexes, whereas monoacylglycerol reduced heat stability due to interfacial protein displacement (McSweeney, Healy, & Mulvihill, 2008). Moreover, the effects of these emulsifiers on droplet characteristics, such as interfacial thickness and density, size, and charge,

subsequently impact lipid oxidation. Fomuso, Corredig and Akoh (2002) found that emulsifier type and concentration influenced oxidation rate of fish oil-based SL emulsions. In addition to emulsifiers, polysaccharides have been found to not only increase emulsion viscosity to stabilize droplets against creaming, but also retard lipid oxidation in O/W emulsions due to free radical scavenging and transition metal chelating capabilities (McClements et al., 2000; Waraho, McClements, & Decker, 2011). Trommer and Neubert (2005) reported that LBG can reduce the level of lipid peroxidation products. Carrageenan was found to increase the oxidative stability of menhaden oil-in-water emulsions since its anionic groups can bind to cationic metals (Chen, McClements, & Decker, 2010).

Response surface methodology (RSM) is a collection of mathematical and statistical methods used to model and optimize the processes in which response variables are affected by multiple independent variables, alone or in combination (Myers, Montgomery, & Anderson-Cook, 2011). This procedure not only determines the interaction between independent variables, but also reduces the number of experimental trails, leading to time and cost saving. To the best of our knowledge, there is no specific research reported on application of SLs in an actual food system—an infant formula emulsion containing dairy proteins, lactose, lipid, vitamins, minerals and other nutrients. Therefore, the objective of this study was to develop a physically and oxidatively stable SL-based IF emulsion to deliver HMF analogues enriched with DHA and SDA for infant nutrition and health. RSM was used to optimize the levels of emulsifiers (lecithin and monoacylglycerol) and thickeners (LBG and carrageenan) needed to achieve a target

value of particle size (~1 μm), the minimum optical change, a suitable viscosity, the lowest loss of DHA and SDA, and the least lipid oxidation products.

Materials and methods

Materials

SDASO was kindly donated by Monsanto Co. (St. Louis, MO). Tripalmitin was purchased from TCI America (Portland, OR), and docosahexaenoic acid single-cell oil (DHASCO) containing 40% DHA was obtained from Martek Bioscience Corp., now DSM Nutritional Products Ltd. (Columbia, MD). *sn*-1,3 specific lipase Lipozyme TL IM and nonspecific lipase Novozym 435 were obtained from Novozymes A/S (Bagsvaerd, Denmark). High heat nonfat dry milk, CM-993X κ/λ-carrageenan, and LBG were generously provided by O-AT-KA Milk Products Cooperative, Inc. (Batavia, NY), Ingredients Solutions, Inc. (Waldo, ME), and TIC Gums, Inc. (Waldo, ME), respectively. Distilled monoacylglycerol, Dimodan HS K-A, was a product from Danisco USA Inc. (New Century, KS). A de-oiled enzyme-modified soy lecithin powder, Alcolec EM, was kindly donated by American Lecithin Co. (Oxford, CT). The main phospholipids present in the lecithin were 24 wt% phosphatidylcholine and lysophosphatidylcholine in total as indicated by the manufacturer. α-Lactalbumin enriched whey protein concentrate and 5120 IF lactose were kindly donated by Hilmar Ingredients (Hilmar, CA). A micronutrient premix mainly consisting of vitamins, minerals and nucleotides (Table 4.1) was custom-formulated by Fortitech, Inc. (Schenectady, NY).

Production and characterization of structured lipids

The DHA and SDA-containing HMF analogue was enzymatically synthesized by a two-step procedure using optimal conditions previously reported (Teichert & Akoh,

2011; Zou & Akoh, 2013). First, structured lipid from SDASO and tripalmitin, called NSL, was produced through interesterification catalyzed by Novozym 435. Second, NSL was reacted with FAs saponified from DHASCO by acidolysis using Lipozyme TL IM as a biocatalyst. SL products were next purified by short-path distillation using a KDL-4 (UIC Inc., Joliet, IL) system as described by (Zou et al., 2013). FA profile, free fatty acid (FFA) percentage, and tocopherol content were determined according to the methods previously reported (Teichert et al., 2011; Zou et al., 2013). All samples were analyzed in triplicate.

Preparation of infant formula emulsion

According to the formulation described in Table 4.2, the SL-based O/W IF emulsion was prepared as follows: high heat nonfat dry milk, α-lactalbumin enriched whey protein concentrate, lactose, and thickeners (LBG and/or carrageenan) were dissolved in deionized water at 60 °C with stirring and then hydrated overnight at 4 °C to ensure complete dispersion and hydration. Lecithin and/or monoacylglycerol were dispersed in SL at 65 °C. The oil phase was added to the aqueous phase while stirring, and the blend was tempered at 60 °C. Then the micronutrient premix and sodium azide (0.01%, w/v) were added. The coarse emulsion was prepared using a Polytron high-speed batch disperser (Kinematica, Inc., Bohemia, NY) at setting 5 for 2 min. The coarse emulsion was then passed 3 times through an EmulsiFlex-C5 high-pressure homogenizer (Avestin, Inc., Ottawa, Canada) at 5000−7000 psi (34.47−48.26 MPa). After adjustment of pH to 6.8 with 0.1 mol/L sodium hydroxide, the mixture was filled into a container and sterilized in an autoclave at 121 °C for 6 min. After sterilization, the emulsion was cooled

down as quickly as possible and stored at room temperature for 28 days. All the emulsion samples were prepared in duplicates.

Measurement of particle size distribution

The particle size distribution was determined using a LS 13 320 MW laser diffraction particle size analyzer (Beckman Coulter, Inc., Miami, FL). To avoid multiple scattering effects, samples were added dropwisely to deionized water in the dispersion cell until obscuration value reached 8−10%. The real and imaginary refractive index (RI) of particle was 1.471 and 0, respectively, and RI of dispersant was 1.332. Each emulsion sample was analyzed in triplicate, and surface-area-average diameter ($D_{3,2}$) was reported.

Optical analysis by Turbiscan

Turbiscan, based on multiple light scattering, is an optical analyzer, which can be used to monitor reversible (creaming and sedimentation) and irreversible (coalescence and flocculation) destabilization phenomena in a concentrated and opaque emulsion without dilution (Mengual, Meunier, Cayre, Puech, & Snabre, 1999). It can detect phase changes much earlier and easier than classic approaches (Mengual et al., 1999).

Seven milliliters of emulsion sample was placed in a flat-bottomed screw-cap cylindrical glass tube, tightly sealed, and stored at room temperature without shaking. The tube was then scanned by Turbiscan Classic MA 2000 (Formulaction Inc., Davie, FL), equipped with a pulsed near infrared light source (λ = 880 nm). Light transmitted through and backscattered by the sample was received by two synchronous detectors at an angle of 0° and 135° with respect to the incident beam, respectively. A plot was produced by the software with transmission (T) or backscattering (BS) signal on the *y*-axis and the sample height (mm) on the *x*-axis. A height of 0 mm corresponded to the

bottom of tube. The same sample measured at different times (day 0 and 28) was recorded in the same file to easily calculate the variations of T or BS signal. In the reference mode of software, after subtracting values of first scan (day 0), mean value kinetics of BS signal with 0% T at the height of 2-12 (creaming layer) and 20-30 mm (aggregation layer) were obtained, respectively. The sum value ($\sum \Delta BS$) was reported to evaluate optical change.

Rheological properties

Steady shear viscosity was measured using a SR-5000 stress-controlled rheometer (Rheometric Scientific, Inc., Piscataway, NJ), equipped with a double-wall couette geometry and a bath temperature controller. Fifteen milliliters of emulsion sample was placed in the double-wall couette, and left for 2 min before starting measurements. A gap of 0.5 mm was used. Steady stress sweep test was performed at 25 °C over a shear stress range of 0.005 to 5 Pa. All the samples (day 0) were tested within 24 h after emulsion preparation. Power law model was used to fit the starting portion ($\dot{\gamma} \leq 20$ s^{-1}) of flow curve

$$\eta = K\dot{\gamma}^{n-1} \quad (1)$$

where η is apparent viscosity, K is consistency index, $\dot{\gamma}$ is shear rate, and n is flow behavior index. At low shear rate of 1 s^{-1} (similar to standing at rest), η is equal to K, which thus can be used to compare shear-thinning ($0 < n < 1$) or shear-thickening ($n > 1$) behavior of non-Newtonian fluids.

Relative content of DHA and SDA

Two milliliters of hexane was added to 300 μL of emulsion sample, mixed thoroughly, and centrifuged at 2000 rpm for 5 min. The upper phase was dried with nitrogen,

methylated, and then analyzed for its fatty acid composition as previously described (Zou et al., 2013). Due to higher stability to oxidation than DHA and SDA, saturated palmitic acid was selected as a reference fatty acid. The concentration ratio of palmitic acid to DHA and SDA ($C_{\text{palmitic acid}} / C_{(\text{DHA+SDA})}$) was calculated as a direct indicator of lipid oxidation.

Measurements of primary and secondary lipid oxidation products

Lipid hydroperoxide, measured as peroxide value (PV), was determined using a modified method of Shanta and Decker (Shantha & Decker, 1994). Briefly, 0.3 mL of emulsion sample was mixed with 1.5 mL of isooctane/2-propanol (3:2, v/v), and vortexed three times for 10 s each. After centrifugation at 2000 rpm for 5 min, 0.20 mL of the clear upper layer (isooctane as a blank) was mixed with 2.8 mL of methanol/1-butanol (2:1, v/v), 15 μL of 3.94 mol/L ammonium thiocyanate solution and 15 μL of 0.072 mol/L ferrous ion solution (freshly prepared from supernatant by mixing an equal amount of 0.132 mol/L $BaCl_2$ and 0.144 mol/L $FeSO_4$ in 0.4 mol/L HCl), and then vortexed. After incubation at room temperature for 20 min, absorbance of the solution in blood-red color was measured at 510 nm by a UV-1601 UV-visible spectrophotometer (Shimadzu Corp., Columbia, MD). PV (expressed as mmol/L emulsion) was quantified using a cumene hydroperoxide standard curve (5, 10, 50, 100, 500, and 1000 μmol/L in methanol), and calculated using the equation

$$PV = \frac{(A-b)\times 1.5\times 0.6}{a\times 0.3\times 1000} \tag{2}$$

where A is absorbance of the sample against blank. a and b are slope and *y*-intercept obtained from the standard curve, respectively.

Para-anisidine value (*p*-AnV) was determined according to the AOCS Official Method Cd 18-90 (AOCS, 2009) with minor modifications. Briefly, 2 mL of emulsion sample was added into a 25-mL volumetric flask and made up to the mark with isooctane. After mixing thoroughly, the sample was transferred to a 50-mL centrifuge tube and vortexed twice for 10 s each. After centrifugation at 5000 rpm for 10 min, absorbance (A_1) of the upper layer was measured at 350 nm against isooctane as a blank. Five milliliters of the upper layer or isooctane was then transferred to a 10-mL test tube. One milliliter of *p*-anisidine solution (0.25% in glacial acetic acid, w/v) was added and vortexed for 10 s. After incubation at room temperature for 10 min, absorbance (A_2) was measured at 350 nm against isooctane containing *p*-anisidine as a blank. *p*-AnV was determined using the equation

$$p\text{-}AnV = \frac{25 \times (1.2 \times A_2 - A_1)}{2} \quad (3)$$

The total oxidation (TOTOX) value was calculated as

$$TOTOX\ value = 2 \times PV + p\text{-}AnV \quad (4)$$

Experimental design and statistical analysis

The effect of four independent variables namely, lecithin (0, 0.2, 0.4 g/100 mL, X_1), monoacylglycerol (0, 0.2, 0.4 g/100 mL, X_2), LBG (0, 0.02, 0.1 g/100 mL, X_3), and carrageenan (0, 0.004, 0.02 g/100 mL, X_4), on the particle size (Y_1), optical change (Y_2), apparent viscosity (Y_3), $C_{\text{palmitic acid}} / C_{(\text{DHA+SDA})}$ (Y_4), and TOTOX value (Y_5), was evaluated by RSM using Modde 5.0 software (Umetrics AB, Umeå, Sweden). A D-optimal design (Table 4.3) generated twenty-seven experiments consisting of twenty-four different combinations and three more replicates of the center point. Experiment at each

design point was randomly performed in duplicate, resulting in a total of fifty-four runs. The relationship between the independent and response variables from the above design was explored by multiple linear regression, and fitted into a second-order polynomial equation

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^{\,2} + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j + \varepsilon_{ij} \quad (5)$$

where Y is the response variable. X_i and X_j are the independent variables. $\beta_0, \beta_i, \beta_{ii}$, and β_{ij} are constant, linear, quadratic, and interaction regression coefficients, respectively. ε_{ij} is the error term. Analysis of variance (ANOVA) was performed to investigate the goodness of model fitting based on coefficient of determination (R^2), adjusted R^2 (R^2_{adj}), and lack of fit test. It was also used to assess the significance of regression as well as each coefficient in the model based on *F*-test at $\alpha = 0.05$. The predictive power of the model was evaluated by Q^2, which was associated with the predicted residual sums of squares. The contour plot was employed to visualize the interaction of independent variables on the responses, and to identify the optimum region for desired responses. The independent variable with the greatest effect was kept on the *x*-axis, the second was placed on the *y*-axis, and the other two with the least effect were held constant. Optimal conditions were generated by optimizer function of the software. Finally, additional confirmation experiments under optimal and another four random conditions were carried out to verify the adequacy of the model. A Chi-squared test was performed to determine significant difference between the predicted and observed values ($\alpha = 0.05$).

Results and discussion

Compositional analysis

As shown in Table 4.2, the total energy density of IF was 65.2 kcal/100 ml, consisting of 5.3, 2.1, and 11.0 g/100 kcal lipid, protein and carbohydrate, respectively. These values, including micronutrient specifications (Table 4.1), meet the nutrient requirements by both FDA regulation 21CFR107.100 (FDA, 1985) and ESPGHAN recommended standards (Koletzko, Baker, Cleghorn, Neto, Gopalan, Hernell, et al., 2005). In addition, α-lactalbumin enriched whey protein concentrate was used to compensate for the protein difference between bovine milk and human milk. The ratio of casein:whey protein was kept at 40:60 to mimic mature human milk status (Wells, 1996). The major FAs of purified SL were palmitic acid (55.9 mol %), oleic acid (9.4 mol %), SDA (8.0 mol %), linoleic acid (7.3 mol %), and DHA (5.4 mol %), with 57.0 mol % palmitic acid esterified at *sn*-2 position, which demonstrates that this HMF analogue with health benefits of omega-3 FAs was successfully synthesized. After short-path distillation, FFA percentage and total vitamin E content of SL were reduced to 0.35% and 51.82 μg/g, respectively. Lecithin mainly contained linoleic acid (62.9 mol %), palmitic acid (19.7 mol %), oleic acid (8.3 mol %), stearic acid (4.1 mol %), and α-linolenic acid (5.0 mol %), whereas the principal FAs present in monoacylglycerol were stearic acid (85.7 mol %) and palmitic acid (14.3 mol %). The FA saturation status may contribute to the differences between solubility and functionality of these two emulsifiers.

Model fitting

As can been seen in Table 4.3, the initial viscosity of IF emulsion ranged from 22.19 to 222.9 Pa·S. After storage for 28 days, the particle size, optical change,

$C_{\text{palmitic acid}}/C_{(\text{DHA+SDA})}$, and TOTOX value ranged from 0.58 to 3.57 μm, 4.18 to 20.93%, 5.51 to 13, and 1.96 to 11.01, respectively. Multiple linear regression was used to fit the experimental data (Table 4.3) into separate second-order polynomial models. ANOVA for the five response variables is shown in Table 4.4. All the regression models were highly significant ($p < 0.01$). The R^2 value, the fraction of variation of the response explained by the model, was 0.860, 0.915, 0.815, 0.920, and 0.892 for particle size, optical change, apparent viscosity, $C_{\text{palmitic acid}}/C_{(\text{DHA+SDA})}$, and TOTOX value, respectively. In other words, more than 80% of the variation in the response variables could be explained by the corresponding models, indicating the good explanatory power of the models. The R^2_{adj} values were calculated to check the adequacy of the models. The closer value of R^2_{adj} to R^2 indicates that non-significant terms were not included in the models. The lack of fit tests with insignificant results ($P > 0.05$) further demonstrates the satisfactory fitness of the models. The Q^2 values, the fraction of variation of the response that can be predicted by the model, were higher than 0.70 (0.735−0.848), indicating that the models had good predictive abilities. The observed versus predicted plots had linear distributions for all the response variables (data not shown). Therefore, the models after eliminating insignificant coefficients ($P > 0.05$) can be written as equations (6) − (10). As shown in Table 4.4, the linear term of lecithin (β_1) affected all the response variables, and the linear term of monoacylglycerol (β_2) was the most significant coefficient.

Particle size (Y_1) =

$$0.781 + 0.327X_1 + 0.538X_2 - 0.243X_4 + 0.431X_3^2 + 0.277X_{12} - 0.144X_{14} - 0.162X_{24} \quad (6)$$

Optical change (Y_2) =

$$6.820 - 0.496X_1 + 0.604X_2 + 0.948X_3 - 1.962X_4 + 3.256X_1^2 - 3.418X_2^2 + 1.661X_3^2 + 2.200X_4^2 + 0.551X_{13} + 1.656X_{14} + 1.866X_{23} - 0.611X_{34} \quad (7)$$

Apparent viscosity (Y_3) =

$$94.184 + 8.929X_1 + 26.884X_2 + 16.657X_3 - 22.385X_2^2 + 10.679X_{12} + 8.741X_{23} \quad (8)$$

$C_{\text{palmitic acid}} / C_{(\text{DHA+SDA})}$ (Y_4) =

$$4.568 - 1.380X_1 - 0.369X_2 + 0.406X_3 + 1.555X_1^2 - 1.533X_2^2 + 1.771X_3^2 + 1.764X_4^2 - 0.700X_{23} + 0.378X_{24} - 0.554X_{34} \quad (9)$$

TOTOX value (Y_5) =

$$2.553 - 1.872X_1 - 0.591X_4 + 1.636X_1^2 - 0.640X_{12} + 0.519X_{14} \quad (10)$$

Particle size

As shown in Table 4.4, the most significant factor determining the variation of particle size was the linear effect of monoacylglycerol, followed by the linear effect of lecithin and interactive effect of monoacylglycerol and lecithin. It was found that the linear term of LBG had a non-significant (P >0.05) effect on the particle size but showed a significant (P < 0.05) effect in the quadratic term. The linear term of carrageenan exhibited a highly significant (P < 0.01) negative effect on the particle size; in contrast, the response value was positively influenced by the linear terms of monoacylglycerol and lecithin. Figure 4.1A illustrates that a higher level of monoacylglycerol and lecithin present in the IF emulsion resulted in a larger particle size of the final product after 28-day storage. The displacement of milk proteins by monoacylglycerol and/or lecithin at the O/W interface may reduce the protein surface coverage of oil droplets and thus contribute to the destabilization (e.g., droplet aggregation), regardless of the small

particle size initially induced by the presence of these non-protein emulsifiers (Euston, Finnigan, & Hirst, 2001; Fligner et al., 1990; McSweeney, 2008).

Optical change

All the parameters were significant ($P < 0.05$) except the interaction term of lecithin and monoacylglycerol, and interaction term of monoacylglycerol and carrageenan (Table 4.4). The most significant factor was the linear term of carrageenan with a negative effect on optical change, followed by a second-order term of monoacylglycerol and LBG with a positive effect. In Figure 4.1B, it can be seen that the optical change was decreased by addition of carrageenan up to about 0.01 g/100 mL while a further addition had little effect. This behavior may be explained by the interaction between carrageenan molecules and protein-coated droplets. After adjusting the pH of emulsion to 6.8 (pH > pI), both of them became negatively charged. Therefore, as the carrageenan concentration in the continuous phase increased, there was stronger electrostatic repulsion between anionic polysaccharides and droplets to avoid bridging flocculation (Cho, Decker, & McClements, 2009; McClements, 2005), hence minimizing optical instability. However, too many polysaccharides present in the continuous phase, either carrageenan or LBG, may cause depletion flocculation (McClements, 2005). As shown in Figure 4.1B, the optimal level of LBG ranging from 0.01−0.06 g/100 mL may give the minimum optical change when both lecithin and monoacylglycerol were kept constant at 0.2 g/100 mL.

Apparent viscosity

All of the emulsions studied exhibited shear-thinning behavior ($0 < n < 1$) over the shear rate range tested (data not shown). As can be seen in Table 4.4, the most

significant factor affecting the viscosity was the linear term of monoacylglycerol, followed by the linear term of LBG. Both of them had positive effects. The linear, quadratic, and interactive effects of carrageenan were shown to be non-significant (P >0.05) on the viscosity, which suggested that there was no significant contribution from either synergetic interaction between carrageenan and LBG, or gelling behavior of κ-carrageenan. Figure 4.1C illustrates that the higher the content of monoacylglycerol and LBG, the higher the viscosity of the emulsions. It also shows that the viscosity was mainly influenced by monoacylglycerol and to a lesser extent by LBG. A possible explanation for the significant positive effect of monoacylglycerol on the viscosity may be due to the formation of self-bodying mesophase structure of non-adsorbing monoacylglycerol in the continuous phase (Dickinson et al., 1989). As a result, the emulsions were more structured, increasing the viscosity. Due to the major contribution of monoacylglycerol (Figure 4.1A and C), viscosity analysis was found to be highly correlated (R = 0.678) with the measurement of particle size distribution (Table 4.5). However, both of them did not correlate well with optical analysis (R = 0.330 and 0.333, respectively). In other words, taking optical analysis as a reference measurement, particle size and initial viscosity may not be good indicators of long-term physical stability by assessing early changes occurring in the IF emulsions.

Relative content of DHA and SDA

The relative content of DHA and SDA was calculated as the concentration ratio of palmitic acid to DHA and SDA. This ratio was expected to decrease with a decrease in oxidation of DHA and SDA. As seen in Table 4.4, the most significant factor was the linear term of lecithin, followed by the interaction term of monoacylglycerol and LBG.

Both of them had negative effects on the oxidation of DHA and SDA. Figure 4.1D also demonstrates that a lower oxidation occurred at a higher level of lecithin. There are two possible explanations. First, this may be due to the formation of lecithin micelles in the continuous phase. When the lecithin exceeds a critical concentration, lecithin micelles may form (Waraho et al., 2011). Cho, McClements and Decker (2002) reported that surfactant micelles could decrease lipid oxidation through their ability to solubilize the irons and keep them in the continuous phase, thus the transition metals (e.g., iron and copper) would be less likely to interact with oxidizable lipids. Nuchi, Hernandez, McClements and Decker (2002) also found that surfactant micelles may solubilize lipid hydroperoxides out of the emulsion droplets which could prevent free radicals formed by hydroperoxide decomposition from attacking unsaturated lipids in the lipid droplet core, thus increasing the oxidative stability of emulsion. Second, this may be attributed to the formation of protein/lecithin complexes at the O/W interface (McSweeney et al., 2008), leading to a thicker adsorbed layer which creates steric hindrance of interactions between lipid droplets and pro-oxidants from the continuous phase as well as free radicals at the interface to inhibit lipid oxidation.

TOTOX value

The TOTOX value is an evaluation of both primary and secondary oxidation products used to estimate oxidative deterioration of food lipids. As shown in Table 4.4, the most significant factor on TOTOX value was the linear term of lecithin having a negative effect, followed by the quadratic term of lecithin having a positive effect. In addition, none of LBG terms were significant ($P > 0.05$), whereas the linear term of carrageenan had a highly significant ($P < 0.01$) negative effect on TOTOX value. Figure

4.1E confirms that TOTOX value decreased with increased carrageenan content. The inhibitory effect of carrageenan on lipid oxidation in O/W emulsion may be probably attributed to its capability to chelate transition metals in the continuous phase (Chen et al., 2010). As shown in Table 4.5, TOTOX value was also found to correlate well (R = 0.674) with $C_{\text{palmitic acid}}/C_{(\text{DHA+SDA})}$, which demonstrates the adequacy of this method to indicate lipid oxidation in IF emulsion. Besides, there was a negative correlation between particle size and TOTOX value (R = -0.329) as well as $C_{\text{palmitic acid}}/C_{(\text{DHA+SDA})}$ (R = -0.409). The results indicate that the rate of lipid oxidation increased as the particle size decreased, but the effect of particle size on lipid oxidation was not significant.

Optimization of variables and verification of models

The criteria of targeted particle size (~1 μm), the minimum optical change, the lowest ratio of palmitic acid to DHA and SDA, and the minimum TOTOX value, were applied in the optimization procedure. The optimal conditions were determined to be 0.2 g/100 mL lecithin, 0.4 g/100 mL monoacylglycerol, 0.045 g/100 mL LBG, and 0.015 g/100 mL carrageenan, resulting in predicted response values for particle size, optical change, viscosity, $C_{\text{palmitic acid}}/C_{(\text{DHA+SDA})}$, and TOTOX value at 1.17 μm, 3.48%, 96.28 Pa·S, 3.33, and 2.17, respectively. Since the computed Chi-squared values (Table 4.6) were smaller than the table value (9.488) at $\alpha = 0.05$ and df = 4, there was no significant difference between the expected and observed values, which indicates the high adequacy of the corresponding models.

Conclusions

A HMF analogue enriched with DHA and SDA was successfully synthesized and incorporated in a ready-to-feed IF to benefit the development and growth of infants. The

ANOVA and verification data demonstrates that RSM could be used to optimize the levels of emulsifiers (lecithin and monoacylglycerol) and biopolymer thickeners (LBG and carrageenan) for the highest physical and oxidative stability. The contour plots showed that lecithin and monoacylglycerol played a more important role than LBG and carrageenan in controlling the emulsion stability. Lecithin content affected all the responses measured, particularly lipid oxidation. An increase in monoacylglycerol concentration was associated with large particle size and high emulsion viscosity. Overall, the results obtained in this study should aid in the formulation of SL-based IF emulsion. Further research will be conducted on assessing oxidative stability of SL-based IF emulsion in the presence of antioxidants.

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Table 4.1 Composition of the micronutrient premix fortified in ready-to-feed structured lipid-based infant formula.

Vitamins	Level [a]	Minerals	Level [a]	Others	Level [a]
Vitamin E (as dl-α-tocopheryl acetate)	2 IU	Manganese (as manganese sulfate·H_2O)	15 µg	GMP (as Disodium Guanosine 5'-monophosphate)	0.25 mg
Pantothenic acid (as calcium d-pantothenate)	0.45 mg	Chloride (as magnesium chloride & sodium chloride)	65 mg	UMP (as disodium uridine 5'-monophosphate)	1.25 mg
Vitamin B_1 (as thiamin mononitrate)	0.1 mg	Phosphorous (as anhydrous dipotassium phosphate)	38 mg	AMP (as adenosine 5'-monophosphate)	1 mg
Vitamin A (as palmitate)	300 IU	Potassium (as anhydrous dipotassium phosphate)	46 mg	CMP (as cytidine 5'-monophosphate)	2 mg
Vitamin D_3 (as cholecalciferol)	60 IU	Calcium (as tricalcium phosphate)	47 mg	Choline (as choline bitartrate)	24 mg
Vitamin B_{12} (as cyanocobalamin)	0.33 µg	Copper (as anhydrous copper sulfate)	80 µg	Inositol	6 mg
Vitamin B_2 (as riboflavin)	0.14 mg	Magnesium (as magnesium chloride)	7 mg	Taurine	12 mg
Vitamin B_6 (as pyridoxine HCl)	75 µg	Selenium (as sodium selenate)	3 µg	L-Carnitine	2 mg
Vitamin C (as ascorbic acid)	10 mg	Sodium (as sodium chloride)	27 mg		
Vitamin K_1 (as phytonadione)	8 µg	Iodine (as potassium iodide)	12 µg		
Niacin (as niacinamide)	1.05 mg	Iron (as ferrous sulfate)	1.5 mg		
Folic acid	15 µg	Zinc (as zinc sulfate)	0.8 mg		
Biotin	4.4 µg				

[a] Unit per 600 mg

Table 4.2 Composition of structured lipid-based infant formula emulsion.

Ingredient	Content (g)	Macronutrient contribution (g)			Energy [a] (kcal)
		Lipid	Protein	Carbohydrate	
High heat nonfat dry milk [b]	20.0	0.2	6.8	10.6	71.4
α-Lactalbumin enriched whey protein concentrate [c]	8.8	1.1	6.9	0.1	37.9
Structured lipid	33.2	33.2	0	0	298.8
Lactose	61.0	0	0	61.0	244.0
Micronutrient premix [d]	3.9	0	0	0	0
Others [e]	873.1	0	0	0	0
Total	1000	34.5 (47.6%) [f]	13.7 (8.4%)	71.7 (44.0%)	652

[a] Energy density for lipid, protein and carbohydrate is 9, 4, and 4 kcal/g, respectively. [b] High heat nonfat dry milk contains 0.76% lipid, 34% protein, and 53% carbohydrate. [c] α-Lactalbumin enriched whey protein concentrate contains 12% lipid, 78% protein, and 1.5% carbohydrate. [d] The usage is 600 mg premix/100 kcal. [e] Others include lecithin, monoacylglycerol, locust bean gum, carrageenan and deionized water, and their contributions to macronutrient and energy are negligible. [f] Energy contribution (%) from each macronutrient is given in parentheses.

Table 4.3 D-optimal design: independent (X_i) and response variables (Y_i).

No.	Independent variables (g/100 mL)				Response variables (mean ± SD, n = 2)				
	Lecithin (X_1)	MAG [a] (X_2)	LBG (X_3)	CG (X_4)	$D_{3,2}$ (μm) (Y_1)	$\sum \Delta BS$ (%) (Y_2)	η (Pa·S) (Y_3)	$C_{\text{palmitic acid}}/C_{(\text{DHA+SDA})}$ (Y_4)	TOTOX value (Y_5)
1	0	0	0	0	0.74±0.09	15.78±0.19	44.47±0.00	8.09±0.02	7.19±0.23
2	0	0	0.1	0.004	0.60±0.00	9.27±0.49	37.45±21.57	11.01±1.81	5.53±0.58
3	0	0	0.02	0.02	0.60±0.00	6.30±0.99	35.10±0.00	8.02±0.42	4.98±0.29
4	0	0	0.1	0.02	0.68±0.04	4.54±0.51	70.01±0.00	10.59±1.40	6.50±0.16
5	0	0.2	0.1	0	1.31±0.18	18.70±3.16	84.12±5.61	11.78±0.31	9.22±2.53
6	0	0.2	0	0.02	0.94±0.02	11.00±0.38	70.03±4.26	11.70±1.84	6.47±1.18
7	0	0.4	0	0	1.29±0.15	10.83±0.78	69.18±6.81	8.67±0.39	9.89±0.08
8	0	0.4	0.1	0	1.29±0.11	18.83±0.10	87.79±0.52	9.07±0.28	9.56±0.45
9	0	0.4	0.02	0.004	1.42±0.22	9.99±0.52	75.34±0.00	6.26±0.01	7.87±0.62
10	0	0.4	0	0.02	1.02±0.28	6.67±0.45	62.01±2.09	10.25±0.04	6.93±2.03
11	0	0.4	0.1	0.02	1.25±0.01	10.80±1.51	85.78±5.41	7.84±0.00	6.18±0.04
12	0.2	0	0.1	0	1.21±0.30	8.91±0.39	69.80±0.00	8.67±0.04	4.50±0.54
13	0.2	0	0	0.02	0.62±0.01	6.71±0.45	33.14±0.00	6.18±0.25	3.68±0.66
14	0.2	0.2	0.02	0	1.26±0.10	11.30±0.71	89.24±1.03	6.00±0.09	3.76±0.59
15	0.2	0.4	0.1	0.02	1.54±0.27	6.69±0.41	147.48±0.73	6.27±0.08	2.99±0.89
16	0.4	0	0.02	0	0.70±0.11	8.02±0.65	29.62±0.00	6.00±0.14	3.87±0.66
17	0.4	0	0.1	0.004	0.83±0.11	9.24±0.54	34.77±12.95	7.08±0.18	3.81±0.27
18	0.4	0	0	0.02	0.97±0.13	9.24±0.98	44.42±0.00	6.00±0.66	3.19±0.27
19	0.4	0	0.1	0.02	0.67±0.12	7.90±0.01	62.15±0.00	7.00±0.14	3.73±0.27
20	0.4	0.2	0	0.004	1.44±0.40	10.09±0.82	84.72±2.11	6.41±0.04	3.71±0.71
21	0.4	0.4	0	0	2.88±0.00	7.37±0.35	80.24±2.02	5.55±0.06	2.53±0.21
22	0.4	0.4	0.1	0	3.49±0.12	13.74±0.40	148.05±2.24	6.21±0.13	3.15±0.20
23	0.4	0.4	0.02	0.02	1.24±0.22	8.01±1.18	76.03±0.66	6.22±0.12	2.80±0.22
24	0.4	0.4	0.1	0.02	1.82±0.37	12.15±0.43	215.44±10.56	6.46±0.01	3.15±0.21
25	0.4	0.4	0.1	0.02	2.66±0.42	13.23±0.76	118.31±7.68	6.19±0.18	2.77±1.15
26	0.4	0.4	0.1	0.02	2.17±0.23	15.94±0.44	117.20±2.40	5.98±0.10	3.73±0.74
27	0.4	0.4	0.1	0.02	2.51±0.95	12.86±0.47	201.20±0.37	6.16±0.16	2.62±0.03

[a] Abbreviations: MAG, monoacylglycerol; LBG, locust bean gum; CG, carrageenan; $D_{3,2}$, particle size; $\sum \Delta BS$, optical change; η, apparent viscosity; $C_{\text{palmitic acid}}/C_{(\text{DHA+SDA})}$, concentration ratio of palmitic acid to DHA and SDA.

Table 4.4 ANOVA for the effects of independent variables on the dependent variables, and corresponding coefficients of the regression models.

Parameters	$D_{3,2}$ (μm, Y_1) [a]		$\sum \Delta BS$ (%, Y_2)		η (Pa·S, Y_3)		$C_{\text{palmitic acid}} / C_{(\text{DHA+SDA})}$ (Y_4)		TOTOX value (Y_5)	
	Coefficient	*P*-value	Coefficient	*P*-value	Coefficient	*P*-value	Coefficient	*P*-value	Coefficient	*P*-value
β_0	0.781	0.020* [b]	6.820	1.4E^{-6}**	94.184	1.6E^{-4}**	4.568	5.7E^{-9}**	2.553	0.004**
Linear										
β_1	0.327	3.1E^{-7}**	-0.496	0.017*	8.929	0.021*	-1.380	2.4E^{-16}**	-1.872	2.8E^{-16}**
β_2	0.538	5.3E^{-12}**	0.604	0.006**	26.884	2.5E^{-8}**	-0.369	0.001**	0.162	0.270
β_3	0.099	0.096	0.948	8.9E^{-5}**	16.657	2.1E^{-4}**	0.406	0.001**	0.092	0.549
β_4	-0.243	6.6E^{-5}**	-1.962	6.8E^{-12}**	2.285	0.553	0.094	0.373	-0.591	1.7E^{-4}**
Quadratic										
β_{11}	0.005	0.970	3.256	2.0E^{-7}**	-13.510	0.173	1.555	8.4E^{-7}**	1.636	5.7E^{-5}**
β_{22}	0.025	0.862	-3.418	1.0E^{-7}**	-22.385	0.029*	-1.533	1.4E^{-6}**	-0.478	0.201
β_{33}	0.431	0.040*	1.661	0.034*	11.730	0.413	1.771	4.9E^{-5}**	0.550	0.305
β_{44}	0.134	0.576	2.200	0.017*	1.738	0.917	1.764	3.9E^{-4}**	1.161	0.069
Interaction										
β_{12}	0.277	2.1E^{-5}**	-0.036	0.869	10.679	0.011*	0.143	0.199	-0.640	1.2E^{-4}**
β_{13}	0.099	0.088	0.551	0.013*	7.970	0.053	-0.043	0.695	0.100	0.504
β_{14}	-0.144	0.015*	1.656	1.4E^{-9}**	1.825	0.647	0.003	0.976	0.519	0.001**
β_{23}	0.097	0.119	1.866	5.1E^{-10}**	8.741	0.048*	-0.700	5.3E^{-7}**	-0.096	0.552
β_{24}	-0.162	0.008**	0.306	0.167	-2.286	0.579	0.378	0.002**	-0.132	0.391
β_{34}	-0.028	0.625	-0.611	0.006**	6.324	0.117	-0.554	8.2E^{-6}**	-0.029	0.847
Regression		0.000**		0.000**		0.000**		0.000**		0.000**
Lack of fit		0.064		0.055		0.588		0.061		0.094
R^2	0.860		0.915		0.815		0.920		0.892	
R^2_{adj}	0.810		0.884		0.749		0.892		0.853	
Q^2	0.764		0.848		0.735		0.838		0.788	

[a] $D_{3,2}$, $\sum \Delta BS$, η, and $C_{\text{palmitic acid}} / C_{(\text{DHA+SDA})}$: see Table 4.3 for explanation of abbreviations. [b] *, significant at α = 0.05; **, significant at α = 0.01.

Table 4.5 Correlation coefficients (R) among methods for assessing the physical and oxidative stability of infant formula emulsion.

Methods	$D_{3,2}$ [a]	$\sum \Delta BS$	η	$C_{\text{palmitic acid}} / C_{(\text{DHA+SDA})}$	TOTOX value
$D_{3,2}$	1.000	0.330	0.678	-0.409	-0.329
$\sum \Delta BS$	0.330	1.000	0.333	0.107	0.318
η	0.678	0.333	1.000	-0.290	-0.296
$\frac{C_{\text{palmitic acid}}}{C_{(\text{DHA+EPA})}}$	-0.409	0.107	-0.290	1.000	0.674
TOTOX value	-0.329	0.318	-0.296	0.674	1.000

[a] $D_{3,2}$, $\sum \Delta BS$, η, and $C_{\text{palmitic acid}} / C_{(\text{DHA+SDA})}$: see Table 4.3 for explanation of abbreviations.

Table 4.6 Expected and observed values for the model verification using a Chi-squared test.

Lecithin (g/100 mL)	MAG [a] (g/100 mL)	LBG (g/100 mL)	CG (g/100 mL)	$D_{3,2}$ (μm)		$\sum \Delta BS$ (%)		η (Pa·S)		$\frac{C_{\text{palmitic acid}}}{C_{(\text{DHA+SDA})}}$		TOTOX value	
				f_e	f_o	f_e	f_o	f_e	f_o	f_e	f_o	f_e	f_o
0.2	0.4	0.045	0.015	1.16	1.71	3.49	4.48	96.38	100.23	3.42	3.77	2.17	3.45
0.2	0.1	0	0.01	0.90	1.88	7.31	8.16	74.59	85.15	5.38	6.68	2.76	2.98
0.35	0.35	0.01	0.02	1.36	1.67	7.84	10.42	89.63	105.2	7.19	7.87	2.72	3.15
0.4	0.4	0	0	2.74	2.91	6.62	7.01	89.38	82.10	5.83	5.61	3.15	3.40
0.05	0.2	0.015	0.004	0.85	1.03	11.92	14.15	80.89	70.12	7.39	8.23	6.13	5.53
$\chi^2 = \sum (f_o - f_e)^2 / f_e$				1.46		1.66		6.38		0.52		0.92	

[a] Abbreviations: MAG, LBG, CG, $D_{3,2}$, $\sum \Delta BS$, η, and $C_{\text{palmitic acid}} / C_{(\text{DHA+SDA})}$: see Table 4.3 for explanation of abbreviations; f_e, expected response value; f_o, observed response value, $n = 2$.

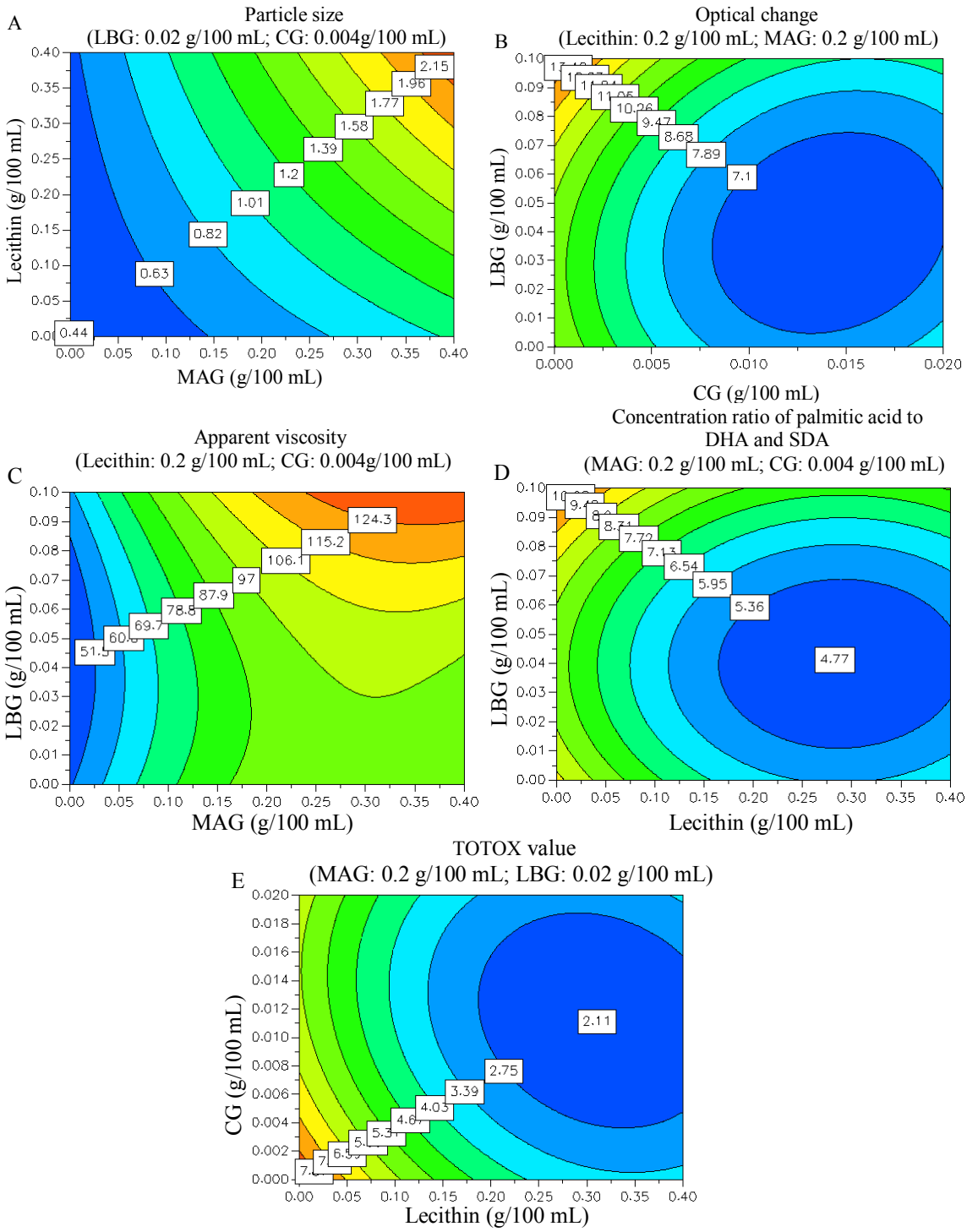

Figure 4.1 Contour plots showing (A) the effect of monoacylglycerols (MAG) and lecithin on particle size, (B) the effect of carrageenan and locust bean gum (LBG) on optical change, (C) the effect of MAG and LBG on apparent viscosity, (D) the effect of lecithin and LBG on concentration ratio of palmitic acid to DHA and SDA, and (E) the effect of lecithin and carrageenan (CG) on TOTOX value, while keeping the left two independent variables constant, respectively.

CHAPTER 5

OXIDATIVE STABILITY OF STRUCTURED LIPID-BASED INFANT FORMULA EMULSION: EFFECT OF ANTIOXIDANTS[1]

[1] Zou, L., and Akoh, C. C. Submitted to *Journal of Agricultural and Food Chemistry*, 05/20/14.

Abstract

The effect of permitted antioxidants, including α-tocopherol, β-carotene, ascorbyl palmitate, ascorbic acid, citric acid, and their combinations, on the lipid oxidation of structured lipid (SL)-based infant formula (IF) emulsion was evaluated. The oil-in-water IF emulsion was formulated with a human milk fat analogue enriched with docosahexaenoic acid and stearidonic acid, and the antioxidants were added at 0.005 and 0.02% of oil. The peroxide values, anisidine values, and hexanal concentrations of emulsion samples stored in the dark at 37 °C were measured over a 28-day period. The results showed that whether a compound exhibited antioxidant, neutral, or prooxidant behavior depended on its mechanism of action, polarity, concentration, oxidation time, method used to determine lipid oxidation, and environmental conditions (e.g., headspace oxygen and pH). The most effective antioxidant was ascorbyl palmitate at 0.005%, and a synergistic antioxidant effect was found between α-tocopherol and β-carotene. A high correlation (r = 0.84) was observed between anisidine value and hexanal content. Our findings have important implications for the successful incorporation of SL into IF products for infant nutrition and health.

Keywords: lipid oxidation; antioxidant; structured lipid; infant formula; emulsion; hexanal; stearidonic acid soybean oil

Introduction

With the increased use of polyunsaturated oils and fortification of mineral nutrients (e.g., iron) for health benefits, lipid oxidation has become a major concern in emulsion-based food products, such as milk, beverages, and ready-to-feed infant formula (IF). It leads to the development of undesirable off-flavors, nutrient loss, and potentially toxic compounds, thus making the foods unsuitable for consumption (McClements & Decker, 2000). One of the most effective and convenient strategies to retard or prevent lipid oxidation is to add antioxidants (Shahidi & Zhong, 2010).

According to the mechanisms of action, antioxidants can be broadly classified as primary antioxidants which scavenge free radicals to break chain-reactions of oxidation, or secondary antioxidants which protect lipids against oxidation mainly by chelating transition metals, quenching singlet oxygen, replenishing hydrogen to primary antioxidants, and/or scavenging oxygen (Reische, Lillard, & Eitenmiller, 2008). Governed by Codex Alimentarius regulations (CAC, 1981), tocopherols, β-carotene, ascorbic acid, ascorbyl palmitate, and citric acid are permitted compounds that may act as antioxidants in regular milk-based IF. α-Tocopherol is an important lipophilic primary antioxidant that protects by donating phenolic hydrogen to lipid free radicals (Eitenmiller & Lee, 2004). Due to the presence of conjugated double bonds in the molecule, the lipid-soluble β-carotene can act as primary antioxidant by scavenging free radicals under low oxygen pressure or as secondary antioxidant by physically quenching singlet oxygen to produce ground-state triplet oxygen and release the excess energy in the form of heat (Reische et al., 2008). Water-soluble ascorbic acid represents a multifunctional antioxidant that exerts its antioxidant effect via inactivation of free radicals, regeneration

of primary antioxidant by hydrogen donation, metal chelating, and scavenging oxygen by reduction (Reische et al., 2008). Ascorbyl palmitate is the hydrophobic derivative of ascorbic acid, but its antioxidant ability is still based on the ascorbic acid group. Citric acid with multiple carboxyl groups is capable of inhibiting metal-catalyzed oxidation by forming a thermodynamically stable complex with transition metal ions (Decker, 2008). Moreover, combinations of antioxidants with different mechanisms of action, such as α-tocopherol/ascorbic acid, α-tocopherol/citric acid, and α-tocopherol/β-carotene, have been reported to exhibit synergistic antioxidant effects (Decker, 2008; Eitenmiller et al., 2004).

Oil-in-water (O/W) emulsion is often more susceptible to oxidation than bulk oil due to its larger surface area that promotes interactions between the lipids and water-soluble prooxidants (Waraho, McClements, & Decker, 2011). Oxidative reactions are believed to be mostly prevalent at the oil-water interface (McClements et al., 2000). Therefore, in addition to its innate potency, the effectiveness of an antioxidant is influenced by its polarity and solubility, which subsequently determine its actual location in O/W emulsion. With respect to interfacial phenomenon, "polar paradox theory" was proposed, which states that polar antioxidants are more effective in less polar media, such as bulk oil, whereas nonpolar antioxidants are more effective in more polar media, such as O/W emulsion (Porter, 1993). The high efficacy of nonpolar antioxidants in O/W emulsion is primarily attributed to their high affinity to orient toward the oil-water interface. However, new evidence suggests that this empirical observation seems to be a particular case of a much wider picture, and more complex factors (e.g., critical concentration) in addition to polarity should be considered to explain antioxidant efficacy

(Shahidi & Zhong, 2011). Furthermore, compounds with antioxidant activity may also exhibit prooxidant behavior under certain conditions (Osborn-Barnes & Akoh, 2003b). Huang, Frankel, and German (1994) reported that whether α-tocopherol acted as an antioxidant or prooxidant depended on the test system, concentration, oxidation time, and the method used to determine oxidation. Therefore, all relevant factors must be taken into account when selecting antioxidants for a particular food system, and experiments should be performed before adding them as functional ingredients.

In our previous study (Zou & Akoh, 2013b), a human milk fat analogue enriched with docosahexaenoic acid (DHA) and stearidonic acid (SDA), which has potential to benefit the development and growth of infants, was synthesized and incorporated into an actual food system—ready-to-feed IF containing dairy proteins, lactose, lipid, vitamins, minerals, and other nutrients. The emulsifiers and thickeners were optimized to achieve the highest physical and oxidative stability. However, since lipid oxidation in O/W emulsion cannot be controlled by just the emulsifiers and thickeners, antioxidants are needed to further protect against rancidity, and their effectiveness in IF with complex matrices (e.g., metals and emulsifiers), which may interact with antioxidants and affect the rate of oxidation, is necessary to be assessed. Moreover, the oxidative stability of structured lipids (SLs) in real foods has seldom been investigated in the literature (Martin, Reglero, & Senorans, 2010). Therefore, as a continuation of our previous work, the objective of this study was to evaluate the effect of permitted antioxidants, including α-tocopherol, β-carotene, ascorbic acid, ascorbyl palmitate, and citric acid, on the lipid oxidation of SL-based IF emulsion (O/W). Mixtures of α-tocopherol/ascorbic acid, α-

tocopherol/ascorbyl palmitate, α-tocopherol/citric acid, and α-tocopherol/β-carotene, were also tested to clarify the mechanism of antioxidant synergism.

Materials and Methods

Materials

Tripalmitin was purchased from TCI America (Portland, OR, USA). SDA soybean oil was kindly donated by Monsanto Co. (St. Louis, MO, USA). DHA single-cell oil containing 40% DHA was purchased from DSM Nutritional Products Ltd. (Columbia, MD, USA). *sn*-1,3 Specific lipase Lipozyme TL IM and nonspecific lipase Novozym 435 were obtained from Novozymes A/S (Bagsvaerd, Denmark). α-Tocopherol (≥ 96% purity), β-carotene (95% purity), ascorbic acid, ascorbyl palmitate, citric acid (≥ 99.5% purity), C7-C30 saturated alkanes, and Tenax porous polymer adsorbent (60-80 mesh) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Hexanal (98% purity) and n-butyl acetate (≥ 99.5% purity) were obtained from Alfa Aesar (Ward Hill, MA, USA). Commercial milk-based Similac Advance ready-to-feed IF (Abbott Nutrition, Lake Forest, IL, USA) was purchased from a local convenience store. The DHA and SDA containing SL, which mimicked human milk fat, was enzymatically produced as previously reported (Zou & Akoh, 2013a). All the solvents were of analytical grade, and purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA) or Fisher Scientific (Norcross, GA, USA).

Preparation of Emulsion Samples

The SL-based IF emulsion consisting of 3.5% lipid at pH 6.8 was prepared according to the optimal formulation and procedures described by Zou et al. (2013b). Antioxidants were added to the emulsion system at 0.02% or 0.005% of the oil. Mixed

antioxidant systems contained equal amounts (0.01 % of the oil) of the antioxidant. α-Tocopherol in hexane was added directly to the oil phase before homogenization. Ascorbyl palmitate was dissolved in ethanol and then added to the oil. β-Carotene was dissolved in hexane before addition to the oil. Organic solvents were evaporated under nitrogen. Ascorbic acid and citric acid were added to the aqueous phase before homogenization. The sample without addition of antioxidants was used as a "control," whereas the sample without addition of antioxidants but flushed under nitrogen was used as another control, named "control-N_2." The commercial ready-to-feed IF was regarded as a "reference."

Oxidation Experiments

The oxidative stability was studied by means of an accelerated storage, which is commonly used to obtain reliable information on product stability. Emulsion samples (50 mL) were placed in capped glass bottles, covered with Parafilm, and allowed to oxidize in a covered water bath at 37 °C for 4 weeks. The primary and secondary oxidation products were measured after 0, 7, 14, 21, and 28 days of storage.

Spectroscopic Measurements of Lipid Oxidation Products

Lipid hydroperoxide, measured as peroxide value (PV), was determined using a modified method of Shanta and Decker (1994). The analytical procedure was performed as described previously (Zou et al., 2013b). *p*-Anisidine value (AV) determines the amount of aldehydes (mainly 2-alkenals and 2,4-alkadienals), which are secondary products from the decomposition of hydroperoxides. The AV of emulsion samples (2 mL) was determined according to the AOCS Official Method Cd 18-90 (AOCS, 2009) with minor modifications as reported previously (Zou et al., 2013b).

Identification of Volatile Oxidation Products by Dynamic Headspace Gas Chromatography-Mass Spectrometry (GC-MS)

Volatile compounds after oxidation were analyzed using a modified method described previously (Yang, Lee, Jeong, Kim, & Kays, 2008). Briefly, emulsion samples (100 mL) were stirred in a specially constructed 1 L glass beaker at 25 °C for 30 min. The beaker was sealed with a ground glass lid containing entry and exit ports, which were wrapped with aluminum foil to minimize losses during sampling. Immediately after stirring, volatiles were then collected on a 10 cm × 4 mm i.d. stainless-steel Tenax trap (Scientific Instrument Services, Inc., Ringoes, NJ, USA) filled with 250 mg Tenax porous polymer adsorbent, using an air sampling pump. Air was purified using a charcoal filter connected to the entry port and passed through the beaker at 150 mL/min for 60 min. A 50 mL Erlenmeyer flask was placed between the exit port and the trap to collect any condensation.

After sampling, the Tenax trap was loaded on TD-5 automated short-path thermal desorption system (Scientific Instrument Services, Inc.), which was connected to the injection port of 6890N/5973 GC-MS (Agilent Technologies Inc., Santa Clara, CA, USA). The volatiles were desorbed from the Tenax trap at 250 °C for 5 min with helium at a flow rate of 10 mL/min and then collected on the first 4 cm of the GC column using a SIS 2-inch cryo-trap (−40 °C) (Scientific Instrument Services, Inc.). After desorption, the cryo-trap was rapidly heated to 200 °C, and the GC separation was achieved with a 30 m × 0.25 mm i.d., 0.25 μm film thickness, J&W DB-5ms fused silica capillary column (Agilent Technologies Inc.). The column temperature was initially maintained at 40 °C, then increased at 1.5 °C/min to 65 °C and held for 1 min, next at 2 °C/min to 120 °C for 1

min, and finally programmed at 15 °C/min to 280 °C for 5 min (total 61.83 min). Carrier gas was helium with purity above 99.999%, and the flow rate was set at 1 mL/min. The injector temperature was maintained at 225 °C, and a split ratio of 0.5:1 was employed. MS detection was performed on a single-quadrupole mass spectrometer in full scan electron impact ionization mode, and conditions were as follows: ion source, 230 °C; electron energy, 70 eV; transfer line, 280 °C; quadrupole, 150 °C; *m/z* range, 35-350; solvent delay, 0 min.

The volatiles were tentatively identified by comparing their mass spectra and relative abundances with NIST 02 and Wiley 7 spectral libraries. Identifications were further confirmed by comparing Kovats retention indices with those reported previously in the literature using similar GC column and temperature program (http://webbook.nist.gov/). Retention indices were calculated by running a standard of C7-C30 saturated alkanes under the same conditions as samples.

Hexanal Analysis by Solid Phase Microextraction (SPME)-GC

Four milliliters of emulsion samples were placed in 40 mL headspace vials, and 10 μL of 50 ug/mL butyl acetate in deionized water was added as an internal standard. The vials were sealed with polytetrafluoroethylene/silicone septa and polypropylene open top caps. The samples were equilibrated with magnetic stirring (500 rpm) at 25 °C for 30 min. Then a preconditioned (250 °C for 30 min) 65 μm thickness polydimethylsiloxane/ divinylbenzene (PDMS/DVB) SPME fiber (Sigma-Aldrich Chemical Co.) was manually injected into the vial headspace at a fixed position and exposed for additional 10 min at 25 °C to adsorb volatiles. Immediately after sampling, the fiber was thermally desorbed at 250 °C for 5 min in the GC injection port, which was initially kept at splitless mode for

3 min and then switched to split mode for another 2 min to prevent carry-over from run to run. The purge flow to split vent was set at 40 mL/min.

Hexanal was measured using a 5890 Series II GC system (Agilent Technologies Inc.), which was equipped with a 0.75 mm i.d. narrow bore inlet liner, a 30 m × 0.32 mm i.d., 1 μm film thickness, J&W DB-5 capillary column, merlin microseal septum, and a flame ionization detector. As the diameter of injection port liner used can affect peak width, a narrow 0.75 i.d. inlet liner was used to make peaks sharper. The merlin microseal septum was used to minimize injection failure. The carrier gas was helium at a constant flow of 1.2 ml/min. The oven temperature was initially kept at 40 °C for 5 min, followed by an increase of 10 °C/min to 150 °C, then an increase of 25 °C/min to 250 °C and held for 5 min to clean the column. The FID temperature was 250 °C. Hexanal was quantified using a matrix-matched internal standard calibration curve by plotting peak-area ratio of hexanal to butyl acetate against hexanal concentration over the range of 0.001-10 μg/mL in a fresh IF matrix. The unspiked and spiked standard solutions were subjected to the same analysis procedures as described for the emulsion samples. The linearity of the calibration curve was determined by least-squares linear regression analysis. The method accuracy was evaluated by analyzing five replicate samples spiked with hexanal at 1 μg/mL, and the recovery rate was reported. The intra-day and inter-day precisions, expressed as relative standard deviation, were determined by analyzing five replicate samples on a single day and on different days, respectively. The limit of detection (LOD) and quantification (LOQ) were determined by measuring a series of matrix-matched standards until the signal-to-noise ratios were ≥ 3 and ≥ 10, respectively.

Fat Extraction and Fatty Acid Profile

The Bligh and Dyer protocol was used to extract the fat from liquid IF samples, following the procedures previously reported (Teichert & Akoh, 2011). The extracted fat was then analyzed for total fatty acid and positional profiles as described previously (Teichert et al., 2011; Zou et al., 2013a).

Oxidative Stability Analysis by Differential Scanning Calorimetry (DSC)

The oxidative stabilities of SL-based IF with optimized formulation and commercial IF were evaluated and compared on the basis of oxidation induction time (OIT) using 204 F1 Phoenix DSC (Netzsch Group, Burlington, MA, USA). Oxygen was used as the purge gas at a rate of 20 mL/min. Liquid samples (about 10 mg) were placed in a crimped aluminum sample pan, and the lid was pierced with two pinholes to facilitate contact with oxygen. The temperature was maintained isothermally at 80 °C for 60 min.

Statistical Analysis

All the experiments were conducted on duplicate samples. The effect of different antioxidants on lipid oxidation over time was evaluated by two-way repeated measures ANOVA of fixed-effects models, using SAS software, version 9.2 (SAS Institute Inc., Cary, NC, USA). Duncan's multiple-range test was performed to determine significant differences of the measurements on the same day ($\alpha = 0.05$). The relationships of PV, AV, and hexanal concentration were determined by Pearson correlation analysis, and Pearson correlation coefficient (r) was reported.

Results and Discussion

Volatile Oxidation Products in IF

As can be seen from Figure 5.1A and Table 5.1, a complex mixture of secondary lipid oxidation volatiles, including 12 aldehydes, 4 ketones, 3 furans, 2 alcohols, 2 aromatics, 2 sulfides, 2 alkenes and 1 terpene, was generated in SL-based IF after 4-week storage. These flavor volatiles, which play important roles in the organoleptic perception of IF products, probably derive from Maillard reactions and lipid autoxidation during sterilization processing and/or storage. Most of the compounds have been previously identified in the headspace of IF products (Fenaille, Visani, Fumeaux, Milo, & Guy, 2003; Hausner, Philipsen, Skov, Petersen, & Bredie, 2009; van Ruth, Floris, & Fayoux, 2006). Hausner et al. (2009) indicated that substantial differences were present among different brands, formula types (e.g., powder and ready-to-feed), and/or different matrices (e.g., milk-based and hydrolyzed milk protein-based) of IF products. Among the 28 volatiles identified (Figure 5.1A), hexanal (no. 5) was the most abundant chemical, followed by 2-ethylfuran (no. 1), 2-pentylfuran (no. 19), and *D*-limonene (no. 23). Hexanal with a green, grassy, fatty aroma (Table 5.1) arises from the oxidation of n-6 polyunsaturated fatty acids (PUFAs) (Romeu-Nadal, Castellote, & Lopez-Sabater, 2004), especially linoleic acid which is abundant in IF. Consistent with the previous results (Fenaille et al., 2003; Hausner et al., 2009; van Ruth et al., 2006), propanal which is considered to be a major breakdown product of n-3 PUFAs (Romeu-Nadal et al., 2004), was not found in our study, albeit a significant amount of DHA and SDA was incorporated (Table 5.2). These observations demonstrated that oxidation of single molecules does not correlate well with that of food systems with complex matrices.

Jimenez-Alvarez et al. (2008) also found that not all the volatile compounds detected during the oxidation of tri-DHA standard were observed in milk containing cod oil during storage. 2-Ethylfuran and 2-pentylfuran may derive from the degradation of 2,4-heptadienal and 2,4-decadienal and/or Maillard reactions (e.g., Strecker degradation) (Hausner et al., 2009). *D*-limonene, the only terpene identified in our study, probably originates from the forage of the cows via the food chain (Hausner et al., 2009). Due to the high level and a low odor threshold of 4.5 μg/kg in water (Table 5.1), hexanal was chosen as a marker to evaluate lipid oxidation in SL-based IF during storage.

Hexanal Analysis by SPME-GC

A SPME-GC method was developed to quantify hexanal in IF samples. According to the SPME manufacturer, PDMS/DVB fiber was selected on the basis of its capacity and sensitivity to small polar and nonpolar molecules. The addition of salt (e.g., NaCl) to aqueous samples prior to SPME extraction is frequently used to decrease the solubility of volatiles in the aqueous phase and drive them into the headspace. However, this "salting out" step was not carried out due to its insignificant enhancement on the relatively nonpolar hexanal extraction (data not shown). A low temperature of 25 °C was used during equilibration and sampling to avoid heat-related alterations. It should be noted that SPME quantification is feasible in non-equilibrium situations once the agitation conditions and adsorption time are held constant (Ai, 1997). Therefore, provided sensitivity is accepted, a short equilibration and sampling time, rather than exhaustive extraction, can be used for quantitative analysis of hexanal. Butyl acetate was used as an internal standard to compensate for the losses of hexanal during sample preparation and injection and facilitate identification of the analyte. Figure 5.1B shows

that hexanal and butyl acetate were baseline separated. To correct for matrix effects, a set of aliquots of fresh IF samples with increased amounts of hexanal was used as standards for calibration. Good linearity ($R^2 = 0.9909$) was observed over a concentration range of 0.001-10 μg/mL for the calibration curve. The LOD and LOQ were found to be 0.4 and 4 ng, respectively, which are comparable to the values reported by other authors (Garcia-Llatas, Lagarda, Romero, Abellan, & Farre, 2007). An acceptable recovery percentage ranging from 104.3-123.0% was obtained. The intra-day and inter-day precisions were 1.4 and 4.0%, respectively, which shows good repeatability of the method. All these validated parameters demonstrated that the developed SPME-GC method is reliable, sensitive, and convenient as a routine technique to determine hexanal in IF products.

Effect of Antioxidants

Comparison of Control Samples

As can be seen from Figures 5.2 and 5.3, primary and secondary oxidation products in the control samples with nitrogen flushing was lower than the corresponding control samples without nitrogen treatments after 7 days of storage, and the difference increased with incubation time. This may be due to the displacement of headspace oxygen by inert nitrogen to decrease the rate of lipid autoxidation. It indicates that nitrogen flushing can be an effective technique to protect IF products against oxidation. We observed a longer induction period of primary and secondary lipid oxidations in the commercial IF references compared to SL-based samples. There are two possible explanations. First, a significant amount of labile fatty acids, including C18:3n-6, C18:3n-3, C18:4n-3, and C22:6n-3, were incorporated in the SL-based samples compared to the commercial product (Table 5.2). Second, the variance in their compositions, such

as emulsifiers, minerals, and antioxidants, may also contribute to the difference in the oxidative stability.

Antioxidant Effects at a Low Addition Level

At a level of 0.005%, no significant difference ($p > 0.05$) was found in lipid hydroperoxide concentrations among α-tocopherol, β-carotene, and citric acid during the entire incubation period (Figure 5.2A). Their antioxidant effects were observed between 7 to 21 days. Ascorbyl palmitate exhibited strong antioxidant effect after 14 days of oxidation, whereas the hydrophilic counterpart, ascorbic acid, showed a small antioxidant activity between 7 to 14 days and prooxidant activity after 21 days of incubation. Sorensen et al. (2011) reported similarly that ascorbic acid in O/W emulsion (5% oil) at pH 7 was efficient at the beginning of storage and acted as a prooxidant towards the end. The prooxidative effect of ascorbic acid on the basis of hydroperoxide formation may be ascribed to its ability to reduce transition metal ions (e.g., Cu^{2+} and Fe^{3+}) that are abundant in IF as mineral nutrients. The reduced metal ions have high abilities to initiate metal-catalyzed lipid oxidation in the presence of oxygen and produce singlet oxygen and hydroxyl radical (Kiokias, Varzakas, Arvanitoyannis, & Labropoulos, 2010) which further promote the formation of hydroperoxides.

On the basis of AVs, no antioxidant effect was observed for α-tocopherol and citric acid (Figure 5.2B). Ascorbic acid showed a little antioxidant activity between 14 to 28 days, whereas β-carotene and ascorbyl palmitate, exhibited antioxidant effect after 21 days of oxidation. It appears that lipophilic ascorbyl palmitate was more active than hydrophilic ascorbic acid as an antioxidant in IF emulsion on the basis of PV and AV measurements, which is in agreement with the "polar paradox theory" (Porter, 1993).

On the basis of hexanal formation, no significant difference ($p > 0.05$) was found between ascorbic acid and ascorbyl palmitate during the entire incubation period (Figure 5.2C). Moreover, all the compounds except citric acid exhibited antioxidant activities after 21-day storage. Osborn-Barnes and Akoh (2003a) also reported that citric acid did not significantly affect the oxidation rate in O/W model emulsion formulated with 10% canola oil/caprylic acid SLs at pH 7. The inefficacy of citric acid on the primary and secondary oxidation (Figure 5.2) can be explained by its week interaction with transition metal ions. As the pH of emulsion (6.8) approaches the p*K*a of citric acid (6.4), its carboxylic groups are not fully ionized and thus the chelating activity decreases. Another possible explanation may be the presence of other metal ions (e.g., calcium) in IF that can compete with the prooxidative metal ions for the binding sites of citric acid.

Antioxidant Effects at a High Addition Level

In contrast to the addition level at 0.005%, no antioxidant effect was observed for all evaluated compounds at 0.02% on the basis of PVs and AVs (Figure 5.3A and B). No significant difference ($p > 0.05$) was found in the PVs and AVs among ascorbic acid, ascorbyl palmitate, and citric acid. Both α-tocopherol and β-carotene showed prooxidant effects on primary and secondary oxidations (AVs) after 28 days of storage. This finding is consistent with that of Osborn-Barnes et al. (2003b), who reported that α-tocopherol and β-carotene at 0.02% exhibited prooxidant activities in SL-based O/W model emulsion stabilized with sucrose fatty acid ester. The prooxidative effect of α-tocopherol at high concentrations may be mainly attributed to its synergism with prooxidants already in the system, such as transition metal ions, high initial levels of hydroperoxides, and reactive oxygen species (Kamal-Eldin & Appelqvist, 1996). The prooxidant chemistry of

β-carotene is highly related to its electron-rich structure with a conjugated polyene chain, which is susceptible to attack from electrophilic reagents, such as lipid peroxyl radicals. The antioxidant efficacy of β-carotene depends on the balance between its radical trapping and autoxidation. At a high concentration, autoxidation reactions in the presence of oxygen are favored to consume β-carotene without scavenging peroxyl radicals, allowing its prooxidant activity to predominate (Decker, 2008; Osborn-Barnes et al., 2003b). Therefore, it is likely that each antioxidant has a critical concentration in a specific matrix for maximum efficacy. Below the critical concentration, the antioxidant effect is below the optimum, and above which the prooxidant effect may dominate, probably by synergizing with other prooxidants present in the substrate. However, more research is required to confirm this hypothesis.

On the basis of hexanal formation, ascorbyl palmitate and α-tocopherol showed antioxidant activities at 0.02% after 21-day incubation (Figure 5.3C), and ascorbyl palmitate was more effective than α-tocopherol. Ascorbic acid, citric acid, and β-carotene exhibited small antioxidant effects between 14 to 28 days.

Antioxidant Synergism

As shown in Figure 5.4, PV, AV, and hexanal concentration were significantly lower ($p < 0.05$) in samples with α-tocopherol/β-carotene mixture than those with α-tocopherol or β-carotene alone after 28-day storage. In other words, the mixture of α-tocopherol and β-carotene showed a strong synergistic effect in inhibiting lipid oxidation. Heinonen, Haila, Lampi, and Piironen (1997) also found a synergistic effect of α-tocopherol and β-carotene on oxidation in 10% O/W emulsion of rapeseed oil. Two mechanisms may be considered to explain the synergism of α-tocopherol and β-carotene.

First, a cycle is operated to regenerate tocopherols from tocopheroxyl radicals by interaction with β-carotene (Eitenmiller et al., 2004). Second, α-tocopherol protects β-carotene from being autoxidized (Kiokias et al., 2010). However, no antioxidant synergism was observed among mixtures of α-tocopherol/ascorbic acid, α-tocopherol/ascorbyl palmitate, and α-tocopherol/citric acid. It has been proposed that the synergistic effect of tocopherols with other antioxidants is mainly due to regeneration of tocopherols from tocopheroxyl radicals by hydrogen donation, thus preventing tocopheroxyl radicals from propagating lipid autoxidation (Decker, 2008; Eitenmiller et al., 2004). However, antioxidants like ascorbic acid and ascorbyl palmitate may have other independent antioxidative potentials (e.g., inactivating free radicals and scavenging oxygen), which are hypothesized to be predominant in IF products, rather than replenishing hydrogen to tocopherols. Therefore, no significant synergism could be observed between these antioxidants and α-tocopherol. The inefficiency of citric acid as a chelator in our study could explain its week interaction with α-tocopherol for a synergistic antioxidant effect.

Antioxidant Selection

The most effective antioxidant in inhibiting lipid oxidation of SL-based IF emulsion was ascorbyl palmitate at 0.005%, as determined by PV, AV, and hexanal content. Jacobsen, Let, Nielsen, and Meyer (2008) also reported that ascorbyl palmitate seemed to work best at low concentrations in fish oil-enriched milk with 1.5% fat, rather than in other tested food systems, including milk drink, salad dressing, and mayonnaise.

Correlations of Oxidation Measurements

Due to the measurements of different stages of oxidation, both AV and hexanal content did not correlate well with PV ($r = 0.50$ and 0.35, respectively). However, a highly significant positive correlation ($r = 0.84$, $p < 0.0001$) was found between AV and hexanal concentration, which also demonstrated the adequacy of headspace hexanal analysis to indicate secondary lipid oxidation in IF emulsion. The high correlation between AV and hexanal analysis has also been reported in the shelf-life evaluation of nuts and frying oils (Tompkins & Perkins, 1999; Zajdenwerg, Branco, Alamed, Decker, & Castro, 2011).

Oxidative Stability of IF Products Measured by DSC

Oxidation reactions are exothermic and thus can be measured by DSC. OIT measures oxidative stability of the materials in an isothermal mode (temperature) under an accelerated oxidative environment. It can be determined by intersection of extrapolated baseline and tangent line of the exothermic curve. The higher the OIT value, the more stable the substrate. As shown in Figure 5.5, the exothermic peak of SL-based and commercial IF appeared after oxidation for 32.9 and 34.0 min, respectively. The shorter OIT of SL-based IF compared to the commercial product is possibly due to the differences in the composition, such as lipid unsaturation (Table 5.2), type and level of antioxidants, emulsifiers, and minerals. In addition, a good agreement of the results with PV, AV, and hexanal content (Figures 5.2 and 5.3) suggests that DSC technique is suitable for oxidation studies of liquid IF products.

In conclusion, the efficacy of permitted compounds as antioxidants in SL-based IF emulsion depends not only on their mechanism of action, but also on polarity,

concentration, oxidation time, method used to determine lipid oxidation, and environmental conditions (e.g., headspace oxygen and pH). A synergistic antioxidant effect was found between α-tocopherol and β-carotene. A high correlation between anisidine value and hexanal content indicates the goodness of hexanal analysis by SPME-GC as an indicator of flavor deterioration in IF products. The most effective antioxidant was ascorbyl palmitate at 0.005%. However, compared to the commercial ready-to-feed IF, our SL-lipid product was slightly less stable on the basis of DSC measurement, but that was due to the high incorporation of unsaturated fatty acids (e.g., DHA and SDA). Nitrogen flushing may be used as a supplemental technique to further protect against oxidation. Overall, this study has important implications for the successful development of SL-based IF products for infant nutrition and health.

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Table 5.1 Volatile Oxidation Compounds of Structured-Lipid Based Infant Formula Identified by Dynamic Headspace GC-MS

No.[a]	compound	RI[b]	identification[c]	odor description[d]	threshold[e] (μg/kg)
aldehydes					
2	pentanal	714	MS,RI	woody, pungent, fruity	12
5	hexanal	793	MS,RI	green, grassy, fatty	4.5
7	*trans*-2-hexenal	836	MS,RI	fatty, green	17
11	*cis*-4-heptenal	888	MS	biscuit-like, fatty, fishy	0.06
12	heptanal	891	MS,RI	fatty, woody	3
14	*trans*-2-heptenal	939	MS	soapy, tallowy	13
15	benzaldehyde	943	MS,RI	bitter almond, aromatic, sweet	350
22	*trans*, *trans*-2,4-heptadienal	999	MS	fatty, rancid	–[f]
21	octanal	994	MS,RI	fatty, sweet, green, orange	0.7
25	*trans*-2-octenal	1046	MS,RI	fatty, green, raw nut	3
27	nonanal	1096	MS,RI	waxy, painty	1
28	*trans*, *trans*-2,4-decadienal	1306	MS,RI	fried fat	0.07
alcohols					
17	1-octen-3-ol	970	MS,RI	mushroom, musty	1
8	1-hexanol	849	MS,RI	green	2500
aromatics					
4	toluene	752	MS	paint	1000
13	2-ethylphenol	909	MS	–	–
ketones					
10	2-heptanone	878	MS,RI	blue cheese, spicy, musty	140
18	6-methyl-5-hepten-2-one	978	MS	–	50
24	3-octen-2-one	1029	MS,RI	rose, fatty, spicy	–
26	3,5-octadien-2-one	1059	MS	fatty, fruity	–
terpenes					
23	*D*-limonene	1015	MS,RI	orange-like	10
furans					
1	2-ethylfuran	708	MS	coffee-like	–
19	2-pentylfuran	981	MS,RI	floral, fruit	6
20	*cis*-2-(2-pentenyl)furan	992	MS	–	–
sulfides					
3	dimethyl disulfide	735	MS	cauliflower, garlic, cabbage-like	7.6
16	dimethyl trisulfide	950	MS	alliaceous, meaty, cabbage-like	0.01
alkenes					
6	3,5-octadiene	806	MS	–	–
9	1,3-*trans*-5-*cis*-octatriene	863	MS	–	–

[a]Numbers correspond to the labeled peaks in Figure 5.1. [b]RI, Kovats retention index. [c]Method of identification: MS, by comparison of the mass spectrum and relative abundance with the NIST and Wiley mass spectral libraries; RI, by comparison of RI with those from the literature. [d]Adapted from literature (Fenaille, Visani, Fumeaux, Milo, & Guy, 2003; Milo & Grosch, 1993; Peres, Denoyer, Tournayre, & Berdague, 2002; Triqui & Bouchriti, 2003; Yang, Lee, Jeong, Kim, & Kays, 2008). [e] Odor threshold in water and adapted from literature (Buttery, Turnbaugh, & Ling, 1988; Fenaille et al., 2003; Milo et al., 1993; Yang et al., 2008). [f] –, unknown.

Table 5.2 Fatty Acid Profiles (Mole Percent) of Extracted Fat from Infant Formulas[a]

fatty acid	commercial infant formula		SL-based infant formula	
	total	*sn*-2	total	*sn*-2
C8:0	3.3±0.0a	0.5±0.1a	0.2±0.0b	ND
C10:0	2.4±0.0a	1.3±0.1a	0.4±0.0b	ND
C12:0	16.6±0.0a	33.9±1.3a	1.2±0.0b	1.0±0.0b
C14:0	6.0±0.0a	4.2±0.1a	3.3±0.0b	3.2±0.0b
C16:0	10.9±0.0b	2.0±0.0b	54.5±0.1a	62.3±0.3a
C18:0	3.7±0.0b	1.5±0.1b	9.2±0.0a	11.9±0.6a
C18:1n-9	33.5±0.1a	32.2±0.8a	8.5±0.2b	5.3±0.1b
C18:2n-6	20.3±0.0a	22.7±0.8a	7.5±0.0b	4.9±0.2b
C18:3n-6	0.1±0.0b	ND	1.8±0.0a	1.5±0.0a
C18:3n-3	1.9±0.0b	1.4±0.1b	2.5±0.0a	1.8±0.0a
C18:4n-3	ND	ND	6.2±0.0a	5.2±0.2a
C20:4n-6	0.2±0.0a	ND	ND	ND
C22:6n-3	0.2±0.0b	0.1±0.1b	3.9±0.0a	2.3±0.1a

[a]Mean ±SD, $n = 3$. Values with different letter in the same row and category (i.e., total or *sn*-2) are significantly different by Duncan's multiple-range test ($p < 0.05$). Trace amounts of C16:1n-7, C17:0, C20:0, C20:1, C22:0, C24:0, and C24:1 were detected. Abbreviations: SL, structured lipid; ND, not detected.

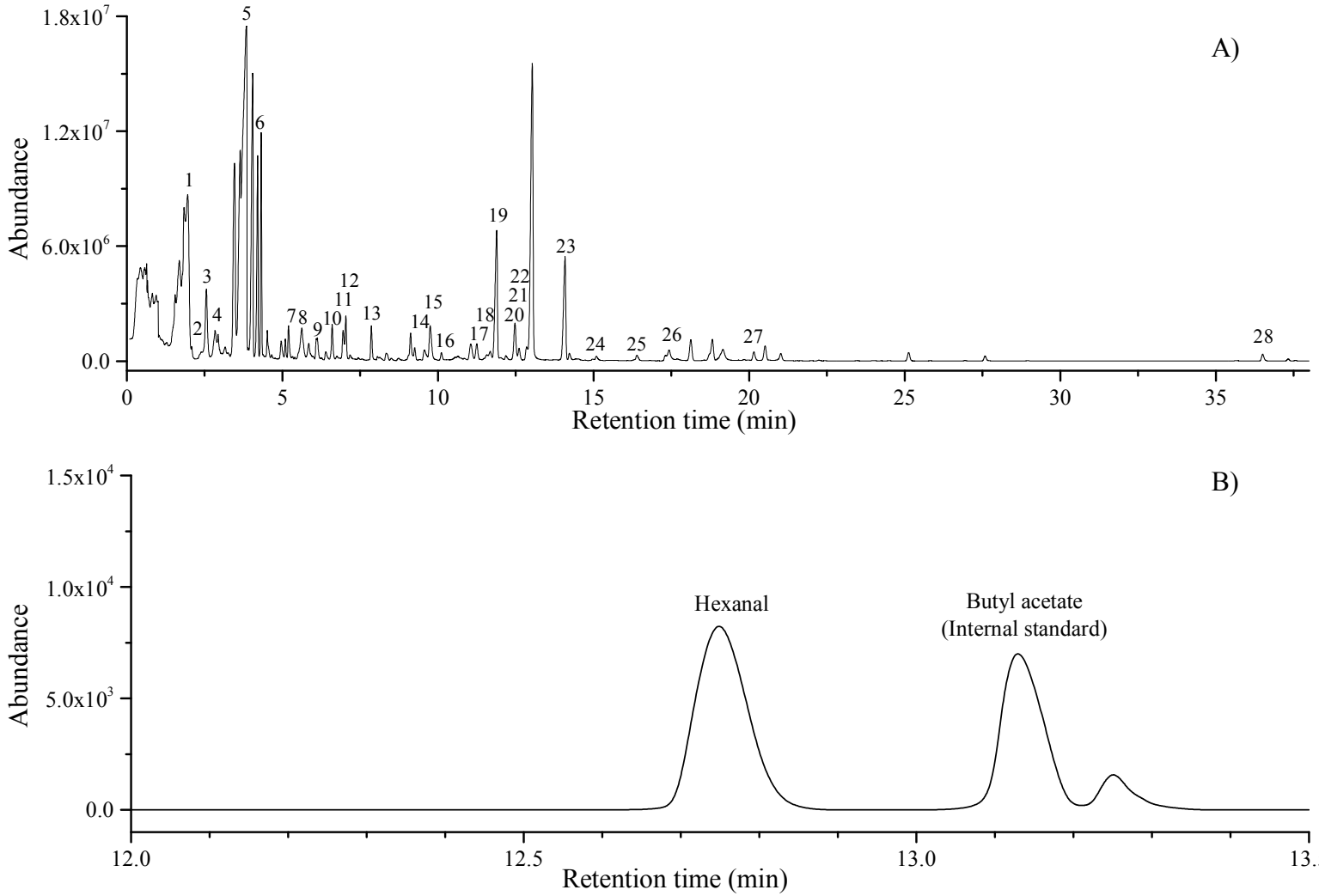

Figure 5.1 (A) Total ion chromatogram of the volatile compounds extracted from structured-lipid based infant formula after 4-week storage at 37 °C by dynamic headspace GC-MS. Peak numbers correspond to the compounds listed in Table 5.1; (B) Chromatogram of a matrix-matched standard solution spiked with hexanal at 0.01 μg/mL analyzed by SPME-GC.

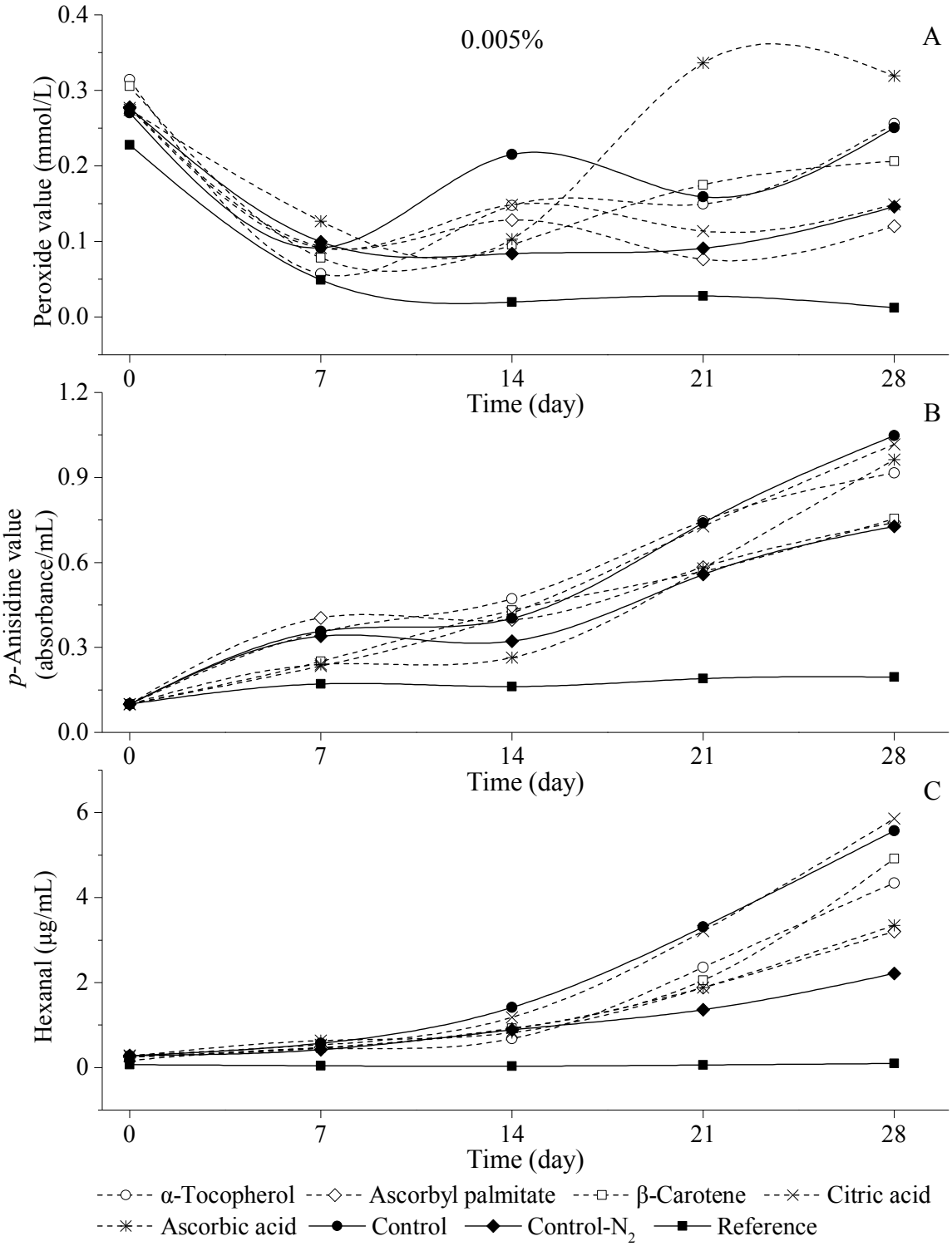

Figure 5.2 Effect of antioxidants (0.005%) on the oxidative stability of structured lipid-based infant formula emulsion over time at 37 °C: (A) peroxide value; (B) *p*-anisidine value; and (C) hexanal content. Data points represent the average of duplicate samples.

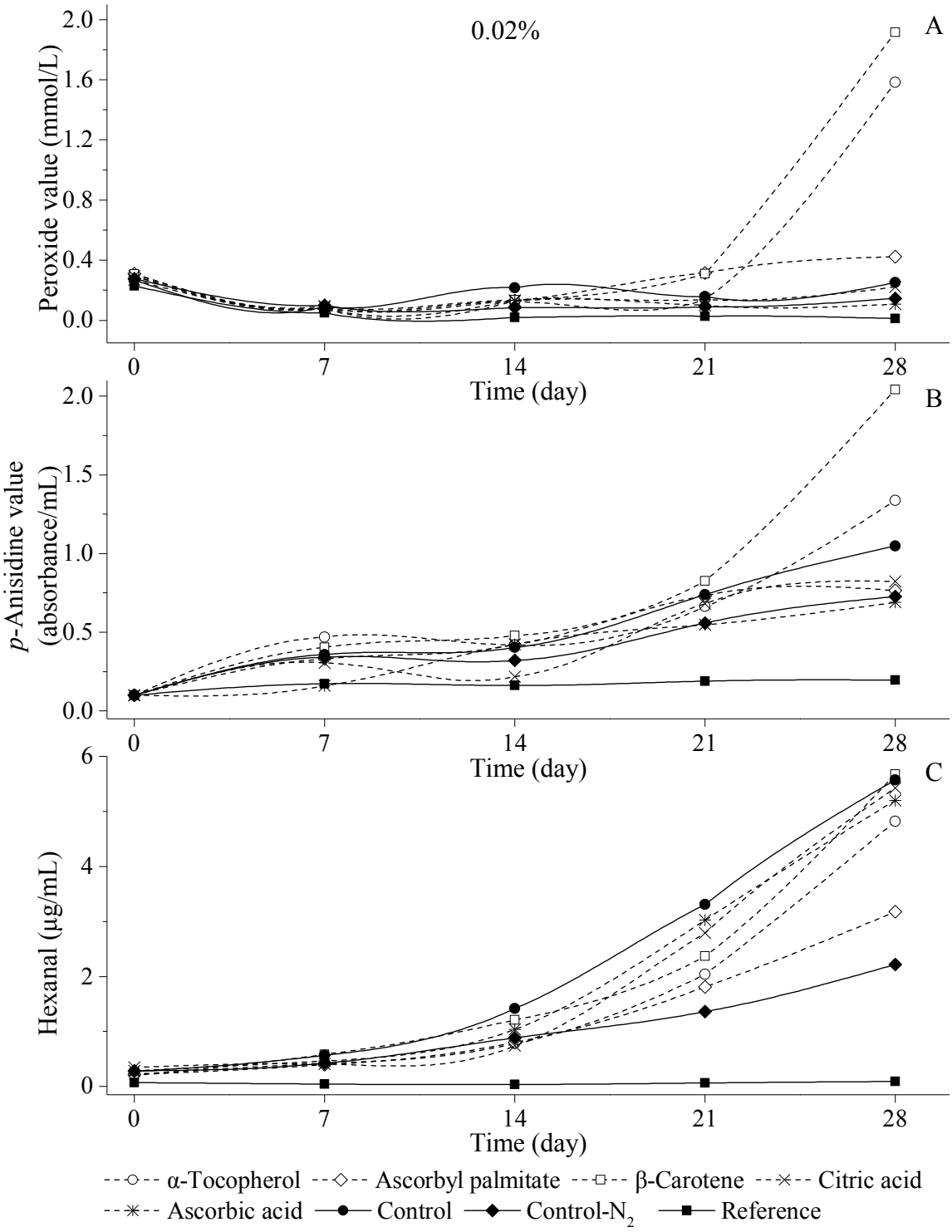

Figure 5.3 Effect of antioxidants (0.02%) on the oxidative stability of structured lipid-based infant formula emulsion over time at 37 °C: (A) peroxide value; (B) *p*-anisidine value; and (C) hexanal content. Data points represent the average of duplicate samples.

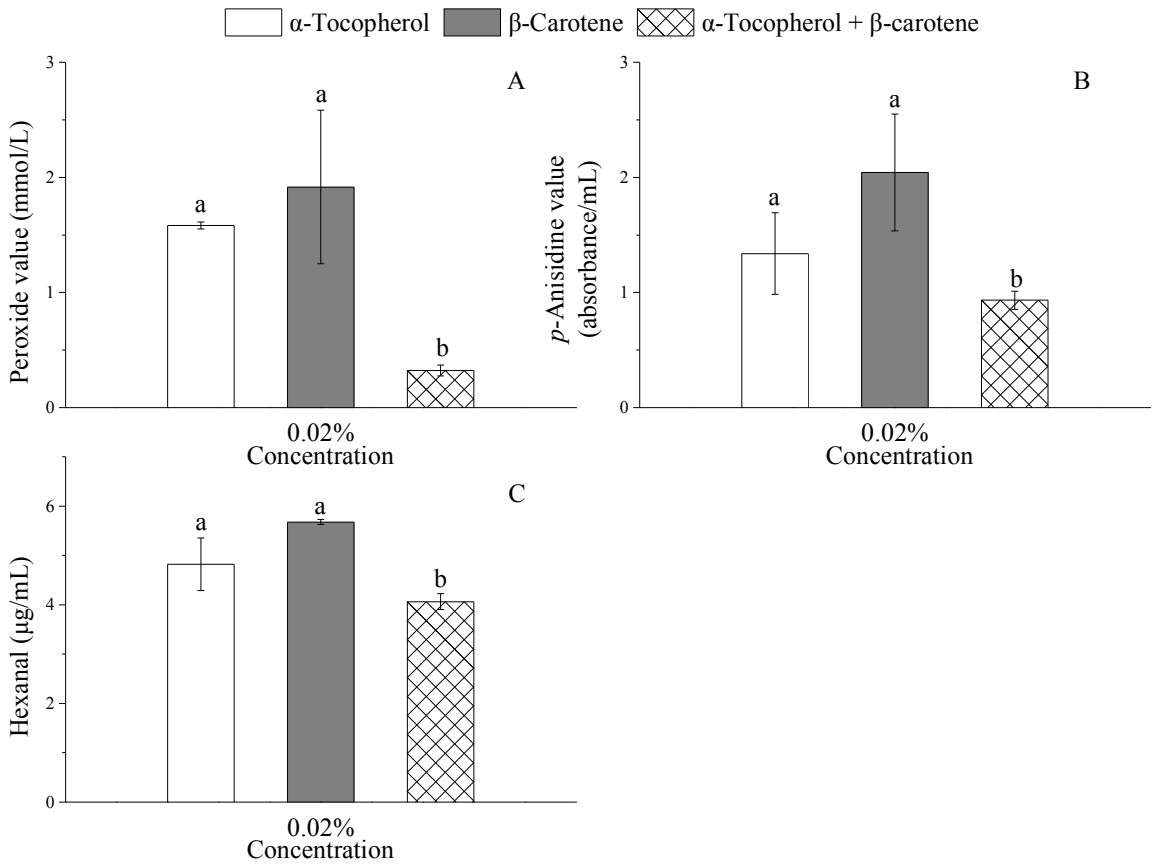

Figure 5.4 Effect of α-tocopherol, ascorbic acid, and their mixture at a ratio of 1:1 (0.02%) on the oxidative stability of structured lipid-based infant formula emulsion after 28 days of storage at 37 °C: (A) peroxide value; (B) *p*-anisidine value; and (C) hexanal content. Different lower-case letters within each category indicate a significant difference ($\alpha = 0.05$).

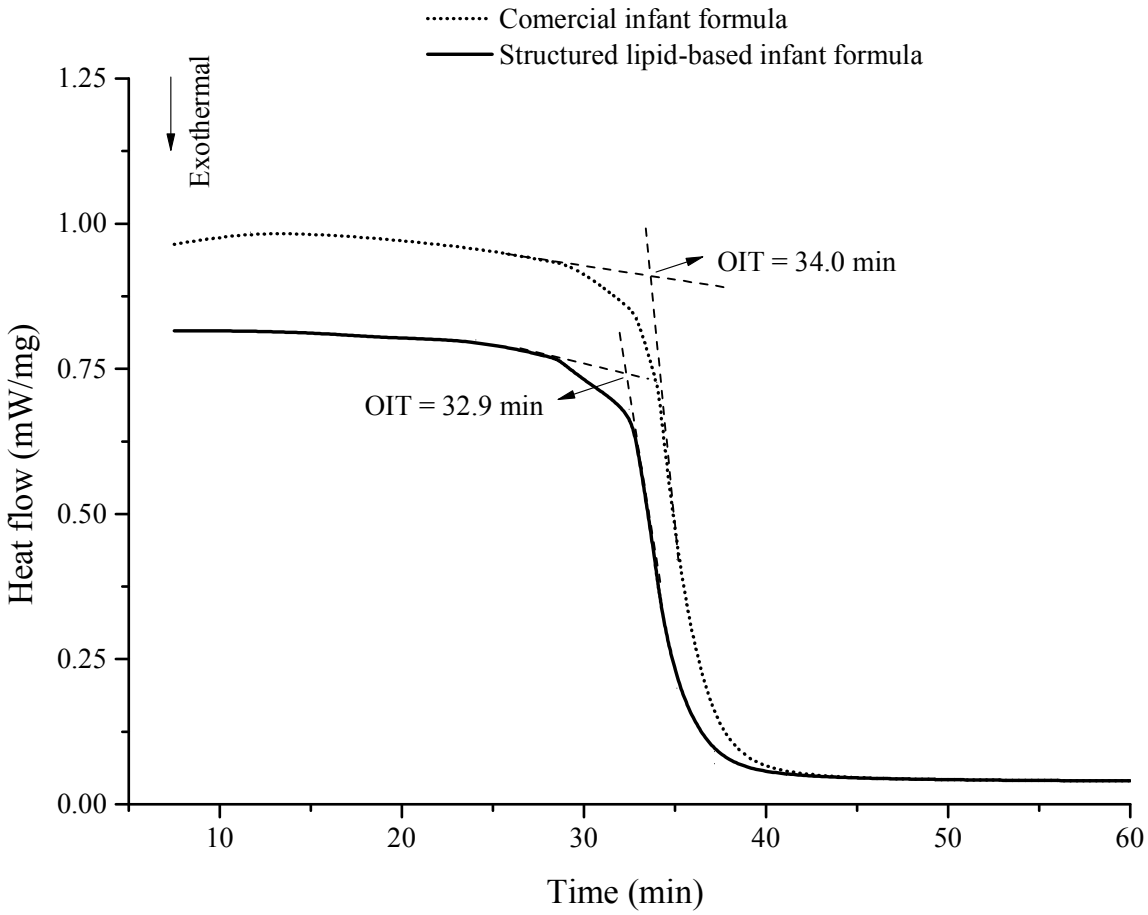

Figure 5.5 Oxidation induction time of structured lipid-based and commercial infant formulas determined by DSC at 80 °C.

CHAPTER 6

ANTIOXIDANT ACTIVITIES OF ANNATTO AND PALM TOCOTRIENOL-RICH FRACTIONS IN FISH OIL AND STRUCTURED LIPID-BASED INFANT FORMULA EMULSION[1]

[1] Zou, L., and Akoh, C. C. Submitted to *Food Chemistry*, 04/13/14.

Abstract

The effectiveness of annatto and palm tocotrienol-rich fractions (TRFs) as natural antioxidants to inhibit lipid oxidation in menhaden fish oil and structured lipid-based infant formula emulsion was evaluated and compared. The peroxide and anisidine values of the bulk oil and oil-in-water emulsion samples stored at 37 °C were measured over a 28-day period. The results showed that annatto TRF was a more effective antioxidant than palm TRF and α-tocopherol in both food systems at 0.02% and 0.05%. Factors, including structural differences in chromanol head and isoprenoid tail, polarity, concentration, oxidation time, and the method used to monitor lipid oxidation, were responsible for the different behaviors of tocopherols and tocotrienols. In contrast to the reported findings in vivo, addition of α-tocopherol (0-75%) did not interfere with the antioxidant activity of tocopherol-free annatto TRF in foods. Our findings may lead to the development of new natural antioxidant products for food applications.

Keywords: tocotrienol; antioxidant; annatto; palm; structured lipid; fish oil; infant formula; emulsion

Introduction

Lipid oxidation in foods has become a major concern with the increased use of polyunsaturated vegetable, fish, or microbial oils for health benefits. It not only produces undesirable off-flavors but also decreases the nutritional quality and safety of food products (McClements & Decker, 2000), which are unacceptable to consumers. Among the methods employed to retard or inhibit oxidation of lipids, addition of antioxidants can be an effective solution (Shahidi & Zhong, 2010; Waraho, McClements, & Decker, 2011). Due to the safety concerns about potentially toxic effects of synthetic additives, there is a worldwide trend toward the use of natural antioxidants (Waraho et al., 2011). Moreover, many natural antioxidants possess additional health-promoting benefits in vivo.

Vitamin E compounds (tocopherols and tocotrienols) are considered to be a major group of natural fat-soluble chain-breaking antioxidants to prevent lipid oxidation in foods and biological systems (Eitenmiller & Lee, 2004). Structurally, they are characterized by a chromanol head with two rings (one phenolic and one heterocyclic) and a lipophilic isoprenoid tail (Figure 6.1). The tocopherols have a saturated phytyl tail, whereas their corresponding tocotrienols have a shorter unsaturated farnesyl tail with three isolated double bonds. The position and number of methyl groups on the chromanol head determine the specific type of tocopherol or tocotrienol homologues, named α, β, γ, or δ. It is widely accepted that the antioxidant activity of tocopherols and tocotrienols is mainly due to their ability to donate phenolic hydrogen to lipid free radicals, with less contribution from singlet oxygen quenching (Eitenmiller et al., 2004). The more methyl substitutes at the ortho- and/or para-position to the hydroxyl group, the more easily the

O-H bond can be cleaved (Kamal-Eldin & Appelqvist, 1996; Wright, Johnson, & DiLabio, 2001). Thus, the relative antioxidant effectiveness of different isomers is originally believed to be in the order of $\alpha > \beta > \gamma > \delta$ on the basis of hydrogen-donating power (Kamal-Eldin et al., 1996). The corresponding tocopherols and tocotrienols are also expected to exert similar antioxidant potential due to the presence of the same chromanol group. However, there is considerable conflicting evidence regarding their antioxidant activities in vivo, in vitro, in model systems, and in specific food matrices (Eitenmiller et al., 2004; Kamal-Eldin et al., 1996; Seppanen, Song, & Csallany, 2010). The reasons behind this confusion have not yet been fully understood. It is recognized that the effectiveness of an antioxidant in foods is not only determined by its structure and chemical reactivity toward lipid radicals but also dependent on other factors including its stability, polarity, molecular size, concentration, environmental conditions (e.g., pH and temperature), physical distribution and mobility in the media (e.g., bulk oil or oil-in-water emulsion), and presence of antagonists or synergists (Decker, Warner, Richards, & Shahidi, 2005; Shahidi & Zhong, 2011; Waraho et al., 2011). Huang, Frankel, and German (1994) also proposed that whether α-tocopherol acted as an antioxidant or prooxidant depended on concentration, oxidation time, the method used to determine oxidation, and physical state (bulk phase or emulsion). Although the antioxidant and biological properties of tocopherols, especially α-tocopherol, have been investigated extensively, little is known about antioxidant properties of tocotrienols in foods (Table 6.1), probably because of the scarcity of these compounds and lower biological vitamin E activity than tocopherols.

Up till now, the commercial sources of tocotrienols are palm, rice bran, and annatto (*Bixa orellana L.*) (Watson & Preedy, 2009). Palm or rice tocotrienol-rich fraction (TRF), is actually a mixture of tocopherols and tocotrienols, typically containing 25-50% tocopherols (mostly as α-tocopherol) (Watson et al., 2009). Annatto TRF, on the other hand, is virtually tocopherol-free and composed of only γ- and δ-tocotrienols (Watson et al., 2009). An expanding body of evidence has demonstrated that tocotrienols possesses powerful cholesterol-lowering, anticancer, and neuroprotective properties that are often not exhibited by tocopherols (Aggarwal, Sundaram, Prasad, & Kannappan, 2010; Watson et al., 2009). Nevertheless, unexpectedly, α-tocopherol has been shown to attenuate tocotrienol health benefits in vivo (e.g., hypocholestrolemic and anticancer effects) (Qureshi et al., 1996; Shibata et al., 2010). This finding immediately suggests a question that needs to be addressed: will α-tocopherol interfere with the antioxidant ability of tocotrienols in foods?

Therefore, the first objective of this study was to determine and compare the antioxidant activities of commercial annatto and palm TRFs in bulk oil and in oil-in-water emulsion, with α-tocopherol, δ-tocopherol, and δ-tocotrienol standards as references. Two actual food systems, fish oil and structured lipid (SL)-based ready-to-feed infant formula, were employed, respectively. Our second objective was to explore whether α-tocopherol can interfere with the antioxidant activity of tocopherol-free annatto TRF in the above foods. Different combinations of α-tocopherol and annatto TRF at ratios of 100:0, 75:25, 50:50, 25:75, and 0:100 were used. A better understanding of antioxidant behavior of tocotrienol products would be of great interest to food

manufacturers and consumers, and may lead to the development of new natural antioxidants in food industry.

Materials and methods

Materials

Tripalmitin was purchased from TCI America (Portland, OR, USA). Stearidonic acid (SDA) soybean oil was kindly donated by Monsanto Co. (St. Louis, MO, USA). Docosahexaenoic acid (DHA) single-cell oil containing 40% DHA was purchased from Martek Bioscience Corp., now DSM Nutritional Products Ltd. (Columbia, MD, USA). *sn*-1,3 Specific lipase Lipozyme TL IM and nonspecific lipase Novozym 435 were obtained from Novozymes A/S (Bagsvaerd, Denmark). α- and δ-Tocopherol standards ($\geq$ 96 and 90% purity, respectively) and menhaden fish oil were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The major fatty acids of fish oil were C16:0 (20.0 mol%), C20:5n-3 (15.2 mol%), C16:1n-7 (13.5 mol%), C22:6n-3 (13.0 mol%), C14:0 (10.5 mol%), and C18:1n-9 (5.8 mol%), as determined by gas chromatography of methyl esters (Zou & Akoh, 2013a). DeltaGold annatto TRF (11% γ-tocotrienol and 89% δ-tocotrienol), palm TRF (23% α-tocopherol, 25% α-tocotrienol, 2% β-tocotrienol, 35% γ-tocotrienol, and 15% δ-tocotrienol), and δ-tocotrienol standard ($\geq$ 99% purity), were generously provided by American River Nutrition, Inc. (Hadley, MA, USA). The DHA and SDA containing SL, which mimicked human milk fat, was enzymatically produced as previously reported (Zou et al., 2013a). All the solvents were of analytical grade, and purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA) or Fisher Scientific (Norcross, GA, USA).

Preparation of bulk oil and oil-in-water emulsion samples

Tocopherols and/or tocotrienols at 0.02% and 0.05% levels were dissolved in hexane and then added to menhaden oil or SL before emulsification. Organic solvents were evaporated under nitrogen. The SL-based infant formula emulsion consisting of 3.5% lipid was prepared according to the optimal formulation and procedures described by Zou and Akoh (2013b). The bulk oil and emulsion samples without addition of antioxidants were used as the controls.

Oxidation experiments

The oxidative stability was studied by means of an accelerated storage. Both bulk oil (5 g) and emulsion (25 mL) samples were placed in capped glass bottles, covered with Parafilm, and allowed to oxidize in a covered water bath at 37 °C for 4 weeks. The primary and secondary oxidation products were measured after 0, 7, 14, 21, and 28 days of storage. Lipid hydroperoxide, measured as peroxide value (PV), was determined using a method described by Chaiyasit, McClements, and Decker (2005). For bulk menhaden oil, the samples (0.01 g) were diluted with 4 mL of isooctane before measurements. Emulsion samples were analyzed by the same procedure described previously (Zou et al., 2013b). *p*-Anisidine value (AV) determines the amount of aldehydes (mainly 2-alkenals and 2,4-alkadienals), which are secondary products from the decomposition of hydroperoxides. The AV of menhaden oil (0.5 g) was determined according to the AOCS Official Method Cd 18-90 (AOCS, 2009). Emulsion samples (2 mL) were analyzed using the same method with minor modifications as previously reported (Zou et al., 2013b).

Statistical analysis

All the experiments were conducted on duplicate samples. The effect of different antioxidants on lipid oxidation over time was evaluated by two-way repeated measures ANOVA of fixed-effects models, using SAS software, version 9.2 (SAS Institute Inc., Cary, NC, USA). Duncan's multiple-range test was performed to determine significant differences of the measurements on the same day ($\alpha = 0.05$).

Results

Antioxidant activity in bulk oil

As can been seen in Figure 6.2A and B, the rate of hydroperoxide formation in the control oil increased steeply after 21 days of storage. No significant difference ($p > 0.05$) was found in lipid hydroperoxide concentrations among the control, annatto TRF, and δ-isomer standards at levels of 0.02% and 0.05% during the entire incubation period (Figure 6.2A and B). In the presence of α-tocopherol, the rate of hydroperoxide formation increased with concentration and incubation time, whereas the PVs were not significantly different ($p > 0.05$) between 0.02% and 0.05% for other compounds. At both concentrations, palm TRF showed prooxidant activity between 14 and 28 days. No antioxidant effect was observed in bulk oil on the basis of hydroperoxide formation.

As shown in Figure 6.2C and D, secondary oxidation products were detected after the appearance of lipid hydroperoxides. Their concentrations fluctuated in the control menhaden oil during the incubation (Figure 6.2C and D). In contrast to hydroperoxide formation, all the compounds exhibited antioxidant activities during initial 14 days of incubation at 0.02% and 0.05% (Figure 6.2C and D). However, α-tocopherol and palm TRF had significantly higher ($p < 0.05$) prooxidant activities than annatto TRF and δ-

isomer standards after 21 days of storage at the two concentrations tested. For all evaluated compounds, increasing concentration from 0.02 to 0.05% did not lead to a pronounced change in the AV curve during the incubation period (Figure 6.2C and D).

Antioxidant activity in oil-in-water emulsion

As shown in Figure 6.3, both primary and secondary oxidations were slower in infant formula emulsion than in bulk oil, as indicated by a longer induction period in all the samples. At 0.02% and 0.05%, δ-tocotrienol and annatto TRF were significantly better ($p < 0.05$) than α-tocopherol, δ-tocopherol, and palm TRF in decreasing the formation of lipid hydroperoxides (Figure 6.3A and B). δ-Tocotrienol showed antioxidant effect on primary oxidation after 28 days of storage at both concentrations. However, annatto TRF showed a small prooxidant activity at 0.02% (Figure 6.3A) and antioxidant activity at 0.05% (Figure 6.3B) on the final day of storage. On the basis of hydroperoxide formation, prooxidant effects were observed for tocopherol standards (α- and δ-) and palm TRF after 28 days of oxidation at the two concentrations tested. An increase in the concentration of the compounds except δ-tocotrienol from 0.02 to 0.05% led to a decrease in the formation of lipid hydroperoxide after 28-day incubation.

No significant difference ($p > 0.05$) was found in the AVs among the control, annatto TRF, palm TRF, and δ-isomer standards at levels of 0.02% and 0.05% during the entire incubation period (Figure 6.3C and D). At both levels, α-tocopherol promoted the formation of secondary oxidation products after 28 days of incubation. No antioxidant effect was observed in oil-in-water emulsion on the basis of hydroperoxide decomposition. However, different from the bulk oil, there was a decrease in the AV

when the concentration was increased from 0.02 to 0.05% for the compounds evaluated after 28-day oxidation.

Effect of α-tocopherol addition on the antioxidant ability of annatto TRF in bulk oil and oil-in-water emulsion

In bulk oil, no significant difference ($p > 0.05$) was found in the PVs among different combinations (100:0, 75:25, 50:50, 25:75, and 0:100) of α-tocopherol and annatto TRF at 0.02% after 28-day storage (Figure 6.4A). However, at 0.05%, annatto TRF alone or combinations of annatto TRF and α-tocopherol were significantly better ($p < 0.05$) than α-tocopherol alone in inhibiting primary oxidation (Figure 6.4A). The same results at both concentrations were also obtained on the basis of hydroperoxide decomposition (Figure 6.4B).

In infant formula emulsion, lipid hydroperoxides in the annatto TRF-containing samples were significantly lower ($p < 0.01$) than samples with α-tocopherol alone at both concentrations tested (Figure 6.5A). Similar to the PV results, AVs were significantly lower ($p < 0.05$ at 0.02%; $p < 0.01$ at 0.05%) in the annatto TRF-containing samples than α-tocopherol treatments (Figure 6.5B). Additionally, increasing the antioxidant concentration from 0.02 to 0.05% led to a decrease in both primary and secondary oxidations after 28-day storage (Figure 6.5A and B). No significant difference ($p > 0.05$) was observed in antioxidant activities among annatto TRF-containing samples at all the conditions tested (Figs. 6.4 and 6.5).

Discussion

In our study, the PV and AV were used to assess and distinguish the effectiveness of commercial annatto and palm TRFs in inhibiting lipid hydroperoxide formation and

decomposition in different media. In bulk oil, δ-tocotrienol, δ-tocopherol, and annatto TRF (about 90% δ-tocotrienol) were more active than α-tocopherol and palm TRF (about 25% α-tocopherol) as antioxidants on the basis of PV and AV measurements (Figure 6.2). The difference in antioxidant effectiveness seems to be mainly attributed to the difference in methyl groups. The finding is consistent with that of Dolde and Wang (2011), who reported that δ type tocopherol or tocotrienol had a higher antioxidant activity in corn oil at 60 °C than α type. Several mechanisms may be considered to explain the difference. First, in contrast to α type containing three methyl groups in the chromanol ring, the corresponding δ type only contains one methyl group, leading to a high polarity (Chaiyasit et al., 2005). As a result, δ type has a high affinity oriented toward the interface of the colloid (water in oil) in bulk oil, and is hence more effective in inhibiting oxidation than the nonpolar α type that is dissolved in the oil phase. This is in agreement with the "polar paradox theory" (Porter, 1993), which states that polar antioxidants are more effective in less polar media, such as bulk oil, whereas nonpolar antioxidants are more effective in relatively more polar media, such as oil-in-water emulsion. Second, as the hydrogen-donating power of α type is high, its stability is low compared to δ type, especially at elevated temperature and oxygen content (Huang et al., 1994; Kim, 2007). Third, the ortho-position 5 with respect to the hydroxyl group (Figure 6.1) is the primary site for radical-radical (termination) reactions (Kamal-Eldin et al., 1996). Therefore, the sterically hindered radical of α type is converted to a non-radical species slower than other isomers, with δ type being the fastest. In addition to that, the oxidation products of δ type, typically diphenyl ether dimer and biphenyl dimer, can be effective as antioxidants, whereas the oxidation products of α type, mainly α-tocopheryl and α-

tocotrienyl quinones, have no antioxidant activity (Huang et al., 1994). Fourth, in the presence of high initial levels of hydroperoxides, α type is expected to decompose them at a faster rate compared to δ type due to its greater potential to donate its phenolic hydrogen (Kamal-Eldin et al., 1996). Fifth, α type tocopherol and tocotrienol are capable of reducing transition metal ions (e.g., Cu^{2+}) to act as prooxidants, but the other forms (β, γ, or δ) are not (Kamal-Eldin et al., 1996; Watson et al., 2009). Compared to the bulk oil, this mechanism is supposed to be more pronounced in infant formula emulsion, as it contains a large amount of metal ions as mineral nutrients.

In oil-in-water emulsion system, the induction periods of primary and secondary oxidations were longer than bulk oil (Figure 6.3). Moreover, compared to the bulk oil, the concentration factor has a higher influence on the antioxidant activity. These observations suggest that lipophilic antioxidants, like annatto and palm TRFs, are more effective in hydrophilic environments (e.g., infant formula emulsion) to protect oil against oxidation, which is consistent with the "polar paradox theory". In oil-in-water emulsion, δ-tocotrienol and annatto TRF (about 90% δ-tocotrienol) were found to be more effective than α-tocopherol, δ-tocopherol, and palm TRF (about 25% α-tocopherol) in inhibiting the hydroperoxide formation, suggesting that the lipophilic isoprenoid tail may be partly responsible for the difference. There are two possible explanations for the higher antioxidant potency of tocotrienols than tocopherols in oil-in-water emulsion. First, due to the presence of three double bonds, tocotrienols form a short "arc" conformation over the chromanol ring (Watson et al., 2009) that renders them more flexible and mobile to cover a large surface area of oil droplets. In other words, tocotrienols are more uniformly distributed in the oil-water interface than tocopherols to protect oil against oxidation.

Second, tocotrienols have a higher recycling efficiency from their chromanoxyl radicals than tocopherols (Serbinova, Kagan, Han, & Packer, 1991), which makes the reaction with lipid radicals more efficient.

Structurally, it appears that although the hydroxyl group confers the antioxidant potency of tocopherols and tocotrienols, the double bonds in the isoprenoid tail and the methyl group at position 5 of the chromanol head may contribute to the net antioxidant effectiveness of tocopherols and tocotrienols in food systems.

Limited studies in vivo (Aggarwal et al., 2010) have shown that both the molecular and therapeutic targets of tocotrienols are distinct from those of tocopherols. For example, suppression of inflammatory transcription factor NF-κB that is closely related to carcinogenesis, inhibition of HMG-CoA reductase that is essential for cholesterol synthesis, and inhibition of mammalian DNA polymerases and certain protein tyrosine kinases, are unique to tocotrienols (Aggarwal et al., 2010). Addition of tocopherols not only compromises the unique functions of tocotrienols, but also inhibits their cellular uptake (Shibata et al., 2010). Effective compositions for the cholesterol-lowering benefit have been suggested to consist of 15-20% α-tocopherol and about 60% γ- or δ-tocotrienols, whereas ineffective preparations consist of 30% or more α-tocopherol and about 45% γ- or δ-tocotrienols (Qureshi et al., 1996). In contrast to the findings in vivo, no inhibitory effect of α-tocopherol (0-75%) on the antioxidant ability of annatto TRF (a mixture of γ- and δ-tocotrienols) was found in menhaden fish oil and infant formula emulsion (Figs. 6.4 and 6.5), which have distinct chemical, physical, and environmental properties. Moreover, similar to the previous results, α-tocopherol/annatto TRF mixtures were again shown to be more effective as antioxidants in more polar

media. From food application's point of view, since the antioxidant ability of annatto TRF alone or α-tocopherol/annatto TRF mixture is better than α-tocopherol, one may use annatto TRF to fully or partially replace α-tocopherol as an antioxidant in food formulations, especially in oil-in-water emulsion systems. However, more research is required to confirm this hypothesis.

Conclusions

It appears that factors like the structural characteristics (e.g., hydroxyl groups, methyl groups, and double bonds) and their resulting polarity, concentration, oxidation time, media, and the method used to monitor lipid oxidation, may determine the antioxidant, neutral, or prooxidant behaviors of annatto and palm TRFs. In our study, annatto TRF was found to be more effective than palm TRF and α-tocopherol in inhibiting lipid oxidation in menhaden fish oil and structured-lipid based infant formula emulsion at 0.02% and 0.05%. Addition of α-tocopherol did not attenuate antioxidant activity of annatto TRF in the foods tested. More studies and applications are needed to explore annatto TRF as a promising natural antioxidant as well as a functional ingredient for the enhancement of food quality and human health.

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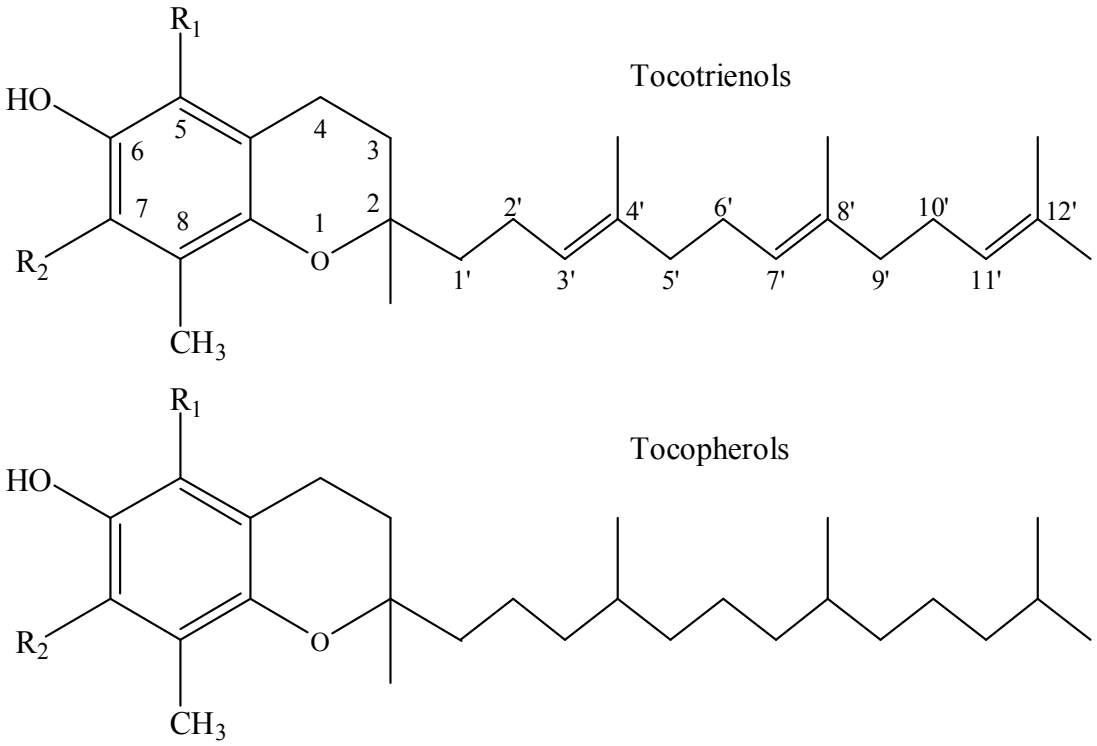
Table 6.1 Summaries of research on the antioxidant activity of tocotrienols in model systems, fats, and oils.

Substrate	Concentration	Oxidation temperature (°C)	Measurement	Result	Reference
Model systems					
Methyl linoleate	0.02%, 0.05%	60	Weight gain	α-T_3 > α-T, γ-T_3> γ-T [a]	Yamaoka, Tanaka, and Kato (1985)
Fats and oils					
Lard	0.02%, 0.05%	110	Refractometric method	Tocotrienols > Tocopherols, δ-T_3 > γ-T_3 > β-T_3 > α-T_3	Seher and Ivanov (1973)
Palm olein	0.02-0.2%	100	Rancimet test	γ-T_3 ≥ δ-T_3 > α-T_3	Top, Ong, Kato, Watanabe, and Kawada (1989)
Palm olein	0.01-0.1%	110	OSI	γ-T_3 ≥ γ-T > α-T_3 ≈ α-T	Feng (1995)
Palm olein	0.04%	110	TOTOX, CD	γ-T_3 > γ-T > α-T_3 ≥ α-T	
Corn oil	0.04%	110	TOTOX, CD	γ-T_3 > γ-T > α-T_3 ≈ α-T	
Soybean oil	0.04%	110	TOTOX, CD	γ-T3 > γ-T > α-T_3 > α-T	
Coconut fat	0.01-0.1%	160	OSI	δ-T_3 > γ-T3 > β-T_3 > α-T_3, δ-T_3 > δ-T, γ-T_3> γ-T	Wagner, Wotruba, and Elmadfa (2001)
	0.01-0.1%	60	PV, CD	δ-T_3 and γ-T_3 as antioxidants, α-T_3 and β-T_3 as prooxidants	
Canola oil	Unfixed	180	OSI	α-T at 432 mg/kg > α-T_3 at 138 mg/kg ≈ α-T at 155 mg/kg	Romero et al. (2007)
Lard	0.01-0.1%	55	Headspace oxygen, PV	δ-T3 > γ-T_3 > β-T_3 > α-T_3	Kim (2007)
Corn oil	0.01-0.5%	60	PV, OSI	γ-T_3 > δ-T_3 ≈ δ-T > α-T_3 ≈ α-T	Dolde and Wang (2011)

[a] Abbreviations: T_3, tocotrienol; T, tocopherol; OSI, oil stability index; TOTOX, total oxidation value; CD, conjugated diene; PV, peroxide value.



Tocotrienols or tocopherols	R_1	R_2
α (5,7,8-trimethyl)	CH_3	CH_3
β (5,8-dimethyl)	CH_3	H
γ (7,8-dimethyl)	H	CH_3
δ (8-monomethyl)	H	H

Figure 6.1 Chemical structures of tocotrienols and tocopherols.

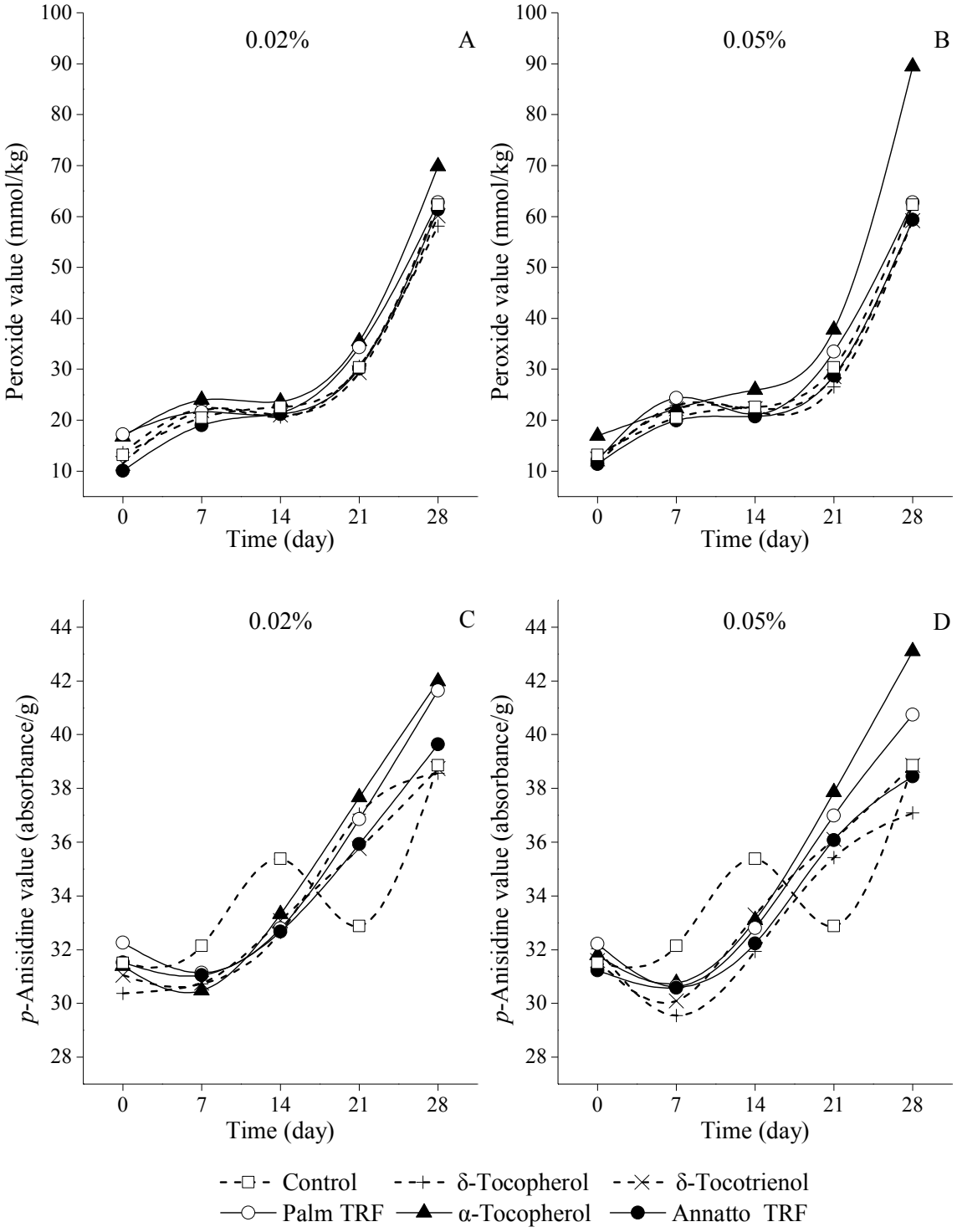


Figure 6.2 Effect of antioxidants (0.02% and 0.05%) on the oxidative stability of menhaden fish oil at 37 °C: (A) and (B) peroxide value; (C) and (D) *p*-anisidine value. Data points represent the average of duplicate samples. (TRF, tocotrienol-rich fraction)

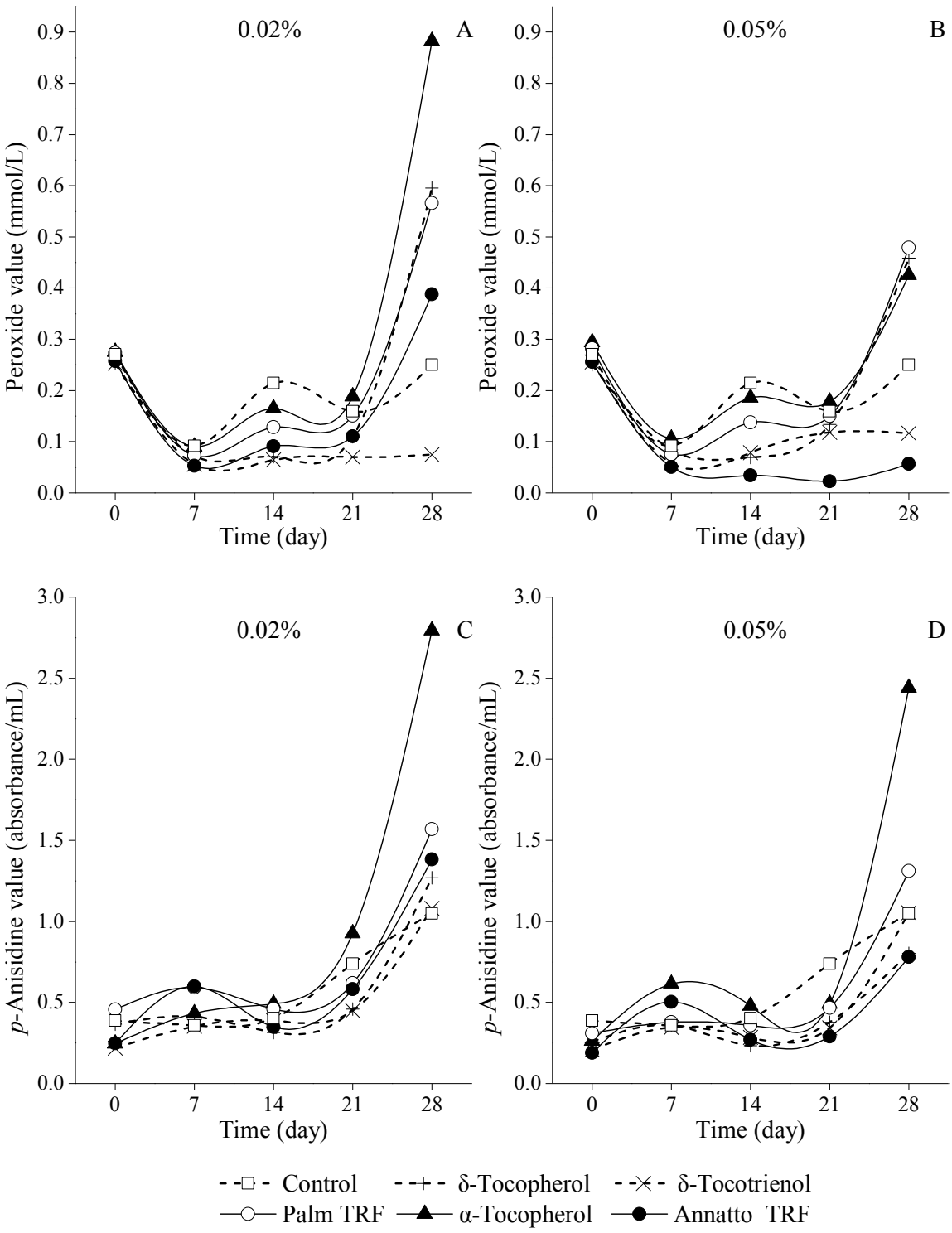


Figure 6.3 Effect of antioxidants (0.02% and 0.05%) on the oxidative stability of structured lipid-based infant formula emulsion at 37 °C: (A) and (B) peroxide value; (C) and (D) *p*-anisidine value. Data points represent the average of duplicate samples. (TRF, tocotrienol-rich fraction)

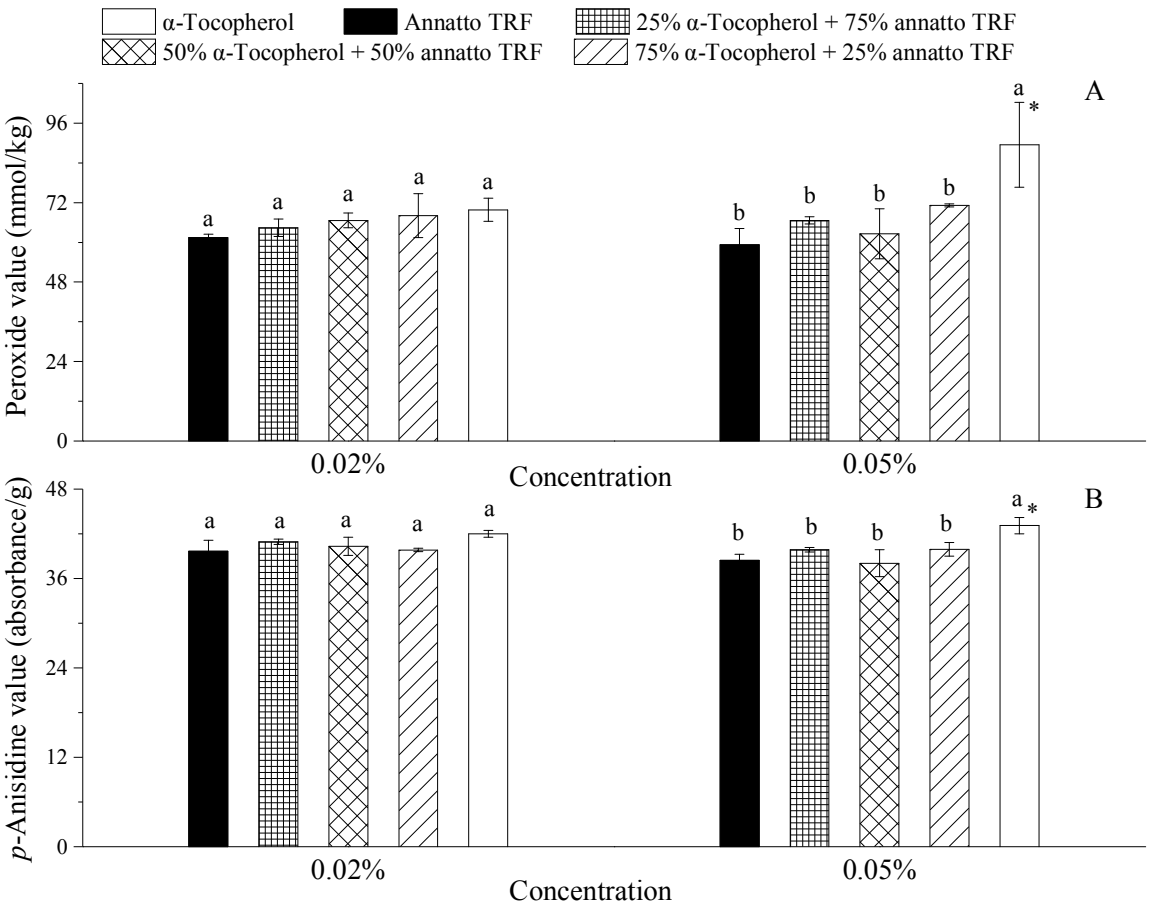


Figure 6.4 Effect of α-tocopherol addition on the antioxidant ability of annatto tocotrienol-rich fraction (TRF) in menhaden fish oil at 0.02% and 0.05% after 28 days of storage: (A) peroxide value; (B) *p*-anisidine value. Different lower-case letters within each concentration group indicate a significant difference (*, $\alpha = 0.05$).

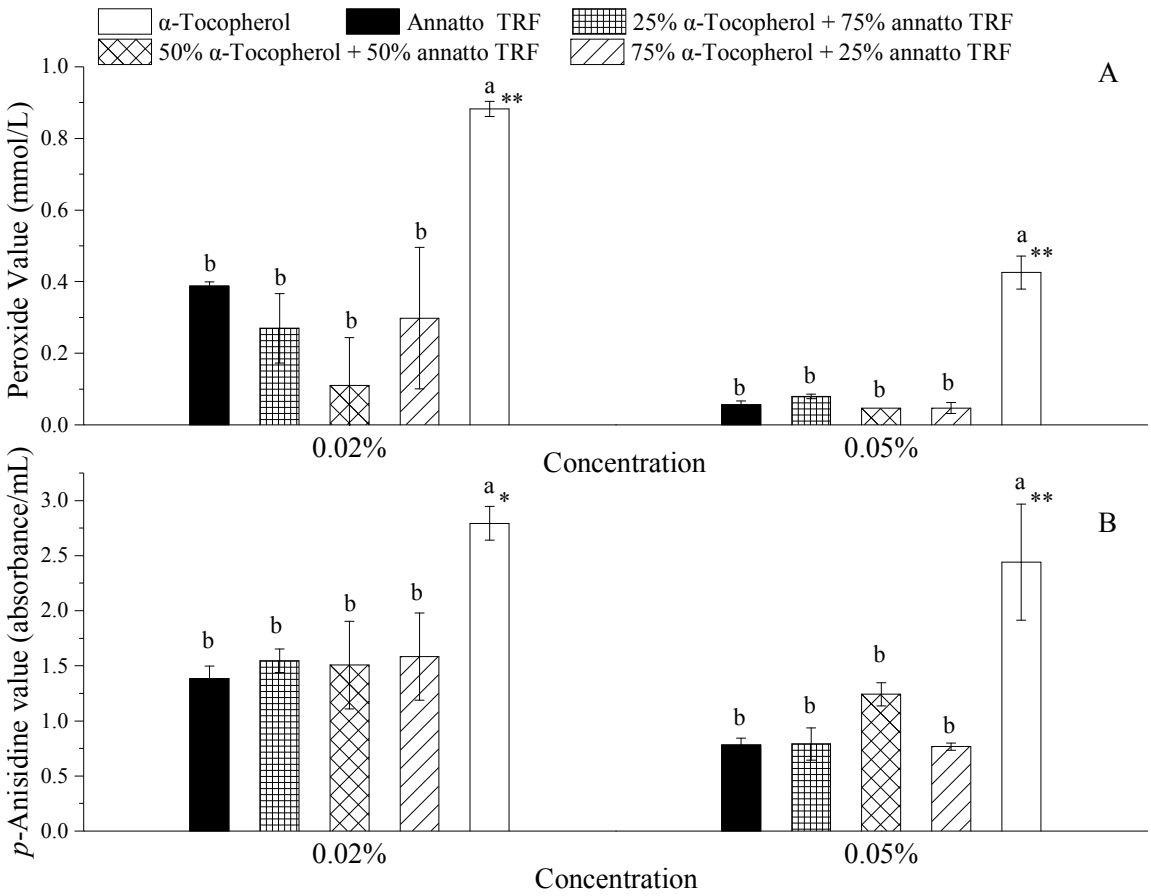


Figure 6.5 Effect of *α*-tocopherol addition on the antioxidant ability of annatto tocotrienol-rich fraction (TRF) in structured lipid-based infant formula emulsion at 0.02% and 0.05% after 28 days of storage: (A) peroxide value; (B) *p*-anisidine value. Different lower-case letters within each concentration group indicate a significant difference (*, $\alpha = 0.05$; **, $\alpha = 0.01$).

CHAPTER 7

CONCLUSIONS

A structured lipid (SL), which mimics the unique structure of human milk fat (HMF) as well as containing omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFAs) in the triacylglycerol backbone, was successfully produced by a two-step enzymatic interesterification and acidolysis using tripalmitin, stearidonic acid (SDA) soybean oil, and docosahexaenoic acid (DHA) as substrates. The SL contained 5.4 mol% DHA and 8.0 mol% SDA, with 57.0 mol% palmitic acid esterified at *sn*-2 position. It was found that the endogenous antioxidants, tocopherols or tocotrienols, in the original oils were mainly lost during enzymatic reactions as fatty acid esters and/or during purification in a free form (>50%). These two types of losses together resulted in a compromised oxidative stability of the SL produced.

After optimizing the type and level of emulsifiers, thickeners, and antioxidants, a SL-based ready-to-feed infant formula (IF) with the highest physical and oxidative stability was successfully developed. The optimal conditions were 0.2 g/100 mL lecithin, 0.4 g/100 mL monoacylglycerol, 0.045 g/100 mL locust bean gum, 0.015 g/100 mL carrageenan, and 0.005% ascorbyl palmitate (of oil). Several important findings about ingredient interactions were observed. First, emulsifiers (lecithin and monoacylglycerol) played a more important role than thickeners (locust bean gum and carrageenan) in controlling the emulsion stability. Lecithin content significantly affected lipid oxidation. An increase in monoacylglycerol concentration was associated with large particle size

and high emulsion viscosity. Second, the efficacy of permitted compounds as antioxidants in SL-based IF emulsion depended not only on their mechanism of action, but also on polarity, concentration, oxidation time, method used to determine lipid oxidation, and environmental conditions (e.g., headspace oxygen and pH). Nitrogen flushing could be used as a supplemental technique to further protect against oxidation.

Compared to palm tocotrienol-rich fraction (TRF) and α-tocopherol, annatto TRF was proved to be a more effective natural antioxidant to inhibit lipid oxidation in menhaden fish oil and SL-based IF emulsion, particularly in the latter case. It appears that the structural characteristics (e.g., hydroxyl groups, methyl groups, and double bonds) of tocotrienols largely contribute to the difference. Addition of α-tocopherol did not attenuate antioxidant activity of annatto TRF in the foods tested. From food application's point of view, one might use annatto TRF to fully or partially replace α-tocopherol as an antioxidant in food formulations, especially in oil-in-water emulsion systems.

All the objectives of this study described in the introduction section (chapter 1) were achieved. This research resulted in a ready-to-feed IF product containing HMF analogues enriched with DHA and SDA to improve infant nutrition and health. The substrate, SDA enriched soybean oil, is a sustainable and oxidatively stable plant-based source of omega-3 fatty acids. The SDA-based SL can compete with flaxseed oil, fish oil, Betapol, and Infat for the lipid source in IF market. Moreover, a better understanding of how emulsifiers, biopolymer thickener, and/or antioxidants at the oil-in-water interface affect the kinetics of lipid oxidation in SL-based emulsion is of great interest to food manufacturers and consumers, and may lead to new strategies to extend shelf-life of omega-3 enriched foods. The findings of annatto TRF as a powerful natural antioxidant

may lead to its application as a functional ingredient in food industry for the enhancement of food quality and human health as well. Overall, this study has important implications for the successful use of SLs for food formulations.

Suggestions for future work:

(1) Find efficient and cost-effective strategies to recover the lost tocopherols and tocotrienols in the distillates and add back to the final oil products (SLs) to improve their oxidative stability.

(2) Verify the health-promoting effects of the HMF enriched with DHA and SDA in animal models by analyzing the absorption of palmitic acid and calcium and bioavailability of omega-3 fatty acids in the tissues (e.g., eyeball and brain).

(3) Evaluate and compare the acceptace of SL-based ready-to-feed IFs with corresponding commercial products through sensory analysis.