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Supplementation of laying hen feed with tocopherols and algal (Haematococcus pluvialis) astaxanthin for egg yolk nutrient enhancement and a novel use of egg yolk protein and lecithin in the formation of artificial oil bodies for stabilizing omega-3 oil

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APPENDIX

LIST OF ABBREVIATIONS

α-TTP	α-Tocopherol Transfer Protein
AOB	Artificial oil body
ASTA	Astaxanthin
ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
CVD	Cardiovascular disease
E_a	Activation energy
EYP	Egg Yolk Protein
FAME	Fatty acid methyl esters
FDA	Food and Drug Administration
GRAS	Generally Recognized As Safe
HDL	High-density lipoproteins
LDL	Low-density lipoproteins
LPC	Lysophospholipid
MUFA	Monounsaturated fatty acid
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SFA	Saturated fatty acid
SM	Sphingomyelin
SPI	Soy protein isolate
TAG	Triglycerides
TBA	Thiobarbituric acid
TBARS	2-Thiobarbituric acid-reactive substances
TCA	Trichloracetic acid
Tocos	Tocotrienols and tocopherols
TPP	Triphenol phosphate
USDA	United States Department of Agriculture

CHAPTER 1. GENERAL INTRODUCTION

INTRODUCTION

Hen's egg is recognized as one of nature's most complete foods. Eggs contain high-quality protein and lipids as well as essential and nonessential nutrients (Huopalahti et al., 2007). Basic elements in egg yolk include: sulfur (0.016%), potassium (0.112-0.360%), sodium (0.070-0.093%), phosphorus (0.0543-0.980%), calcium (0.121-0.262%), magnesium (0.032-0.128%), and iron (0.0053-0.011%) (Stadelman and Cotterill, 1995). It is also one of the most versatile foods. Eggs are widely used in the food industry for their diverse properties including, but not limited to foaming, gelling, and emulsification (Aluko and Mine, 1998). Eggs contain 70 calories per egg and have been connected with healthy eating habits (American Egg Board, 2011). Egg yolk protein has been tested against milk casein where egg protein was shown, *in vitro*, to be digested faster than milk casein (Sakanaka et al., 2000). The nutrients in eggs can help with weight management, muscle strength, pregnancy health, brain function, eye health, and more (Weigle et al., 2005). Diets with increased high-quality protein, like the protein found in eggs, with a constant carbohydrate intake reduces the amount of energy intake by 441 ± 63 kcal/day and a decrease in body weight by 4.9 ± 0.5 kg over a 16-week human study (Weigle et al., 2005).

Eggs are made up of yolk (36%) with 50-52% of water, and the remaining is the egg white with 90% water (Huopalahti et al., 2007; Aro et al., 2009). Egg yolk possess unique physical and chemical characteristics that can be manipulated by pH, heating, freezing, and drying. Major uses of egg yolk occur in the medical, pharmaceutical, cosmetic, nutraceutical, and biotechnological industries (Huopalahti et al., 2007; Aro et al., 2009). Egg yolk is used for food products like mayonnaise, salad dressings, baked goods, pasta, etc. (Aton el al., 2003).

Yolk lipids are primarily exist in lipoprotein assemblies made of 62% triglycerides, 33% phospholipids, and less than 5% cholesterol (Huopalahti et al., 2007). Fatty acid composition of lipids, based on a standard feed, are as seen in Table 1. Fatty acid

composition can be altered due to modifications in the dietary fatty acids. Polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA) can be more affected by diet yet saturated fatty acids (SFA) proportions are only slightly affected. Triacylglycerols are typically esterified by palmitic acid on position 1, oleic or linoleic acids on position 2, and oleic, palmitic, or steric acids on position 3 (Huopalahti et al., 2007).

Table 1. Composition of Egg Lipids Found Based on a Standard Feed

Fatty Acid	Percentage
SFA	30-35
MUFA	40-45
PUFA	20-25
Oleic acid (18:1)	40-45
Palmitic acid (16:0)	20-25
Linoleic acid (18:2)	15-20

Phospholipids contain one hydrophilic head group either consisting of phosphoric acid plus alcohol, amino acids, or polyol, and one hydrophobic group containing two fatty acids (Huopalahti et al., 2007). Phospholipids in eggs are primarily phosphatidylcholine (76-80%) and phosphatidylethanolamine (11-22%) (Aluko and Mine, 1998; Palacios and Wang, 2005). Of the remaining phospholipids, phosphatidylinositol, phosphatidylserine, sphingomyelin, cardiolipins, lysoPC, and lysoPE are present in low quantities. Choline is an essential nutrient for fetal development, influencing brain and spinal cord structures and reducing the risk of neural tube defects (Zeisel, 2006). Choline is also an important compound for neurons in the brain (Zeisel, 2006). Cholesterol is found in the egg yolk present in low-density lipoproteins. The compounds arise from feed and synthesis in the hen liver (Huopalahti et al., 2007).

Carotenoids concentration can range from 1.4-12.6 μg/g of yolk which give the yolk's color and represent less than 1% of total yolk lipids (Surai et al., 2001; Huopalahti et al., 2007). Carotenoids present in the yolk include carotene, lutein, cryptoxanthin, and zeaxanthin (Huopalahti et al., 2007). Lutein and zeaxanthin accumulate in the eye where the compounds have been suggested to reduce the risk for cataracts (up to 20%) and age-related macular degeneration (up to 40%) (Moeller et al., 2000).

Proteins are the last important group found in egg yolk. Yolk proteins are present in free and apoproteins. On a basis of dry matter, yolk has five major protein constituents

which are as follows: 68% low-density lipoproteins (LDL) or also known as lipovitellins, 16% high-density lipoproteins (HDL), 10% globular proteins (livetins), 4% phosphoproteins (phosvitins), and 2% minor proteins (Huopalahti et al., 2007; Aluko and Mine, 1998; Aton et al., 2003; Jolivet et al., 2006). Low density lipoproteins are the main constituent in egg yolk making up approximately 2/3 of the dry material made of 87% lipids and 12% proteins (Huopalahti et al., 2007; Aton et al., 2003). These proteins are similar to the lipoproteins found in chicken blood when the blood enters the ovaries, the lipoproteins are transferred to the yolk (Huopalahti et al., 2007; Jolivet et al., 2006). Described to be a polypeptide chain folded in a globular configuration, these proteins form spherical particles with the lipid molecule within the protein and a variable amount of lipid on the surface (Aluko and Mine, 1998; Aton et al., 2003; Jolivet et al., 2006). High-density lipoproteins are the second group, accounting for 1/6 of the dry yolk matter and 36% of the protein (Huopalahti et al., 2007). These proteins are made up of 75-80% proteins and 20-25% lipids (Huopalahti et al., 2007). The third group are phosphoglycoproteins called phosvitin. Of the known proteins for this group, 50% of the amino acids are serine and of which, 90% is phosphorylated (Huopalahti et al., 2007). Due to the unique chemical characteristics, phosvitins act as phospholipid antioxidants (Huopalahti et al., 2007). The last group of proteins are the globular proteins also known as livetins. These proteins have different fractions including α-, β-, and γ-livetin at the ratio of 2:5:3, respectively.

RATIONALE FOR NUTRIENT FEEDING PROJECT

Egg composition can be altered by heredity genes, diet, and laying hen age (Frediksson et al., 2006). Egg yolk color is attributed to the presence of β-carotene, zeaxanthin, crypotoxanthin, and lutein naturally found in yolks (Huopalahti et al., 2007).

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is a dark-red pigment, member of the carotenoid family. Astaxanthin is the main carotenoid found in algae, such as *Brevibaterium, Mycobacterium lacticola, Agrobacterium auratim, Haematococcus pluvialis,* and *Phaffia rhodozyma* (Domínguez-Bocangegra et al., 2004). Aquatic animals including salmon, trout, shrimp, lobster, zooplankton, and birds, such as flamingo and quail may

contain astaxanthin from algae consumption directly or from the food chain (Johnson and An, 1991). Astaxanthin has a strong antioxidant activity, 10 times higher than β-carotene and more than 300 times more effective compared to α-tocopherol (Rao et al., 2009; Higuera-Ciapara et al., 2006; Miki, 1991). Astaxanthin from *Haematococcus pluvialis* is an excellent source not only for pigmentation, but also for protection against lipid oxidation, UV photoxidation, inflammation, cardiovascular disease, cellular health, and cancer (Guerin et al., 2003).

Tocotrienols and tocopherols are fat-soluble vitamins and generically named as vitamin E, whose antioxidant properties are well studied. Within the vitamin E analogs, α-tocopherol is considered the most active form (Theriault et al., 1999). Bioavailability of vitamin E is greatly influenced by food matrix, formulation, and food processing (Reboul et al., 2006). Vitamin E is thought to prevent atherosclerosis (Stephens et al., 1996), protect against coronary disease (Stampfer et al., 1993), oxidative stress, development of Alzheimer's disease (Li et al., 2001), reduce bone loss, have immunomodulatory functions, gastroprotective effects, anti-aging, and skin protective effects (Aggarwal et al., 2010).

Previous work on astaxanthin has shown successful pigmentation to egg yolks, salmon, trout, and chicken meat. A study by Akiba et al. (2000) examined the effects of feeding a red yeast, *Phaffia rhodozyma*, that contained astaxanthin to increase the color of egg yolk. The authors used very low concentrations of astaxanthin, 0, 2, 4, 8, and 16 ppm for four weeks to feed hens. The yolk color score was increased from 6.6 (golden yellow) to 12.7 (orange) on the Roche color fan. Dike et al. (1992) completed a similar study using laying hens with 0.3% *Phaffia rhodozyma,* broken or intact yeast cells for eight months to increase the Roche color fan score by only 1 point (intact cells) and 2 points (broken cells) taking the color from a yellow to a more golden yellow. Johnson et al. (1980) used the same yeast, *Phaffia rhodozyma*, for laying hens and Japanese quail, and examined the various concentrations of astaxanthin in the diet. They also examined the effect of intact red yeast cells, mechanically fractured, and oil extracted from the yeast on individual chickens. The astaxanthin from the fractured cells and oil was deposited in the yolk. Another study by Nelson and Baptist (1967) fed quail astaxanthin with or without lutein. Results concluded

less total pigment was needed when astaxanthin and lutein were combined than with lutein alone. The authors also examined the effects of astaxanthin pigment in yellow and white corn diets. Astaxanthin in white corn was more effective with yolk pigmentation than in yellow corn diet due to the other naturally occurring carotenoids. Lastly, a study completed by Takahashi et al. (2004) evaluated the effect of astaxanthin feed containing 0, 50, or 100 mg/kg of astaxanthin from *Phaffia rhodozyma* over two weeks on broilers. From tissues examined for astaxanthin concentration, the highest concentration resulted in the small intestine followed by subcutaneous fat, abdominal fat, spleen, liver, heart, kidneys, and skin. The lowest astaxanthin concentration resided in the muscle. Despite the low levels in the muscle, Takahashi et al. (2004) were able to show that astaxanthin was transported to different tissues. All of these studies demonstrated successful incorporation of astaxanthin in a variety of tissues, from eggs to muscle tissues and plasma levels.

Astaxanthin has also been used to pigment other tissues besides egg yolks. One study by Gobantes et al. (1998) used 5% astaxanthin in the diet to feed rainbow trout (*Oncorhynchus mykiss*) to examine the color stability in vacuum packaging over storage time. This study found considerable color variations among the neck, tail, and back parts along the fish but failed to maintain the color stability over 15-day storage time. Another fish pigmentation study completed by Barbosa et al. (1999), evaluated the blood of rainbow trout (*Oncorhynchus mykiss*) by feeding the fish two different sources of astaxanthin, green algae *Haematococcus pluvialis* and commercial beadlets of 8% astaxanthin. Four diets were used, two of which with the astaxanthin from algae, 129.8 and 142.0 mg/kg of feed dry matter, and two from the synthetic source, 110.1 and 119.7 mg/kg of feed dry matter. They were able to astaxanthin achieve serum levels from 5.3 to 9.0 μg/ml. Higher levels in the serum were achieved with high lipid levels in the diet independent of astaxanthin source. Johnson et al. (1977) used *Phaffia rhodozyma* as a dietary supplement, 15% wt/wt, for salmonoids and crustaceans. They used lobster feed either at two different yeast concentrations, or a diet of brine shrimp that contained astaxanthin. The results showed the lobsters fed the yeast diets did not grow or accumulate as much astaxanthin as the lobsters fed the shrimp. Also within this study, the authors examined rainbow trout fed yeast diets that also increased tissue

pigmentation. However, there is little research completed to incorporate astaxanthin at concentrations where health benefits can be reached. The study in chapter 3 will try to answer the dose in feed versus the concentration in egg yolk relationship.

Vitamin E transfer analysis from supplemented feed to hen eggs is another topic for this study. Sen et al. (2006) noted for all the vitamin E papers in PubMed there is less than 1% related to tocotrienols. There have been several feeding studies dealing with vitamin E enriched feed for the nutrient to transfer to eggs. One study by Flachowsky et al. (2002) examined the effect of vitamin E supplementation on α-tocopherol concentration on foods of poultry origin. This study fed 0, 100, 1000, 10,000, and 20,000 mg vitamin E/kg of feed. Eggs had the highest levels of α-tocopherol. Another study by Franchini et al. (2002) used DL-α-tocopheryl acetate, 100 or 200 mg/kg of feed, and ascorbic acid, 500 or 1000 mg /kg of feed to increase levels in eggs, and were successfully incorporated α-tocopherol into eggs. Galobart et al. (2001) added 0, 50, 100, and 200 mg/kg α-tocopheryl acetate to a base diet containing 5% linseed oil to determine the amount transferred to eggs. They were able to achieve a dose-dependent manner with a range of 16-132 μg/g in egg. The last example is by Grobas et al. (2002) added α-tocopheryl acetate and vitamin A to the diet to assess the supplementation levels in the egg yolk. Kang et al. (1998) used palm oil, 1.5% or 3.5%, to increase the α- and γ-tocotrienol content in eggs, liver, meat, and adipose tissues of hens. They showed that α-tocopherol was the major tocopherol isomer in egg lipid, and increasing α-tocotrienol from 1.1 μg/g in control diet to 10.8 μg/g (in the 1.5% palm oil diet) and 6.2 μg/g (in the 3.5% palm oil diet) and γ-tocotrienol from 2.4 μg/g in control diet to 5.2 μg/g (in the 1.5% palm oil diet) and 4.7 μg/g (in the 3.5% palm oil diet). Concentrations of α- and γ-tocotrienol in the liver, white meat, dark meat, and adipose tissues were generally higher in the increased diets with palm tocotrienols and tocopherols (tocos) but not always significant from the control diet (Kang et al., 1998).

Tocotrienols should not be avoided for feed enhancement studies as these compounds possess powerful neuroprotective, anticancer, and cholesterol lowering properties that are not often shown by tocopherols. A study by Osakada et al. (2004) used palm oil to investigate the effects of vitamin E on oxidative stress and apoptosis using primary neuronal cultures from

rat striatum. Each of the different analogs were purified from the palm oil and examined. All the tocotrienols significantly decreased hydrogen peroxide induced neurotoxicity, but α-tocopherol did not. α-, γ-, and δ-Tocotrienols had protection against cytotoxicity of superoxide donors, paraquat, and nitric oxide donors.

Another study evaluated γ-tocotrienol antitumor activity on human hepatoma Hep3B cells (Sakai et al., 2006) and found low levels of γ-tocotrienol and shorter treatment was able to inhibit cell proliferation better than α-tocotrienol. Lastly, a study completed by Lee et al. (2009) examined the effect on exercise endurance and oxidative stress on forced swimming rats that were fed tocotrienol-rich or D-α-tocopherol rich diets. They concluded the tocotrienol was able to improve the physiological condition and reduce exercise-induced oxidative stress in rats. Tocotrienols have specifically been linked for neuroprotection, cholesterol, blood pressure lowering, anti-aging, antioxidant, anticancer, anti-angiogenic, anti-inflammatory, immune function, improving obesity, osteoporosis, eye, and bone health (Sen et al., 2006). Sen et al. (2006) has a comprehensive list of the studies dealing with these health issues. Because of many health benefits of tocotrienols, these compounds should be examined for their transfer to a widely used food product, this is where the current study can fill the literature gap.

RATIONALE FOR ARTIFICIAL OIL BODY RESEARCH

Lipid oxidation and secondary oxidation product generation have been a serious concern for food industry relating to food quality and consumer health. One factor influencing lipid oxidation is the degree of unsaturation. Several studies illustrate linoleic acid oxidation was 20-40 times faster than oleic acid (Kolakowska, 2003; Frankel, 1980; Fatemi and Hammond, 1980). Polyunsaturated fatty acids are a significant amount of total lipid due to the natural occurrence and presence in fortified formulations and more prone to oxidation than other lipids. Another factor that can influence the rate of oxidation is transition metal ions, copper and iron, which are commonly found in food emulsions (Wang and Wang, 2008). Copper is less likely to be found in food but has been shown to be more active accelerating primary oxidation product decomposition (Yoshida and Niki, 1992).

Other factors, such as oxygen availability, storage temperature, and pH can also affect oxidation rate.

Naturally occurring oil bodies protect lipids from oxidation and can be utilized by food manufactures for the same purpose. Artificial oil bodies can serve similar purposes, such as carriers for hydrophobic molecules like nutraceutical compounds, pharmaceutical drugs, probiotics, or healthy fatty acids (Chen et al., 2004; Peng et al., 2003; Chiang, 2010). Plant oil bodies are very stable at neutral pH values. They do not aggregate or coalesce *in vivo* during seed maturation or after centrifugation (Haung, 1994). Oil bodies consist of three basic components, proteins, phospholipids, and triglycerides (Huang, 1992). They have a matrix composed of triacylglycerols covered by a phospholipid monolayer with unique proteins, such as oleosins, caleosins, and steroleosins (Bhatla et al., 2010; Frandsen et al., 2001; Huang, 1994). Oil bodies have been observed from mature seeds of rape (*Brassica napus L.),* mustard *(Brassica juncea L.)*, cotton *(Gossypium hirsutum L.)*, flax *(Linus usitatis simum)*, maize *(Zea mays L.)*, peanut *(Arachis hypogaea L.)*, and sesame *(Sesamum indicum L.)* (Tzen et al., 1993). Plants use the oleosomes as energy sources for germination and post-germinative seedling growth (Tzen et al., 1998; Peng et al., 2003). Oleosomes are held together by simple physical forces, steric hindrance and negative surface charge by oleosin (Huang, 1992; Chiang, 2010). Oleosomes are stable both *in vitro* and *in vivo* (Chiang, 2010).

Hen egg yolk is typically marketed whole providing low added value (Anton and Gandemer, 1997) despite the range of various functional properties and composition. The proteins in egg yolk typically are hydrophilic, but also contain hydrophobic groups that can be absorbed into oil-water interface (Daimer and Kulozik, 2010). Egg yolk consists of a mixture of proteins with wide ranges of molecular weights, pI, and roles in emulsions (Daimer and Kulozik, 2010). Egg yolk proteins can provide stabilizing effects in food systems with low pH (Nilsson et al., 2007). For this study, egg lecithin was used since it contains less linoleic, almost no linolenic acid, and more long-chain polyunsaturated fatty acids as compared to soy lecithin (Wang and Wang, 2008).

With the ease of PUFAs oxidation, it is the goal of this study to create artificial oil bodies with egg yolk protein to mimic naturally occurring oleosomes to protect oil from oxidation.

THESIS ORGANIZATION

The contents of this thesis include a general introduction, literature review for both studies, manuscripts prepared for journal submissions for both studies, and general conclusion chapter.

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CHAPTER 2. LITERATURE REVIEW-NUTRIENT FEEDING STUDY

GENERAL INTRODUCTION

Carotenoids are naturally found in egg yolk which give its yellow color. The color can range from a pale yellow to a dark orange. Color plays as a quality criterion thus carotenoid content is an important to egg quality because good yolk color is desirable (Huopalahti el al., 2007). Typically, the carotenoids found in egg yolk are from the xanthophyll family. Lutein, cryptoxanthin, and zeaxanthin are found in the yolk with the concentration of lutein and zeaxanthin combined are ten times greater than cryptoxanthin (Huopalahti et al., 2007). Carotene is the main carotenoid commonly found in corn-based feed. After ingestion of the feed, carotene is converted to the oxidized form of xanthophylls (Huopalahti et al., 2007).

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is a dark-red pigment and a member of the carotenoid family. The source of astaxanthin for this study was from algae, *Haematococcus pluvialis,* astaxanthin dry mass concentration of 1.35% (Cyanotech Spec Sheet, 2010). Astaxanthin is used in industry as a feed pigment for poultry, trout, salmon, and shrimp (Bolin et al., 2010). Specifically in salmonids, astaxanthin is used over canthaxanthin because astaxanthin pigmentation is more nature-identical and more efficiently deposited in the tissues (Barbosa et al., 1999). Other applications have been growing in the medical field showing potential use as an antioxidant, photoprotectant, cardiovascular disease preventer, and immune booster. Astaxanthin has been shown *in vitro* studies to have antioxidant properties up to 500 times of other commercial carotenoids like vitamin E. This carotenoid's structure is related to β-carotene, zeaxanthin, cryptoxanthin, and lutein.

Tocotrienols and tocopherols are fat-soluble vitamins and generically named as vitamin E. Their antioxidant properties have been well studied. Within the vitamin E group, α-tocopherol is considered the most active form, reverses vitamin E deficiency symptoms, and is the most abundant form in nature (Theriault et al., 1999; Brigelius-Flohé and Traber, 1999). Bioavailability of vitamin E is greatly influenced by food matrix, formulation, and

food processing (Reboul et al., 2006). Vitamin E food sources include sunflower seeds, almond, and hazelnuts (Reboul et al., 2006). Tocopherols are present in corn, wheat, and soybeans while tocotrienols are present in barley, oats, palm, and rice bran (Aggarwal et al., 2010). Vitamin E cannot be synthesized in the human body, therefore must be obtained from the diet (FDA: GRAS 307). Vitamin E has been known to be an essential nutrient since 1922 (Brigelius-Flohé and Traber, 1999).

This review will focus on astaxanthin, tocopherols, tocotrienols, their health benefits, safety of these compounds, bioavailability, and digestibility.

ASTAXANTHIN

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is a dark-red pigment, member of the carotenoid family, and the main carotenoid found in algae and aquatic animals including salmon, trout, shrimp, lobster, zooplankton, and birds, such as flamingo and quail (Johnson and An, 1991). Although most of these animals cannot synthesize it *de novo* from other carotenoids, they can obtain it from natural sources of astaxanthin. Natural sources of the pigment are also from bacteria such as *Brevibaterium, Mycobacterium lacticola, Agrobacterium auratim, Haematococcus pluvialis,* and *Phaffia rhodozyma* (Domínguez-Bocanegra et al., 2004). Astaxanthin is used for pigmentation and feed additive for poultry, salmon, trout, and shrimp (Higuera-Ciapara et al., 2006). The pink or red pigmentation is a direct result of astaxanthin consumption (Holtin et al., 2009). The pigment is commercially produced by *Haematococcus pluvialis*, a microalgae where the pigment can accumulate between 0.2-4% dry

Table 1. Composition of Astaxanthin

Components	Composition (%)
Natural Astaxanthin	1.35
Total Fat	20-25
Protein	20-35
Carbohydrates	30-55
Dietary Fibers	18
Moisture	<9
Ash	<17
Free Astaxanthin	1.9
Monoester	84.25
Diester	13.85
Di-cis-astaxanthin	2.22
Trans-astaxanthin	74.07
9-cis astaxanthin	6.66
13-cis astaxanthin	8.15
15-cis astaxanthin	0.74

Adapted from ALGAtechnologies (1998) spec sheet, Cyanotech certificate of analysis (2010) and Cyanotech analysis report (2011).

weight basis (Steinbrenner and Linden, 2003; Johnson and An, 1991). *Haematococcus pluvialis* is a member of the family Chlorophyceae and is known to accumulate carotenoids under stress conditions (Rao et al., 2009). This study used BioAstin, a dry flake/powder of natural astaxanthin, from Cyanotech Corporation in Hawaii. Natural astaxanthin exists in free (alcohol) form, but primarily as a monoester, and diester. The BioAstin composition is reported in Table 1.

Structure of Astaxanthin. Typical carotenoids are 40 carbon atoms hydrocarbons with two terminal rings joined by a chain of conjugated double bonds. Xanthophylls are oxygenated derivatives (Higuera-Ciapara et al., 2006). Due to the conjugated chain, each double bond can exist in two geometric configurations, *cis* or *trans*. *Cis*-isomers are thermodynamically less stable than the *trans* isomer. Because of the isomer stability most carotenoids found in nature predominantly exist in the all-*trans* isomer (Higuera-Ciapara et al., 2006). Astaxanthin incorporates oxygen as OH group and as oxi-groups. It exists in five stereoisomers but mostly in the 3S, 3'S conformation in Haematococcus pluvialis (Misawa et al., 1995; Holtin et al., 2009). The stereoisomers are shown in Figure 1. Unique features of astaxanthin are the hydroxyl and keto endings of each ring explaining the ability to be

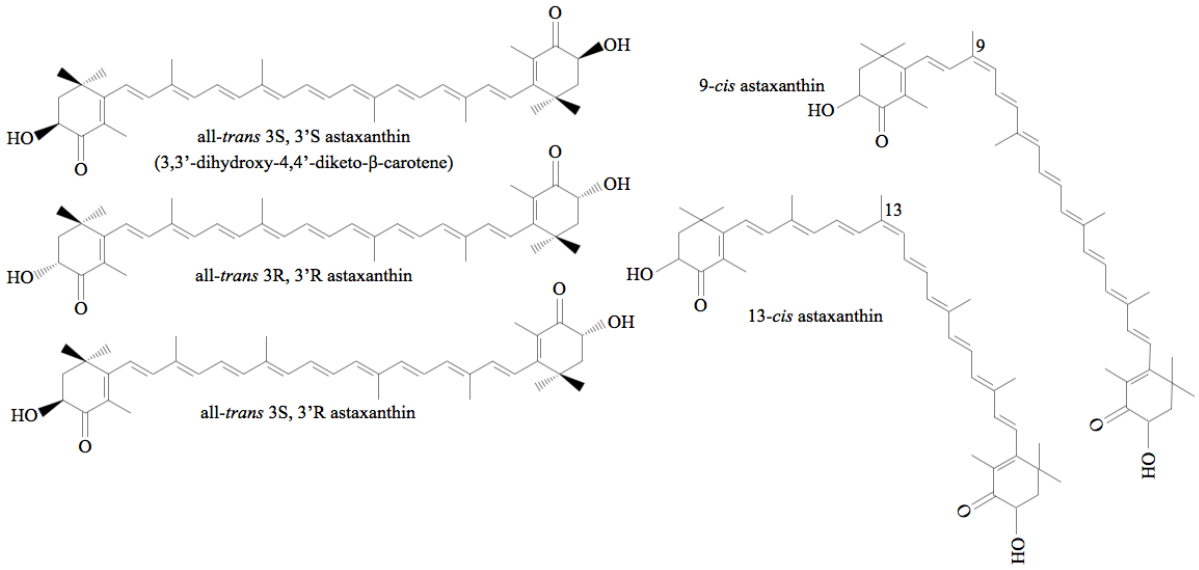


Figure 1. Astaxanthin stereoisomers of the three all-*trans* (3S 3'S, 3R 3'R, and 3S 3'R) and the two *cis* isomers (9-cis and 13-cis). Figure adapted from Holtin et al., 2009.

esterified, the high antioxidant activity, and more polar characteristics than other carotenoids. Astaxanthin in the all-trans formation is unstable and readily transforms to the cis conformation by heat, light, or metal ions (Holtin et al., 2009).

Haematococcus pluvialis Life Cycle. The astaxanthin for this study is from freshwater microalgae, *Haematococcus pluvialis*, a unicellular alga from the class Chlorophyceae (Goodwin and Jamikorn, 1953). The microalgae has a 2-week life cycle consisting of four stages: vegetative cell growth, encystment, maturation, and germination (Figure 2) (Kobayashi et al., 1997). Each stage is uniquely recognized by the ratio of carotenoid/chlorophyll and the intracellular protein content (Kobayashi et al., 1997). The first stage, vegetative cell growth, cells exists as ellipsoidal in shape ranging in size for 8 to 50 µm (Boussiba, 2000) in diameter and is capable of swimming with two equal length flagella with a characteristic green color (Kobayashi et al., 1997). The flagella emerge at a wide angle from the anterior papilla where the cell wall is greatly thickened. The cellular structure consists of a cup-shaped chloroplast with numerous scattered pyrenoids and contractile irregular vacuoles near the cell wall (Boussiba, 2000). There are high levels of chlorophyll and protein present but low levels of carotenoids (Kobayashi et al., 1997). The cellular structure remains the same during the mobile stage (Boussiba, 2000).

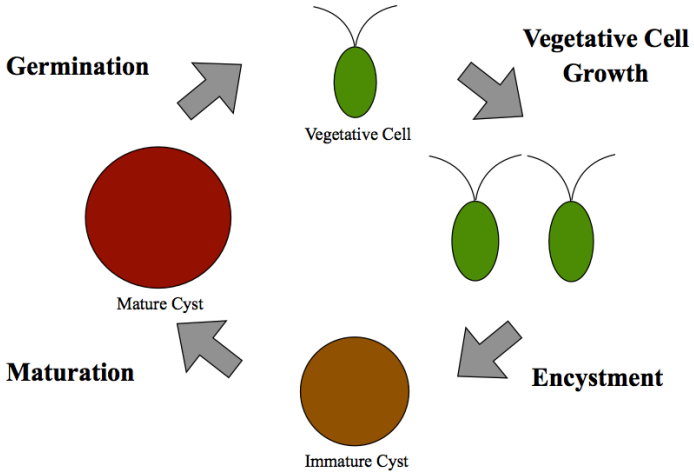


Figure 2. Life cycle schematic of *Haematococcus pluvialis*.

At the second stage the cells become spherical, immotile cysts form, called the 'palmella' stage (Kobayashi et al., 1997; Boussiba, 2000). Once growing conditions become less favorable, cells increase their volume, entering a resting stage, develop a heavy and resistant cellulose wall made from sporopoline-like substances, and become brown in color (Boussiba, 2000). Degradation of chlorophyll and protein occurs also during the encystment

stage. During maturation, carotenoid biosynthesis significantly increases as does protein degradation (Kobayashi et al., 1997). This stage is easily recognized as the protoplast takes on a more characteristic red color due to the increase of astaxanthin (Boussiba, 2000; Goodwin and Jamikorn, 1954; Harker et al., 1996). Peak cellular astaxanthin concentration is achieved after 78 hours of high light stress (Kim et al., 2006). Astaxanthin is thought to be a photoprotectant reducing the amount of light to light-harvesting pigment protein complexes thus reducing the risk of photo-inhibition (Harker et al., 1996). Astaxanthin may also provide a physiochemical protective barrier preventing free-radical damage within the cell (Harker et al., 1996). During the increased astaxanthin concentration stage, there are a number of proteins that are induced in the red cyst cells compared to the green vegetative cells (Kim et al., 2006). Two of the important proteins are isopentenyl pyrophosphate isomerase and lycopene β-cyclase appear to be involved with carotenoid accumulation within the cytoplasm (Kim et al., 2006).

After the maturation stage, intracellular daughter cells are released from the mature cyst cell during germination (Kobayashi et al., 1997). Chlorophyll and protein synthesis occur while carotenoid degrades. The daughter cells then grow mixotrophically into the vegetative cell stage (Kobayashi et al., 1997).

Carotenogenesis in Haematococcus pluvialis. In organisms with photosynthetic electron transport chain there is an unavoidable consequence for the production of reactive oxygen species (ROS). Chloroplasts are a rich source of ROS because of the abundant oxygen supply and highly energetic reactions forming ATP and NADPH. When environmental conditions become more stressful, ROS will be produced when energy balance between absorption of solar energy and CO_2 fixation is quenched (Boussiba, 2000). Excessive irradiance can cause the generation of triplet chlorophyll that can form $^1O_2$ (Boussiba, 2000). This reaction can continue as long as the triplet chlorophyll has not been quenched by carotenoids. Kobayashi et al. (1993) have shown ROS and $^1O_2$ may have some involvement with astaxanthin accumulation. Light is not necessary for the production of astaxanthin as shown by Droop (1955). Other environment stresses that cause

carotenogenesis in *Haematococcus pluvialis* are mineral deprivation, salt stress, excess light, low temperatures, drought, and aging.

Carotenoid pigments are synthesized in the chloroplasts of algae. In *Haematococcus pluvialis* contains a set of enzymes responsible for the conversion of β-carotene to canthaxanthin to astaxanthin. The specific steps of astaxanthin biosynthesis in *Haematococcus pluvialis* as seen in Figure 3 are carried out by two enzymes, β-carotene ketolase (BKT) and carotenoid hydroxylase (CHY) (Steinbrenner, 2003). Formation of astaxanthin takes place by the initial formation of canthanxanthin with the precursor being β-carotene. Unique to *Haematococcus pluvialis,* unlike other photosynthetic organisms, the accumulation of β-carotene and derived xanthophylls reside in the chloroplast, astaxanthin is found in the cytosol. Enzyme activities specific to astaxanthin biosynthesis occur in the cytoplasm whereas the enzymes required for general carotenoid pathway are located in the

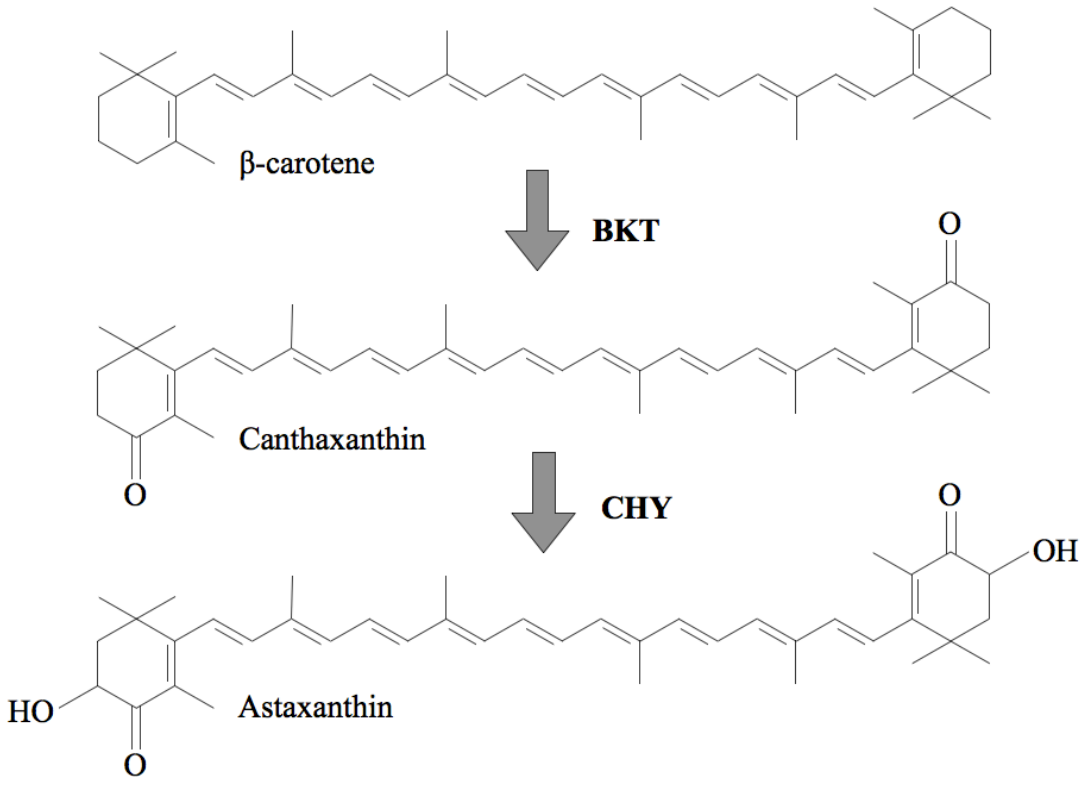


Figure 3. Biosynthetic pathway of astaxanthin in *Haematococcus pluvialis*. The enzymes in the pathway are β-carotene ketolase (BKT) and carotenoid hydroxylase (CHY) (Steinbrenner, 2003).

chloroplast (Steinbrenner, 2003). Unique to *Haematococcus pluvialis* astaxanthin exists in three different forms that can be classified as free (~5%), monoesters (~70%), and diesters (~25%) (Kamath et al., 2008). The different forms can be seen in Figure 4. Typical fatty acids attached in the esterified form are C18:3, C18:2, C18:1, and C16:0 (Hotin et al., 2009).

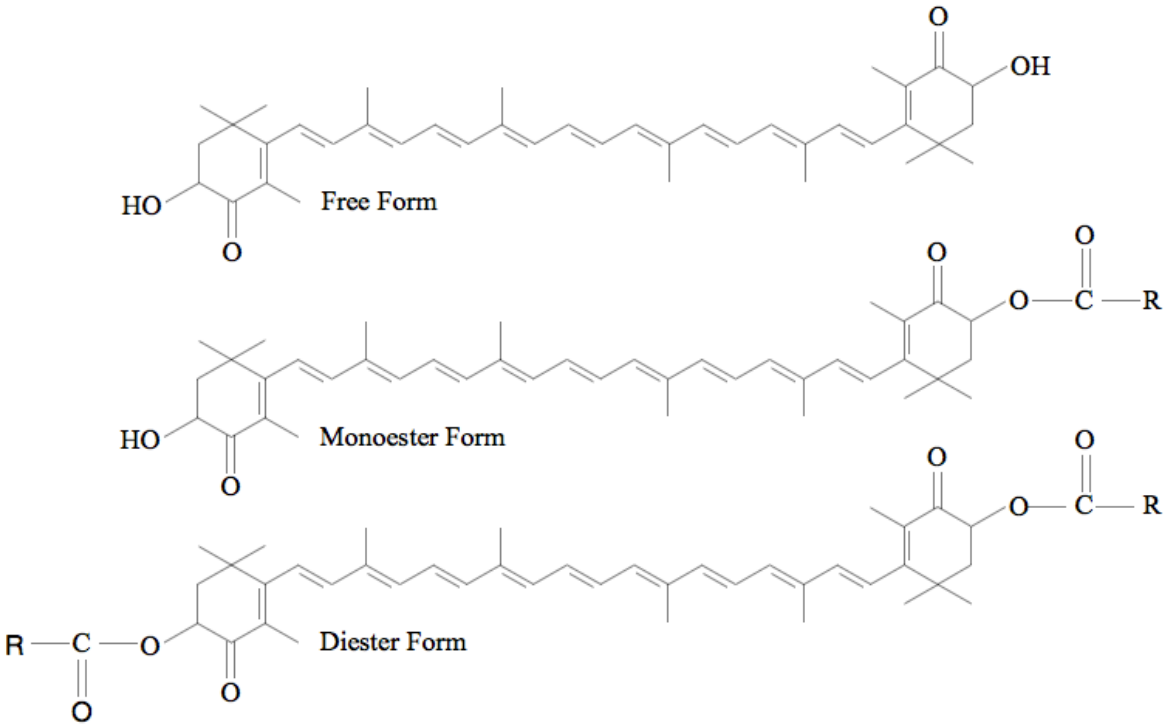


Figure 4. Structure of astaxanthin in the free, monoester, and diester form. R can be saturated or unsaturated acyl chain. Figure adopted from Kamath et al., 2008.

Mechanism of Antioxidation. Astaxanthin acts as a quencher of singlet oxygen and other free radicals by absorbing the excited energy onto the polyene electron-rich chain thus preventing cellular components from damage. This absorption results in an excitation of the carotenoid to a triplet state. Dissipation of the extra energy is given off by heat thus returning the compound to ground state (Algatech, 2004). Results from Miki (1991) illustrate the number of carbonyl and hydroxyl groups in the carotenoids that are important for quenching activities. Astaxanthin is most likely to reside in the phospholipid bilayer so the polar OH-groups in the rings are near the membrane surface and the polyene chain in the interior (Goto et al., 2001). With this orientation the heads of the molecule can scavenge

reactive oxygen species at or near the surface and the polyene chain can inhibit radical chain reactions inside the membrane (Goto et al., 2001). Potential intermolecular hydrogen bonds can be formed between the polar ends of astaxanthin and the polar groups of the surrounding phospholipids (Goto et al., 2001). Another possible way for astaxanthin to bond with the surrounding phospholipids is by the hydroxyl and carbonyl groups of astaxanthin readily forming intramolecular hydrogen-bonded five-membered ring, therefore increasing the hydrophobicity allowing for further advantages within the bilayer, like stabilizing the carbene radicals (Goto et al., 2010). Goto et al. (2001) also suggested the C3 methine is the radical scavenging site of the terminal ring. The radical scavenging ability of the terminal ring is not likely to be affected by the methyl groups and polyene change, therefore the rings and chain radical scavenging activity are independent of each other (Goto et al., 2001). The terminal ring has the unique property to scavenge radicals both on the surface and in the interior of the membrane while their chain can only scavenge inside the membrane (Goto et al., 2001).

HEALTH BENEFITS OF ASTAXANTHIN

Astaxanthin as an Antioxidant. Free radicals like hydroxyl and peroxyl radicals are highly reactive forms of oxygen and are produced by the body during normal metabolic reactions and processes. Physiological stress, exposure to chemicals or ultraviolet light, air pollution, or tobacco smoke, can magnify the production of free radicals. Free radicals can do damage to DNA, proteins, and lipid membranes *in vivo*. All the constant bombardment leads to oxidative stress. Antioxidants such as carotenoids may help to prevent harm by absorbing the energy of the singlet oxygen into the carotenoid molecule avoiding damage of surrounding tissues or cellular components (Guerin et al., 2003; Goto et al., 2001).

Astaxanthin is an effective protecting compound for membrane phospholipids and other lipids against peroxidation. Astaxanthin has a strong antioxidant activity, 10 times higher than β-carotene and more than 300 times more effective to α-tocopherol (Rao et al., 2009; Higuera-Ciapara et al., 2006; Miki, 1991). Astaxanthin is also more effective in scavenging hydroxyl radicals than β-carotene (Karppi et al., 2007). Astaxanthin is able to reduce the endogenous oxidative stress level. Wolf et al. (2010) suggest astaxanthin does not

directly scavenge reactive oxygen species like superoxides or peroxides. Astaxanthin is extremely lipophilic thereby unable to cross membranes so it protects cells against oxidative damage in the membrane (Wolf et al. 2010). A study completed by Bolin et al. (2010) suggests astaxanthin displays anti-inflammatory effects by preserving redox-sensitive and essential structures of human lymphocytes *in vitro*. There was data that also suggest the astaxanthin dosage level (5 μM) hindered lymphocyte proliferation by the increase in nitric oxide formation, but it would be improbable to achieve such high levels of astaxanthin *in vivo*. This and other studies also suggest astaxanthin has the capacity to scavenge reactive oxygen species *in vivo* (Bolin et al., 2010; Rao et al., 2009; Kamath et al., 2008). The antioxidant scavenging activity with IC_{50} of ~8 μg/mL and reducing power of $59*10^3$ U/g *in vitro* (Kamath et al., 2008).

A study completed by Kim et al. (2009) examined astaxanthin efficacy against high-glucose-induced oxidative stress, inflammation and apoptosis in proximal tubular epithelial cells by assessing lipid peroxidation, total reactive species, superoxide, nitric oxide, peroxynitrite, and inflammatory proteins. Results of this study showed astaxanthin had a protective property in reducing oxidative stress, inflammation, and apoptosis caused by high glucose exposure for these cells.

Astaxanthin as a Photoprotectant. Photons coming from the sun are constantly bombarding earth with 56% being infrared light (780-5000 nm), 39% visible light (400-780 nm) and 5% UV light (González et al., 2008). Exposure to light, especially ultraviolet A (320-400 nm) and ultraviolet B (290-320 nm), can lead to production of free radicals and photo-oxidative damage to lipids and tissues (Lee et al., 2000). Carotenoids have an important role in protecting these tissues against UV-light photo-oxidation and they are usually found in tissues directly exposed to sunlight, like the skin (Lee et al., 2000; O'Connor and O'Brien, 1998). Astaxanthin particularly is efficient at eliminating peroxilipidic radicals and inhibiting the concentration of free polyamines and protecting fibroblasts from photo-induced damage (González el al., 2008).

A study by Kobayashi et al. (2000) examined the protective role of astaxanthin against UV in *Haematococcus pluvialis.* Astaxanthin-rich mature cyst cells were six folds

more tolerant to UVB and three folds more tolerant to UVA than immature cysts. Kobayashi el al. (2000) concluded there is evidence for astaxanthin to protect against UV light.

O'Connor and O'Brien (1998) assessed the ability of β-carotene, lutein, and astaxanthin in protecting against UVA-induced oxidative stress in rat kidney fibroblasts. Tissue cells were exposed to UVA light (320-400 nm) at a dose of 5.6 mW/cm^2. Results illustrated that a much lower concentration of astaxanthin (10 nM) compared to β-carotene (1 μM) and lutein (1 μM) was required to decrease superoxide dismutase and catalase activities. This study concluded astaxanthin protects against UVA light-induced oxidative stress *in vitro*.

Another study by Camera et al. (2008) used human dermal fibroblasts in the presence of astaxanthin, canthaxanthin, or β-carotene for 24 hours prior to UVA exposure. There was a higher uptake of astaxanthin by the fibroblasts compared to the other compounds. Astaxanthin was determined to protect against UVA-induced alterations to a significant level. The authors concluded astaxanthin has a superior preventive effect against changes from photo-oxidation in cell culture.

Astaxanthin and Cardiovascular Disease (CVD). Oxidative stress and inflammation play important roles in the different aspects of CVD, including endothelial dysfunction, functional lipid disorders, periprocedural myocardial damage, and atrial fibrillation. Triggers like hypertension, dyslipidemia, diabetes, and obesity increase oxidative stress. Astaxanthin reduces LDL oxidation in humans in contrast to other antioxidants (Pashkow et al., 2008). Studies have shown significant reductions in oxidative stress, hyperlipidemia, and inflammatory markers of cardiovascular disease by oral supplementation of astaxanthin in animal case studies. There were few studies investigating the potential benefits of astaxanthin and CVD on humans except an ongoing study (Fassett et al., 2008) involves 66 renal transplant recipients investigating the effect of oral astaxanthin supplementation of 12 mg/day for one year on arterial stiffness, oxidative stress, and inflammation. The results of this study may lead to a larger intervention trial to assess cardiovascular morbidity and mortality (Fasset et al., 2008).

Anticancer Properties of Astaxanthin. Colon cancer is one of the major causes of mortality worldwide. Colonic crypt cell hyperproliferation has been suggested to play a

significant role in the formation and development of this type of cancer (Prabhu et al., 2009). ROS plays a role in the various stages of colon carcinogenesis and tumorigenesis. With carotenoid's antioxidant properties, they have a chemopreventive agents (Prabhu et al., 2009). The study by Prabhu et al. (2009) concluded that astaxanthin administered to rats orally at a concentration of 15 mg/kg body weight suppressed the total number of aberrant crypt foci and the development of multi crypt foci.

A study by Tanaka et al. (1994) tested the effects of astaxanthin (50 ppm) on mice given *N*-butyl*N*(4-hydroxybutyl) bitrosamine, a urinary bladder carcinogen, for 20 weeks. The mice who were given the astaxanthin had smaller preneoplastic lesions in their bladder and a significantly reduced incidence of bladder cancer than the control group. The authors concluded astaxanthin is a possible chemopreventive agent for bladder carcinogenesis by suppressing cell proliferation.

Astaxanthin has also been associated with relieving other types of diseases or conditions such as: age-related macular degeneration, Alzheimer's and Parkinson's diseases, high cholesterol, inflammatory diseases, dyspepsia, muscle function, anti-hypertension, stress management, and repair of damage caused by lack of oxygen from a stroke (Lorenz and Cysewski, 2000; Guerin et al., 2003). This review of astaxanthin's health benefits is only a small portion of the potential this compound has to offer.

SAFETY CONCERNS WITH ASTAXANTHIN

The FDA in 1987 conducted safety and toxicology studies on astaxanthin. Acute and subacute (10-day) toxicology study in rats (up to 1.2 g/kg/day for 91 days) and in dogs (up to 162 mg/kg/day for 91 days) demonstrated minor effects in rats and no effects in dogs at the highest dose. Reproductive toxicology was evaluated in rats and rabbits with no effects from the doses up to 1 g/kg/day. Since the FDA's approval, astaxanthin has been used as a feed additive primarily for salmon. Other published human clinical trials using astaxanthin have also proven the safety of the carotenoid (Pashkow et al., 2008). Astaxanthin supplements have been available commercially since 2000. Comparisons with 62 over-the-counter drugs including aspirin or ibuprofen, astaxanthin was reported to be as effective or more effective

in 76% of the comparisons for relieving sore muscles, back pain, rheumatoid arthritis, and macular degeneration etc. (Guerin et al., 2003). However, the high effectiveness must be taken with caution, as placebo effects cannot be ruled out (Guerin et al., 2003). In 2010 FDA accepted *Haematococcus pluvialis* extract containing astaxanthin esters to be on the Generally Recognized As Safe (GRAS) list (GRAS 294, 2011).

A study by Mera Pharmaceuticals (1999) used 33 adult volunteers whom were given natural astaxanthin over 29 days where participants were given 3.85 mg or 19.25 mg of astaxanthin a day (Guerin, 2003). From the study, no ill effects or toxicity was found from the astaxanthin supplement (Guerin, 2003).

Spiller and Dewell (2003) used 35 healthy adults ranging in age from 35-69 years in a randomized, double-blind, placebo-controlled trial for 8 weeks. Participants were given three gelcaps per day to be taken with meals. Each gelcap contained 2 mg of astaxanthin in safflower oil for the treatment group and the placebo had safflower oil only gelcaps. Blood pressure, blood chemistry test including comprehensive metabolic panel and blood cell count were analyzed at the beginning, after four weeks, and after eight weeks during the study. Only slight differences were found, yet the differences did not have any clinical importance. The researchers concluded 6 mg of astaxanthin from *Haematococcus pluvialis* per day can be consumed safely by healthy adults (Spiller and Dewell, 2003).

HUMAN BIOAVAILABILITY OF ASTAXANTHIN

Bioavailability of lipophilic carotenoids varies from 10% in raw uncooked vegetables to 50% in oily solutions or synthetic commercial formulations (Odeberg et al., 2003). This difference in bioavailability can be explained by incomplete release from food matrices or by the dissolution limitations of the gastrointestinal fluids (Odeberg et al., 2003). Another possible explanation is the limits of absorption (Parker, 1996). Astaxanthin, like other xanthophylls, are typically present in esterified forms, mono and diesters, and must be hydrolyzed before absorption (Zaripheh and Erdman, 2002). Dissolution from the food matrix and incorporation into micelles are two important steps before absorption across the membrane. Once the xanthophylls have been absorbed by the mucosal cells they are

incorporated into chylomicrons (Zaripheh and Erdman, 2002). The xanthophylls that are not incorporated into chylomicrons are thought to be returned to the intestinal lumen (Zaripheh and Erdman, 2002).

A study by Odeberg et al. (2003) used 32 healthy male subjects with age range of 20-46 and were given three different treatments of lipid based gelatin capsules. All three treatments enhanced the bioavailability of astaxanthin by 1.7 to 3.7 fold. Another study by Okada et al. (2009) examined bioavailability and timing of either after or before eating a meal and the effects of smoking habits. This study involved 15 males (age 18-60) and 5 females (26-32) with the a single dose of 40 mg. Some subjects were given the astaxanthin either 2 hours before or 10 minutes after the prepared meal. Subjects fed the astaxanthin pill after the meal had an increase of two times in absorption than the group given the pill before the meal. Smoking also affected the astaxanthin in the blood which decreased compared to the nonsmokers (Okada et al., 2009).

In conclusion, bioavailability depends not only on the structure of astaxanthin ingested and must be converted to free form for absorption to occur, but also be attributed to when astaxanthin is consumed, and lipid composition in food (Odeberg et al. 2003).

TOCOPHEROLS AND TOCOTRIENOLS

Tocotrienols and tocopherols are fat-soluble vitamins and generically named as vitamin E, whose antioxidant properties are well studied. Within the vitamin E analogs, α-tocopherol is considered the most active form (Theriault et al., 1999). Bioavailability of vitamin E is greatly influenced by food matrix, formulation, and food processing (Reboul et al., 2006). Vitamin E food sources include, sunflower seeds, almond, and hazelnuts (Reboul et al., 2006). Vitamin E cannot be synthesized in the human body therefore must be obtained from the diet (GRAS 307, 2011). Tocopherols are present in corn, wheat, and soybeans where tocotrienols are present in barley, oats, palm, and rice bran (Aggarwal et al., 2010). For our study, we used Tocomin® 50%, an oil extracted from virgin crude palm oil (*Elaeis guineensis)*, containing predominately α, β, γ, δ tocotrienol. The Tocomin® 50% composition is illustrated in Table 2.

Vitamin E Deficiency. A lack of vitamin E in the diet can result in degenerative disease such as ataxia, Duchenne muscular dystrophy-like muscle degeneration, and infertility (Aggarwal et al., 2010). Vitamin E deficiency impairs myelination of axons, increasing lipid peroxidation of the mitochondrial membranes of sensory neurons (Brigelius-Flohé and Traber, 1999; Meydani et al., 2003). The nerve cells' death is likely due to the increase of nitric oxide production that is characteristically seen in ataxia, vitamin E deficiency syndrome (Meydani et al., 2003). Anemia occurs predominantly in infants due to free radical damage likely because of insufficient vitamin E stores (Meydani et al., 2003). Hypercholesterolemic subjects who are marginally or severely deficient in vitamin E have decreased erythrocyte life span and increased susceptibility to peroxide-induced hemolysis (Brigelius-Flohé and Traber, 1999). In cystic fibrosis patients who have vitamin E deficiency, the plasma levels of malondialdehyde and hydroperoxide, lipid peroxidation, and protein carbonyls, are markers for protein damage (Meydani et al., 2003). Skeletal muscle fiber damage is done by the loss of calcium homeostasis leading to dystrophic conditions (Meydani et al., 2003). Goss-Sampson et al. (1998) observed the defects in retinal function in experimental animals. The irreversible loss of long-chain PUFA from the retina, increased lipid peroxidation, and alternation in membrane fluidity were observed.

Table 2. Composition of Tocotrienols and Tocopherols in Tocomin® 50%

Analogs	Composition in Palm Extract (mg/g)
d-α-tocotrienol	115
d-β-tocotrienol	15
d-γ-tocotrienol	210
d-δ-toctrienol	55
d-α-tocopherol	115
d-β, γ, δ-tocopherol	3
Total tocotrienol tocopherol	513

Adapted from Carotech Inc. spec sheet.

The deficiency can occur as a result of genetic abnormalities in the α-tocopherol transfer protein (α-TTP) (Brigelius-Flohé and Traber, 1999). People with the genetic modification of α-TTP have dramatically lower plasma vitamin E levels and will likely have neurological disorders, characteristic of the deficiency (Brigelius-Flohé and Traber, 1999).

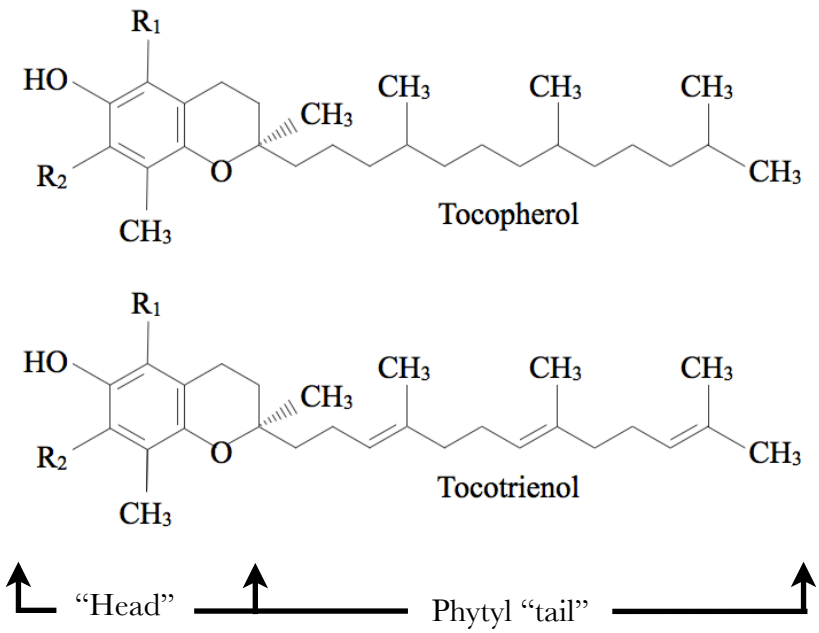
The mutation in the *ttpA* gene causes the protein to not function properly (Manor and

Morley, 2007). The extreme hydrophobicity of the tocopherols poses a major thermodynamic barrier for its distribution and transportation through the cytosol and circulation. Vitamin E plasma levels do not respond linearly, suggesting other factors may regulate vitamin E levels (Manor and Morely, 2007).

Typically vitamin E deficiency rarely occurs in humans with the cause not from dietary deficiencies as it typically takes years for normal individuals to develop clinical symptoms (Brigelius-Flohé and Traber, 1999; Meydani et al., 2003). Disorders associated with vitamin E deficiency include: chronic cholestatic liver disease, abetalipoproteinaemia, cystic fibrosis, chronic pancreatitis, progressive systemic sclerosis, short-bowel syndrome, isolated vitamin E deficiency syndrome, and other malabsorption disorders (Meydani et al., 2003). Individuals most at risk besides those with the previously mentioned disorders are newborn infants, particularly premature babies with low body weight, and the elderly. For infants, the placental transfer of vitamin E is poor for the developing fetus and newborns usually have about one-third plasma concentrations compared to adults (Meydani et al., 2003). Premature babies can have even lower concentrations. Vitamin E status can be boosted by breast feeding (Meydani et al., 2003). Elderly are at risk as a result of low overall dietary intake or low availability of high-quality food (Meydani et al., 2003). The symptoms caused by vitamin E deficiency, can be ameliorated by administering vitamin E doses up to 2000 mg/day orally or through intramuscular injection over long time periods (Brigelius-Flohé and Traber, 1999; Meydani et al., 2003).

Structure of Tocopherols and Tocotrienols. Structurally, tocopherols and tocotrienols share some resemblance with a common chromanol head and side chain occurring at the C2 position, only distinguishable by their side chains (Theriault et al., 1999). Tocopherols have a saturated phytyl tail while tocotrienols have an unsaturated isoprenoid side chain (Figure 5) (Theriault et al., 1999). These compounds are further separated to individual compounds by Greek letter prefixes (α, β, γ, δ). The structural name for α-tocopherol is 2,5,7,8-tetramethyl-2-(4’,8’,12’-trimethyltricecyl)-6-chromanol and for α-tocotrienol is 2,5,7,8-tetramethyl-2-(4’,8’,12’-trimethyltrideca-3’,7’,11’-trienyl)-6-

chromanol. The different analogues are known together as vitamin E (Aggarwal et al., 2010).



Analog	R_1	R_2
α-Tocotrienol/Tocopherol	CH_3	CH_3
β-Tocotrienol/Tocopherol	CH_3	H
γ-Tocotrienol/Tocopherol	H	CH_3
δ-Tocotrienol/Tocopherol	H	H

Figure 5. Tocopherol and tocotrienol structures. The 4 tocotrienols and 4 tocopherols share common R_1 and R_2 groups. Tocotrienols are different due to the unsaturated phytyl side chain as tocopherols have a saturated side chain. Figure adapted from Theriault et al., 1999 and Burton and Ingold, 1986.

Mechanism. The tocopherol compound is considered to be a first line of defense against cell membranes at early stages of free radical attack (Lien et al., 1998). Vitamin E is an antioxidant which falls into the chain-breaking antioxidant category as it can interfere with one or more of the propagation steps in autoxidation (Burton and Ingold, 1986). Vitamin E neutralizes peroxyl and alkoxyl radicals that are generated during lipid oxidation (GRAS 307, 2011). Tocopherols and tocotrienols can interact by binding directly or

indirectly at the transcriptional, translational, or post-translational levels (Aggarwal et al., 2010).

HEALTH BENEFITS OF VITAMIN E

Vitamin E as an Antioxidant. In the general steps of oxidation, α-tocopherol can intercept the peroxyl radicals quicker than polyunsaturated fatty acids. Figure 6 illustrates the three phases of the free radical chain mechanism for lipid peroxidation. α-Tocopherol donates a phenolic hydrogen atom to the radical then converting it to a hydroperoxide. The new tocopheroxyl radical formed is removed from the cycle by reacting with another peroxyl radical to form an inactive, non-radical compound (Burton and Traber, 1990). Burton and Traber (1990) also stated α-tocopherol can react approximately 200 times faster than butylated hydroxytoluene (BHT) and the head of the molecule is entirely responsible for

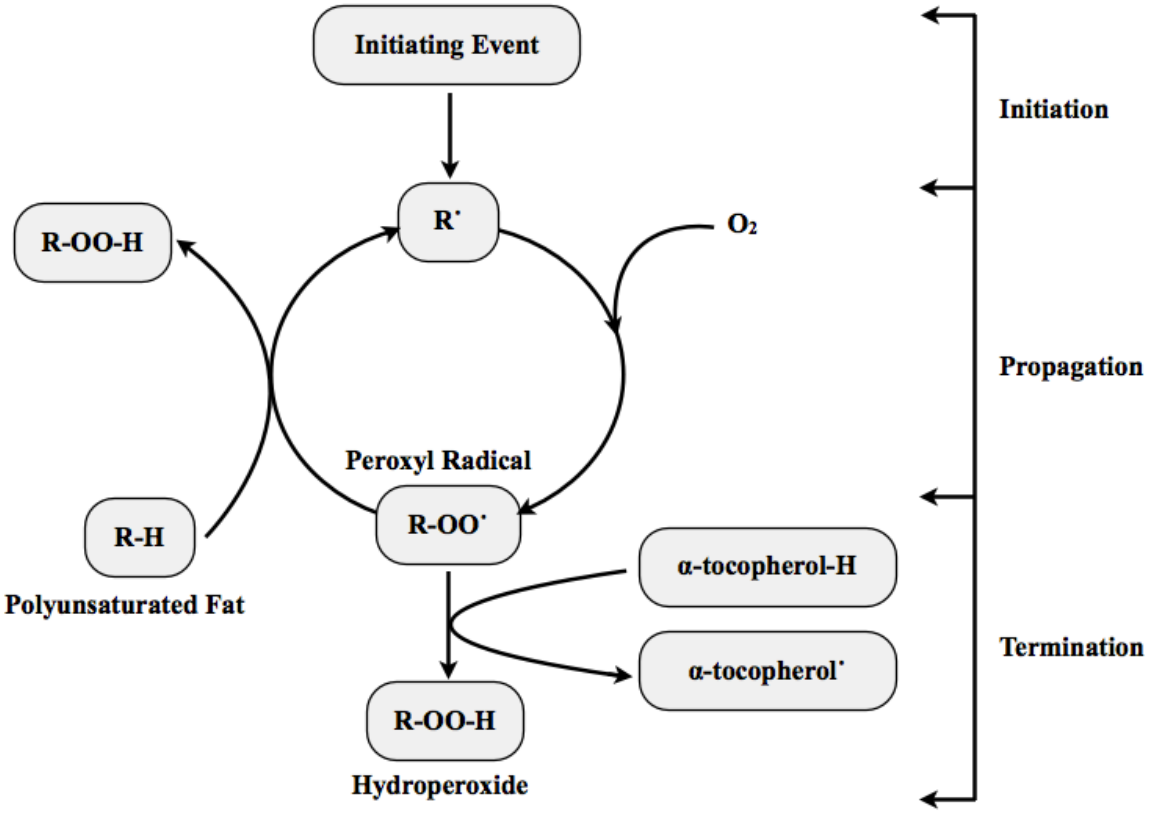


Figure 6. Scheme of free radical chain mechanism for lipid peroxidation using α-tocopherol as termination.

antioxidant properties. With this ability a small amount of α-tocopherol is needed to protect lipid molecules. In biological membranes α-tocopherol exists approximately one part per 1,000 lipid molecules (Burton and Traber, 1990). Recent studies suggest α-tocotrienol is at least three times more efficient than α-tocopherol as a peroxyl radical scavenger due to its more uniform distribution in membrane (Lanari et al., 2004). Antioxidant activities are mediated through the induction of antioxidant enzymes like superoxide dismutase, NADPH:quinone oxidoreductase, and glutathione peroxidase (Aggarwal et al., 2010).

A study completed by Lee et al. (2009) examined the effect on exercise endurance and oxidative stress on forced swimming rats. The rats were fed 25 mg/kg tocotrienol-rich fraction, 50 mg/kg tocotrienol-rich fraction, or 25 mg/kg d-α-tocopherol. The rats fed the tocotrienol-rich fractions were able to swim significantly longer than the control. Other biological factors were also measured. The authors concluded the tocotrienol was able to improve the physiological conditions and reduce exercise-induced oxidative stress in rats.

Another study performed on rats induced with chronic lung lipid peroxidation by paraquat, a herbicide extremely toxic to plants and animals, was completed by Asmadi et al. (2005). Tocotrienols and α-tocopherol were supplemented at 150 mg/kg pellet and 34 mg/kg pellet respectively. Both diets significantly reduced lung malondialdehyde levels but did not affect lung edema. Asmadi et al. (2005) concluded tocotrienols and α-tocopherol provide protection against paraquat toxicity.

A study by Naguib et al. (2003) examined the antioxidant activities of natural d-α-tocopherol, mixed tocopherols and tocotrienols, with a formulation comprising of all forms of vitamin E, all providing 400 IU using oxygen radical absorbance capacity assay. Their findings showed antioxidant activities of the mixed tocopherol and tocotrienols exhibited higher antioxidant activities than d-α-tocopherol alone.

Vitamin E and Cardiovascular Disease (CVD). Cardiovascular disease is a major cause of death in the United States (American Heart Association, 2011). Vitamin E is thought to have a role in preventing atherosclerosis by inhibiting oxidation of low-density lipoproteins. In a study completed by Stephens et al. (1996) examined the effects of high dose treatment of α-tocopherol on the reduction of myocardial infarction and cardiovascular

death in patients with established ischaemic heart disease. A double-blind, placebo-controlled study with 2,002 patients with proven coronary atherosclerosis were enrolled. Five hundred forty-six patients were given 800 IU daily, 489 were given 400 IU daily, and 967 received the placebo. The patients who received the treatment showed an increase in plasma α-tocopherol levels. From this study the investigators concluded that patients with coronary atherosclerosis, α-tocopherol treatment significantly reduced the rate of nonfatal myocardial infarction with beneficial effects after one year of treatment.

Another study completed by Stampfer et al. (1993) examined the protective potential of vitamin E against coronary disease. The observational study used 87,245 females nurses from 34-59 years of age and were free from any diagnosed cardiovascular disease or cancer. The study lasted eight years. The data gathered did not prove a cause-and-effect relationship between coronary disease and vitamin E intake, however, it did suggest middle-aged women who used vitamin E supplements had a reduced risk of coronary heart disease.

Lastly, a study completed by Lee et al. (2005) on 39,876 healthy U.S. women of at least 45 years of age were randomly assigned to receive vitamin E, aspirin, or a placebo. Patients who received the vitamin E were given 600 IU alternate days. Results from this study had a significant reduction for cardiovascular death with no significant reduction with myocardial infarction or stroke.

Anticancer Properties of Vitamin E. Vitamin E has had great success to suppress proliferation and induce apoptosis in tumor cells in tissues such as breast, colon, liver, lung, stomach, skin, pancreas, and prostate (Aggarwal et al., 2010). Vitamin E can act by extrinsic or intrinsic pathways. Extrinsic pathways involve the induction of death receptors, and intrinsic pathways involve mitochondrial depolarization (Aggarwal et al., 2010). Another way vitamin E can cause apoptosis is by DNA fragmentation in certain cells (Aggarwal et al., 2010). Some of the forms, γ- and δ-tocotrienols exhibit higher anticancer activity, than that of α- or β-tocotrienol (Aggarwal et al., 2010).

A study by Nesaretnam et al. (2008) examined the effect of tocotrienols on apoptotic signals in androgen-independent PC-3 human prostate cancer cells. The researchers investigated the effect of tocotrienol-rich fraction from palm oil, α-tocopherol, α-tocotrienol,

γ-tocotrienol, and δ-tocotrienol on PC-3 cell growth. After discussing at several other factors, Nesaretnam et al. (2008) came to the conclusion tocotrienols are able to induce apoptosis and cell cycle arrest in PC-3 cells with increased expression of Fas receptor, Fas ligand, caspase 8, caspase 3, and bax. From this study they suggest tocotrienols have a potential role in chemoprevention of prostate cancer.

A study examined the synergistic effects of γ-tocotrienol and statin on mammary tumor cells. Statins are a drug used typically for lowering high blood cholesterol, yet there have been recent developments showed that the compound can induce cell cycle arrest *in vitro* and inhibit tumor growth *in vivo* (Wali and Sylvester, 2007). The problem with using statin is due to their high-dose toxicity. Wali and Sylvester (2007) studied the combination of low dose treatment of γ-tocotrienol and statin on mouse mammary epithelial cells. A combined treatment dose of 0.25-2 μM resulted in a dose-response inhibition. Tests with the statins or γ-tocotrienol alone had no effect, therefore, the conclusion was the combination of a low-dose treatment of γ-tocotrienol and statin may have a potential to treat breast cancer without causing myotoxicity.

Another study evaluated γ-tocotrienol anti-tumor activity on human hepatoma Hep3B cells. Sakai et al. (2006) studied α-, δ-, and γ-tocotrienol (25, 50 and 75 μM) and found low levels of γ-tocotrienol and shorter treatments than α-tocotrienol were able to inhibit cell proliferation. The researchers also found caspase-8 and caspase-9 were also involved in apoptosis induction. Bax and Bid, pro-apoptotic proteins, were involved in the regulation of apoptosis induced by γ-tocotrienol (Sakai et al., 2006).

Neuroprotective Properties of Vitamin E. The brain responses to injury by activating glia, releasing pro-inflammatory cytokines, and other glia-derived factors. Once the activated microglia accumulate, it contributes to neurotransmitter systems, such as glutamate, γ-aminobutyric acid, and serotonin (Li et al., 2001). Understanding these pathways will help identify therapeutic targets and potential treatment strategies for neurodegenerative conditions. There is evidence that vitamin E protects neurons against oxidative stress and might help prevent the development of Alzheimer's disease (Li et al., 2001). The authors examined the interactions between microglial-neuronal and pathogenesis of

neurodegenerative diseases like Alzheimer's disease. They used a microglial-neuronal coculture. From the study, they concluded that vitamin E provides direct antioxidant protection for neurons and may provide neuroprotection *in vivo* through suppression of signaling events.

A study by Shichiri et al. (2007) examined the effect of tocotrienols and tocopherols on methylmercury neurotoxicity. Cerebellar granule cells were used to determine the methylmercury induced neurotoxicity. Significant protection against cell death was observed with tocopherols and tocotrienols. Tocotrienols also illustrated to be multi-fold more potent than tocopherols. When micromolar concentrations were examined, tocotrienols exhibited protection by antioxidant mechanisms. Both tocotrienols and tocopherols did show a protective effect on the cerebellar granule cells against the toxicity from methylmercury, therefore, both tocopherols and tocotrienols offered pharmacological protection for the brain against methylmercury (Shichiri et al., 2007).

A study by Osakada et al. (2004) used palm oil to investigate the effects of vitamin E on oxidative stress and apoptosis using primary neuronal cultures from rat striatum. They found vitamin E significantly reduced hydrogen peroxide induced neuronal death. Each of the different analogs was purified from the palm oil and examined individually. All the tocotrienols significantly decreased hydrogen peroxide induced neurotoxicity, but α-tocopherol did not. α-, γ-, and δ-Tocotrienols had protection against cytotoxicity of superoxide donors, paraquat, and nitric oxide donors. α-Tocotrienol only prevented oxidative stress-independent apoptotic cell death, DNA cleavage, and nuclear morphological changes. Among all the analogs and the examined oxidative stress and apoptosis, α-tocotrienol exhibited the most potent neuroprotective properties with the rat striatal cultures (Osakada et al., 2004).

Vitamin E has also been linked to reduce bone loss, immunomodulatory functions, gastroprotective effects, anti-aging, and relieving skin disease (Aggarwal et al., 2010). Tocotrienols have specifically been linked to neuroprotection, cholesterol lowering, blood pressure lowering, anti-aging, antioxidant, anticancer, anti-angiogenic, anti-inflammatory,

immune function, modulation obesity, osteoporosis, eye, and bone health (Sen et al., 2006). Sen et al. (2006) has a comprehensive list of the studies on these health issues.

SAFETY CONCERNS OF TOCOTRIENOLS AND TOCOPHEROLS

Currently tocotrienols are not listed on the GRAS-affirmed substance list in the U.S., however, the dietary supplements containing tocotrienols have been on the market since 1999 under the Dietary Supplements Health and Education Act of 1994. Palm tocotrienols have been tested for safety and toxicity. A study of chicks for 5 weeks indicated safe intake levels up to 1,000 mg/day (GRAS 307, 2011). Other animal studies have included both female and male rats with no abnormalities over varying lengths of time ranging from 14 days to 52 weeks. A study by Tasaki et al. (2008) observed no abnormal effects in either male or females rats with 0.4% palm tocotrienol rich fractions in the diet.

Besides numerous animal studies, there have been a number of clinical trials conducted using tocotrienol and tocopherols with few side effects reported (GRAS 307, 2011). Daily consumption level reported by the FDA up to 330-360 mg of palm tocotrienol was not associated with any adverse effects in healthy persons and persons with hypercholeterolemia or non-insulin-dependent diabetes mellitus (GRAS 307, 2011).

HUMAN BIOAVAILABILITY OF TOCOTRIENOLS AND TOCOPHEROLS

Once ingested, tocotrienols and tocopherols are incorporated into chylomicrons in the intestine. The newly absorbed tocotrienols and tocopherols are incorporated into very low-density-lipoproteins in the liver. The liver is responsible for the release and control of vitamin E into the blood. α-Tocopherol is the most plentiful and most biologically active form of these compounds (Burton and Ingold, 1986). Despite similar antioxidant functions for all the forms of tocotrienols and tocopherols, non-α-tocopherols are poorly recognized by the hepatic α-tocopherol transfer protein (Traber and Atkinson, 2007; Manor and Morley, 2007). Table 3 illustrates the relative biological activities of vitamin E in rat resorption-gestation test (GRAS 307, 2011). Absorption efficiency of lipophilic micronutients, like vitamin E, is highly variable and dependent due to the affinity of the α-TTP.

In a recent study by Leonard et al. (2005), it was demonstrated γ-tocopherol is more rapidly metabolized in women than in men, where α-tocopherol is maintained in the plasma with little being metabolized. Despite the low affinity for tocotrienols, there is evidence that oral supplementation of tocotrienols results in an increase of plasma tocotrienol concentration to 1 μM (O'Byrne et al., 2000).

GALLUS DOMESTICUS DIGESTION

Absorption efficiency from feed depends on a variety of factors. For mammals, absorption can be summarized into four stages: digestion of food matrix, formation of lipid micelles, uptake into the intestinal mucosal cells, and finally delivery to the lymph system. For laying hens, this process is slightly different due to the lymphatic system is not developed (Surai et al., 2001). Because of the lack of lymphatic system, carotenoids are most likely delivered to the liver and other tissues by portomicrons, lipid-rich lipoproteins that are released from the intestinal cells directly into the portal vein (Surai et al., 2001). Once feed is ingested, carotenoids are released from the feed matrix by digestive enzymes. The newly free carotenoids are emulsified with bile salts and pancreatic esterases to form oil droplets. Since astaxanthin exists in free as esterified forms in the algae biomass, ester hydrolysis by pancreatic esterases occurs on the water-lipid interface (Surai et al. 2001). Tyczkowski and Hamilton (1986 a) also reported the diester was partially hydrolyzed in the intestine to both monoester and free form. These droplets are too large to gain proximity to the small intestine surface for absorption (Surai et al., 2001). The oil droplets are then degraded to form mixed micelles containing

Table 3. Relative Vitamin E Biological Activities in Rat Resorption-Gestation

Compound	Relative activity in prevention of fetal resorption (%)
d-α-Tocopherol	100
d-β-Tocopherol	25-50
d-γ-Tocopherol	1-11
d-δ-Tocopherol	1-3
d-α-Tocotrienol	29-30
d-β-Tocotrienol	5
d-γ-Tocotrienol	Unknown
d-δ-Tocotrienol	Unknown

Adopted from GRAS 307.

nonpolar and nonamphiphilic lipids where the micelles are able to interact with the intestinal lumen (Surai et al., 2001). The jejunum is the major site of lipid absorption in birds, yet some absorption also occurs in the ileum (Surai et al., 2001). Tyczkowski and Hamilton (1986 b) studied the effect of absorption of zeacarotene, cryptoxanthin, and lutein. They concluded cryptoxanthin compared to lutein in the serum was 15.8:1 and 14.2:1 in the toe webbing. Results from the same study were able to illustrate lutein and zeacarotene were absorbed in different areas of the intestine. Zeacarotene was mainly absorbed in the ileal or middle region of the intestine, where lutein was absorbed mainly in the duodenum and jejunum or upper region of the intestine (Tyczkowski and Hamilton, 1986 b). The lipid can diffuse into the glycoprotein layer surrounding the microvilli and encounter the epithelial cells (Surai et al., 2001). The type of feed can determine bile secretion and micelle formation (Surai et al., 2001). The pH of the intestinal lumen can also affect absorption by changing the surface charges of the micelles and luminal cell membrane. Low pH values allow for better diffusion (Surai et al., 2001). Other soluble proteins and peptides from feed can aid with carotenoid dispersion. Other fat in the diet provides a vehicle for transportation of the carotenoids or other lipid molecules from the gizzard to the intestinal tract (Surai et al., 2001). Once the carotenoids have been absorbed, they are transported to the liver where the portomicrons partly undergo some transformation in the liver. Most likely they are released into the circulation unchanged or with minor changes (Surai et al., 2001). Carotenoids accumulate in the liver as a complex mixture of lutein monoesters and diesters as determined by Tyczkowski and Hamilton (1986 a) in young broiler chickens. Carotenoid absorption is a delicate process, which can be hindered by impairing any stage in the process or promoted by lipid solubilization or micelle formation (Surai et al., 2001).

Nine factors are identified that influence carotenoid bioavailability. These factors include: species of carotenoid, molecular linkage, amount of carotenoids consumed from the feed, feed matrix, absorption effectors and bioconversion, nutrient status of the organism, genetic factors, host-related factors, and nutrient interactions (Castenmiller and West 1998). Naturally occurring carotenoids in plant foods are in the all-*trans* isomer. *Cis* isomers are more polar, less prone to crystallization, and more soluble in oil and hydrocarbon solvents, in

general. Molecular linkage is important for carotenoid esters. The intestinal tract is unable to absorb esterified forms of carotenoids but has the necessary esterases to make the cleavage for the enterocyte to absorb the nutrients (Castenmiller and West, 1998). The amount of carotenoids in the feed relates to the dose-response relationship between the carotenoid and the serum concentrations. More carotenoids can be absorbed when consumed throughout the day rather than just a one time administration (Castenmiller and West, 1998). The food matrix can drastically influence the bioavailabilities of astaxanthin, tocopherols or tocotrienols. Lipid soluble carotenoids dissolved in oil are more readily absorbed than in a non-lipid matrix (Castenmiller and West, 1998). Optimal uptake of vitamin E and α- and β-carotene requires a limited amount of fat where lutein esters require more fat (Roodenburg et al., 2000). Zeaxanthin, pigment found in corn, is three times better absorbed than astaxanthin (Schiedt et. al., 1985; Goodwin, 1986). The availability and the interactions with other carotenoids can affect the absorption of these compounds (van het Hof et al., 2000). Absorption of carotenoids can be drastically impacted by the variety of other nutrients consumed together. Since vitamin E and astaxanthin are fat soluble the absorption and bioconversion could be greatly reduced with a low fat intake. Dietary fiber can also affect the absorption by methyl esterification of pectin in hens which is important in determining absorption inhibition. Dietary fiber change the bile acid interactions, resulting in increased fecal excretion of bile acids, thus, decreased carotenoid absorption (Castenmiller and West, 1998). Cofactors needed for the bioconversion of the carotenoids are important otherwise inhibition occurs not allowing the nutrient to get into the serum (Castenmiller and West, 1998). Genetic factors can play a role, where one organism will have lower concentrations in the plasma even if it consumes the same amount as another organism (Castenmiller and West, 1998). Lastly, host-related factors include the effect of gender, sex, fat maldigestion and malabsorption (Castenmiller and West, 1998).

Generally carotenoid absorption and accumulation in chickens is low. In broilers, 94% of astaxanthin supplied by the diet were excreted and only 0.4% were deposited in the skin (Hencken, 1992). In laying hens, egg yolk deposition of astaxanthin is only 14% and excretion about 70% (Hencken, 1992). The free alcohol form would deposit in the egg yolk

(Tyczkowski and Hamilton, 1986 a). Astaxanthin transfer depends on the source. Astaxanthin from yeast *Phaffia rhodozyma*, and marigold extract added to quail diet transferred only 4% and 3%, respectively to the egg yolk (Johnson et al., 1980). In laying hens tissue carotenoid concentrations are found in decreasing order from the liver, abdominal fat, kidney, spleen heart, lung, and muscle (Surai et al., 2001). Unused astaxanthin is not oxidatively metabolized but reduced to idoxanthin and crustaxanthin, which are quickly disposed of by the liver (Goodwin, 1986).

α-Tocopherol may interfere with tocotrienol absorption therefore increasing the binding capacity over γ-tocopherol and α-tocotrienol by 8-11 times (Lanari et al., 2004). A protein, α-tocopherol transfer protein (αTTP) has been identified to bind vitamin E analogs and transfer the compounds between membranes in the liver cytosol of animals. Hosomi et al. (1997) examined the affinities of αTTP toward different tocos and calculated taking α-tocopherol as 100%: β-tocopherol 38%, γ-tocopherol 9%, δ-tocopherol 2%, and α-tocotrienol 12%. The αTTP specifically sorts for the RRR-α-tocopherol (Brigelius-Flohé and Traber, 1999). Packer et al. (2001) also concluded that liver preferentially enriches VLDL with α-tocopherol, discriminating other tocopherols and tocotrienols. The metabolism of vitamin E is unclear. Excess α-tocopherol is converted into α-CEHC (2′-carboxyethyl-6-hydroxychroman) and excreted where other tocopherols, γ- and δ-tocopherol, are degraded and excreted through the urine as the corresponding CEHCs (Traber and Atkinson, 2007).

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CHAPTER 3. SUPPLEMENTATION OF LAYING HEN FEED WITH TOCOPHEROLS AND ALGAL (*HAEMATOCOCCUS PLUVIALIS)* ASTAXANTHIN FOR EGG YOLK NUTRIENT ENHANCEMENT

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ABSTRACT

Adding supplements to hen feed can increase egg nutritional value. Research on astaxanthin, tocotrienols, and tocopherols indicates this compound is a potent antioxidants and provides health benefits to humans. We hypothesized that the addition of these nutrients to hen feed will result in egg yolks with increased nutrient content with minimum change in functional properties. Laying hens (W-36 breed) were fed four diets with different supplementation levels of palm toco concentrate and algae (*Haematococcus pluvialis*) biomass containing astaxanthin for eight weeks. Egg yolks were analyzed for physical (color and Haugh unit), chemical (fatty acid composition, phospholipids by ^{31}P NMR, quantification of tocotrienols, tocopherols, and astaxanthin by HPLC), and functional properties (texture, emulsification, viscosity, and sensory evaluation). Feed with the highest nutrient concentration was also studied for stability of these antioxidants using the Arrhenius approach. No significant differences were observed among functional properties, except emulsification capacity and sensory characteristics among eggs from different diet treatments. Emulsification capacity decreased as the concentration of feed additives increased. Changes in egg yolk color achieved maximum values at day 8. Incorporation of

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tocopherols and tocotrienols increased until day 8 and astaxanthin incorporation increased until day 10, then decreased slightly thereafter. Feed nutrients resulted in a dose-response relationship of these in the egg yolk. Transfer efficiency ranged from 0-9.9% for tocotrienols and tocopherols and 7.6-14.9% for astaxanthin for their peak values. Sensory evaluation of cooked eggs showed a significant difference in perceived sulfur, fishy flavor, astringency, and lumpy levels. No significant difference was shown with dryness and hardness characteristics. Results from the Arrhenius accelerated stability study showed significant differences in shelf-life of various nutrients and such results can be used to properly formulate the feed materials.

Key words: Astaxanthin, egg quality, nutrient transfer, tocopherols, tocotrienols

INTRODUCTION

Hen's egg is recognized as one of nature's most complete foods. Eggs contain high-quality protein and lipids as well as essential and nonessential nutrients. They are an inexpensive food, food ingredient, and consumed worldwide. Egg composition can be altered by heredity genes, diet, and laying hen age (Fredriksson et al., 2006). Egg yolk contains natural carotenoids, and its yellow color is attributed to the presence of β-carotene, zeaxanthin, crypotoxanthin, and lutein naturally found in commercial feed (Huopalahti et al., 2007).

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is a dark-red pigment, and a member of the carotenoid family. It is the main carotenoid found in algae, such as *Brevibaterium, Mycobacterium lacticola, Agrobacterium auratim, Haematococcus pluvialis,* and *Phaffia rhodozyma* (Domínguez-Bocangegra et al., 2004), and aquatic animals including salmon, trout, shrimp, lobster eggs, zooplankton, and birds, such as flamingo and quail (Johnson and An, 1991). Astaxanthin has a strong antioxidant activity, 10 times higher than β-carotene and more than 300 times more effective compared to α-tocopherol (Rao et al., 2009, Higuera-Ciapara et al., 2006; Miki, 1991). Astaxanthin from *Haematococcus pluvialis*

is an excellent source not only for pigmentation, but also to protect against lipid oxidation, UV photoxidation, inflammation, cardiovascular disease, and cancer (Guerin et al., 2003).

Tocotrienols and tocopherols (tocos) are fat-soluble vitamins and generically regarded as vitamin E, for which the antioxidant properties are well studied. Within the vitamin E analogs, α-tocopherol is considered the most active form (Theriault et al., 1999). Bioavailability of vitamin E is greatly influenced by food matrix, formulation, and food processing (Reboul et al., 2006). Vitamin E is thought to prevent atherosclerosis (Stephens et al., 1996), protect against coronary disease (Stampfer et al., 1993), prevent neurons from oxidative stress and the development of Alzheimer's disease (Li et al., 2001), reduce bone loss, modulate immunomodulatory functions, protect GI tract, slow aging, and skin disease development (Aggarwal et al., 2010).

Previously astaxanthin has been used successfully in adding pigmentation to egg yolks, salmon, trout, and chicken meat. A study by Akiba et al. (2000) examined the effects of feeding yeast, *Phaffia rhodozyma*, that contains astaxanthin to increase the color of egg yolk. They used very low concentrations of astaxanthin, 0, 2, 4, 8, and 16 ppm for 4 weeks to feed hens and increased the yolk color score from 6.6 (golden yellow) to 12.7 (orange) on the Roche color fan. Dike et al. (1992) completed a similar study using laying hens with 0.3% *Phaffia rhodozyma* for 8 months and increased the Roche color fan score by only 1 point taking the color from a yellow to a more golden yellow. Takahashi et al. (2004) evaluated the effect of astaxanthin from *Phaffia rhodozyma* feed containing 0, 50, or 100 mg/kg of astaxanthin to broilers over two weeks. Broiler tissues were examined for astaxanthin concentration with the highest concentration in the small intestine followed by subcutaneous fat, abdominal fat, spleen, liver, heart, kidneys, and skin. The lowest astaxanthin concentration resided in the muscle.

Astaxanthin has been used to pigment other tissues besides egg yolks. A study by Gobantes et al. (1998) used 5% astaxanthin to fed rainbow trout (*Oncorhynchus mykiss*) to examine the color stability in vacuum packaging over storage time. They found considerable color variations among the neck, tail, and back parts along the fish but failed to maintain the color over 15 day storage time. Another fish pigmentation study by Barbosa et al. (1999)

evaluated the blood of rainbow trout (*Oncorhynchus mykiss*) by feeding the fish two different sources of astaxanthin, green algae *Haematococcus pluvialis* and commercial beadlets of 8% astaxanthin. They were able to achieve serum levels of astaxanthin from 5.3-9.0 μg/ml, and higher astaxanthin serum levels were achieved when fish were fed a high lipid content diet, independent of astaxanthin source. Therefore, the majority of the past research with astaxanthin is for pigmentation, not for nutrient enrichment.

Regarding vitamin E, there have been several feeding studies on vitamin E enriched feed for the nutrient to transfer to eggs, current literature is not comprehensive on transfer evaluation among the different vitamin E analogs. Flachowsky et al. (2002) examined the effect of α-tocopherol concentration on foods of poultry origin, and found that eggs had the highest levels of α-tocopherol. A study by Franchini et al. (2002) successfully used DL-α-tocopheryl acetate, to increase its level in eggs. Galobart et al. (2001) also used α-tocopheryl acetate to a base diet containing linseed oil to determine its transfer to eggs and they showed a dose-dependent enrichment with the range of 16-132 μg/g in whole egg. Grobas et al. (2002) used α-tocopheryl acetate and vitamin A to the diet to assess the supplementation levels in the egg yolk, and found that α-tocopherol was deposited in the egg yolk. All these feeding studies were focused on α-tocopherol transfer to eggs, not the tocotrienols, which have health benefits that are different from those offered by tocopherols (Aggarwall et al., 2010).

Since tocotrienols possess powerful neuroprotective, anticancer, and cholesterol lowering properties that are not often shown by tocopherols. Osakada et al. (2004) showed that all the palm tocotrienols significantly decreased hydrogen peroxide induced neurotoxicity, but α-tocopherol did not. γ-Tocotrienol antitumor activity on human hepatoma Hep3B cells, was shown by Sakai et al. (2006). Lee et al. (2009) showed that rats fed the tocotrienol-rich fractions were able to swim significantly longer than those fed α-tocopherol. Tocotrienols was also linked to cholesterol lowering, blood pressure lowering, anticancer, anti-angiogenic, anti-inflammatory, and immune function modulation (Sen et al. 2006). Despite knowledge of many health benefits of tocotrienols, we intended to examine their transfer from feed to egg yolks.

It is expected that successful incorporation of both astaxanthin and tocos in eggs will further improve egg nutrition and human health. We proposed to test how well these nutrients can be transferred to eggs. We hypothesized the addition of astaxanthin, tocotrienols, and tocopherols will result in egg yolks with increased nutrient content with minimum change in functional properties.

MATERIALS AND METHODS

Feeding Experiments

An 8-week feeding experiment was performed with 72 laying hens (Hy-Line W-36) of 35 weeks old at the start of the experiment. Iowa State University Institutional Animal Care and Use Committee approved all techniques for this experiment. The hens were allowed to adjust to the new surroundings and feeding system for one week. An environmentally-controlled room (temperature of 16-31 °C) with 24 cages (8 stacks x 3 tiers per stack), three birds per cage (as the experimental unit) was used to house the hens (Appendix 1, Figure 1). Eighteen hens were assigned per treatment (6 replicates for each treatment) for a total of three treatments and 18 hens for a control diet. Daily lighting program conditions were controlled to 16 hours of light and 8 hours of darkness, as used in commercial operations. Hen housing allowed the water containers to be shared but feed containers was allowed only for one cage. With this arrangement, no feed could be cross-contaminated between cages. Slightly slanted floors allowed the eggs to roll for easy collection.

The base diet formulation met the white laying hens NRC (1994) nutrient recommendations. A vertical feed mixer combined the base diet (Table 1) for 30 minutes. From the base diet ingredients, some corn and animal rendered oil was held back to mix in the Tocomin® 50% (Carotech Inc., Edison, NJ) (Table 2) and BioAstin, dry flake/powder (1.35% natural astaxanthin Cyanotech Corporation, Kailua-Kona, HI) (Table 3) in a Hobart mixer (model H-600; Hobart, Troy, OH). The algae product is designed to be used for human, fish, and animal consumption. Tocomin® contains naturally occurring mixture of

tocotrienols and tocopherols extracted and concentrated from virgin crude palm oil and palm fruits (*Elaeis guineensis*).

The base diet was divided into four equal parts for each treatment. To make Diet A, control, the withheld corn and fat were added back to the base diet. The lowest diet concentration, Diet B, with 0.49% algae (as-is basis) and 0.012% Tocomin® added would yield astaxanthin and toco 1,081 ppm in yolk lipid assuming 100% transfer efficiency. The middle diet concentration, Diet C, with 1.47% algae and 0.036% Tocomin® would yield astaxanthin and toco 3,245 ppm in yolk lipid assuming 100% transfer efficiency. The highest diet concentration, Diet D, with 2.94% algae and 0.072% Tocomin® would yield astaxanthin and toco 6,490 ppm in yolk lipid assuming 100% transfer efficiency. The horizontal mixer combined each diet for 20 minutes. Feed was stored at 4°C when not used at the poultry farm. Approximately a 6-day supply was brought out to the farm when the feed levels became low. The birds were fed and watered *ad-libitum*. Daily measurements included feed intake, egg production, and egg weight. Weekly hen weight was also measured.

Egg Physical Properties

Egg Production Measurements. Scheduled daily egg collection resulted in the previous 24-hour production data. The number of eggs and feed consumption from one-week's collection were combined to calculate egg production rate and daily feed intake. No egg collection was done during the adaption period, of 1 week. Once the study began, eggs from day 0, 2, 4, 6, 8, 10, 12, 14, 16, 17, 21, 28, 35, 42, 49, and 56 were collected for egg yolk total lipid extraction, color analysis, and egg moisture content from each cage. Each three-hen cage eggs were combined to be one unit. Throughout the experiment whole egg and egg yolk weight were recorded.

Eggs collected from different days after reaching steady-state color enrichment were used for different tests to compare diet effect, because it is not possible to perform all analyses with the small number of eggs produced from one day.

Color Analysis. Egg yolk separation from albumen was completed with a small strainer and a micro spatula. Manual stirring homogenized the sample of combined three-hen

cage eggs. A LabScan XE spectrophotometer with Hunter Lab system (Hunter Associate Laboratory Inc., VA) was used for color analysis. The system conditions were set to D65 (daylight 65), 10° standard observer, 2.00-inch port size, and 1.75-inch diameter area view conditions. Certified standard black and white plates were used to calibrate the system. The CIE L*a*b* scale was used for measurements where L* represents the lightness, (value of 100 was white and 0 was black), positive a* represents red, negative a* represents green, positive b* represents yellow, and negative b* represents blue. Approximately ten grams of fresh sample was placed into a plastic petri dish (60 x 15 mm) with white tape covered cardboard sleeve to avoid light escaping the system.

HunterLab color analysis was also performed with hard-boiled yolk samples from collection day 39, 40, and 41. Approximately seven grams of cooked yolk was used, because this quantity matched the height from ten grams of fresh yolk samples. Cooking was done by boiling eight eggs for eight minutes. Egg yolks were carefully separated from the white and shell, then mixed together for each cage to make a homogeneous mixture. Samples were gently pressed into the petri dish to decrease surface area and light scattering. All samples, fresh or hard-boiled, were measured once.

Haugh Unit. Eggs collected on days 51, 52, and 53 were used for Haugh unit measurement. Eggs were analyzed after an 24 hour equilibration period in an 18 °C cooler. A standard USDA method (USDA Handbook: 75, 2000) was followed. Each egg was weighed and broken onto a glass top over a reflective mirror. Thick albumin measurements were taken immediately after the breaking the egg using a micrometer. The Haugh unit was calculated using egg weight and thick albumin height, with the following equation:

$$HU = 100 \log (H - 1.7*W^{0.37} + 7.6)$$

Where: HU=Haugh unit, H=Observed height of the albumen (mm), W=Weight of egg (g). Each egg was measured once then averaged within the cage for a unit value.

Egg Yolk Functional Properties

Raw Egg Yolk Viscosity Measurement. Viscosity measurement was completed with eggs collected on day 44 with a Haake RS 150 Rheometer (ThermoOrion, Karlsruhe,

Germany). Careful separation of egg yolk from the white was done to not break the yolk membrane. The yolk was rolled in filter paper (Whatman cellulose filter paper 4) to remove any remaining albumen. The yolks from the same cage were combined and mixed by a stirring rod. Approximately ten drops of egg yolk was used for each measurement. Samples were placed between a plate (diameter of 35 mm) and attachment (PP35T) with a gap of 1.000 mm. The rheometer was maintained at 25 °C. Samples were examined with two viscosity tests. The first test with changing shear rate linearly from 0.10/s to 60.00/s for 30 seconds. These results were fitted to the following equation:

$$\tau = \kappa * (\dot{\gamma}/\dot{\gamma}_o)^n$$

Where τ [Pa] as shear stress, $\dot{\gamma}$ [s^{-1}] as shear rate, $\dot{\gamma}_o$ as constant shear rate [s^{-1}], κ [Pa] as consistency index, and n as flow index (Buxmann et al. 2010). The second test used a constant shear rate at 60.00/s for 60 seconds to give the apparent viscosity measure. Each sample was subsampled in duplicate for each test.

Egg Yolk Texture Measurement. Egg yolk texture profile was measured on eggs collection day 38 and 45. TA-XT2i texture analyzer (Texture Technologies, Scarsdale, NY) was used to evaluate hard-boiled egg yolk texture profile using Texture Expert software. Eggs were boiled as described before in the color analysis method. The shell and white were removed carefully not to break the yolk. The whole yolk was measured using a modified two-bite model (TPA) method of Juliano et al. (2007). The system was fitted with probe TA-4 (3.8-cm diameter acrylic cylinder 20 mm tall), pretest speed of 2.0 mm/s, test speed of 1.0 mm/s, posttest speed of 2.0 mm/s, and distance of 15.0 mm. Each egg was measured once and then averaged within the cage for a unit value.

Characteristics measured include fracturability, hardness, cohesiveness, adhesiveness, springiness, and chewiness. Fracturability (g) was determined as the force at the first significant break in the curve. Hardness (g) was calculated by measuring the peak force obtained during the first compression cycle. Cohesiveness (dimensionless) was determined by the ratio of the positive force area during the second compression to that of the first compression. Adhesiveness (g*s) was determined by the first bite's negative area, representing the work necessary to pull the probe away from the sample. Springiness (mm)

was defined as the height the yolk recovers during the time lapse between the first and second bites. Chewiness (g) was calculated by the product of hardness, cohesiveness, and springiness (Bourne 1978).

Egg Yolk Emulsification Properties. The method were adapted from Wang and Wang (2009). Egg yolk, from collection day 47, 1.5% dwb (liquid yolk) suspension in 25 mL was mixed with dyed (Sudan Red 2B about 4 ppm) soybean oil at constant rate of oil addition, 5 mL/min. A Bamix brand handheld blender (Switzerland) continuously mixed the oil and protein dispersion at the "low" speed until apparent phase inversion was observed, at which point the oil-in-water emulsion system lost viscosity. The amount of oil added until phase inversion was used to calculate the emulsification capacity (EC, g oil/g yolk).

For emulsion stability measurement, the same dyed vegetable oil 8 mL was added into 32 mL of 1.5% yolk dwb suspension and mixed with the same handheld blender for one minute on the "high" speed setting. From the resulting emulsion, 10 mL were transferred into a 15 mL plastic centrifuge tube with 0.5 mL marked intervals. Emulsion stability (ES, %) was calculated by dividing non-separated volume by total volume after one day standing at ambient temperature, and it is expressed as percentage.

Sensory Evaluation. Thirteen panelists (7 males and 6 females college-aged students) completed sensory evaluation training lasting three hours. For the training, characteristic standard definitions were defined and standard samples were placed along an unstructured 15-cm line scale (Figure 1). The training session included egg yolks from a local grocery store, diet A, diet D fresh eggs, and 2-week old diet D eggs stored at 4 °C. The panelists agreed on the standard sample placement on the line during training. Three tests replicates (three separate sessions) were completed in a standard sensory test facility under red light to reduce visual cues. Characteristics measured include flavor profile (sulfur and fishy) and texture profile (hardness, astringent, lumpy, and dry) modified from Juliano et al. (2006). Sulfur was described as rotten egg smell, fishy as seafood or fishy smell, astringent as puckering quality, lumpy as cottage cheese texture, and dry as how little moisture is perceived from the yolk. A general preference (likeness) test was also conducted using a rating scale of overall quality (10 excellent to 1 very poor) and overall flavor intensity (10

bland to 1 extreme) (AOCS Cg 2-83. 1993). A sample sensory test sheet is shown in Figure 1. Eggs were cut in half exposing the yolk and one half from each of the feed Diets, A, B, C, and D, were given to the panelists. Samples were placed in a random order. Each panelist was tested three times with each sample. A 3-digit random number identified egg samples with the key only known to the researchers in the sensory kitchen.

Egg Chemical Properties

Egg Yolk Lipid Extraction. Egg yolk was separated and mixed as described before. Total lipids were extracted by using Folch extraction method (Folch et al., 1957). Briefly, 6 grams of fresh yolk was extracted three times with 2:1 chloroform:methanol. DI water was then added to the separatory funnel volume ratio of 8:4:3 of chloroform:methanol:water and time was allowed for phase separation. The organic layer was roto-evaporated and the resulting oil was weighed and transferred with hexanes into glass 22-mL vials. Vials were stored at 4 °C in the dark until further analysis. Each sample was extracted once.

Egg Yolk Lipid GC Fatty Acid Composition Analysis. Collection day 10 egg oil samples were determined for fatty acid methyl ester (FAME) composition. The method was adapted as described by Hammond (1991). Two mL of benzene were added to the oil aliquots (3 mL) in hexanes to remove any remaining water and solvent was evaporated with nitrogen until dry. One mL of 1 M sodium methoxide was added to the vials and react for five hours at ambient room temperature. Three mL of DI water and two mL of hexanes were added to stop the reaction and separate the FAME from the rest of the solution. The FAMEs were analyzed by GC (Hewlett-Packard 5890 Series II) with a flame ionization detector, a silica capillary column (15 m long, 0.25 mm internal diameter, 0.2 μm film thickness, SP-2330, Supelco, Bellefonte, PA). The chromatographic parameters were: injector and detector temperature of 230 °C, oven temperature program was 140-200 °C at 10 °C/min with no hold time. The carrier gas (He) was set at 5.4 mL/min, the auxiliary gas (He) at 19.4 mL/min, H_2 was at 13.9 mL/min and air at 426 mL/min. The split ratio was 24:1 (Gerde et al., 2011).

^{31}P NMR Phospholipid Quantification. Concentrated egg oil samples (80-90 mg/ mL) from day 10 were used for the analysis. The internal standard, triphenol phosphate (TPP) was added to the oil (approximately 10 mg was dissolved in 1 mL chloroform-d, 1 mL methanol, and 1 mL Cs-EDTA (0.2 N, pH 8.5)). After vigorous shaking, the samples were centrifuged at 1,250 x g for 2.5 minutes. The lower phase was transferred to a 5 mm NMR tube (Kimble/Kontes, Vineland, NJ). A Varian VXR-400 spectrometer (Varian, Inc., Palo Alto, CA) with a Bruker Magnet (Bruker BioSpin, Billerica, MA) operating at 162 MHz was used to obtain ^{31}P NMR spectra. Egg lipid samples were analyzed with an inverse gated decoupling pulse sequence to suppress any nuclear overhauser effect. The NMR scan conditions were as follows: pulse width, 22 μs; sweep width 9718 Hz; acquisition time, 1.2 s; relaxation delay, 10 s; and 256 scans. All chemical shifts were recorded relative to TPP (δ -17.8). MestReNova software (Mestrelab Research SL, Spain) was completed for data processing. The phospholipid content (percent) in the extracted egg oil was calculated as follows: phospholipid content (%) = 100* [(phospholipids (g) in the concentrated oil)/ (starting oil weight (g))] (Yao and Jung, 2010).

Tocotrienols and Tocopherols HPLC Quantification. Normal-phase HPLC (Waters Alliance 2695, Waters Corporation, Milford, MA) was used to determine tocotrienol and tocopherol concentrations of all oil samples dissolved in 1 mL hexanes. The detector (Waters 474 Fluorescence, Waters Corporation, Milford, MA) was set to an excitation wavelength of 292 nm and emission wavelength of 335 nm. Injection volume was 5 μL and run time of 22 minutes. The column (Phenomenex, Luna 3μ NH_2 100Å, 150 mm long x 3.0 mm internal diameter) was set to 30 °C. Tocopherol standards (Matreya) and tocotrienol standards (Davos Life Science) with standards concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2.5, and 5 ppm had 0.995 linearity or greater coefficient for the calibration curves. The mobile phases were HPLC grade 100% isopropanol (solvent A) and 100% hexanes (solvent B). The HPLC ran 2% solvent A and 98% solvent B isocratically.

Astaxanthin HPLC Quantification. Reverse-phase HPLC (32 Karat 8.0, Beckman Coulter Gold HPLC, Beckman Coulter Inc.) was performed to determine astaxanthin level in all extracted egg yolk oil samples dissolved in 1 mL 50:50 ethyl acetate:ethanol. One mL oil

samples previously suspended in hexanes were evaporated with nitrogen in amber colored vials. Samples were then placed into a vacuum oven to remove any remaining hexanes from the samples then weighted. The UV/Vis detector (System Gold 16 Detector, Beckman Coulter Inc.) was set 450 nm wavelength. Injection volume was 20 µL and run time of 50 minutes at a flow rate of 1 mL/min. The column (YMC carotenoid, Waters, 3µ particle size, 250 mm long x 4.6 mm internal diameter, Waters Corporation, Milford, MA) was set to 25 °C. A linear gradient mobile phase of 81:15:4 methanol: methyl t-butyl ether: water (solvent A) and 6:90:4 methanol: methyl t-butyl ether: water (solvent B) was used for lipid separation. The gradient program adapted from Sander et al. (1994) was used: linear decrease from 100% of A to 77.8% A in 20 minutes, linear increase to 100% B from 20 to 23 minutes, then holding at 100% B until 35 minutes. For preparation for the next sample, linear decrease from 100% to 0% B was completed in 5 minutes, and 10 minutes conditioning at 100% A.

Due to the lack of astaxanthin standard, β-carotene standard was used for quantification. A standard curve was generated using 50, 100, 300, 600, 1000, 2000, 5000, and 7000 ppm β-carotene solutions and their HPLC areas. Astaxanthin area was converted to β-carotene equivalents using extinction coefficient correction. Astaxanthin's extinction coefficient was reported as $E^{1\%}_{1\ cm} = 2{,}100$ for free, monester, and diester forms, while β-carotene's extinction coefficient was $E^{1\%}_{1\ cm} = 2{,}600$ (Barbosa et al., 1999; Yuangsoi et al., 2008).

Astaxanthin Identification by Liquid Chromatography-Mass Spectroscopy. Negative ion mode APCI mass spectrometer (6540 UHD Accurate-Mass Q TOF LC-MS, Agilent Technologies, Schaumburg, IL) with a diode array LC detector (450 nm) was used for egg oil and algae biomass extract xanthophyll identification with a method modified from Holtin et al. (2009). The same separation conditions were used as described for the HPLC astaxanthin quantification. The LC system (Agilent 1200 Series) delivered the solvent at a flow rate of 1 mL/min and 1 µL sample injection. The MS was tuned and calibrated using the Agilent APCI tune mix. Agilent MassHunter Software (Version B.0.3) was used to process data and identify the five astaxanthin isomers.

Nutrient Transfer Efficiency into Egg Yolk

Transfer efficiency calculation for each of the additives was completed as the ratio of milligrams of the additive transferred in the egg at the peak value to the milligrams of additive consumed during the same period. The calculation of milligrams of additive consumed was concentration in feed (mg additive/kg feed) multiplied with the feed consumed (Galobart et al., 2001).

Shelf-life Study of the Feeding Material Using Arrhenius Approach

Arrhenius Approach Degradation Kinetics. The highest enrichment diet was used for this study for more easily detectable changes in concentration. Approximately 100 g samples of Diet D were placed into four different ovens set at 50, 60, 70, and 80 °C. Time interval for sampling at each oven temperature were based on the $Q_{10} = 2$ rule. Samples were taken every seven days for the 50 °C sample, five days for the 60 °C sample, three days for the 70 °C sample, and two days for the 80 °C sample. The Arrhenius equation is as follows:

$$k=Ae^{-Ea/RT}$$

where k is the rate coefficient, A is a constant, E_a is the activation energy, R is the universal gas constant, and T is the temperature in Kelvin. By linearly correlating ln(k) vs. 1/T, the activation energy can be estimated and shelf life of the nutrient at any temperature can be estimated using the Arrhenius equation.

Feed Lipid Extraction. Diet D feed total lipids were extracted using the same Folch method mentioned for egg yolk oil extraction with a minor modification. A 9 gram sample was extracted using 50 mL of 2:1 chloroform:methanol for one hour covered to avoid light deterioration. Mixtures were then filtered (Whatman cellulose filter paper 1) and extract was repeated two more times. Solvent extracts were combined for Folch wash in a separatory funnel and covered with aluminum foil to minimize nutrient degradation for phase separation.

HPLC Quantification of Astaxanthin in Feed Lipid. Reverse-phase HPLC was also performed on the feed lipid samples for astaxanthin degradation rate determination. The same

sample preparation as for egg yolk astaxanthin lipid quantification was used. The same HPLC systems, software, column, and solvents were also used. The gradient was the same as found in Sander et al. 1994 method, which was different from egg yolk astaxanthin quantification. Briefly, 100% solvent A changed to 100% solvent B linearly over 90 minutes. Ten minutes of 100% solvent A followed each sample run to condition the column for the next run. Astaxanthin in feed and algae biomass were mainly in the monoester forms. The total area of all the ester peaks were used for quantification.

Statistical Analysis

Statistical analysis was completed using SAS (version 9.2, SAS Institute Inc., Cary, NC) One-way analysis of variance (ANOVA) was used for mean comparisons, and Fisher's least significant differences were calculated at $P< 0.05$ ($LSD_{0.05}$). All treatments had six replicates.

RESULTS AND DISCUSSION

Feeding Experiment

After mixing the feed, the astaxanthin induced a color in the feed from a pale yellow to a burnt red color as seen in Figure 2. The color is directly related to the amount astaxanthin-containing in the algae biomass added to the diet; the higher astaxanthin biomass added the more red the feed became.

Visual Observations at the Farm. Visual observations were made of the hen's appearance. Appendix Figures 2 and 3 shows observed color changes in the hens' combs and feet respectively, during week 7 of the study. The redness coloration differences can be linked to the astaxanthin pigment as seen in a broiler feeding study that showed similar results in meat with higher astaxanthin concentrations in the feed leading to more red coloration in the skin and muscle tissues (Akiba et al., 2001; Takahashi et al., 2004). Appendix Figure 4 shows the hens' fecal matter darkening from the different treatment diets, as a result of incomplete absorption of astaxanthin. The red intensity increased due to the

astaxanthin increase in feed. Hencken (1992) found that 70% of astaxanthin fed was excreted.

Egg Physical Properties

Hen Performance and Egg Quality. Laying performance and egg physical quality parameters are shown in Table 4. Laying rate, hen weight, egg yolk weight, moisture content, and Haugh unit for all the diets had no significant difference among the diets. The mean laying rate was above 90% for all the diets. Laying rate percent (or hen-day egg) was calculated by number of eggs produced/number of hens x 100. Whole egg weight was not statistically significant among Diets A, B, and D, but there was a difference with Diet C, whose eggs weighted slightly less than the other diets. Egg yolk weight for all the diets had a mean of 16 g/egg yolk and no statistical difference was found. Yolk moisture content for all the diets was about 50% which matches the value found in literature (Huopalahti et al., 2007; Aro et al., 2009). Haugh unit values were 88-89 for all the diet and these values are comparable to a study completed by Franchini et al. (2002). Feed intake was similar for all diets ranging from 104-106 g/day/hen. Yet, it increased slightly (not statistically significant) to a mean of 108 g/day/hen for Diet D. Similar feed intake was seen with a study by Carrillo-Domínguez et al. (2005) fed red carb meal, containing astaxanthin to laying hens. Prayitno et al. (1997) reported that a higher intensity of red color encouraged the hens to consume more feed, and this may explain the seemingly higher feed consumption because of Diet D's higher intensity of red color.

Yolk Color Enrichment. Color analysis exhibited some interesting characteristics. All fresh egg yolk samples changed in L*, a*, or b* values but they became relatively constant after day 8. A similar observation was made by Fredriksson et al. (2006) where *N. oculata* was added to their feed. A glimpse of the color progression is displayed in Appendix Table 1. Yolk color changes were noticeable by day 3. Color started with the outer membrane of the yolk and further continued inward until the entire yolk was uniform. Samples decreased in L* value, to day 8 where each diet plateaued at different values, with the highest concentration diet being the darkest (Figure 3). The a* values of yolk increased

drastically when the samples became more red with increasing feeding time and concentration, with Diet D treatment having the highest value of redness (Table 5). The b* values exhibited smaller and slower changes among the different diets as compared to the L* and a* values. These trends for the L*, a*, and b* values were similarly reported in Fredriksson et al. (2006) who fed the hens *Nannochloropsis oculata,* a green algae and Herber-McNeill and Elswyk (1998) who fed an enriched marine microalgal product. Akiba et al. (2000) fed *Phaffia rhodozyma,* which contained astaxanthin and obtained similar trends as observed in this study, but their color change were not as drastic as the current study due to lower ppm concentrations fed to the hens. Hard-boiled yolk samples showed the same trends in color analysis except the values were lower for a* and b*, and higher for L* (Table5) possibly due to cooking and protein denaturation.

Egg Yolk Functional Properties

Effect of Diet Enrichment on Yolk Viscosity. The viscosity of raw egg yolks illustrated a shear thinning, pseudoplastic profile, as reported by Ibarz and Sintes (1989). The apparent viscosity showed no significant difference among the diets (Table 6). The viscosity coefficients or consistency indexes were the same Diets A, B, and D, yet Diet C showed a higher value from unknown causes, because the amount of oil and moisture content were similar in eggs from all diets. Viscosity coefficient is defined as the viscosity at a shear rate or stress of 1 s^{-1}. From the power law model, the exponent, flow index, had no significant difference among diets. The flow index is a measure of how a non-Newtonian fluid is. If the index is 1 the fluid is Newtonian, index between 0 and 1 the fluid is shear-thinning, and index greater than 1 is shear-thickening.

Effect of Diet Enrichment on Emulsification Properties. Emulsification capacity and emulsion stability measure the amount of oil the protein suspension can emulsify and how stable the emulsion formed with a specific amount of oil, respectively. There were no significant differences among the treatments for the stability test (Table 6). The capacity did exhibited differences among the diets. Diet A, the control, had the highest emulsification capacity Diet C and D had lowest levels. This decrease cannot be explained. The possibility

of the nutrient incorporation causing a different interaction between the oil and yolk protein would be low. The possibility of the proportion of various types of yolk protein may have changed by diet may exist. However, the focus of this study was only on lipid components and how the yolk as a whole may have different properties, so the potential protein type, proportion or functionality change may be a focus for future studies.

Effects of Diet Enrichment on Egg Yolk Texture. Texture measurements obtained were fracturability, hardness, adhesiveness, cohesiveness, springiness, and chewiness. The egg yolks were analyzed individually, then averaged for cage values as unit values with 6 replicates. The texture test showed no significant difference among the diets for each of the parameters measured (Table 7). Results were also similar to a study by Kassis et al. (2010) that used krill oil containing astaxanthin, for egg enrichment, resulting in no significant differences in springiness, cohesiveness, gumminess, chewiness, and resilience. Large standard deviations can be explained by uneven cooking, yolk's natural texture, or if the yolk started to break before the test was completed.

Sensory Evaluation. Appendix Figure 5 shows the hard boiled eggs after hard boiling used for the sensory panel. Data from the sensory panel are shown in Table 8. Perceived sulfur levels were noticeably lower with an increase of fishy flavor for Diet D treatment. The normal egg flavor change with this diet is due the high algae biomass concentration and the addition of the highly unsaturated fatty acids contained in the algae. Typical egg flavor is comprised from many compounds. Macleod and Cave (1976) identified cooked egg yolk flavor compounds including 9 saturated and 8 unsaturated straight chain hydrocarbons, 1 alcohol, 3 carbonyls, 5 alkyl, 5 alkenylbenzenes, 3 phenols, 4 indans, 3 indoles, 1 sulfide, 4 pyrazines, 3 pyrroles, and 4 nitriles. The predominant compounds identified were 2-methylbutanal, 5-heptadecene, indole and toluene. Algal biomass has a fishy characteristic therefore the egg flavor was affected by the astaxanthin addition. No observed differences were found among diets for hardness. This also correlates with the texture analysis performed. Astringency was significantly different for Diet B compared to the other diets, which was less astringent than the rest of the eggs, and the reason is unknown. Astringent compounds in egg yolks are phenols like o-cresol, m-cresol, and p-

cresol (Maga, 1982). The lumpy characteristic was lower for Diet A, where Diet D was significantly the most lumpy, cottage cheese-like characteristic. There was no significant difference in dryness among the diets. The last two parameters from the sensory panel (Table 8) were not from trained evaluation, but were given as preliminary indicators of consumer acceptance. The overall quality was statistically significantly higher for Diet A and B than for Diet D, but there no significant difference with overall intensity flavor. These differences are small, and may not be practically different. A consumer level test should be done to assess acceptability.

Egg Chemical Properties

Fatty Acid Composition. Appendix Figure 6 illustrates a typical chromatogram from egg lipid FAME analysis. Table 9 illustrates the GC results of fatty acid composition from day 10 of the feeding study. All egg yolk oils were not significantly different and composition percentages are similar to reported values from USDA (2010) for fresh egg yolk.

Diet Effect on Egg Yolk Phospholipids. Figure 4 illustrates a typical spectra from the ^{31}P NMR analysis. There was good separation of various polar lipids classes as presented by Yao and Jung (2010) for identification and class quantification. Cardiolipin, phosphatiylethanolamine, sphingomyelin, lysophophatidylcholine, phosphatidylinositol, and phosphatidylcholine were identified from the ^{31}P NMR chromatogram. Table 10 illustrates the phospholipid class composition and concentration relative to total oil from day 10 eggs. These phospholipid compositions are not significantly different among treatments.

Astaxanthin Identification. The monester and free astaxanthin HPLC peaks from algae lipid extract are shown in Figure 5A. The astaxanthin extract chromatogram is similar to that reported by Holtin et al. (2009) and Miao et al. (2006), showing astaxanthin in algae are mainly monoesters. However, hens are able to convert the esters to free astaxanthin and deposit the free form in eggs. The five isomers shown in Figure 5C are due to the *cis* and *trans* double bond configuration difference and the hydroxyl group orientation (Holtin et al., 2009). All astaxanthin peaks were confirmed by LC-MS (Appendix Figure 7). The five isomers of astaxanthin are all-*trans* 3S, 3'S astaxanthin, all-*trans* 3R, 3'R astaxanthin, all-

trans 3S, 3'R astaxanthin, 9-*cis* astaxanthin, and 13-*cis* astaxanthin. The sum of areas of all five peaks was used to calculate astaxanthin amount in egg yolk lipid extract.

Diet Effect on Nutrient Concentration in Egg Yolk. HPLC quantification was completed for tocotrienols, tocopherols, and astaxanthin (Figure 5B, C). All toco analogs were well separated, as well as the major isomers of astaxanthin. The additives' concentration in yolk lipid increased as their concentration in feed increased (Figure 6 and 7) except for γ- and δ-tocopherols. The increase in concentration continued until day 8 for Diets B, C, and D for all tocotrienols. However, tocopherols enrichment showed different profiles. At the end of the 56-day feeding, many tocopherol concentrations tended to be lower than those at the start of the trial, especially for δ- and γ-tocopherols, which showed a steady reduction and for α- and β-tocopherol from the control and Diet B treatment. The reason could be that in the palm toco concentrate (Table 2), there were negligible amount of β-, δ-, and γ-tocopherols (a total of 3 mg/g, 0.6% of total tocos). However, the feed itself had its own natural tocopherols from the natural ingredients. With the added tocotrienols and α-tocopherol, the absorption of the δ- and γ-tocopherols in the feed itself was greatly suppressed. Because tocotrienols are not typically found in the natural ingredients in feeds, their feed enrichment led to a steady enrichment in eggs, and in a dose-response relationship. There seems to be a feedback or competitive inhibition for all the nutrients. With α-tocopherol there was a decrease between 30-65% depending on the diet. Other compounds exhibited a more dramatic decrease, with δ-tocopherol having 75% reduction compared to the initial value. Astaxanthin has a smaller decrease of 38-43% between Diet D and Diet B. A study by Galobart et al. (2002) observed a decrease in α-tocopherol of 10-12% while Meluzzi et al. (2000) and Franchini et al. (2002) saw reductions similar to the current study of 30% and 20-55%, respectively. Differences among the studies could be due to several factors including genetics, laying parameters, type and concentration of tocopherol used, experimental conditions, basal diets, and presence of other antioxidants (Meluzzi et al., 2000).

Astaxanthin increased in concentration up to day 10 for diets B, C and D and there was also a slight reduction thereafter (Figure 7). Astaxanthin had smaller decreases of

38-43% relative to peak values from Diet D to Diet B. This may indicate that the feedback inhibition for this antioxidant is not as strong as for tocopherols.

Nutrient Transfer Efficiency into Egg Yolk

From HPLC quantification, transfer efficiency can be calculated. Since day 8 and 10 of the study exhibited the highest nutrient concentration for tocos and astaxanthin in yolk lipid, respectively, this data were used for the calculation of the highest efficiency. Another two times were also selected to show the lower efficiencies and the results are shown in Table 11. The highest transfer efficiency occurred at the lowest feed concentration for most of the compounds. Efficiency variation for different compounds can be attributed to the difference of bioavailability and biochemical processes. A protein, α-tocopherol transfer protein (αTTP) has been identified to bind vitamin E analogs and transfer the compounds between membranes in the liver cytosol of animals. Hosomi et al. (1997) examined the affinities of αTTP toward different tocos and calculated relative affinity taking α-tocopherol as 100%: β-tocopherol 38%, γ-tocopherol 9%, δ-tocopherol 2%, and α-tocotrienol 12%. Packer et al. (2001) also concluded the liver preferentially enriches VLDL with α-tocopherol, discriminating other tocopherols and tocotrienols. The present study confirmed this trend, with the highest vitamin E analog transferred was α-tocopherol. This transfer protein's specificity could be the cause for this study failing to enrich the egg yolks sufficiently with tocotrienols. The low affinity of the transfer protein for α-tocotrienol in the presence of α-tocopherol resulted in small amount of α-tocotrienol transferred from the feed to the egg. The other tocotrienols transferred were even lower (< 1%). However, there is an interesting observation comparing the relative transfer efficiency of α-tocotrienol to α-tocopherol's at different feed concentration: 17, 36, and 41% for Diet B, C and D. Apparently, at higher concentration, more tocotrienols may be forced into the circulation system. These values are also higher than the 12% as reported by Hosomi et al. (1997).

The α-tocopherol transfer efficiency exhibited the highest transfer at 9.9% in Diet B to 3.4% in Diet D. Galobart et al. (2001) also observed a decrease from 41.8% with 50 mg/kg in the diet down to 26.7% with 200 mg/kg noting the highest transfer efficiency was

obtained with the lowest dietary level of α-tocopherol. In our study, the amount of added total tocos in Diet B was 60 mg/kg, in C was 180 mg/kg, and in D was 360 mg/kg. However, since α-tocopherol was only 22.5% of the total tocos, the added α-tocopherol in the three diets were 13.5, 40.5, and 81 mg/kg. Therefore, our feed enrichment level is similar to that of Galobart el al. (2001) but with much lower transfer efficiency. Similar trends were also confirmed in other vitamin E supplementations studies by Flachowsky et al. (2002) and Shahriar et al. (2008).

Astaxanthin transfer efficiency rate was determined to be as high as 14.9% at the low enrichment level. Its transfer efficiency reduced to 7.6% when the enrichment was increased 6-fold in the diet. Although the total concentration in yolk was increased by the increase in enrichment, however, the efficiency is greatly reduced. Akiba et al. (2000) observed 3.6% efficiency from their study using *Phaffia rhodozyma* as a source of astaxanthin. A study by Johnson et al. (1980) using the same yeast also observed from 3 to 4% efficiency in laying quails. Our data showed a higher transfer efficiency from algae biomass compared to red yeast.

It should be noted that we used the peak concentration of the nutrients to discuss the maximum transfer efficiencies. In reality, a steady state concentration, if observed, should have been used for such calculation. Since our concentrations showed steady reductions, and no plateaus were observed after 56 days, especially for the high enrichment concentrations, we decided to use the peak values for comparing the differences among different treatments.

Shelf-life Study of the Feeding Material using Arrhenius Approach

A shelf life study of Diet D was completed using the Arrhenius approach. Figure 8 and Appendix Figure 8 shows the shelf-life study. The slopes from Arrhenius plots were used for activation energy determination. Summary of the kinetics is shown in Table 12. γ-Tocopherol had an apparent zero order degradation reaction. α-Tocopherol, α-tocotrienol, and δ-tocopherol were determined to have an apparent first order degradation reaction. All the remaining compounds, β-tocopherol, β-tocotrienol, γ-tocotrienol, δ-tocotrienol, and astaxanthin, appeared to have an apparent second order degradation reaction. Activation

energy ranged from 28 to 307 kJ/mol. The half-life of each of the nutrient compounds are also presented in Table 12. The half-lives of the nutrient compounds show that certain compounds had very short half-lives, such as γ-tocopherol. The longest half-lives were exhibited by astaxanthin and δ-tocopherol. The low R^2 values may be attributed to the 70 °C oven temperature fluctuation causing irregular degradation trend.

CONCLUSION

This study shows astaxanthin and tocos can be incorporated into the egg yolk by different degrees of success by adding the ingredients into the feed. The higher the concentration in the feed the higher the concentration in the resulting egg yolk, however with lower transfer efficiency. Even though tocotrienols are nutrients with unique health benefits, they cannot be effectively transferred into eggs as α-tocopherol.

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Table 1. Composition of Base Diet for Laying Hens

Ingredient	Composition (%)
Corn	60.79
Soybean Meal	24.56
Coarse Calcium Carbonate	5.87
Fine Calcium Carbonate	3.92
Dicalcium Phosphate	2.03
Animal Rendered Oil	1.58
Vitamin and Trace Mineral Premix	0.68
Sodium Chloride	0.38
DL-Methionine	0.18
Crude Protein	16.05
Metabolizable Energy, kcal/kg	2,825
Crude fat	3.94
Linoleic acid	1.82
Calcium	4.20
Phosphorus (nonphytate)	0.48
Sodium Chloride	0.18
Chloride	0.26
Lysine (digestible)	0.77
Methionine (digestible)	0.41
Methionine + Cystine (digestible)	0.63

Premix includes per kilogram of diet: vitamin A, 9,000 IU; vitamin D_3, 3,000 IU; vitamin E, 20 IU; cobalamine, 13 µg; riboflavin, 6 mg; niacin, 45 mg; pantothenic acid, 12 mg; choline, 487 mg; menadione, 1.2 mg; folic acid, 1.5 mg; pyridoxine, 1.2 mg; thiamine, 1.5 mg; biotin, 45 µg; magnesium, 136 mg; manganese, 136 mg; zinc, 136 mg; iron, 140 mg; copper, 14 mg; and selenium, 0.27 mg.

Table 2. Composition of Tocomin® 50%

Analogs	Composition (mg/g)
d-α-Tocotrienol	115
d-β-Tocotrienol	15
d-γ-Tocotrienol	210
d-δ-Tocotrienol	55
d-α-Tocopherol	115
d-β, γ, δ-Tocopherol	3
Total Tocotrienol/Tocopherol	513

Composition of Tocomin® 50% from Carotech. Information adapted from Carotech product specification sheet.

Table 3. Composition of BioAstin

Components	Composition (%)	Components	Composition (%)
Natural Astaxanthin	1.35	**Natural Astaxanthin:**	
Total Fat	20-25	Free Astaxanthin	1.9
Protein	20-35	Monoester	84.3
Carbohydrates	30-55	Diester	13.9
Dietary fibers	18		
Moisture	<9	**Astaxanthin Isomers:**	
Ash	<17	Di-cis-astaxanthin	2.22
Other Carotenoids (relative to total Astaxanthin):		Trans-astaxanthin	74.07
β-carotene	2.22	9-cis astaxanthin	6.66
Canthaxanthin	1.48	13-cis astaxanthin	8.15
Lutein	2.96	15-cis astaxanthin	0.74

Information adapted from ALGAtechnologies (1998) spec sheet, Cyanotech certificate of analysis (2010) and Cyanotech analysis report (2011).

Table 4. Laying Performance and Egg Quality Parameters with Different Levels of Supplementation

Diet [1]	Laying Rate (%) (n=8)	Hen Weight (kg/hen) (n=12)	Daily Feed Intake (g/day/ hen) (n=8)	Whole Egg Weight (g) (n=348)	Egg Yolk Weight (g) (n=168)	Haugh Unit (n=18)[2]	Moisture Content (%) (n=15)
A	91.2 ± 4.0	1.46 ± 0.10	104.2 ± 10.2	60.47 ± 0.80 [a]	15.85 ± 0.53	88.89 ± 3.80	50.3 ± 1.4
B	93.1 ±2.0	1.50 ± 0.09	106.2 ± 9.7	60.81 ± 1.11 [a]	16.57 ± 0.74	88.72 ± 1.73	50.1 ± 1.5
C	92.7 ± 3.2	1.43 ± 0.08	105.1 ± 10.1	59.36 ± 1.05 [b]	16.10 ± 0.64	89.07 ± 3.45	50.4 ± 1.8
D	93.0 ± 2.7	1.46 ± 0.06	108.4 ± 9.9	60.74 ± 1.03 [a]	16.03 ± 0.96	89.26 ± 3.33	49.5 ± 1.4
P-value	NS	NS	NS	**	NS	NS	NS

Values are mean ± standard deviation.

[1] Diets are A=no supplements, B enriched with 0.012% tocomin and 0.49% algae biomass; C enriched with 0.036% tocomin and 1.47% algae biomass; and D enriched with 0.072% tocomin and 2.94% algae biomass.

[2] Haugh Unit analysis was completed on collection days 51, 52, and 53.

Different letters in the same column indicate significant different at 95% confidence interval.

**P-value ≤ 0.01; NS - not significant.

Table 5. Color Analysis of Fresh and Hard Boiled Egg Yolk Samples with Different Levels of Supplementation

	Fresh Yolk[2] (n=84)			Boiled Yolk[3] (n=18)		
Diet [1]	**L* value**	**a* value**	**b* value**	**L* value**	**a* value**	**b* value**
A	65.39 ± 1.37 [a]	9.45 ± 1.66 [c]	73.24 ± 3.32 [a]	76.90 ± 3.33 [a]	2.90 ± 0.90 [d]	43.04 ± 5.79 [b]
B	47.86 ± 2.23 [b]	42.75 ± 1.91 [b]	69.27 ± 3.67 [b]	62.67 ± 3.95 [b]	31.28 ± 4.85 [c]	46.74 ± 4.06 [a]
C	40.86 ± 1.18 [c]	48.42 ± 0.87 [a]	64.85 ± 2.96 [c]	56.43 ± 4.82 [c]	39.08 ± 2.82 [b]	48.78 ± 3.84 [a]
D	35.57 ± 1.20 [d]	49.67 ± 0.81 [a]	59.68 ± 3.60 [d]	53.71 ± 3.64 [c]	41.78 ± 3.22 [a]	50.34 ± 2.24 [a]
P-value	**	**	**	**	**	*

Values are mean ± standard deviation.

[1] Diets are A=no supplements, B enriched with 0.012% tocomin and 0.49% algae biomass; C enriched with 0.036% tocomin and 1.47% algae biomass; and D enriched with 0.072% tocomin and 2.94% algae biomass.

[2] Mean and standard deviation include day 8-56, once steady state incorporation has been reached.

[3] Mean and standard deviation are from collection day 39, 40, and 41.

Different letters in the same column indicate significant different at 95% confidence interval.

*P-value ≤ 0.05; **P-value ≤ 0.01

Table 6. Viscosity and Emulsification Egg Yolk Samples with Different Levels of Nutrient Supplementation

Diet[1]	Viscosity (kg/s*m) (n=12)[2]	Consistency Index (Pa) (n=6)[2]	Flow Index (n=6)[2]	Emulsification Capacity (average g oil/g yolk) (n=6)[3]	Emulsion Stability (%) (n=6)[3]
A	0.86 ± 0.32	4.26 ± 1.68	0.66 ± 0.08	62.00 ± 3.14 [a]	62.23 ± 6.71
B	0.75 ± 0.20	2.90 ± 1.35	0.64 ± 0.07	56.35 ± 2.16 [b]	61.00 ± 9.45
C	0.93 ± 0.22	8.08 ± 6.15	0.55 ± 0.13	53.98 ± 5.61 [c]	63.50 ± 7.18
D	0.82 ± 0.26	4.21 ± 4.76	0.64 ± 0.13	51.53 ± 1.21 [c]	58.17 ± 10.07
P-value	NS	NS	NS	*	NS

Values are mean ± standard deviation.

[1] Diets are A=no supplements, B enriched with 0.012% tocomin and 0.49% algae biomass; C enriched with 0.036% tocomin and 1.47% algae biomass; and D enriched with 0.072% tocomin and 2.94% algae biomass.

[2] Viscosity parameters analysis was completed on collection day 44.

[3] Emulsification properties analysis was completed on collection day 47.

Different letters in the same column indicate significant different at 95% confidence interval.

*P-value ≤ 0.05; NS - not significant.

Table 7. Texture Analysis of Hard Boiled Egg Samples with Different Levels of Nutrient Supplementation

Diet [1]	Fracturability (g) (n=12)	Hardness (g) (n=12)	Adhesiveness (g*s) (n=12)	Cohesive-ness (ratio) (n=12)	Springiness (mm) (n=12)	Chewiness (g) (n=12)
A	400 ± 90	649 ± 187	-34.2 ± 14.9	0.2 ± 0.1	6.4 ± 1.0	1034 ± 694
B	416 ± 74	825 ± 656	-40.3 ± 40.6	0.2 ± 0.2	6.2 ± 1.1	1282 ± 1221
C	408 ± 101	999 ± 1174	-32.1 ± 20.5	0.3 ± 0.1	6.2 ± 0.9	2236 ± 4108
D	441 ± 74	716 ± 270	-32.6 ± 21.4	1.8 ± 5.6	6.0 ± 0.6	8807 ± 26117
P-value	NS	NS	NS	NS	NS	NS

Values are mean ± standard deviation are from collection days 45 and 46.

[1] Diets are A=no supplements, B enriched with 0.012% tocomin and 0.49% algae biomass; C enriched with 0.036% tocomin and 1.47% algae biomass; and D enriched with 0.072% tocomin and 2.94% algae biomass.

NS - not significant.

Table 8. Sensory Panel Evaluation of Egg Yolks with Different Levels of Nutrient Supplementation

Diet [2]	Sulfur (cm)[1,3] (n=39)	Fishy (cm)[1,3] (n=39)	Hard (cm)[1,3] (n=39)	Astringent (cm)[1,3] (n=39)	Lumpy (cm)[1,3] (n=39)	Dry (cm)[1,3] (n=39)	Overall Quality[1] (n=39)	Overall Intensity[1] (n=39)
A	8.7 ± 4.8 [a]	2.5 ± 3.2 [b]	6.8 ± 4.0	6.7 ± 4.0 [a]	5.6 ± 4.5 [a]	8.1 ± 4.3	8 ± 1 [a]	2 ± 1
B	7.6 ± 4.7 [a]	3.3 ± 4.3 [b]	7.1 ± 4.6	5.1 ± 3.7 [b]	7.0 ± 5.2 [ab]	6.5 ± 3.7	8 ± 1 [a]	2 ± 1
C	7.8 ± 4.5 [a]	3.0 ± 3.6 [b]	7.6 ± 4.3	7.4 ± 4.2 [a]	7.4 ± 4.5 [ab]	7.7 ± 4.4	7 ± 2 [ab]	2 ± 1
D	5.6 ± 4.1 [b]	5.9 ± 4.2 [a]	8.0 ± 3.9	7.4 ± 4.1 [a]	8.6 ± 4.1 [b]	6.7 ± 4.4	7 ± 1 [b]	2 ± 0
P-value	*	**	NS	*	**	NS	*	NS

[1] Values are mean ± standard deviation from collection day 56.

[2] Diets are A=no supplements, B enriched with 0.012% tocomin and 0.49% algae biomass; C enriched with 0.036% tocomin and 1.47% algae biomass; and D enriched with 0.072% tocomin and 2.94% algae biomass.

[3] Measurements used 15-cm long line scale; the higher the value the higher the intensity. Overall quality (10 excellent to 1 very poor) and overall flavor intensity (10 bland to 1 extreme).

Different letters in the same column indicate significant different at 95% confidence interval.

*P-value ≤ 0.05; **P-value ≤ 0.01; NS - not significant.

Table 9. Fatty Acid Composition of Egg Yolk Lipids

Fatty Acid	Diet A (%)	Diet B (%)	Diet C (%)	Diet D (%)	P-value
14:0	0.06 ± 0.14	0.06 ± 0.15	0.06 ± 0.01	0.00 ± 0.00	NS
16:0	26.05 ± 1.60	24.16 ± 1.68	24.97 ± 2.48	23.81 ± 3.56	NS
16:1	2.69 ± 1.37	2.51 ± 1.32	2.65 ± 1.33	3.12 ± 0.27	NS
18:0	9.92 ± 0.61	9.19 ± 0.48	9.24 ± 0.69	8.95 ± 1.38	NS
18:1	40.94 ± 3.18	38.84 ± 3.05	40.14 ± 2.79	38.60 ± 1.60	NS
18:2	14.23 ± 1.18	14.09 ± 1.09	14.18 ± 1.38	13.62 ± 1.60	NS
18:3	0.09 ± 0.22	0.00 ± 0.00	0.13 ± 0.31	0.18 ± 0.44	NS

FAME analysis of egg oil samples from collection day 10.

Diets are A=no supplements, B enriched with 0.012% tocomin and 0.49% algae biomass; C enriched with 0.036% tocomin and 1.47% algae biomass; and D enriched with 0.072% tocomin and 2.94% algae biomass.

Values are mean ± standard deviation.

*P-value NS - not significant.

Table 10. Phospholipid Composition of Egg Yolk Lipid as Determined by ^{31}P NMR

Phospholipids	Diet A (weight %)	Diet B (weight %)	Diet C (weight %)	Diet D (weight %)	P-value
CL	1.22 ± 1.36	0.36 ± 0.88	1.32 ± 1.46	0.83 ± 1.29	NS
PE	19.86 ± 0.73	19.09 ± 1.22	19.62 ± 1.58	18.37 ± 1.62	NS
SM	1.75 ± 0.27	1.57 ± 0.28	1.56 ± 0.53	1.72 ± 0.23	NS
LPC	0.04 ± 0.09	0.04 ±0.11	0.08 ± 0.20	0.00 ±0.00	NS
PI	1.73 ± 0.17	0.84 ± 0.95	1.66 ± 0.25	1.39 ± 0.70	NS
PC	75.40 ± 0.60	78.10 ± 2.11	75.75 ± 2.20	77.69 ± 1.83	NS
Total in oil	**31.18 ± 0.79**	**29.15 ± 4.19**	**28.30 ± 4.94**	**30.61 ± 2.32**	**NS**

Phospholipid composition analysis of egg oil samples from collection day 10. CL-cardiolipin, PE-phosphatidylethanolamine, SM-sphingomyelin, LPC-lysophophatidylcholine, PI-phosphatidylinositol, PC-phosphatidylcholine.

Diets are A=no supplements, B enriched with 0.012% tocomin and 0.49% algae biomass; C enriched with 0.036% tocomin and 1.47% algae biomass; and D enriched with 0.072% tocomin and 2.94% algae biomass.

Values are means ± standard deviation.

*P-value NS - not significant.

Table 11. Nutrient Transfer Efficiency to Egg Yolk (%)

Day	Component	Diet B	Diet C	Diet D	P-value
8 or 10	**α-Tocopherol**	9.90 ± 1.85 [a]	3.83 ± 1.30 [b]	3.43 ± 0.95 [ab]	*
	α-Tocotrienol	1.72 ± 0.20 [a]	1.39 ± 0.12 [b]	1.41 ± 0.15 [b]	*
	β-Tocotrienol	0.52 ± 0.60 [a]	0.75 ± 0.05 [b]	0.72 ± 0.10 [b]	**
	γ-Tocotrienol	0.16 ± 0.02 [a]	0.13 ± 0.01 [ab]	0.13 ± 0.02 [b]	*
	δ-Tocotrienol	0.00 ± 0.00 [a]	0.06 ± 0.02 [b]	0.05 ± 0.01 [c]	**
	Astaxanthin	14.89 ± 2.20 [a]	10.59 ± 1.83 [b]	7.60 ± 1.26 [c]	*
35	**α-Tocopherol**	3.95 ± 2.18 [a]	-0.27 ± 1.30 [b]	-0.19 ± 0.76 [b]	**
	α-Tocotrienol	0.89 ± 0.13 [a]	0.45 ± 0.24 [b]	0.54 ± 0.08 [b]	**
	β-Tocotrienol	0.54 ± 0.33	0.34 ± 0.17	0.46 ± 0.06	NS
	γ-Tocotrienol	0.09 ± 0.01 [a]	0.06 ± 0.03 [b]	0.07 ± 0.01 [b]	**
	δ-Tocotrienol	0.00 ± 0.00 [a]	0.01 ± 0.02 [a]	0.04 ± 0.01 [b]	**
	Astaxanthin	11.95 ± 4.02 [a]	9.46 ± 2.25 [ab]	6.31 ± 1.16 [b]	**
56	**α-Tocopherol**	-1.11 ± 3.03	-1.48 ± 0.85	-0.74 ± 0.59	NS
	α-Tocotrienol	0.56 ± 0.08 [a]	0.38 ± 0.07 [b]	0.36 ± 0.73 [b]	**
	β-Tocotrienol	0.00 ± 0.00 [a]	0.30 ± 0.07 [b]	0.31 ± 0.07 [b]	**
	γ-Tocotrienol	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	NS
	δ-Tocotrienol	0.00 ± 0.00 [a]	0.00 ± 0.00 [a]	0.02 ± 0.01 [b]	**
	Astaxanthin	9.26 ± 1.77 [a]	7.08 ± 1.64 [b]	5.12 ± 1.02 [c]	**

Feeding transfer resulting in egg yolks at maximum amount on day 8 values for all tocos, and day 10 for astaxanthin.

Diets are A=no supplements, B enriched with 0.012% tocomin and 0.49% algae biomass; C enriched with 0.036% tocomin and 1.47% algae biomass; and D enriched with 0.072% tocomin and 2.94% algae biomass.

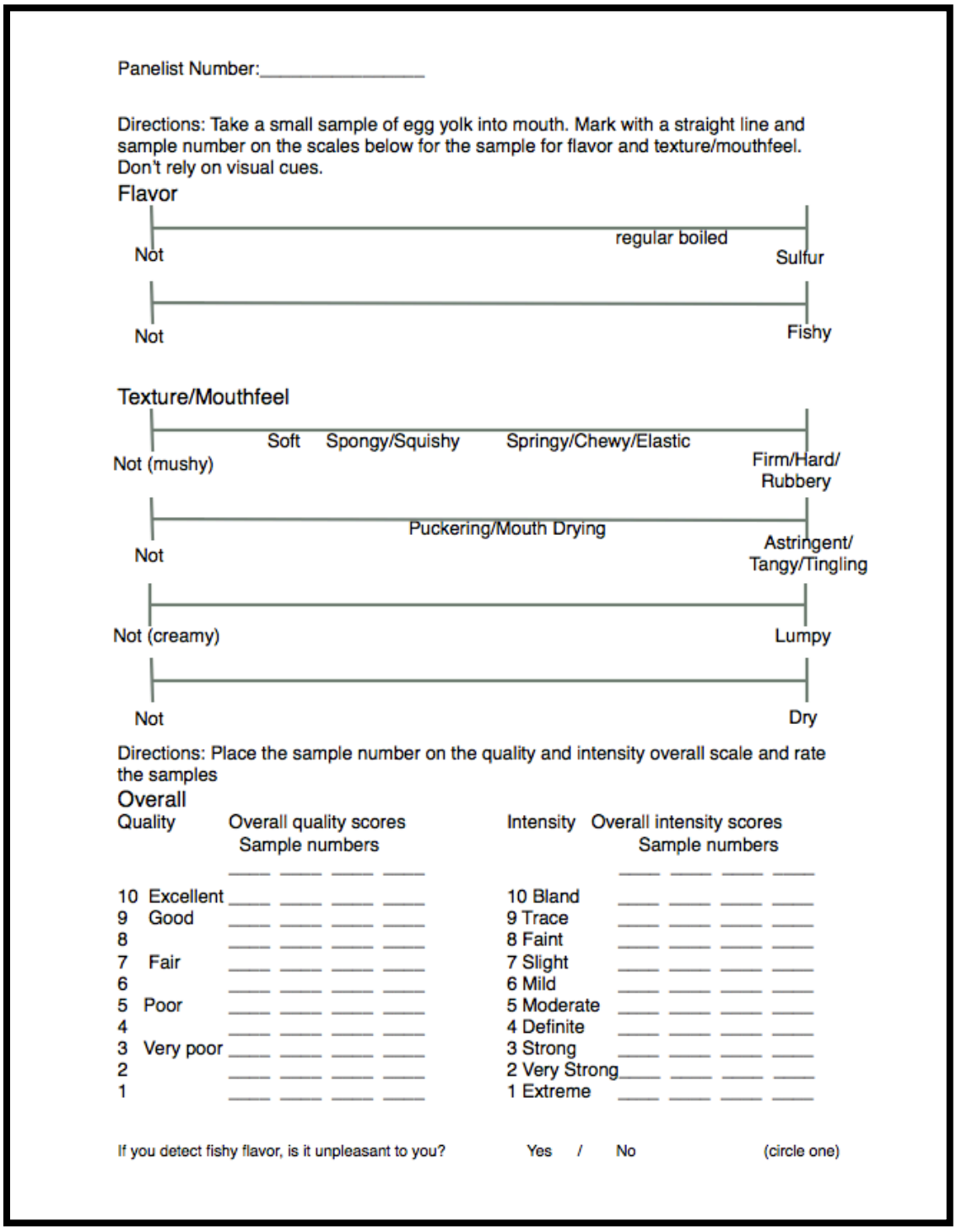
Values are means ± standard deviation.

*P-value ≤ 0.05; **P-value ≤ 0.01.

Table 12. Kinetics Parameters of Hen Feed Using Arrhenius Approach

Component	Apparent Order	Activation Energy (kJ/mol)	ln(A)	R^2	k (day^{-1}) at 25 °C	$t_{1/2}$ (days) at 25 °C
α-Tocopherol	First	28.8	7.05	0.4936	1.0×10^{-2}	67.5
α-Tocotrienol	First	307.6	7.70	0.6958	1.3×10^{-2}	53.9
β-Tocopherol	Second	48.5	11.91	0.7549	4.8×10^{-4}	72.8
β-Tocotrienol	Second	29.5	4.22	0.9656	4.5×10^{-4}	23.1
γ-Tocopherol	Zero	36.7	14.11	0.8057	5.0×10^{-1}	0.3
γ-Tocotrienol	Second	49.0	10.01	0.8162	5.9×10^{-5}	39.5
δ-Tocopherol	First	48.8	13.76	0.663	2.7×10^{-3}	254.6
δ-Tocotrienol	Second	50.9	10.31	0.9071	3.6×10^{-5}	56.5
Astaxanthin	Second	106.7	28.37	0.9607	4.2×10^{-7}	307.1

Results of HPLC for tocotrienol, tocopherol, and astaxanthin analysis of highest diet level by determining the apparent order, activation energy, k value, and half-life value. The highest concentration diet was used for the experiment.



Panelist Number:_____

Directions: Take a small sample of egg yolk into mouth. Mark with a straight line and sample number on the scales below for the sample for flavor and texture/mouthfeel. Don't rely on visual cues.

Flavor

Not — regular boiled — Sulfur

Not — Fishy

Texture/Mouthfeel

Not (mushy) — Soft — Spongy/Squishy — Springy/Chewy/Elastic — Firm/Hard/Rubbery

Not — Puckering/Mouth Drying — Astringent/Tangy/Tingling

Not (creamy) — Lumpy

Not — Dry

Directions: Place the sample number on the quality and intensity overall scale and rate the samples

Overall

Quality	Overall quality scores Sample numbers				Intensity	Overall intensity scores Sample numbers			
	____	____	____	____		____	____	____	____
10 Excellent	____	____	____	____	10 Bland	____	____	____	____
9 Good	____	____	____	____	9 Trace	____	____	____	____
8	____	____	____	____	8 Faint	____	____	____	____
7 Fair	____	____	____	____	7 Slight	____	____	____	____
6	____	____	____	____	6 Mild	____	____	____	____
5 Poor	____	____	____	____	5 Moderate	____	____	____	____
4	____	____	____	____	4 Definite	____	____	____	____
3 Very poor	____	____	____	____	3 Strong	____	____	____	____
2	____	____	____	____	2 Very Strong	____	____	____	____
1	____	____	____	____	1 Extreme	____	____	____	____

If you detect fishy flavor, is it unpleasant to you? Yes / No (circle one)

Figure 1. Descriptive sensory panel ballot developed during panelist training sessions and used for evaluation of hard-boiled eggs. The line scale is 15-cm unstructured for each descriptor.

Figure 2. Feed samples after mixing the diets. The diets are as follows: D (0.072% tocomin and 2.94% algae biomass), C (0.036% tocomin and 1.47% algae biomass), B (0.012% tocomin and 0.49% algae biomass) and A (control) as seen from left to right.

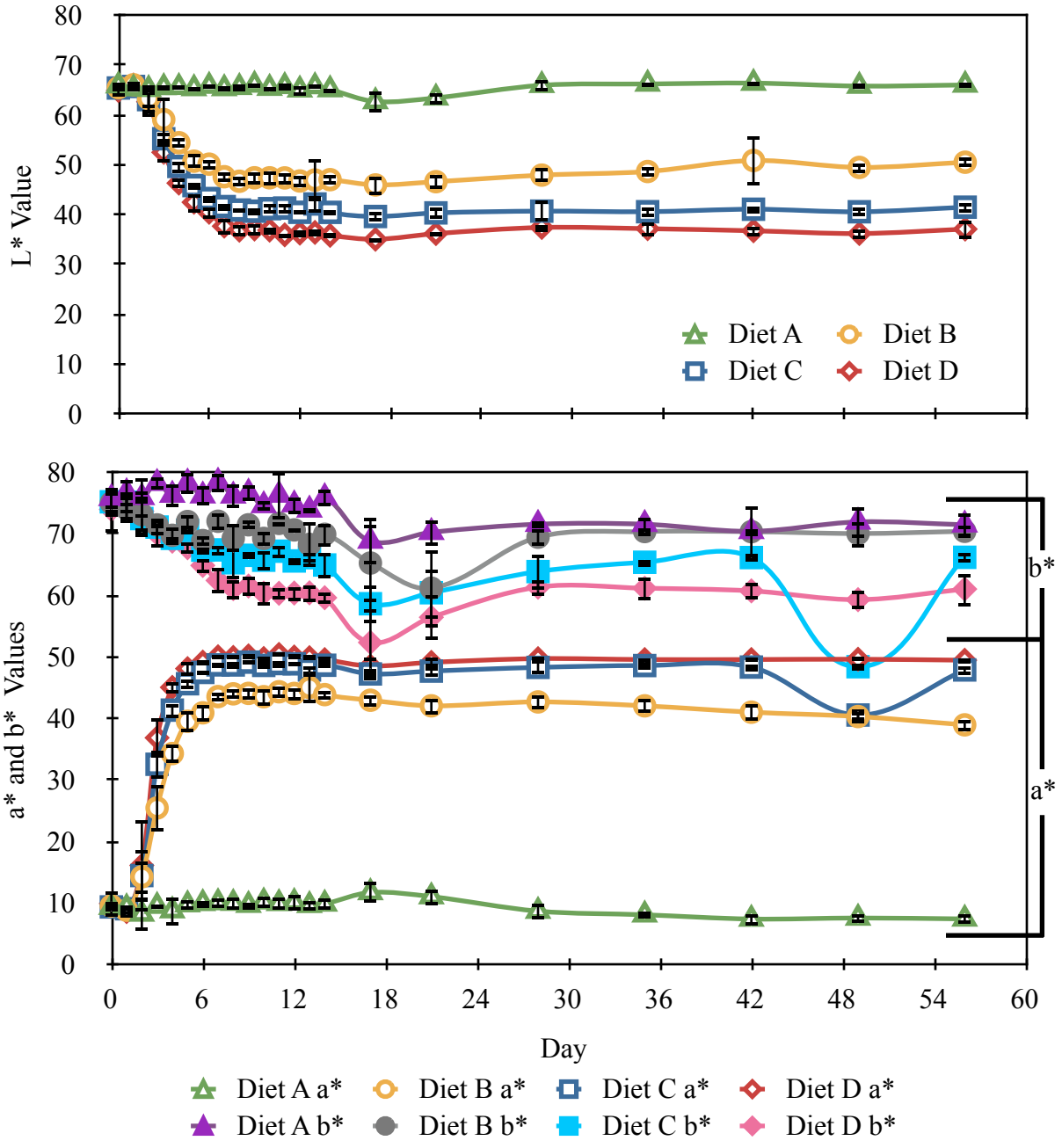


Figure 3. Color change of egg yolks from day 0 of study through day 56 for fresh egg yolk samples. Open symbols are a* values and closed symbols are b* values. Standard deviation bars are shown in all charts have the mean and standard deviation bars. Diets are A=no supplements, B enriched with 0.012% tocomin and 0.49% algae biomass; C enriched with 0.036% tocomin and 1.47% algae biomass; and D enriched with 0.072% tocomin and 2.94% algae biomass.

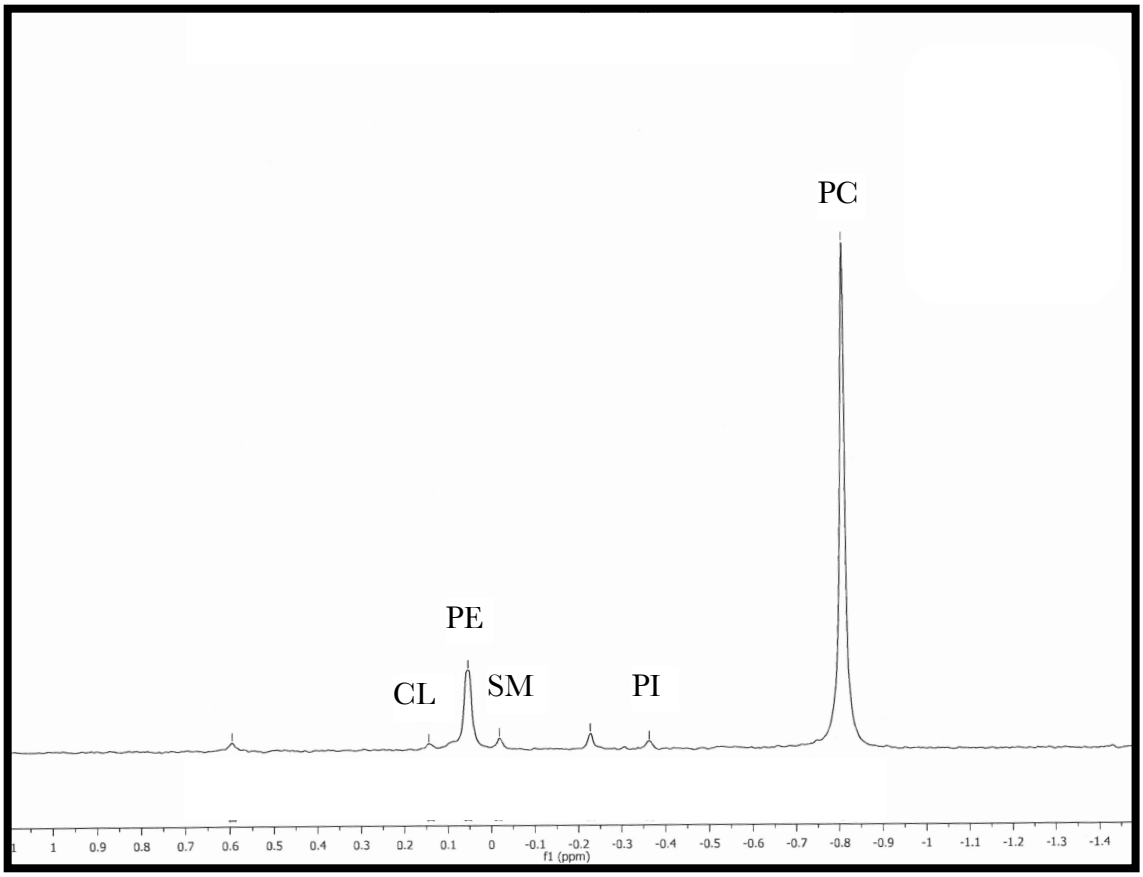


Figure 4. Typical ^{31}P NMR spectrum from day 10 egg oil samples. CL-cardiolipin, PE-phosphatidylethanolamine, SM-sphingomyelin, PI-phosphatidylinositol, PC-phosphatidylcholine.

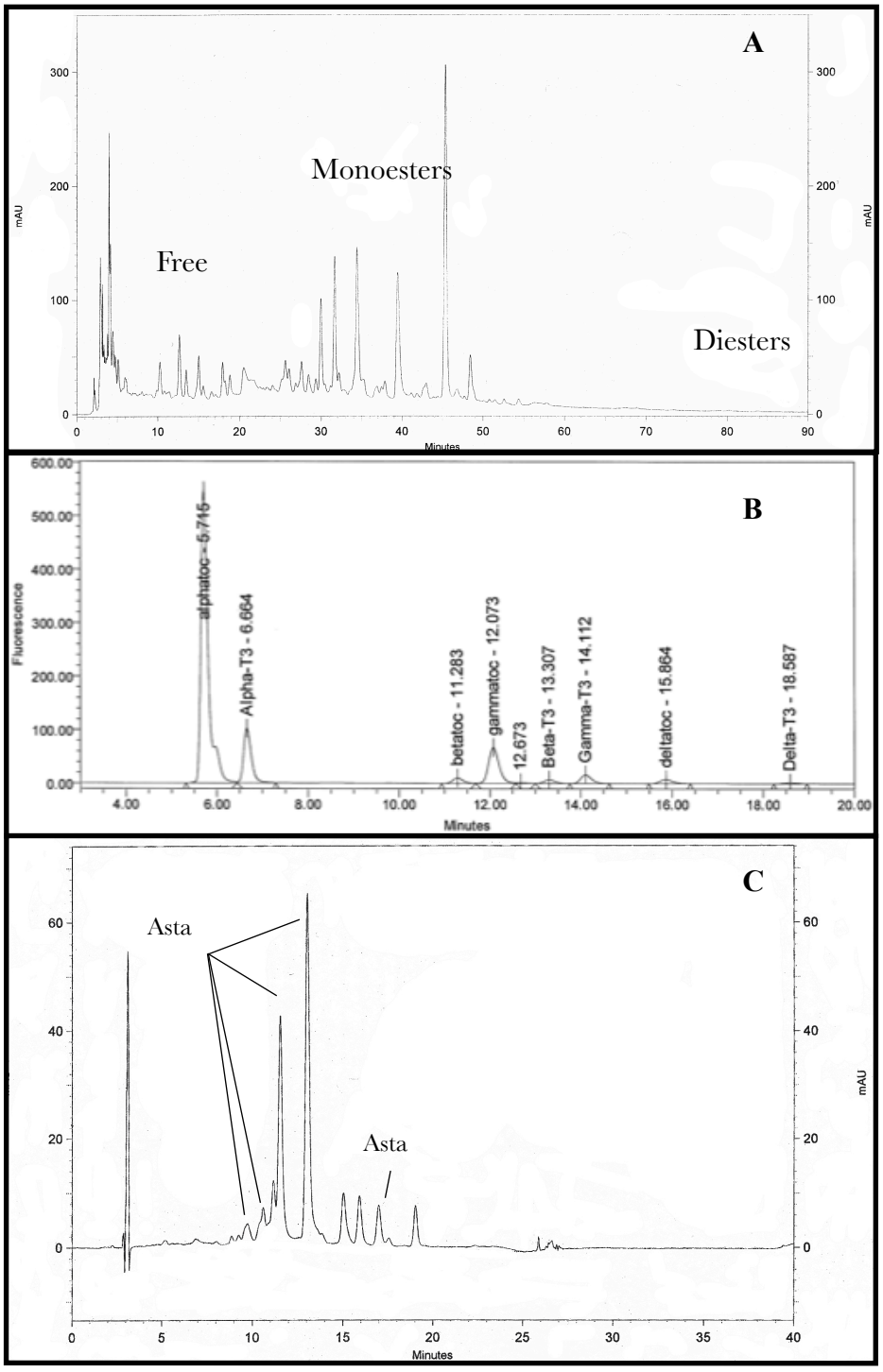


Figure 5. Chromatogram of A) algal biomass lipid extract; B) Tocopherols and tocotrienols; C) Astaxanthin yolk lipid extract.

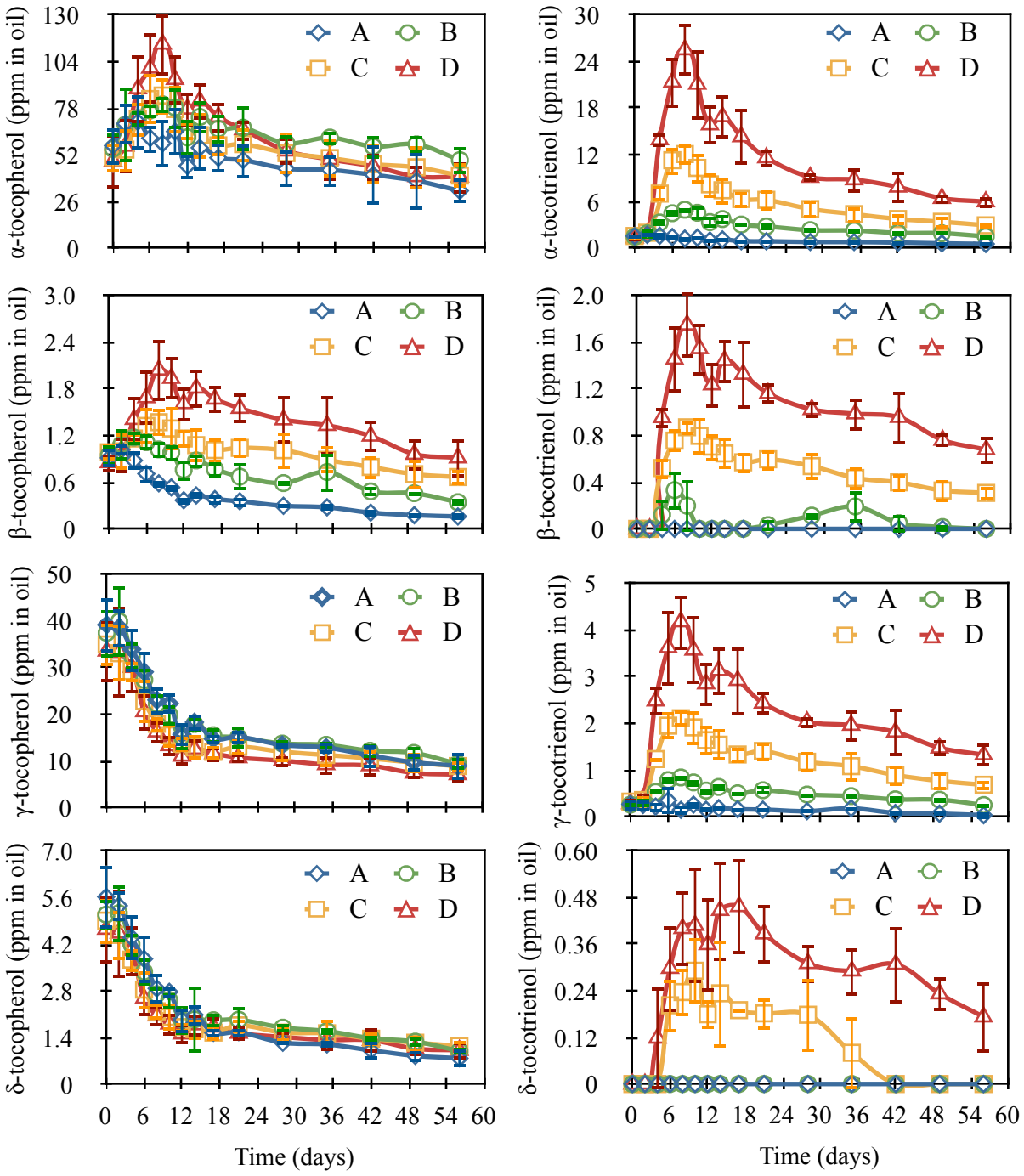


Figure 6. HPLC quantification of tocotrienols and tocopherols over the 56 day feeding period, showing means and standard deviations from 6 replicates. Diets are A=no supplements, B enriched with 0.012% tocomin and 0.49% algae biomass; C enriched with 0.036% tocomin and 1.47% algae biomass; and D enriched with 0.072% tocomin and 2.94% algae biomass.

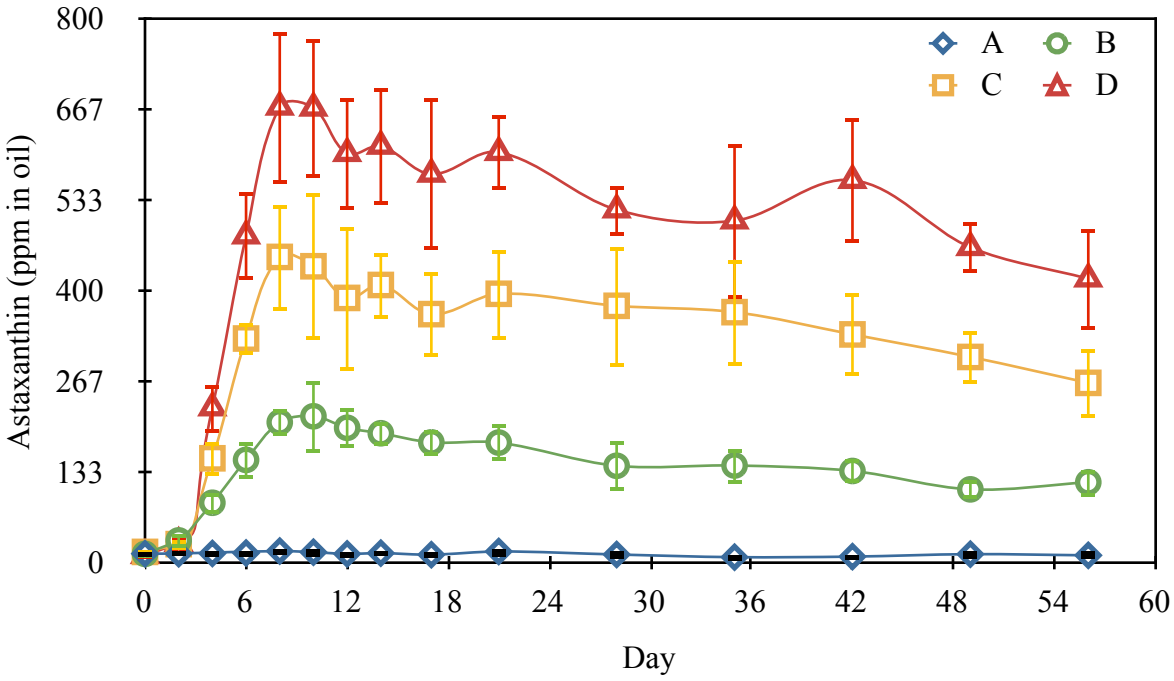


Figure 7. HPLC of astaxanthin quantification over the 56 day feeding period, showing means and standard deviations from 6 replicates. Diets are A=no supplements, B enriched with 0.012% tocomin and 0.49% algae biomass; C enriched with 0.036% tocomin and 1.47% algae biomass; and D enriched with 0.072% tocomin and 2.94% algae biomass.

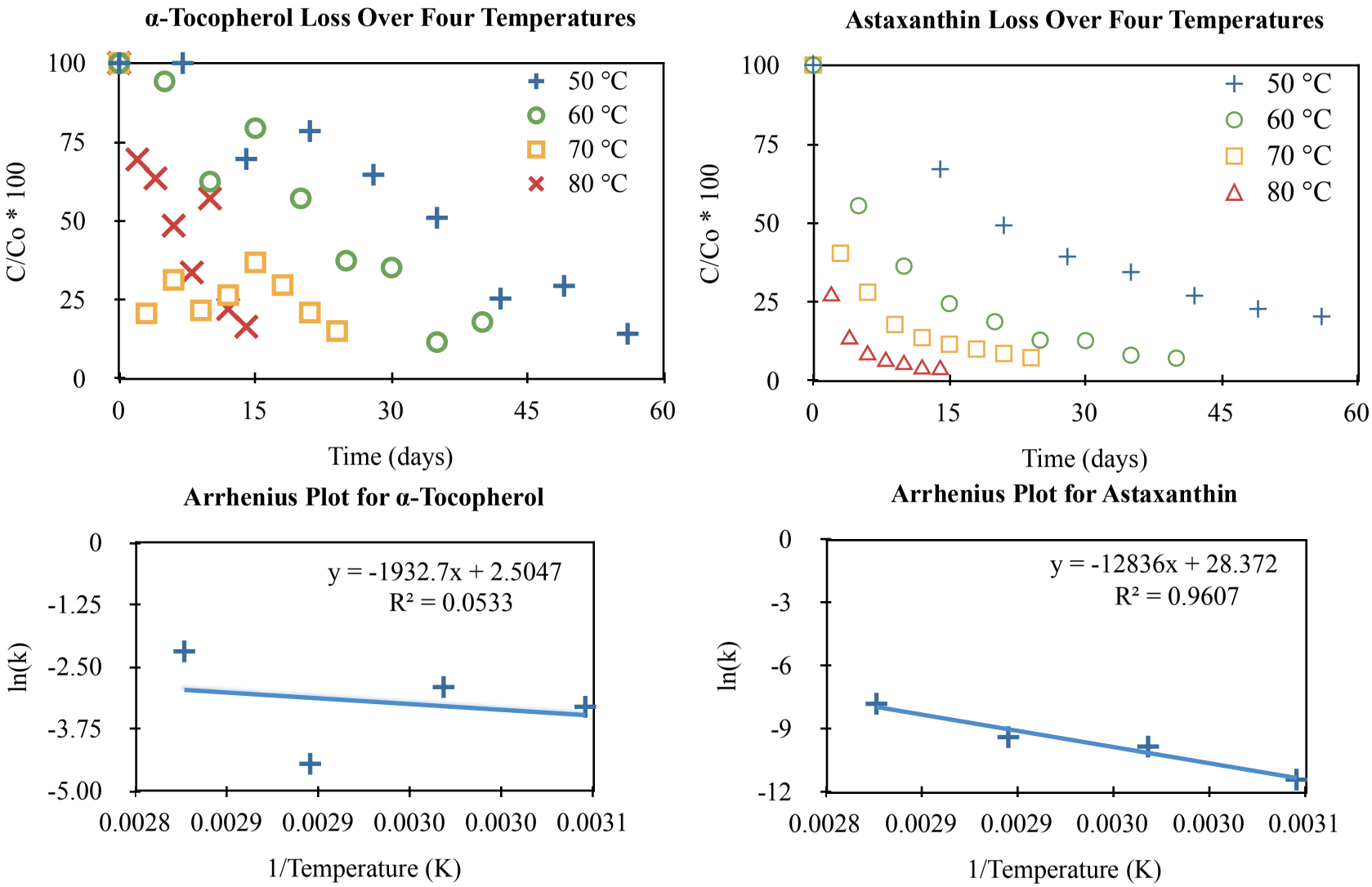


Figure 8. Arrhenius shelf-life study of Diet D showing the loss of α-tocopherol and astaxanthin over time at four elevated storage temperatures.

CHAPTER 4. LITERATURE REVIEW-ARTIFICIAL OIL BODY

GENERAL INTRODUCTION

Oil naturally occurs in plant cells, pollen, spores, and seeds where lipid can be stored in sub-cellular lipid particles, oil bodies (Frandsen et al., 2001; Huang, 1994). Oil bodies can be found in seeds and pollen of angiosperms and primitive plants, like gymnosperms and fern spores (Jiang et al., 2009). Lipid particles are found in mammal tissues, eggs of some nematodes, yeast, or algae (Huang, 1994). The particles have been called lipid bodies, oil bodies, oleosomes, or spherosomes (Huang, 1992). Spherosomes is an old term used to describe sub-cellular particles where their matrix content is uncertain (Huang, 1992). This review will use oleosomes and oil bodies interchangeable when referring to naturally occurring structures. This literature review will examine naturally occurring oleosomes, oleosin, egg yolk protein, egg phospholipids, artificial bodies, and lipid oxidation.

NATURALLY OCCURRING OLEOSOMES

Plant oil bodies are very stable at neutral pH values. They do not aggregate or coalesce *in vivo* during seed maturation or after centrifugation (Haung, 1994). Oil bodies are made up of three basic components, proteins, phospholipids, and triglycerides (Huang, 1992). Oil bodies have a matrix composed of triacylglycerols (TAG) bond by phospholipid monolayer with unique proteins, oleosins, caleosins, and steroleosins (Bhatla et al., 2010; Frandsen et al., 2001; Huang, 1994). Oleosin and caleosin are considered to contribute to structural stability of oleosomes, but not steroleosin (Liu et al., 2009). Generally, oleosomes in situ are spherical, yet they can be pressed into irregular shapes by other cellular structures (Bhatla et al., 2010). Oil bodies are typically 0.5-2.5 μm in diameter where the size can be directly affected by the nutritional status and environmental factors (Frandsen et al., 2001; Tzen et al., 1998). Oil bodies have been observed from mature seeds of rape (*Brassica napus L.),* mustard *(Brassica juncea L.)*, cotton *(Gossypium hirsutum L.)*, flax *(Linus usitatis simum)*, maize *(Zea mays L.)*, peanut *(Arachis hypogaea L.)*, and sesame *(Sesamum indicum*

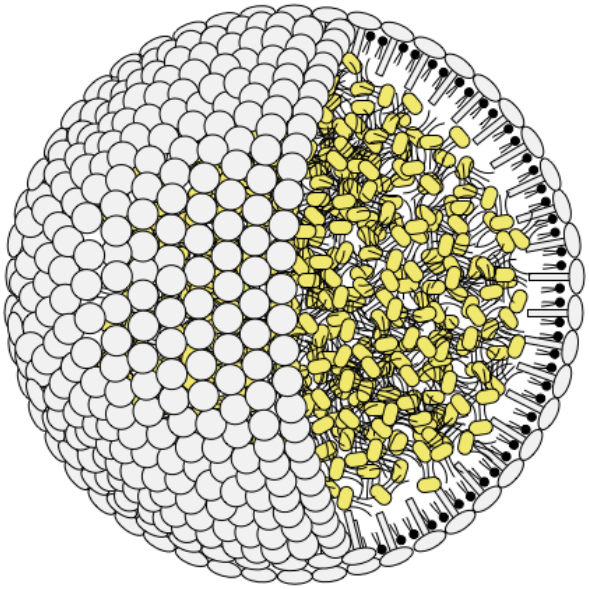


Figure 1. Schematic of oil body. Outer layer is proteins with phospholipids and inside are triglycerides.

L.) (Tzen et al., 1993). Plants use the oleosomes as energy sources for germination and post-germinative seedling growth (Tzen et al., 1998; Peng et al., 2003). Oleosomes are held together by simple physical forces, steric hindrance and negative surface charge by oleosin (Huang, 1992; Chiang, 2010). Oleosomes are stable both *in vitro* and *in vivo* (Chiang, 2010). It is generally understood the oil body has a matrix of TAG surrounded by a layer of phospholipids embedded with alkaline proteins, oleosins (Figure 1) (Tzen et al., 1998). The phospholipids form a monolayer where the acyl moieties face inward to interact with the hydrophobic TAG within the matrix while the hydrophilic head groups exposed to the cytosol (Tzen et al., 1993). The proteins have three structural domains, N-terminal amphipathic domain, central hydrophobic domain, and C-terminal amphipathic domain. The hydrophobic portion penetrates the phospholipid layer into the TAG matrix and the hydrophilic region rest on the phospholipid layer exposed to the cytosol (Tzen et al., 1993). There are minor proteins of higher molecular mass also embedded in the phospholipid monolayer (Chen and Tzen, 2001). The three minor proteins termed Sops 1-3, have been identified in sesame oil bodies, but their functions remain unknown (Chen and Tzen, 2001). Five seed-specific oleosins have been identified in *Arabidopsis thaliana* ranging in size from 14-21 kDa (Gohon et al., 2011). Sop1, also known as caleosin for its calcium-biding capacity, is very similar to oleosin in structure, containing an N-terminal hydrophilic domain, central hydrophobic anchoring domain, and a C-terminal hydrophilic domain (Chen and Tzen, 2001). Caleosin and oleosin are very different when examining the sequence and only share 26% of the sequence (Gohon et al., 2011). Oleosin S5 is the smallest at 14.9 kDa due to a shorter N and C termini and is the most hydrophobic oleosin (Gohon et al., 2011). Oleosin

S3 (186 kDa) is the most abundant protein in *Arabidopsis thaliana* with longer N and C termini than S5 making the protein less hydrophobic (Gohon et al., 2011). S5 and S3 share 63% sequence identity (Gohon et al., 2011).

During seed maturation, oil bodies are formed on the rough endoplasmic reticulum by budding TAG between the two phospholipid layers and oleosins simultaneously are synthesized on polyribosomes, which are bound to the endoplasmic reticulum (Frandsen et al., 2001; Huang, 1994). The resulting oleosins move to the budding TAG particle where the hydrophobic domain guides the placement on the oil bodies (Huang, 1994; Chen and Tzen, 2001). Typically an oil body consists of 94-98% (w/w) neutral lipids, 0.6-2% phospholipids and 0.6-3% proteins (Tzen et al., 1993). The neutral lipids are in the TAG form where the composition can vary greatly among different species (Tzen et al., 1993).

OLEOSIN

Oleosin is unique and principal membrane protein to oleosomes (Bhatla et al., 2010; Frandssen et al., 2001; Huang, 1994). There are two isoform classes, H- and L-oleosin with the main difference being the insertion of 18 residues in the C-terminal domain in the H-oleosins (Tai et al., 2002). These proteins are alkaline in nature with low molecular masses, 15-25 kDa (Haung, 1992). In typical oleosomes the oleosin account for approximately 1-4% of the total weight (Huang, 1992). The protein has a tripartite structure with a hydrophobic domain of 70 residues located at the center which interacts with the oils in the oleosome is highly conserved among species including sesame, *Arabidopsis thaliana,* maize and rice within the same isoform class (Tai et al., 2002; Liu, 2009). A proline knot motif lies in the center of the hydrophobic region of three prolines within a 12-

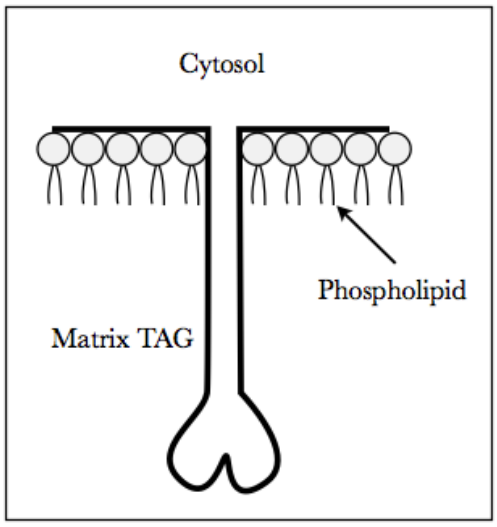


Figure 2. Schematic of oleosin protein on the surface of oil body. Figure adapted from Huang, 1994.

residue sequence taking the secondary structure of two β-strands in antiparallel formation (Abell et al., 2004; Huang, 1994; Tzen et al., 1998). The proline knot is the section of the protein that is inserted into the oil body TAG matrix like a hairpin (Frandsen et al., 2001). The proline knot has also been suggested to play an essential role to target oil bodies (Chen and Tzen, 2001). The N- and C-termini are two hydrophilic domains with secondary structure of α-helix that reside on the oil body surface (Abell et al., 2004; Frandsen et al., 2001). These domains can vary among oleosins from different species (Huang, 1994). Oleosin is synthesized in the endoplasmic reticulum membrane both *in vitro* and *in planta* (Abell et al., 2004). Literature suggests oleosin may be synthesized on specific TAG-synthesizing subdomains (Abell et al., 2004). TAG accumulates between the endoplasmic reticulum phospholipid bilayer to produce droplets (Frandsen et al., 2001). Oleosin exists in two distinct oleosin isoforms, molecular mass of 18 and 16 kDa as found in rice but can range from 15-26 kDa depending on the isoform and plant species (Tzen et al., 1998). The central hydrophobic region is highly conserved among species (Tzen et al., 1998).

Oleosin functions as a shield of the phospholipids and stabilize to the oil bodies (Frandsen et al., 2001). The topology of oleosin exposed to the cytosol gives the oil bodies a negatively charged surface which helps to prevent aggregation and coalescence (Tzen et al., 1993). The surface charge can be changed, by lowing the pH, will result in a neutral oil body surface causing aggregation (Frandsen et al., 2001). Oleosins are only required for long-term storage as oleosins are not found in olives and avocados. The oil bodies in these examples are not long-term storage organelles (Frandsen et al., 2001). The relative size of the oil bodies can be controlled by the ratio of oil to oleosin. Maize kernels with high oil content have high ratio oil to oleosin will have large oleosomes, while low-oil breeds with a low ratio will have smaller oil bodies and irregular surfaces (Frandsen et al., 2001).

EGG YOLK PROTEINS

Egg yolk poteins have a range of functional properties. Yolk proteins are present in free and apoproteins. On a basis of dry matter, yolk has five major protein constituents which are as follows: 68% low-density lipoproteins (LDL) or also known as

lipovitellins, 16% high-density lipoproteins (HDL), 10% globular proteins (livetins), 4% phosphoproteins (phosvitins), and 2% minor proteins (Huopalahti et al., 2007; Aluko and Mine, 1998; Aton el al., 2003; Jolivet et al., 2006). Low density lipoproteins are the main constituent in egg yolk making up approximately two-thirds of the dry material made of 87% lipids and 12% proteins (Huopalahti et al., 2007; Aton et al., 2003). These proteins are similar to the lipoproteins found in chicken blood when the blood enters the ovaries, the lipoproteins are transferred to the yolk (Huopalahti et al., 2007; Jolivet et al., 2006). Described to be a polypeptide chain folded in a globular configuration, these proteins form spherical particles with the lipid molecule within the protein and a variable amount of lipid on the surface (Aluko and Mine, 1998; Aton et al., 2003; Jolivet et al., 2006). High-density lipoproteins are the second group, accounting for one-sixth of the dry yolk matter and 36% of the protein (Huopalahti et al., 2007). These proteins are made up of 75-80% proteins and 20-25% lipids (Huopalahti et al., 2007). The lipids are composed of 65% phospholipids, 30% triglycerides, and 5% cholesterol (Huopalahti et al., 2007). The third group are phosphoglycoproteins called phosvitins. Of the known proteins for this group, 50% of the amino acids are serine and of which, 90% is phosphorylated (Huopalahti et al., 2007). Due to the unique chemical characteristics, phosvitins act as phospholipid antioxidants (Huopalahti et al., 2007). The last group of proteins are the globular proteins also known as livetins. These proteins have different fractions including α-, β-, and γ-livetin at the ratio of 2:5:3, respectively.

Dyer-Hurdon and Nnanna (1993) found proteins that exist in the plasma, low-density lipoproteins and livetins, had better emulsifying activity and stability at low ionic strength, <0.3 M sodium chloride. Its been reported that protein emulsifying properties are due to the large range of proteins and experimental conditions (Anton et al., 2003). A study completed by Anton and Gandemer (1997) fractioned yolk into granules and plasma and found there was no difference when emulsions were made with yolk, granules, or plasma. They also noted the contribution of proteins to the emulsifying activity was higher than phospholipids. At 80% solubilization, Anton and Gandemer (1997) noted the granules formed a more stable emulsion than yolk or plasma due to the composition of granules. Granules contained half

the lipids and cholesterol and double the proteins as compared to yolk and plasma (Anton and Gandemer, 1997).

EGG PHOSPHOLIPIDS

Of the total lipids in egg yolk, approximately 30% are phospholipids and often referred as egg yolk lecithin (Stadelman and Cotterill, 1995). There are several compounds that make up phospholipids including two main compounds, phosphatidylcholine (PC, 76%) and phosphatidylethanolamine (PE, 22%) (Aluko and Mine, 1998). Lysophospholipid (LPC, 3-6%), sphingomyelin (SM, 3-6%), and phosphatidylinositol (PI, 0.6%) are also found in egg yolk phospholipids as minor components (Rhodes and Lea, 1957). Phospholipids are used as emulsifiers in food products and industry applications. Phospholipids coat oil droplets therefore reduce the surface tension between the immiscible phases. There are various forces stabilizing the oil-in-water emulsion, such as Van der Waals force, steric interaction, electrostatic force, hydration interaction, and hydrophobic interaction (Levine et al., 1989).

ARTIFICIAL OIL BODIES

The natural oleosomes contain oil surrounded by a membrane of phospholipids or lecithin and a layer of hydrophobic proteins, usually oleosin. It is mechanically feasible for artificial oil body formation using the three essential components, neutral lipids (TAGs) 94-98%, phospholipids 0.5-2%, and proteins 0.5-3.5% (Peng et al., 2007). The use of egg lecithin is growing as a food product in industry primary as emulsifiers. With the increased demand of lecithin, the abundance of by-product, yolk protein, is also increasing. Yolk protein contains high nutritional value and may also have the property to form artificial oil bodies, which this research will explore. Egg yolk proteins are low and high density lipoproteins within the yolk. With the addition of yolk protein and lecithin, a protective outer monolayer will form highly oxidizabable omega-3 oils. The success of this study may lead to new applications for egg yolk proteins to produce artificial oil bodies where the lipid core can be protected from oxidation.

LIPID OXIDATION

Lipid oxidation is a great concern for industry producing lipid-containing foods. Oxidation not only produces offensive odors and flavors but also decrease nutritional value. Oxidation can be initiated by autoxidation, photoxidation, or enzyme catalyzed reaction. Typically lipid oxidation occurs by autoxidation since the oxidation rate increases as the reaction continues (Fennema, 1976). Other mechanisms can occur if the oxidants or enzyme control the oxidation. Free-radical oxidation mechanism involves three stages: initiation, propagation, and termination. Autoxidation occurs when oxygen reacts with organic substrates (RH) to yield hydroperoxides (Fennema, 1976; Halliwell and Chirico, 1993). In the first stage, initiation, the hydrocarbon losses a hydrogen forming a radical. If oxygen is present in the system the oxygen will add to a double bond forming a peroxyl radical (Fennema, 1976; Halliwell and Chirico, 1993). The initiation step will produce two free radicals (Fennema, 1976). The susceptibility of the substrate depends on the relative ease to donate a hydrogen. With natural lipids there are often double bonds where the availability of allylic hydrogens will dictate oxidation. The more double bonds, like PUFAs, in a fatty acid chain, the easier for radical to form (Halliwell and Chirico, 1993). Peroxyl radicals can combine if they meet, attack membrane proteins, or obtain a hydrogen from an adjacent fatty acid chain propagating the reaction (Halliwell and Chirico, 1993). Oxidation can be initiated by different molecules. Reactive $OH^{\cdot}$ can be formed by high-energy irradiation in solution or by transition-metal salts reacting with H_2O_2 to form $OH^{\cdot}$ (Halliwell and Chirico, 1993). In lipid oxidations studies, copper and iron ions are used to stimulate oxidation. Singlet oxygen is another way to generate lipid peroxides by singlet O_2 interaction directly with the fatty acid (Halliwell and Chirico, 1993; Frankel, 1980). Singlet oxygen can be generated many ways, yet one important way is by light exposure (Frankel, 1980). Propagation continues the reaction to form peroxy radicals, hydroperoxides, and new hydrocarbon radicals (Fennema, 1976). Propagation length can depend on many factors including the lipid-protein ratio, fatty acid composition, oxygen concentration, and the presence of antioxidants (Halliwell and Chirico, 1993). If two radicals interact or if an antioxidant is present, termination occurs.

Hydroperoxides can be decomposed by high-energy radiation, thermal energy, metal catalysis, or enzyme activity (Fennema, 1976). The first step of decomposition in an unsaturated hydroperoxide is the homolytic cleavage of the oxygen-oxygen bond to yield alkoxy and hydroxy radical (Frankel, 1980). Carbon-carbon bonds are cleaved to result in aldehyde esters and aldehydes (Frankel, 1980). Decomposition of hydroperoxides will result in the formation of additional radicals which can start oxidation initiation events (Fennema, 1976). Decomposed hydroperoxides result in several secondary products including aldehydes, alcohols, and acids (Fennema, 1976) causing rancid flavor.

Lipid oxidation can be measured by loss of unsaturated fatty acids, primary product quantification, or secondary product quantification. HPLC or GC methods can be used to measure not only the loss of unsaturated fatty acids but also to identify the product distributions (Halliwell and Chirico, 1993). Other methods for lipid peroxides quantification include glutathione peroxidase, cyclooxygenase, and iodine liberation. Secondary products are most commonly measured by 2-thiobarbituric acid-reactive substances because its simply and inexpensive (Halliwell and Chirico, 1993).

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CHAPTER 5. A NOVEL USE OF EGG YOLK PROTEIN AND LECITHIN IN THE FORMATION OF ARTIFICIAL OIL BODIES FOR STABILIZING OMEGA-3 OIL

Laurie Walker[1] and Tong Wang[1, 2]

ABSTRACT

Egg yolk protein is a co-product from egg lecithin isolation. We hypothesize egg yolk protein and egg lecithin can be used to make artificial oil bodies (AOB) mimicking the naturally occurring oleosomes with similar stability and oil protection from oxidation. Egg yolk protein, soy protein isolate, and naturally occurring oleosomes were used for a 30-day oxidation study. Egg yolk protein and soy oleosin were also used for a 2-week accelerated oxidation test with fish oil to determine the effect of environment (pH 3 and 7) and in the presence of a pro-oxidant (0 and 50 ppm $CuSO_4$) on lipid oxidation. Both experiments were evaluated for primary oxidation (peroxide value), secondary oxidation TBARS (2-thiobarbituric acid-reactive substances), particle size, and turbidity. Native soy oleosomes had lowest primary and secondary oil oxidation. Yolk protein created more stable AOB than soy protein isolate. All three oil bodies were not significantly different for turbidity. pH and pro-oxidant had effects on the particle size and turbidity of the accelerated 2-week oxidation, but was unable to determine statistical significance because the AOB were not stable and oil was separated from the system.

Key words: Artificial oil body, egg yolk protein, oleosome, oleosin

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INTRODUCTION

Lipid oxidation and the generation of secondary oxidation products have been a serious concern for food industry in terms relating to food quality and consumer health. Lipids with higher unsaturation are easier for oxidation. Several studies illustrate linoleic acid oxidation was 20-40 times faster than oleic acid (Kolakowska, 2003; Frankel, 1980; Fatemi and Hammond, 1980). Polyunsaturated fatty acids (PUFAs) are a significant amount of total lipid due to their natural occurrence and presence in fortified formulations, so they present more challenges for oxidation prevention. Other factors can influence the rate of oxidation, such as transition metal ions commonly found in food emulsions like copper and iron, can initiate or accelerate oil oxidation (Wang and Wang, 2008). Copper is less likely to be found in food but has been shown to be more active accelerating primary oxidation product decomposition (Yoshida, 1992). Other factors, such as oxygen availability, storage temperature, and pH can also affect oxidation rate (Wang and Wang, 2008).

Oil bodies, or oleosomes, is nature's way to protect lipids from oxidation. Oil bodies are made up of three basic components, proteins (1-4%), phospholipids (1-4%), and triglycerides (92-98%) (Huang, 1992). Oil bodies have a matrix comprised of triacylglycerols bound by phospholipid monolayer mixed with unique proteins, oleosins, caleosins, and steroleosins (Bhatla et al., 2010; Frandsen et al., 2001; Huang, 1994). Oleosin and caleosin are considered to contribute to structural stability of oleosomes, but not steroleosin (Liu et al., 2009). Generally, oleosomes in situ are spherical, yet they can be pressed into irregular shapes by other cellular structures (Bhatla et al., 2010). Oil bodies are typically 0.5-2.5 μm in diameter where the size can be directly affected by the nutritional status and environmental factors (Frandsen et al., 2001; Tzen et al., 1998). Oleosomes from *E. plantagineum* were the most stable form of dispersed oil, even more so than bulk oil extracted after incubating at 40 °C for one week (Gray et al., 2010). Although oil body suspension can become unstable and aggregate over a pH range of 4-6 and salt concentrations greater than 25 mM (Chen et al., 2010), oleosomes are in general, stable both *in vitro* and *in vivo* (Chiang, 2010).

Artificial oil bodies (AOB) can serve as carriers for hydrophobic molecules like neutraceuticals, pharmaceutical drugs, probiotics, or healthy fatty acids like omega-3 (Chen

et al., 2004; Peng et al., 2003; Chiang, 2010). Hen egg yolk is marketed as low value product (Anton and Gandemer, 1997) and its protein may be an ideal candidate for making AOB. The proteins in egg yolk typically are hydrophilic, but also contain hydrophobic groups that can be absorbed into oil-water interface (Daimer and Kulozik, 2010). Egg yolk proteins can provide stabilizing effects in food systems with low pH (Nilsson et al., 2007). For this study, egg yolk protein, yolk lecithin, and soybean oil were used to make AOB with a ratio that is naturally occurring in soybean oleosomes. Egg lecithin was used since it contains less linoleic and almost no linolenic acid as compared to soy lecithin (Wang and Wang, 2008). Egg lecithin contains mainly phosphatidylethanolamine (PE, 18.1%) and phosphatidylcholine (PC, 78.7%) (Wang and Wang, 2008).

With the ease of PUFAs oxidation, it is the goal of this study is to create AOB to mimic naturally occurring oleosomes to protect the encapsulated oil from oxidation. We hypothesize the artificial oil bodies created with egg yolk protein and phospholipids will have an increased stability compared with that prepared from other types of protein.

MATERIALS AND METHODS

Starting Material Preparation

Soy Protein Isolate. Soy protein isolate (SPI) (90.89% protein) was obtained from ADM (Lot #10013011, 2010, USA).

Defatting of Egg Yolk Proteins. Full-fat spray-dried egg yolk material was obtained from Rembrandt Enterprises (Rembrant, IA). Ten grams of the egg yolk powder was mixed with 10 mL of hexanes and placed into a column (1.5 cm wide x 16 cm long) with silica gel at the bottom of the column. The top of the column was covered with aluminum foil to avoid evaporation of the hexanes. Fractions were collected for each additional 10 mL of hexane added to the top of the column. Hexanes were allowed to run through the column under the force of gravity. Once the fractions of oil had been collected, the yolk proteins (67.13% protein) were left in the fume hood to dry and then placed in the vacuum oven overnight to remove hexanes completely at ambient temperature.

Soybean Oleosome Extraction and Purification. Oleosomes were obtained from dehulled, full-fat soybean flour (2008 crop year) from Natural Products Inc. (Grinnell, IA). The method was modified from Kapchie et al. (2011) from a pilot-plant scale to a lab bench scale. Soy flour of 25 grams were mixed 150 mL with 0.1 M potassium acetate pH 4.6 containing 0.5 M sodium chloride and 0.4 M sucrose. Enzymes of 3% (vol/wt) (Multifect® Pectinase FE, Multifect® GC, Multifect® CX B) from Genencor International (Rochester, NY) was used. Multifect® Pectinase FE from *Aspergillus niger* is reported to contain pectinase, cellulase and hemicellulase activities (145 – 180 U/g); Multifect® CX B is a β-glucanase derived from a selected strain of *Trichoderma reesei,* with activity of 2,250 BGLU/ mL (β-glucanase unit per milliliter); Multifect® GC from *Trichoderma reesei* is a cellulase with a minimum activity of 82 GCU/g (Kapchie et al., 2011). The extraction mixture was incubated at 57 ºC overnight with a constant stirring at 35 rpm (shaker model 3582, Thermo Scientific, USA). After incubation, the mixture was mixed using a single speed blender (Model 700G, Waring Commercial, USA) for three minutes. The mixture was then centrifuged at 3,000 g for 45 minutes. The fat pad was removed from the top of the supernatant and pellet. The fat pad was resuspended in 0.1 M tris hydrochloric acid pH 8.6 containing 0.4 M sucrose and 0.5 M sodium chloride by mixing with a stir bar until no clumps were present. The resuspended oleosomes were centrifuged at 3,000 g for 45 minutes and separated from the supernatant and pellet, and the procedure was repeated until no pellet formed after centrifugation.

The oleosomes were then purified by adapting the methods of Kapchie et al. (2011) and Kapchie et al. (2008). The unpurified oleosomes were weighed and washing solution of 0.1 M Tris hydrochloric acid, 0.4 M sucrose, and 0.5 M sodium chloride at pH 8.6 was added at a ratio of 1:10 (oleosomes:washing solution). The mixture was centrifuged at 10,000 g, 4 ºC, for 30 minutes (Avanti® J-20 Series, Beckman Instruments Inc., Fullerton, CA). Oleosomes floating on the top were collected and resuspended in the same amount of washing solution. A total of three wash cycles were carried out. The final washed oleosomes underwent dialysis in 10 mM sodium phosphate buffer, pH 7.0, to remove the remaining salt

and sugar from the oleosome dispersion overnight with periodic changing of the water in the 1 L beaker.

Oleosin Extraction. The oleosomes were freeze-dried before hexane extraction. Ten grams of the freeze-dried oleosomes was mixed with 10 mL of hexanes. Oleosin was extracted from the washed and purified oleosomes in a similar manner to the method mentioned for egg yolk protein extraction. The dispersion of oleosomes and hexanes was filled in the glass column and extracted for a total of eight times. The defatted material was dried in a vacuum oven overnight at ambient temperature, to remove remaining hexanes.

Oxidation Study

Stability of Natural Soy Oleosome and Artificial Oil Bodies Created by Using Egg Yolk Protein and Soy Protein Isolate. Oleosomes were placed into 50 mL erlenmeyer flasks at the same concentration as the AOB created with egg yolk protein and the SPI. The AOBs were created by using a method according to Tzen et al. (1998) and Chen et al. (2005). The 40 mL suspension contained 18 g of soybean oil extracted from oleosomes, 0.18 g of egg lecithin, and 0.27 g of protein (dwb), either egg yolk protein or SPI. The liquid for the suspension was 10 mM sodium phosphate buffer, pH 7.0. Egg yolk protein or SPI, egg lecithin, and buffer were first added to the a 50 mL conical centrifuge tube and sonicated (Sonicator XL2020, Misonix Inc. Farmingdale, NY) for 1 minute at 20 second intervals with 30 seconds rest in between sonication cycles at an amplitude of 35% using a flat tip (0.5 inch diameter). Samples were placed in an ice-water bath to dissipate heat during sonication. The use of the sonicator is to break up large protein particles so that the smaller particles may expose hydrophobic groups allowing for the more interaction on the oil-water interface (Diamer and Kulozik, 2010). Soybean oil extracted from oleosomes was added to mixture and sonicated again in the same manner. Samples were then transferred to 50 mL erlenmeyer flasks. All flasks were covered with aluminum foil with small holes punched and were placed into an oven at 55 ºC (FED 53, forced convection, Binder Inc, NY) for 30 days. Each day throughout the study buffer was added to equal the amount of evaporation (typically 0.5 g) over the past 24 hours. Samples were shaken by hand to homogenize the sample each day

after added back evaporated buffer, unless there was noticeable oil where the samples were sonicated for 20 seconds to incorporate the oil back into the AOB system. A flask of soybean oil, bulk oil system, was used a control. Each treatment was completed in duplicate.

Effect of AOB Protein and Environment on Fish Oil Oxidation. Egg yolk protein and the extracted oleosin protein were used for this experiment. Both proteins were used with the same ratio relative to lecithin and oil as for above except fish oil was used. There were four environment treatments in 10 mM sodium phosphate buffer, 0 ppm $CuSO_4$ pH 7, 50 ppm $CuSO_4$ pH 7, 0 ppm $CuSO_4$ pH 3, or 50 ppm $CuSO_4$ pH 3. A flask of fish oil, bulk oil system, was used a control. Each treatment was completed in duplicate.

Evaluation Analysis

Images of samples. Light microscopy images were taken every 3 days for the soybean oil experiment and every 2 days for the fish oil experiment to monitor the changes under 100x magnification.

Turbidity Test. A 1 mL sample was taken from each treatment and used for turbidity test after the initial sonication, according to a method of Tzen et al. (1998). The mixture was placed in a disposable cuvette of 1.8 mL capacity. Dilutions were made with 10 mM sodium phosphate buffer, pH 7 or 3 depending on the sample's environment so the sample had absorbance below 0.9. The sample was subjected to minimal disturbance and measured with a spectrophotometer (DU 720 UV/Vis, Beckman Coulter) set at 600 nm with readings taken every 5 minutes for 3 hours. The starting measurement was denoted as A_o and the time specific readings as A. The relative turbidity was expressed as (Tzen et al., 1998):

$$T/T_o=10^{A}/10^{Ao}$$

Particle Size. Two mL sample was taken from each treatment and used for this test after the initial sonication. A particle size analyzer (Mastersizer 2000 S, Malvern Inc., Worcestershire, United Kingdom) was used to determine particle size distribution of the oleosomes and AOBs. The sample was suspended in approximately 800 mL of DI water in a Hydro 2000 MU (A) at 2,040 rpm. Five readings were averaged to give a result. Each

sample was measured twice. The particle size distributions were calculated using a refractive index of 1.46. The laser obscuration was approximately 12-14%.

Lipid Hydroperoxide Determination by Iron Oxidation Test. This method was adopted from Stine et al. (1953). From each treatment, 1.5 mL uniform sample was removed and placed into a glass vial with 6 mL of 2:1 chloroform:methanol to extract the oil. Store bought vegetable oil (Canola oil) was oxidized by heating, weighed, and used to calculate peroxide value as determined by the iodiometric method (AOCS method Cd 8-53). This oil was also measured by the iron oxidation test to establish a standard curve for PV. Samples were stored at -20 °C until analyzed. A small amount of oil sample was obtained, 10-300 mg accurately weighed, was placed into a 10 mL volumetric flask and filled with 2:1 chloroform:methanol (v/v) and swirled until well mixed then filled to 10 mL. Ammonium thiocyanate solution (30% w/v) of 60 µL was added to a10 mL volumetric flask and then 60 µL of ferrous chloride (~0.014 M) was added to the flask and timed for 10 minutes before reading on the spectrophotometer at 515 nm (DU 720 UV/Vis, Beckman Coulter). The solution was shaken periodically. The standard curve using canola oil was diluted using 2:1 chloroform:methanol (v/v) to several concentrations. These solutions were placed into 10 mL volumetric flask and followed the rest of the procedure as for the samples.

Secondary Oxidation Products. A method used to determine the amount of 2-thiobarbituric acid-reactive substances (TBARS) from Wang and Wang (2008) was used. Briefly, 15% (w/v) trichloracetic acid (TCA) and 0.375% (w/v) thiobarbituric acid (TBA) were dissolved in 0.25 M hydrochloric acid aqueous solution by mild heating and agitation. Butylated hydroxytoluene (BHT), 3 mL of 2% in absolute ethanol, was added to 100 mL of the TCA/TBA stock solution. An oil sample of approximately 400 µL was accurately weighted and placed in a test tube and 4 mL of TCA/TBA solution was added and mixed thoroughly with a Vortex mixer. The samples were placed in a boing water bath for 15 minutes and allowed to cool to room temperature. Absorption was measured at 535 nm with a blank containing only the TCA/TBA reagent and mineral oil. Concentrations of TBARS were determined using a standard curve prepared using 1,1,3,3-tetraethoxypropane ranging from 0 to 4.2 nmol/mL.

Solubility Curve

The oleosin and egg yolk proteins' solubility behavior was measured. The protein was dispersed in water with a concentration of 0.75%. Samples were placed on a magnetic stirrer (250 rpm) for 30 minutes for full protein suspension before 1 mL samples were taken from the solution. pH was adjusted by adding either 2 N NaOH or 2 N HCl. Samples were allowed to equilibrate for 30 minutes. Removal of 1 mL sample, pH adjustment, and 30 minute equilibration time was repeated until all pH values were taken ranging from 1-13 with increase or decrease of 1 pH intervals. Samples were centrifuged for 10 minutes at 10,000 x g. Supernatants were evaluated for soluble protein by using the Lowry method (Lowry et al., 1951). Briefly, samples were placed into tubes and 200 µL of 1 N NaOH was added along with 2 mL of Lowry solution, 100:1:1 of 2% sodium carbonate: 2% sodium potassium: 1% copper sulfate. Samples were vortexed and allowed to stand at room temperature for 10 minutes. Folin-Ciocalteu phenol reagent solution (1N) of 200 µL was added to the samples, vortexed, and allow to stand at room temperature for 30 minutes in the dark. Samples were then read on a spectrophotometer at 750 nm. Standard protein of bovine serum albumin was used for standard curve development with concentration range from 0 to 0.05 mg/mL. The experiment was done in duplicate.

Statistical Analysis

Statistical analysis was completed using SAS (version 9.2, SAS Institute Inc., Cary, NC) One-way analysis of variance (ANOVA) was used for mean comparisons, and Fisher's least significant differences were calculated at $P< 0.05$ ($LSD_{0.05}$).

RESULTS AND DISCUSSION

Defatting of Egg Yolk Proteins

Egg yolk proteins were extracted with hexanes to remove the lipids. The results of the amount of oil in each extraction step are seen in Figure 1. The amount of oil extracted during the 1st fraction had most of the oil, 70.7%. As the fractions progress there was a

significant decrease in oil content. In the last fractions, there was 0.0% of total oil, indicating a complete oil removal.

Stability of natural soy oleosomes and AOB created by egg yolk protein and SPI

At the start of the experiment, all samples appeared visually the same. All emulsions were a creamy fully-dispersed systems with no visual free soybean oil similar to the samples created by Chen et al. (2005) using caleosin to encapsulate cyclosporine A. By the end of 30 days, our samples had greatly changed. There was free soy oil apparent in all samples except for oleosomes. The egg yolk protein emulsion broke at day 28 where a clear lower water layer and an oily layer were observed. SPI emulsion broke at day 23 when similar separation was observed. Therefore, although yolk protein did not create a stable AOB as the oleosomes, it performed better than SPI.

Turbidity Test. The turbidity measures the stability of the dispersion. All samples were not significantly different when comparing the slope (Figure 2). This result illustrates egg yolk protein and SPI under these experimental conditions and over three hours did not lose emulsion stability. The stability of the oleosomes was similar as observed by Chen et al. (2004) and Choi and Chang (2009).

Particle Size. Particle size among the different oil bodies were significantly different from each other. Oleosomes had the smallest diameter of particles and also had a wider range of particle size distribution (Figure 3). Egg yolk protein had the next largest average diameter followed by soy protein isolate. Egg yolk protein size ranged from 0.1-10 μm and soy protein isolate from 0.1-100 μm. A study conducted by Chiou and Lai (2009) used caleosin, another protein found in oleosomes, to make AOB to encapsulate a photosensitizing drug. They were able to successfully encapsulate the drug and had smaller particle size, 122 nm diameter.

Microscope Images. Figure 4 shows images taken from the beginning and the end of the experiment. At the beginning of the experiment all samples had similar particle appearance but with some larger particles in the AOB samples. This observation is confirmed with the particle size analysis. Images taken at the end of the experiment revealed

significant changes over 30 days. Oleosomes appear to maintain the numbers and also maintained a similar size to that at the beginning of the experiment. Egg yolk protein oil bodies appear to have significantly lost in numbers with the few remaining appearing to have a similar size to the oleosomes with some oil bodies with larger diameters. SPI oil bodies appeared to be mostly free oil. Peng et al. (2003) studied the effect of temperature on sesame oleosomes and they were thermostable up to 60 °C. The same conclusion was made with AOBs made of caleosin, decomposing at temperatures higher than 70 °C (Liu et al., 2009). Therefore our oxidation temperature of 55 °C should not be a significant factor in oleosome or AOB breakage. However, the ability of the protein used to form AOB is the main factor.

Oxidative Stability of the Encapsulated Soybean Oil. Primary oxidation of soybean oil is shown in Figure 5. Oleosomes exhibited very little increase if any in lipid oxidation. The soy oil started to increase in peroxide value towards the last 15 days. The oil is a crude unrefined oil having the natural antioxidants, so it is relatively stable, particularly when oil is dispersed in water as an emulsion, its oxidation can be accelerated by many factors, such as large surface area and pro-oxidants in water. Egg yolk protein AOB had an increased peroxide value and it peaked at day 28. Soy protein isolate AOB's PV increased at a much faster rate and peaked at day 26. Day 26 was used for statistical analysis where all treatments were significantly different from each other with p-value of 0.0002. This result suggests that neither yolk protein nor soy protein successfully created AOB when compare to the natural oleosome. However, yolk protein may have performed better than soy protein. Secondary oxidation had a results similar to primary oxidation. Oleosomes and soy oil had a slow increase over the 30 days. Egg yolk protein treatment had a lower TBARS than SPI treatment. Day 26 was used for statistical analysis with all the treatments significantly different from each other with a p-value of 0.0001.

Effect of AOB Protein and Environment on Fish Oil Oxidation

At the start of the experiment, all samples appeared the same, creamy fully-dispersed systems with no visual free fish oil. There were some interesting observations: egg yolk protein with 0 ppm $CuSO_4$ at pH 7 had no phase separation unlike egg yolk protein with 0

ppm $CuSO_4$ at pH 3 which had a clear water and cream layer separation. Egg yolk protein with 50 ppm $CuSO_4$ at pH 7 had two distinct cream layers whereas the egg yolk protein with 50 ppm $CuSO_4$ at pH 3 had a clear water and cream layer separation but not to the same degree as the egg yolk protein treatment of 0 ppm $CuSO_4$ at pH 7 or 3. Oleosin with 0 ppm $CuSO_4$ at pH 7 and 3 and 50 ppm $CuSO_4$ at pH 7 had two distinct cream layers. The oleosin with 0 ppm $CuSO_4$ at pH 3 had a clear water and cream layer separation. By the end of the 2-week experiment, the samples had greatly changed. All the samples had visible oil and phase separation. Samples with the 50 ppm $CuSO_4$ have a darker color oil than the samples with 0 ppm $CuSO_4$. A study by Choi and Chang (2009) examined the effects of pH with AOB formed with oleosin and concluded that the negatively charged AOB possess electronegative repulsion at pH 7, but tend to aggregate when the surface negative charges were neutralized at pH lower than 6. Therefore the aggregate samples with pH 3 can be explained by this charge neutralization fact.

Turbidity Test. Figure 7 shows the stability of AOB created with the oleosin and egg yolk protein. For yolk protein AOB, the pH environment and $CuSO_4$ addition did not affect the initial stability of the emulsion. However, for oleosin AOB, the particles were very unstable at pH 3 compared to at pH 7 and the $CuSO_4$ addition seemed to have slightly reduced the stability. Peng et al. (2007) used recombinant oleosin expressed by *E.* coli. using the same ratio of protein, phospholipid, and oil as this present study and observed the AOB made with the oleosin was comparable to the turbidity exhibited by the sesame seed oleosomes. Another study by Chen et al. (2004) showed a larger decrease in stability from their turbidity test over three hours using oleosin, under similar conditions at pH 7.5 with stability reduced to 85%. This study's oleosin at pH 7 only decreased to approximately 92% relative turbidity.

Particle Size Distribution. Particle size measurement is presented in Table 1. Oleosin 0 ppm $CuSO_4$ pH 3 had the largest diameter size particle and egg yolk protein 0 ppm $CuSO_4$ pH 7 had the smallest. All the samples had a narrow peak similar to the peaks of natural soy oleosomes. As shown in Table 1 the particle size for all treatments are bigger at pH 3 than at pH 7. Egg yolk AOB are smaller particles than oleosin. Therefore, egg yolk

protein seemed to have outperformed oleosin for creating physically stable AOB. Kiosseoglou (2003) observed a similar trend where yolk emulsion (10% corn oil and 1% yolk) had a larger particle size at pH 3.8 than compared to pH 7. Magnusson and Nilsson (2011) also had similar results when they compared egg yolk livetin emulsions at pH 4.0, 4.5, 6.0, and 8.0 had smaller particle sizes observed at pH 6.0 and 8.0 than compared to pH 4.0 and 4.5.

Microscopic Images. Images of the samples were taken at the beginning and the end of the 2-week time span. The beginning images can be seen in Figure 8. These observations in general correlate with the particle size measurement at the beginning of the experiment. By the end of the 2 weeks all samples had drastically changed because not many oil bodies can be found in the samples. Egg yolk protein and oleosin with 0 ppm $CuSO_4$ at pH 7 appeared still stable at day 7 of the experiment under the light microscope. Magnusson and Nilsson (2011) observed very similar results to our own at day 7 of the experiment by using egg livetin for emulsion formation.

Oxidative Stability of Fish Oil Encapsulated in AOB Created by Egg Yolk Protein and Oleosin. The peroxide value and TBARS were completed for only the first nine days of the study due to the lack of any trend and the breakage of the emulsions. Peroxide value changes are shown in Figure 9. The only sample that exhibited a typical peroxide value trend was the fish oil control. The variation of peroxide values can be attributed to the homogeneity of the oxidized free oil when the samples were taken from the broken emulsions.

A similar trend can be seen for the TBARS values as seen in Figure 10. Fish oil sample displayed the typical pattern for TBARS, since a gradual increasing line of TBARS was formed. This oxidative stability trial is thus a failed experiment because the AOB was not very stable and the release of free oil made the sampling for oxidation measurement impossible.

Solubility Curve

Oleosin protein and egg yolk protein solubility curve is shown in Figure 11. Oleosin has a very low solubility at pH <10. There is no difference in solubility shown where experiment was conducted at pH 3 and 7. The egg yolk protein solubility was also similar at pH 3 and 7. Therefore, this curve alone cannot explain the charge effect on emulsion stability due to pH change.

CONCLUSION

In this study, fish oil and soybean oil were encapsulated by using egg yolk protein, a novel use for this protein. From the experiments shown, the environment and protein can have effects on the stability of the AOB formed. AOB constitution by egg yolk protein showed a great promise to encapsulate and protect oil from oxidation. More research needs to be done to fully explore such potential.

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Table 1. Particle Size of AOB Created by Egg Yolk Protein and Oleosin

Sample[1]	Surface Weight Mean (µm) (n=4)
Egg Yolk Protein 0 ppm pH 7	1.069 ± 0.115 ^{d}
Egg Yolk Protein 0 ppm pH 3	6.101 ± 1.960 ^{b}
Egg Yolk Protein 50 ppm pH 7	1.367 ± 0.061 ^{cd}
Egg Yolk Protein 50 ppm pH 3	2.048 ± 0.113 ^{cd}
Oleosin 0 ppm pH 7	2.365 ± 0.259 ^{c}
Oleosin 0 ppm pH 3	27.456 ± 1.169 ^{a}
Oleosin 50 ppm pH 7	2.162 ± 0.275 ^{cd}
Oleosin 50 ppm pH 3	5.155 ± 1.026 ^{b}
P-value	**

[1]ppm concentration are of $CuSO_4$.

Values are mean ± standard deviations.

** P-value ≤ 0.0001

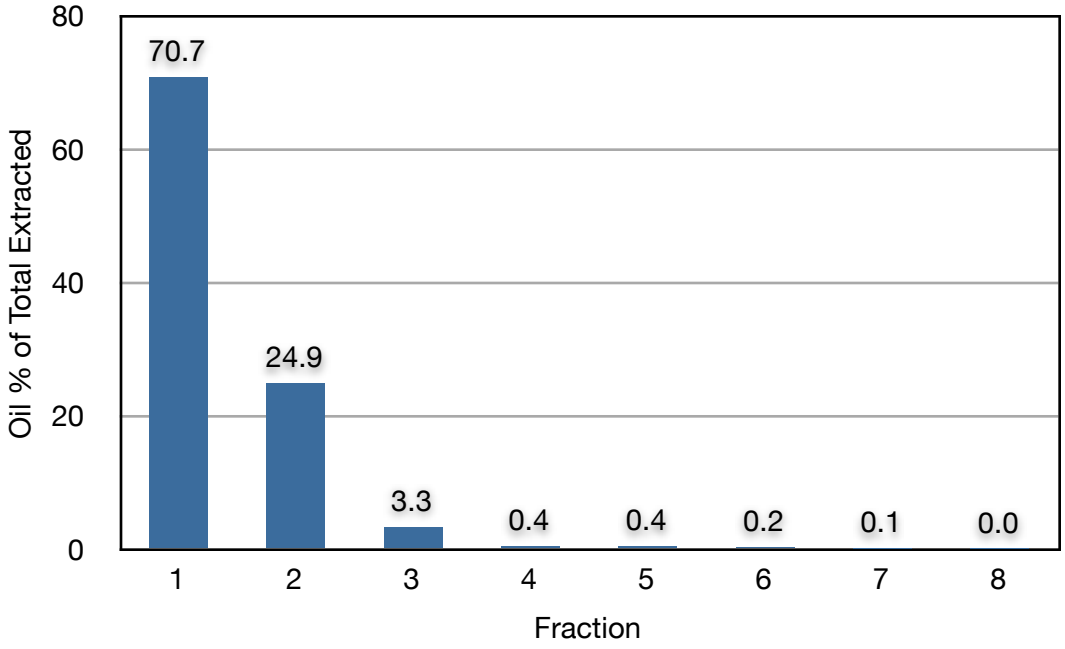


Figure 1. Defatting of egg protein by using hexanes for each of the fractions collected. Values are shown with standard deviations.

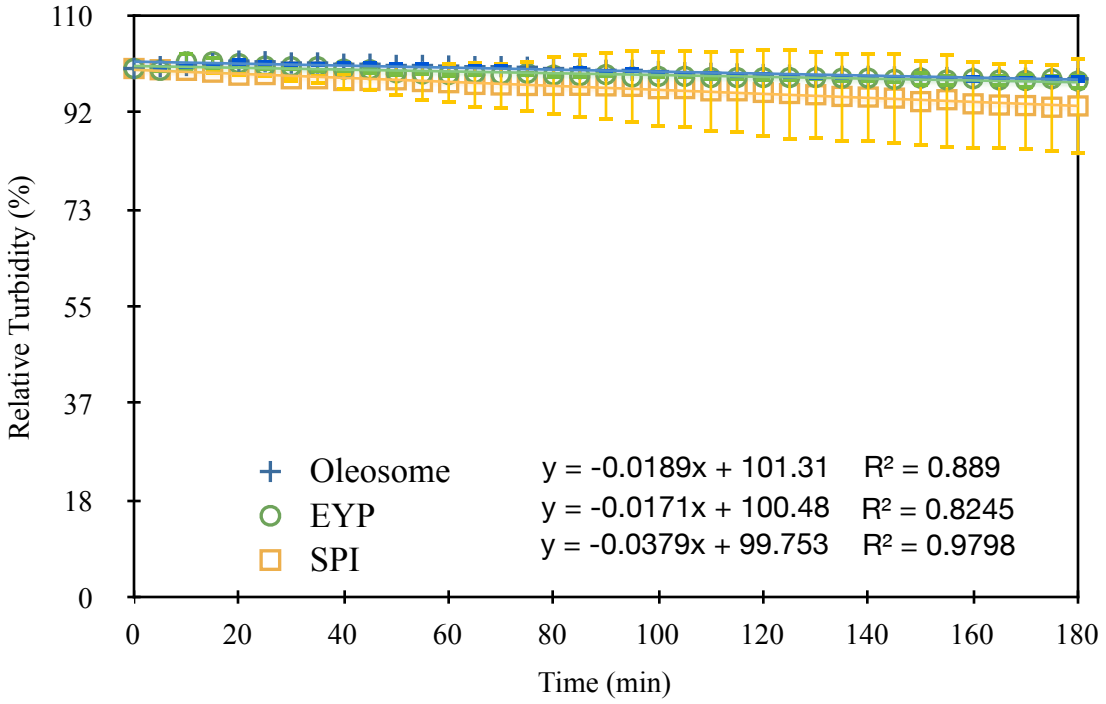


Figure 2. Stability of naturally occurring soy oleosomes, AOB made with egg yolk protein, and SPI. There was no significant difference among the samples. Chart has mean and standard deviation bars.

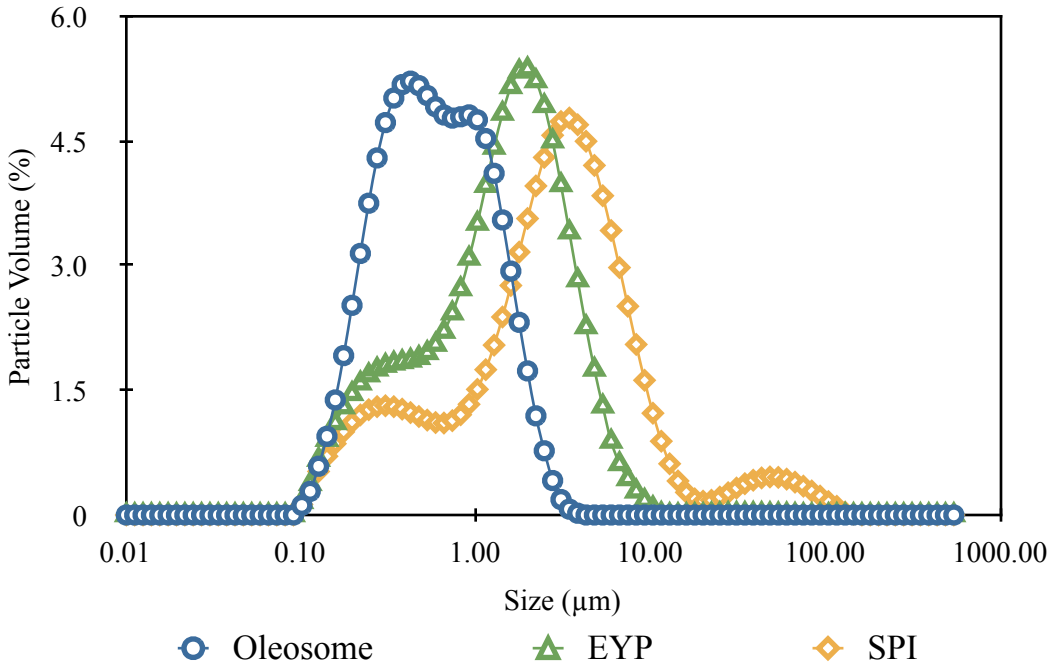


Figure 3. Particle size distribution of native soy oleosomes and AOB made with egg yolk protein and SPI at the start of the experiment.

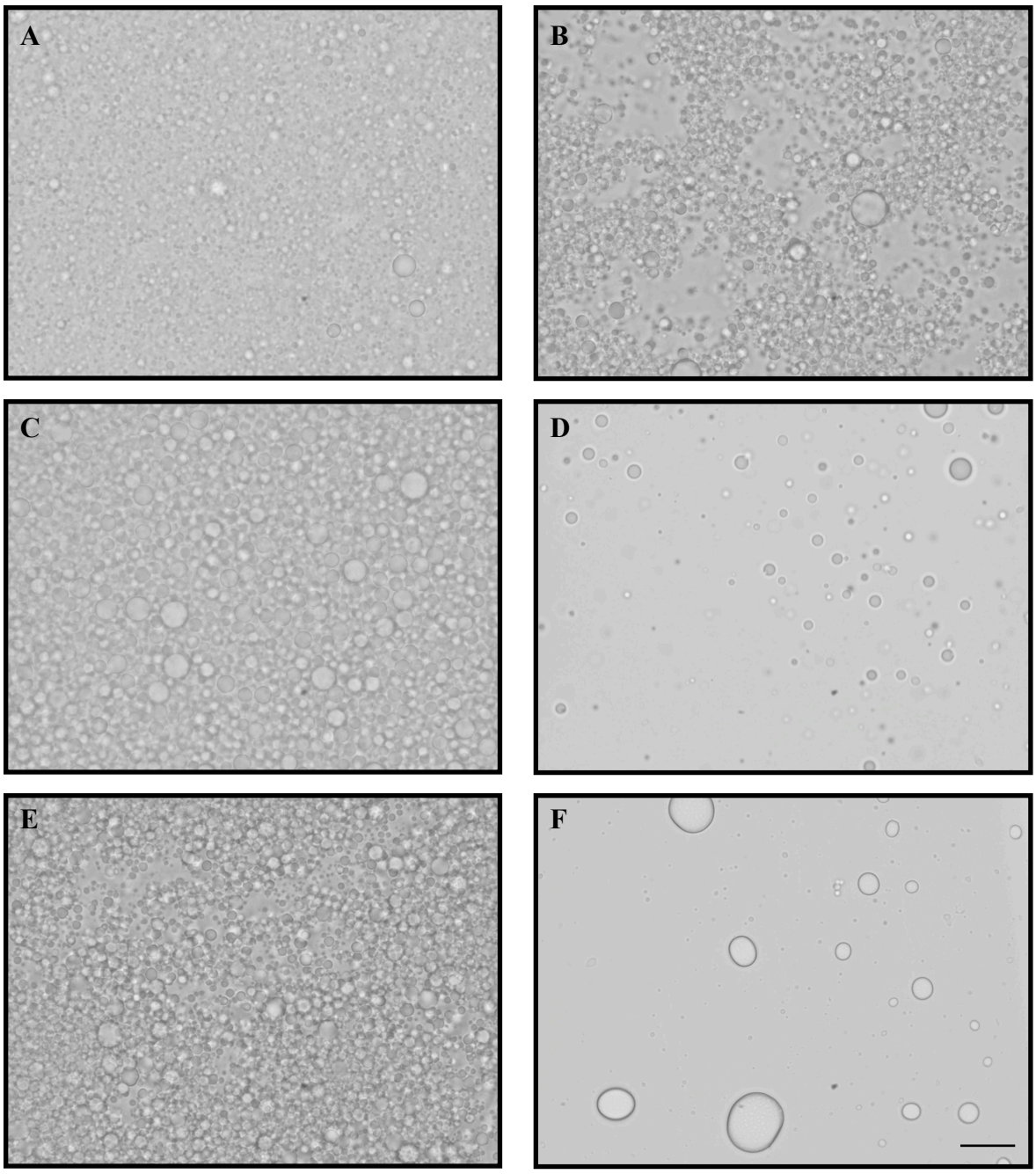


Figure 4. Microscopic images under 100x magnification. Soy oleosomes day 0 (A), soy oleosomes day 30 (B), egg yolk protein day 0 (C), egg yolk protein day 30 (D), SPI day 0 (E), and SPI day 30 (F). The line represents 10 μm.

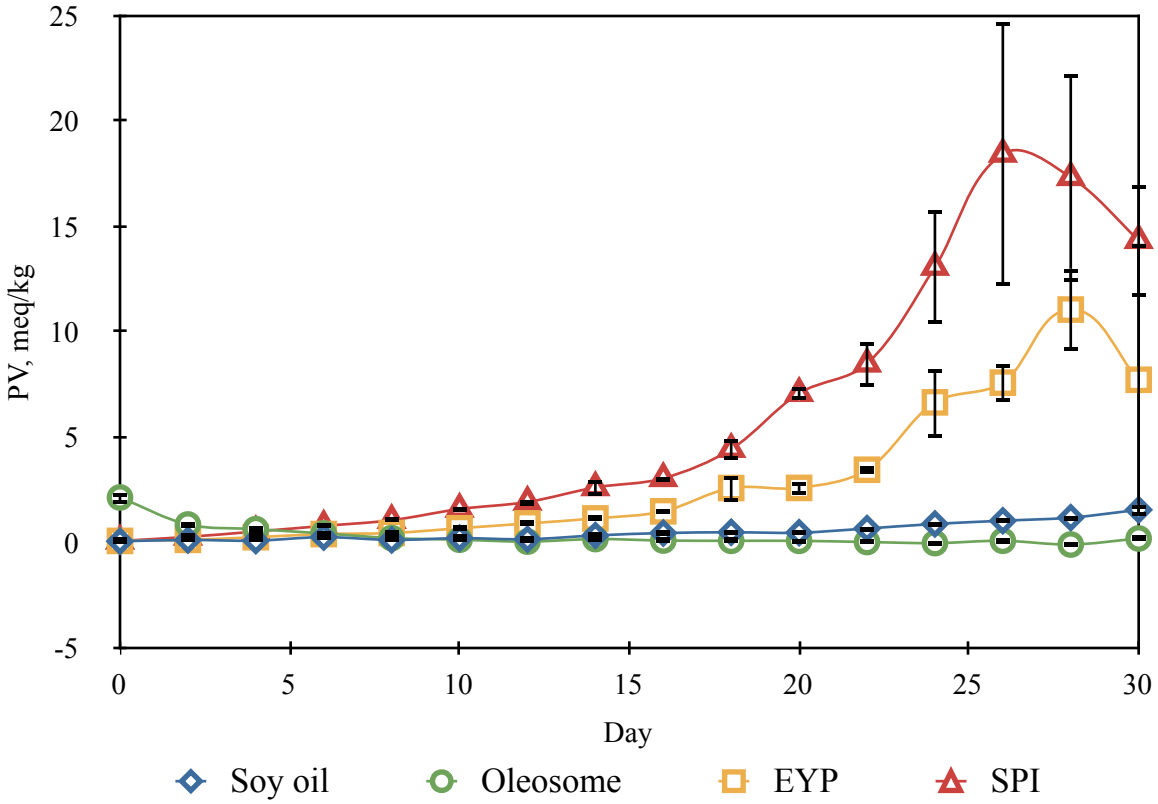


Figure 5. Lipid hydroperoxide of soybean oil in naturally occurring oleosomes and AOB made with egg yolk protein, and SPI over 30 days. Soybean bulk oil was used a control. Standard deviation bars are shown.

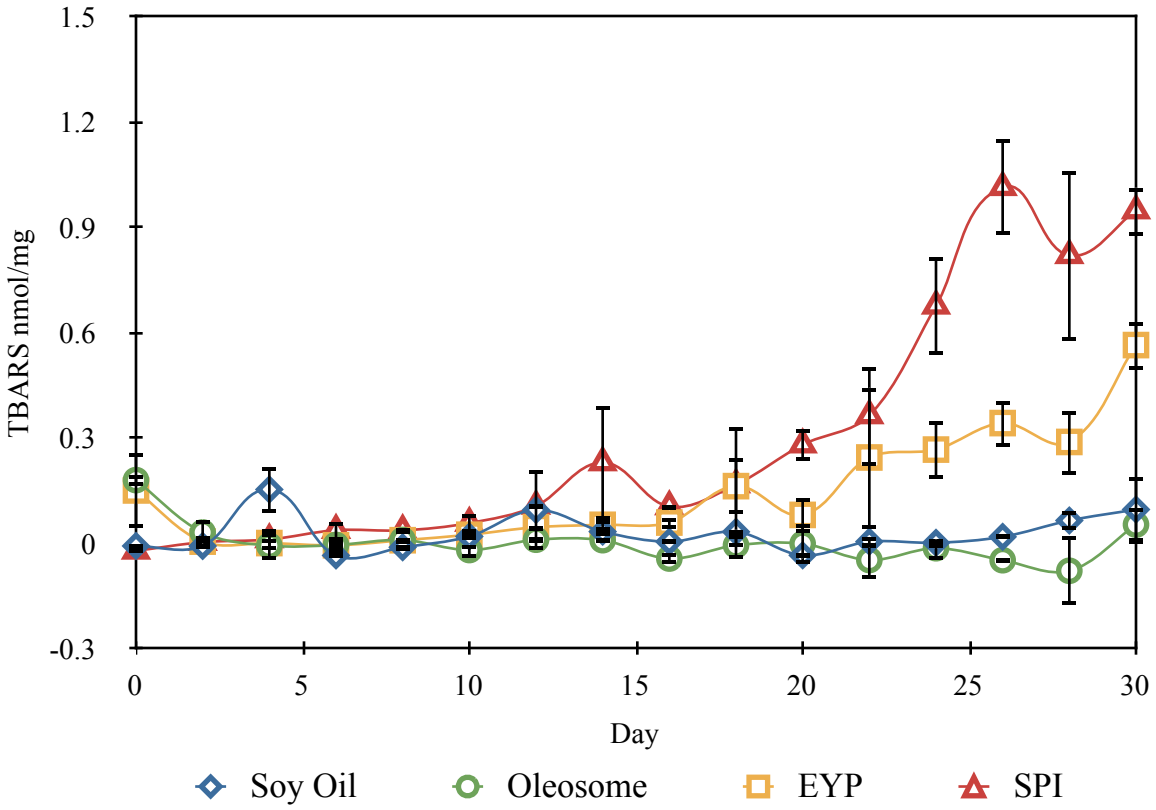


Figure 6. Secondary oxidation analysis by 2-thiobarbituric acid-reactive substances (TBARS) for of soybean oil in naturally occurring oleosomes and AOB made with egg yolk protein, and SPI over 30 days. Standard deviation bars are shown.

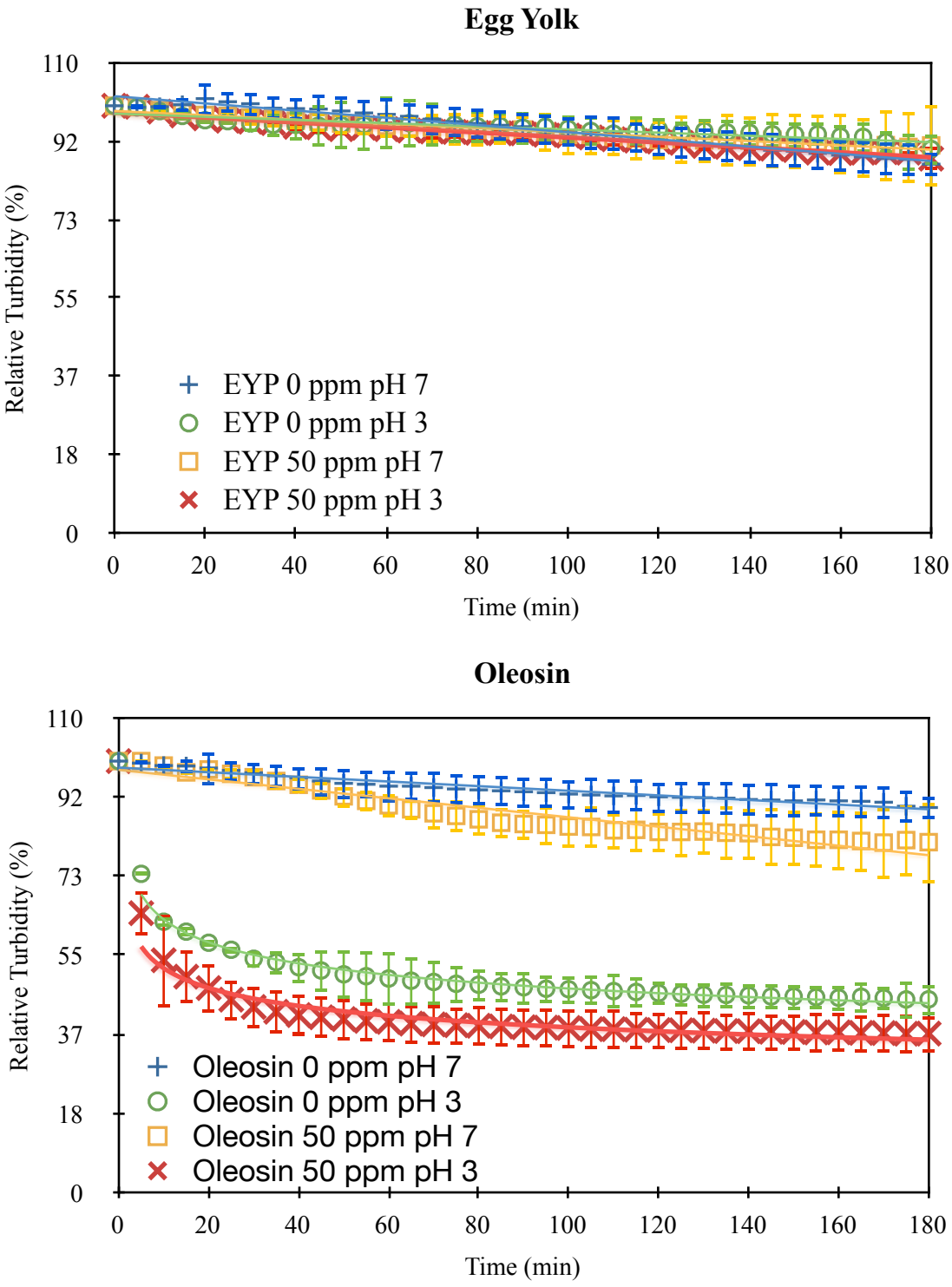


Figure 7. Stability of AOB made with egg yolk protein and oleosin with varying treatments of pH 7 or 3, and 0 or 50 ppm $CuSO_4$. All charts have mean and standard deviation bars.

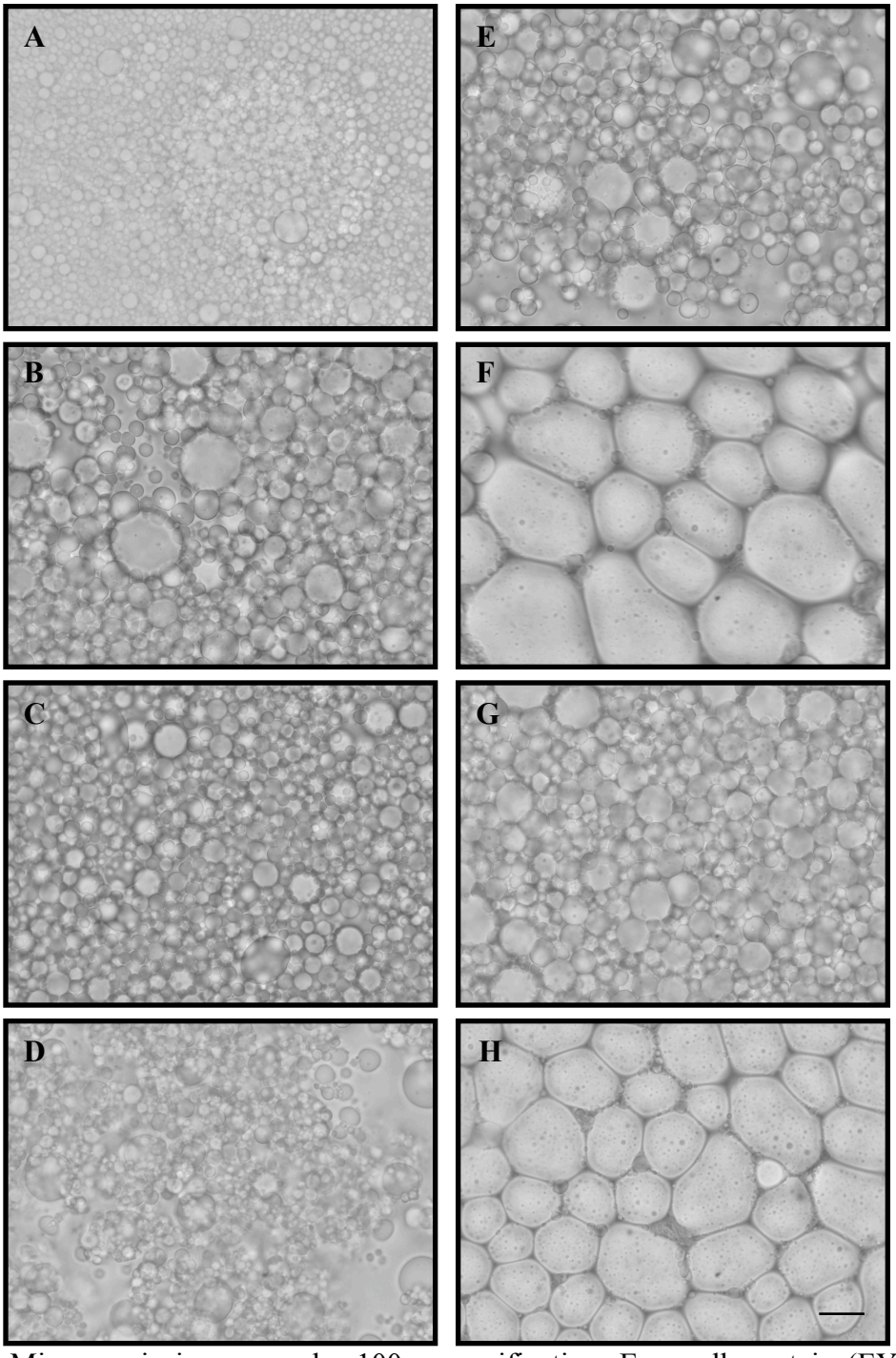


Figure 8. Microscopic images under 100x magnification. Egg yolk protein (EYP) 0 ppm $CuSO_4$, pH 7 (A); EYP 0 ppm $CuSO_4$, pH 3 (B); EYP 50 ppm $CuSO_4$, pH 7 (C); EYP 50 ppm $CuSO_4$, pH 3 (D); oleosin 0 ppm $CuSO_4$, pH 7 (E); oleosin 0 ppm $CuSO_4$, pH 3 (F); oleosin 50 ppm $CuSO_4$, pH 7 (G); and oleosin 50 ppm $CuSO_4$, pH 3 (H) at the experiment beginning. The line represents 10 μm.

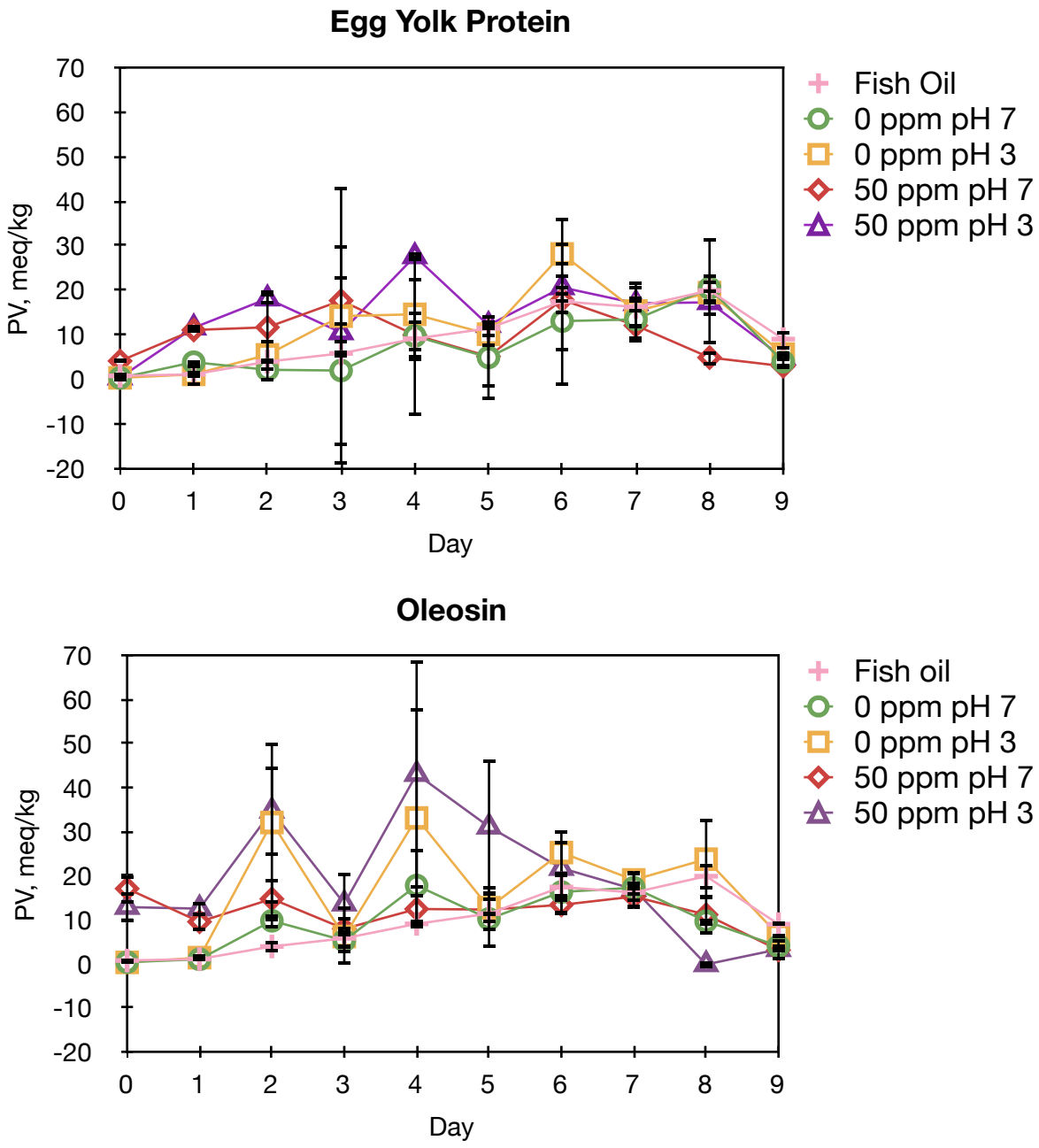


Figure 9. Primary oxidation analysis for peroxide value comparing egg yolk protein (EYP) 0 ppm $CuSO_4$, pH 7; EYP 0 ppm pH 3; EYP 50 ppm pH 7; EYP 50 ppm pH 3; oleosin 0 ppm, pH 7; oleosin 0 ppm pH 3; oleosin 50 ppm pH 7; and oleosin 50 ppm pH 3 over 2 weeks. All charts have mean and standard deviation bars.

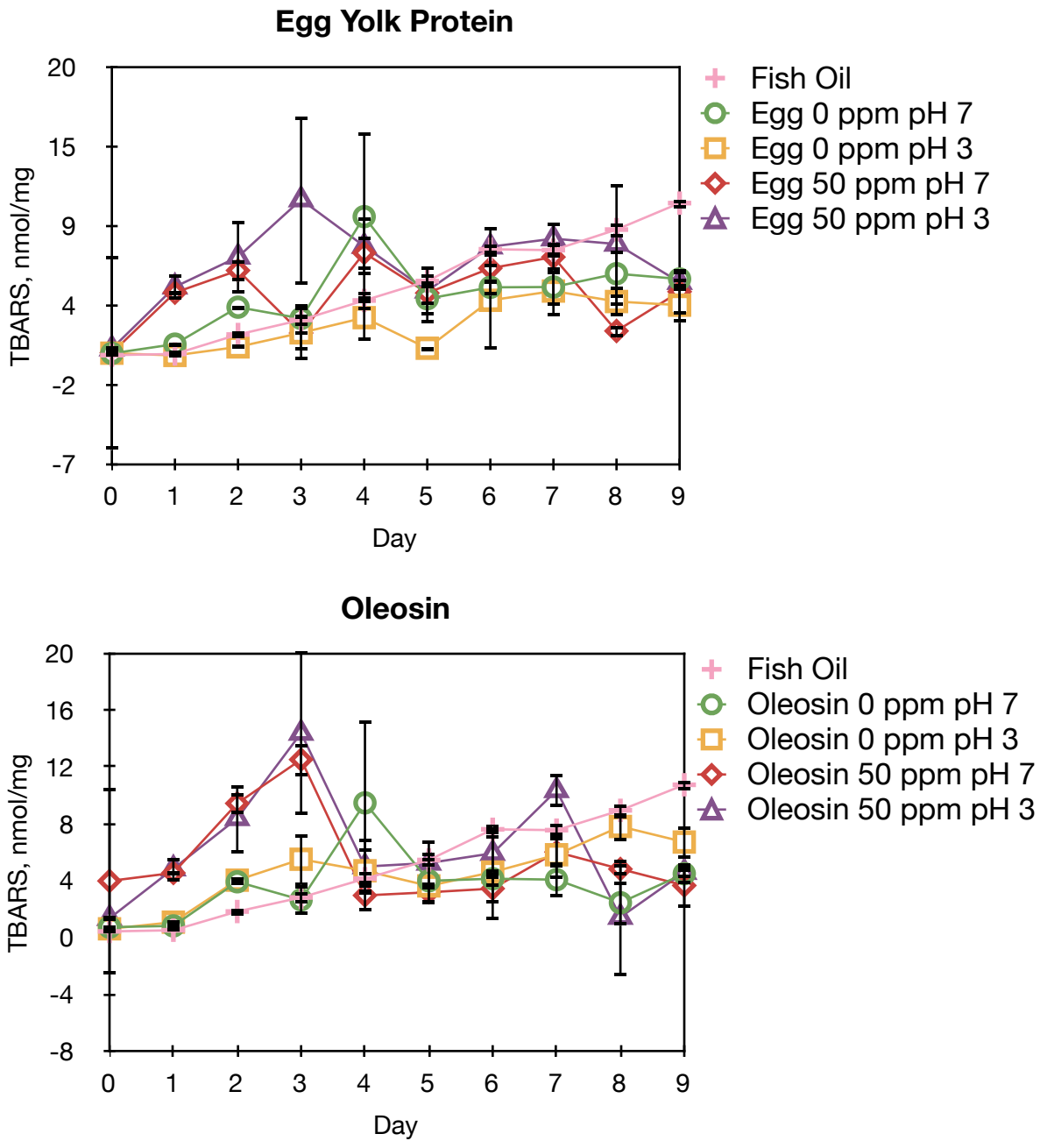


Figure 10. Secondary oxidation analysis by 2-thiobarbituric acid-reactive substances (TBARS) for experiment 2: comparing egg yolk protein (EYP) 0 ppm $CuSO_4$, pH 7; EYP 0 ppm $CuSO_4$, pH 3; EYP 50 ppm $CuSO_4$, pH 7; EYP 50 ppm $CuSO_4$, pH 3; oleosin 0 ppm $CuSO_4$, pH 7; oleosin 0 ppm $CuSO_4$, pH 3; oleosin 50 ppm $CuSO_4$, pH 7; and oleosin 50 ppm $CuSO_4$, pH 3 over 2 weeks. All charts have mean and standard deviation bars.

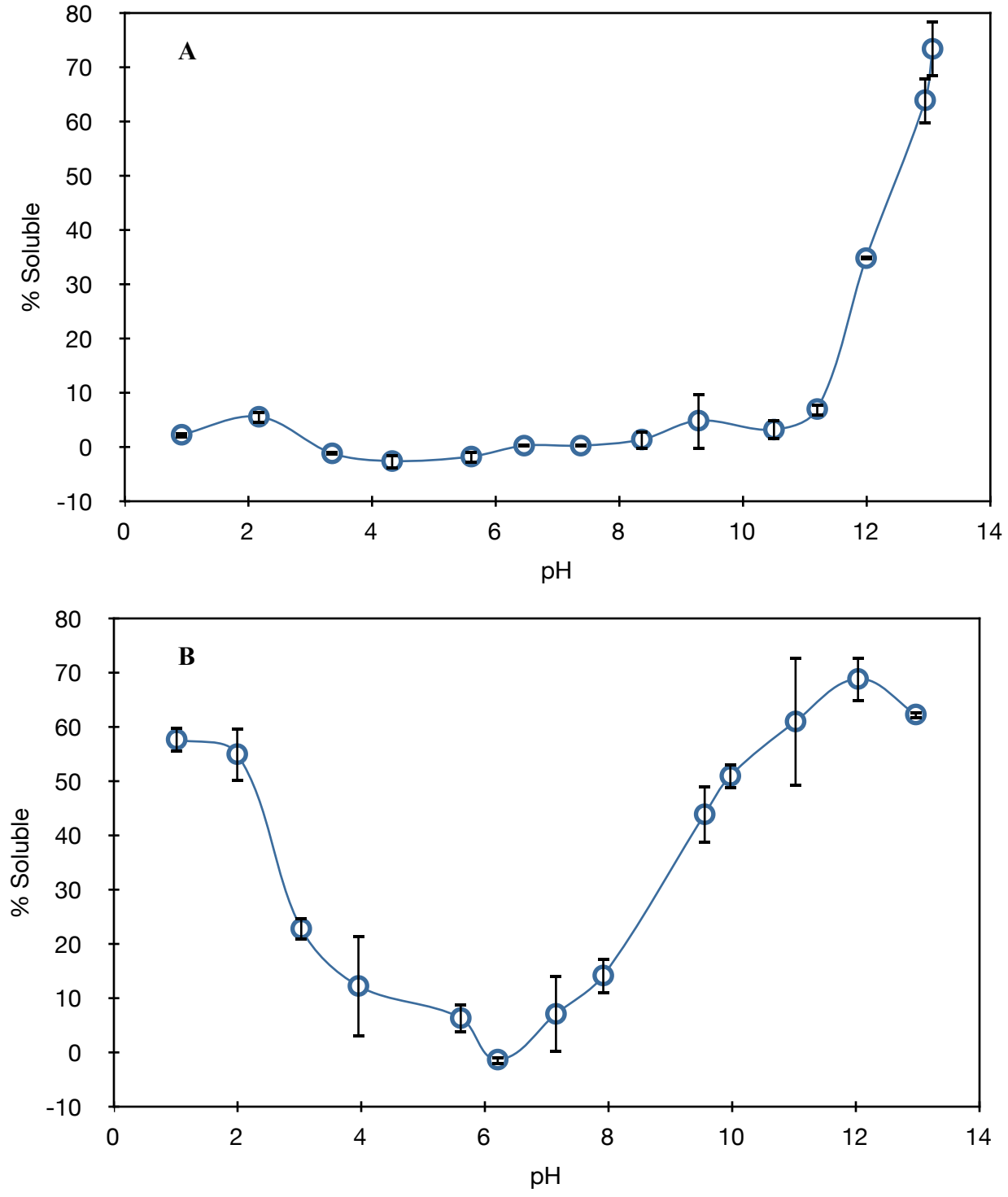


Figure 11. Oleosin (A) and egg yolk protein (B) solubility curves extracted by hexanes. Charts has mean and standard deviation bars.

CHAPTER 6. GENERAL CONCLUSIONS

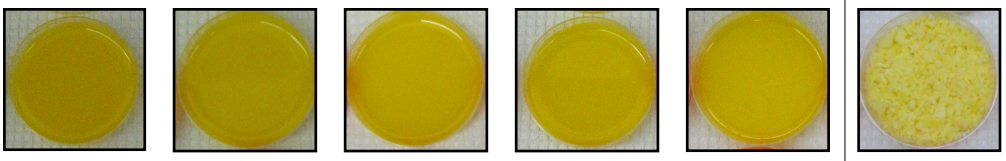
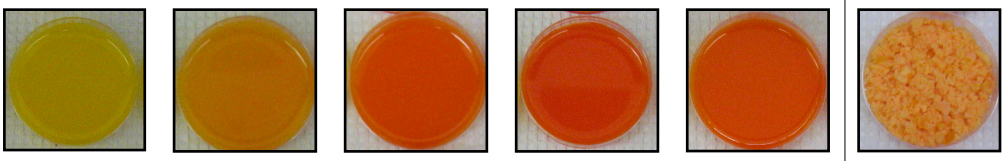
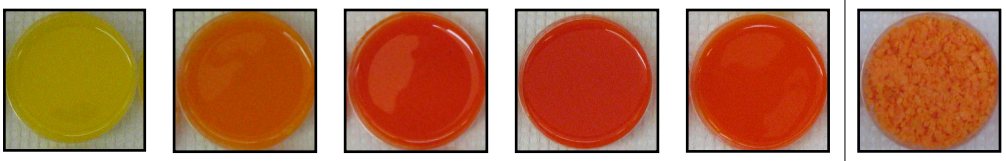
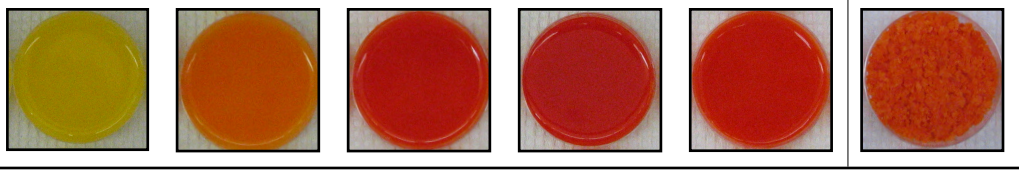
The proteins in egg yolk typically are hydrophilic, but also contain hydrophobic groups that can be absorbed into oil-water interface. Therefore this property was exploited for artificial oil body formation. In this study, soybean oil was successfully encapsulated by using egg yolk protein, a novel use for this protein. From the experiments shown, the environment and protein can have significant effects on the stability of the AOB formed. Egg yolk protein did create a better AOB than soy protein isolate. The experiment with fish oil stabilization using the different proteins and environments was able to clearly illustrate the effect pH and presence of an oxidizer has on the system. The samples with a pH of 3 were less stable than the samples at pH 7. AOB constitution by egg yolk protein may be a suitable technique to encapsulate oil that is easily oxidized therefore allowing the healthy oil to be consumed.

The feeding study with astaxanthin and vitamin E was able to illustrate the success of astaxanthin incorporation at higher concentrations than previously seen in other studies where color enhancement was the goal. This study shows astaxanthin and tocopherols can be successfully incorporated into the egg yolk by adding the ingredients into the feed. The higher the concentration in the feed the higher the concentration in the resulting egg yolk, however with lower transfer efficiency. The reason could be that in the palm toco concentrate contained negligible amounts of β-, δ-, and γ-tocopherols (a total of 3 mg/g, 0.6% of total tocos). However, the feed itself had its own natural tocopherols from the natural ingredients. Because tocotrienols are not typically found in the natural ingredients in feeds, their feed enrichment led to a steady enrichment in eggs, and in a dose-response relationship. There seems to be a feedback or competitive inhibition for all the nutrients. Despite the lack of tocotrienol transfer, this study was able to illustrate there was no signifiant changes with the egg weight, texture properties, fatty acid composition, and phospholipids. Changes in yolk color was related to the amount of astaxanthin in the diet. Changes in the yolk color will need to be examined for consumer acceptance.

Overall both studies illustrated the novel use of egg proteins and ways to manipulate the egg nutrient content by changing the feed composition, thus confirming both of our original hypotheses.

APPENDIX

Table 1. Visual Progression of Color Change Throughout the Feeding Study

Diet	Fresh Yolk					Cooked Yolk
	Day 0	Day 3	Day 6	Day 9	Day 42	Day 40
A						
B						
C						
D						

Diets are A no supplements, B enriched with 0.012% tocomin and 0.49% algae biomass; C enriched with 0.036% tocomin and 1.47% algae biomass; and D enriched with 0.072% tocomin and 2.94% algae biomass.

Figure 1. Setup of the chicken housing arrangements. The water trays are in the middle of the rack and the feed is on the outside so there was no mixing of feeding diets. Eggs were collected below the feed and drinking containers.

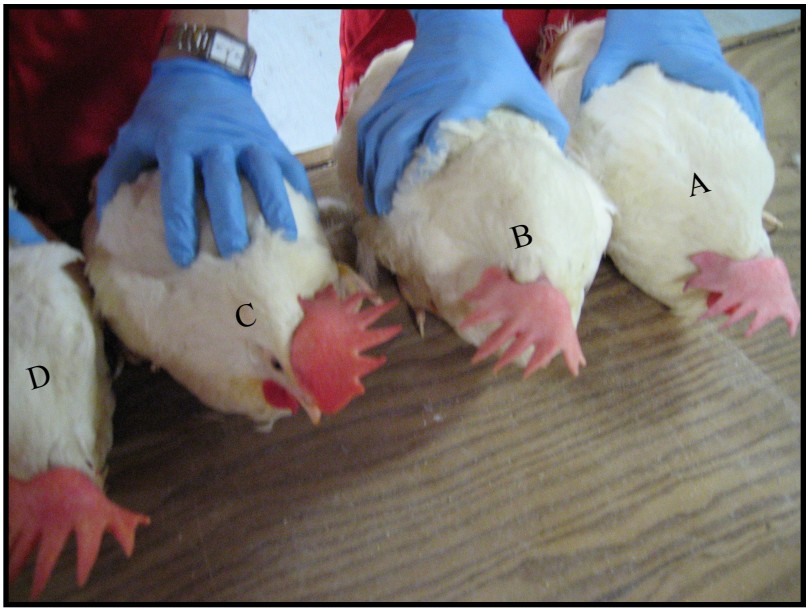


Figure 2. Image of chicken combs from each of the four different diets during week 7 illustrating the coloration changes. Chickens are labeled above with their respective diet starting on the left with Diet D and the right Diet A. Diets are A no supplements, B enriched with 0.012% tocomin and 0.49% algae biomass; C enriched with 0.036% tocomin and 1.47% algae biomass; and D enriched with 0.072% tocomin and 2.94% algae biomass.

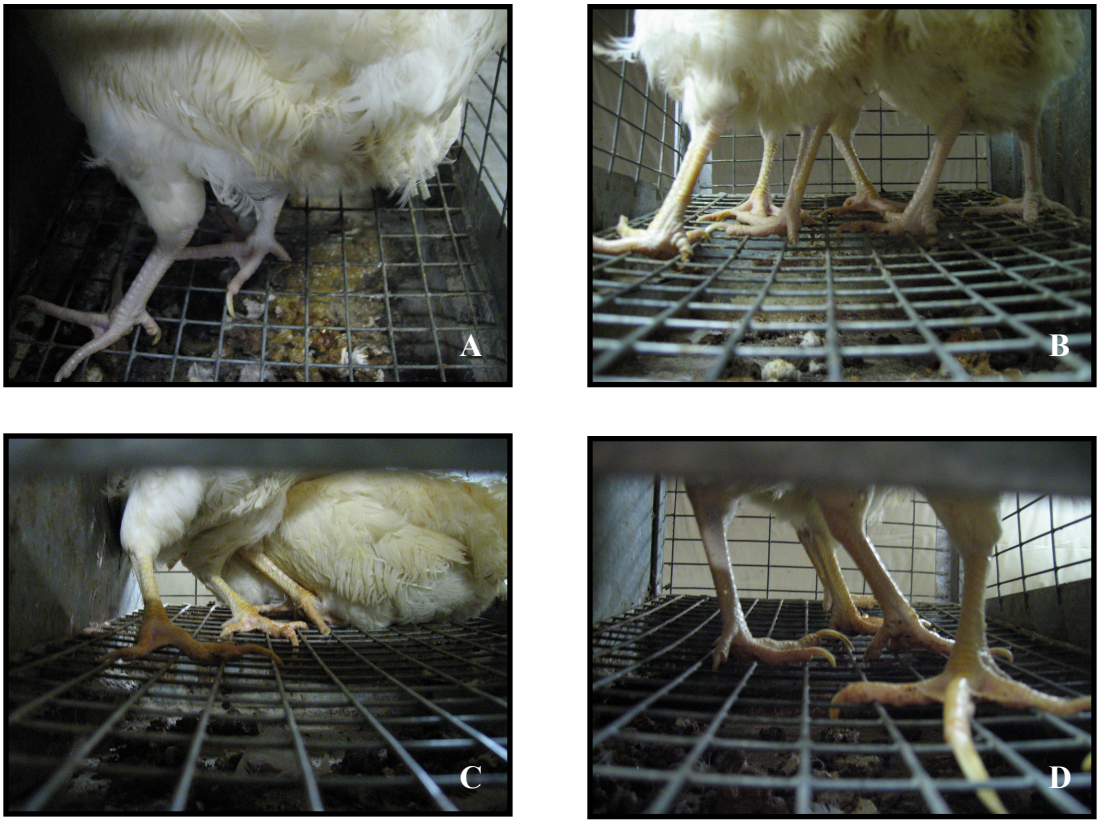


Figure 3. Images are of chicken feed from the end of the study during week 7 illustrating the coloration changes. Upper left is Diet A (no supplements), upper right Diet B (0.012% tocomin and 0.49% algae biomass), lower left Diet C (0.036% tocomin and 1.47% algae biomass), and lower right Diet D (0.072% tocomin and 2.94% algae biomass).

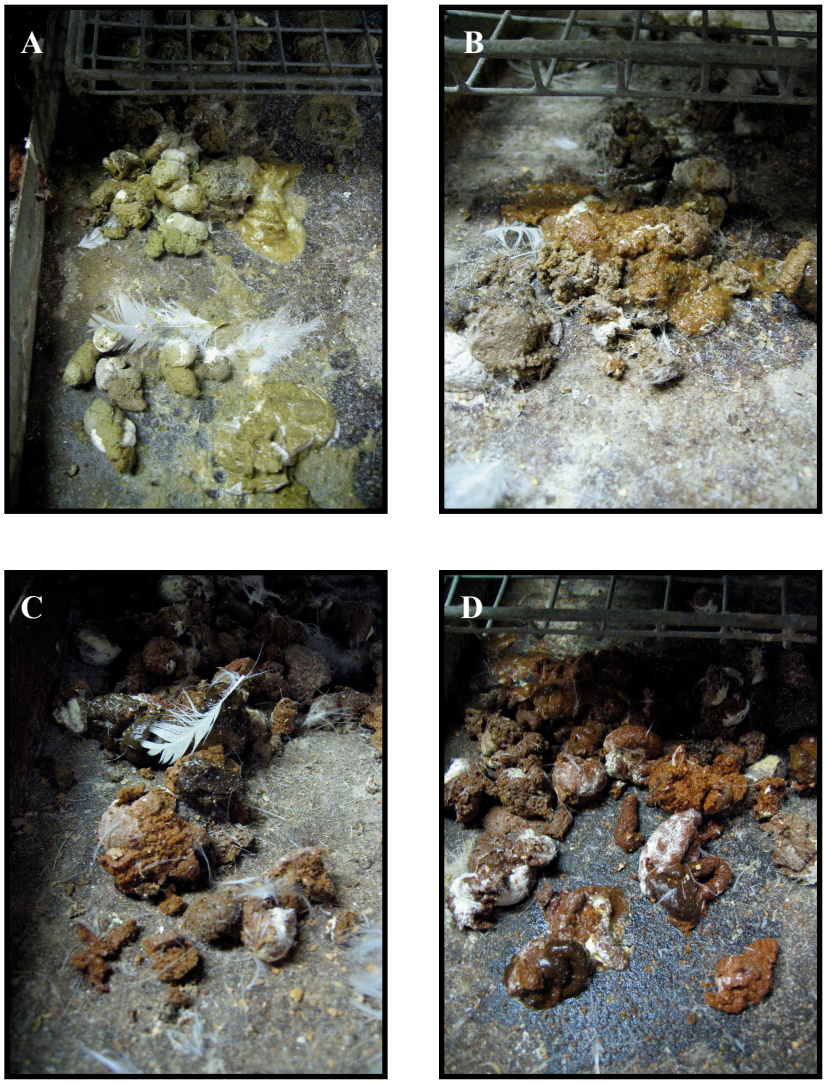


Figure 4. Images are of fecal matter from the chickens at the end of the study during week 7 illustrating the coloration changes. Upper left is diet A (no supplements), upper right Diet B (0.012% tocomin and 0.49% algae biomass), lower left Diet C (0.036% tocomin and 1.47% algae biomass), and lower right Diet D (0.072% tocomin and 2.94% algae biomass).

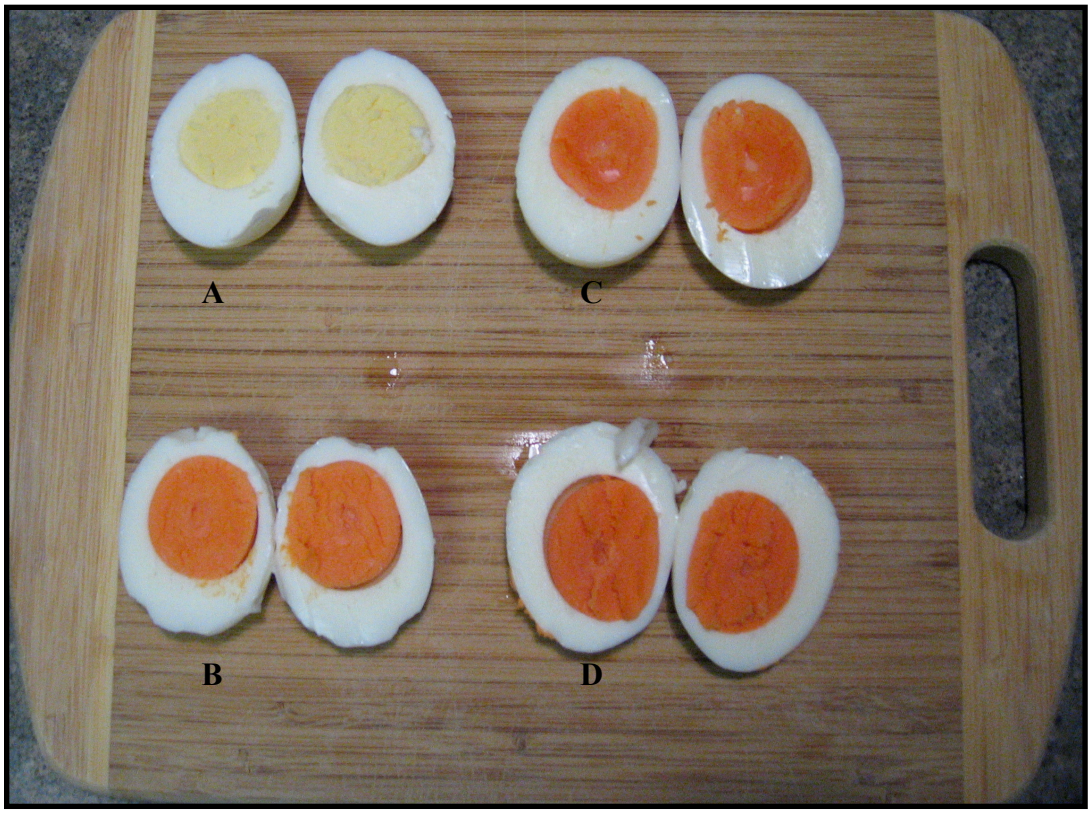


FIGURE 5. Image of hard boiled eggs used for sensory panel testing. Upper left is diet A, lower left diet B, upper right diet C, and lower right diet D. Diets are A no supplements, B enriched with 0.012% tocomin and 0.49% algae biomass; C enriched with 0.036% tocomin and 1.47% algae biomass; and D enriched with 0.072% tocomin and 2.94% algae biomass.

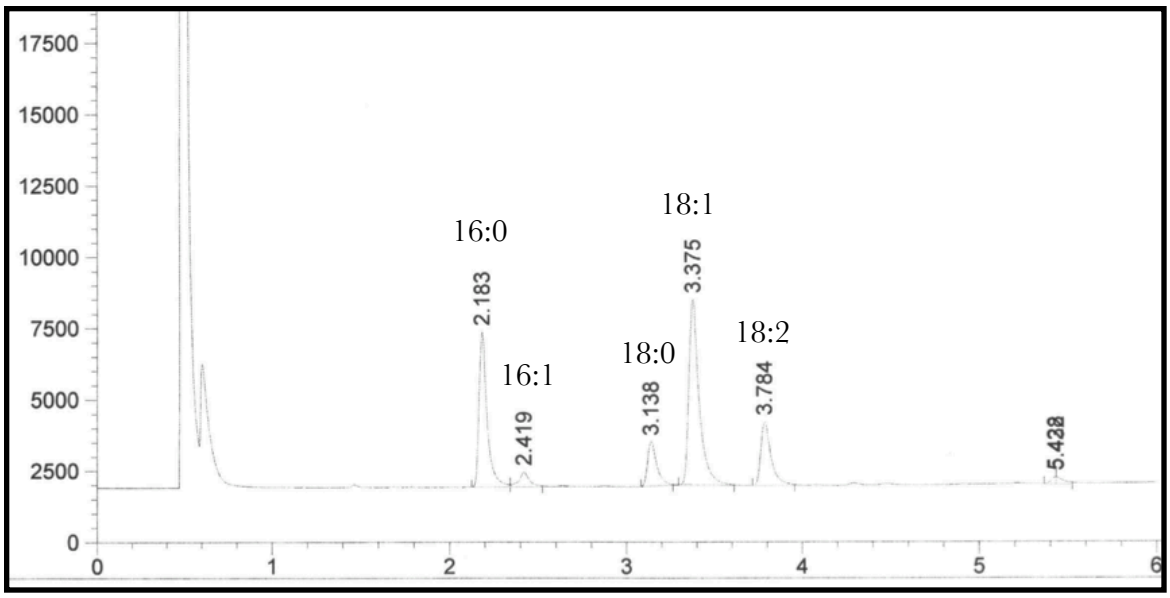


Figure 6. Typical gas chromatography chromatogram from the fatty acid methyl ester analysis of day 10 egg oil samples.

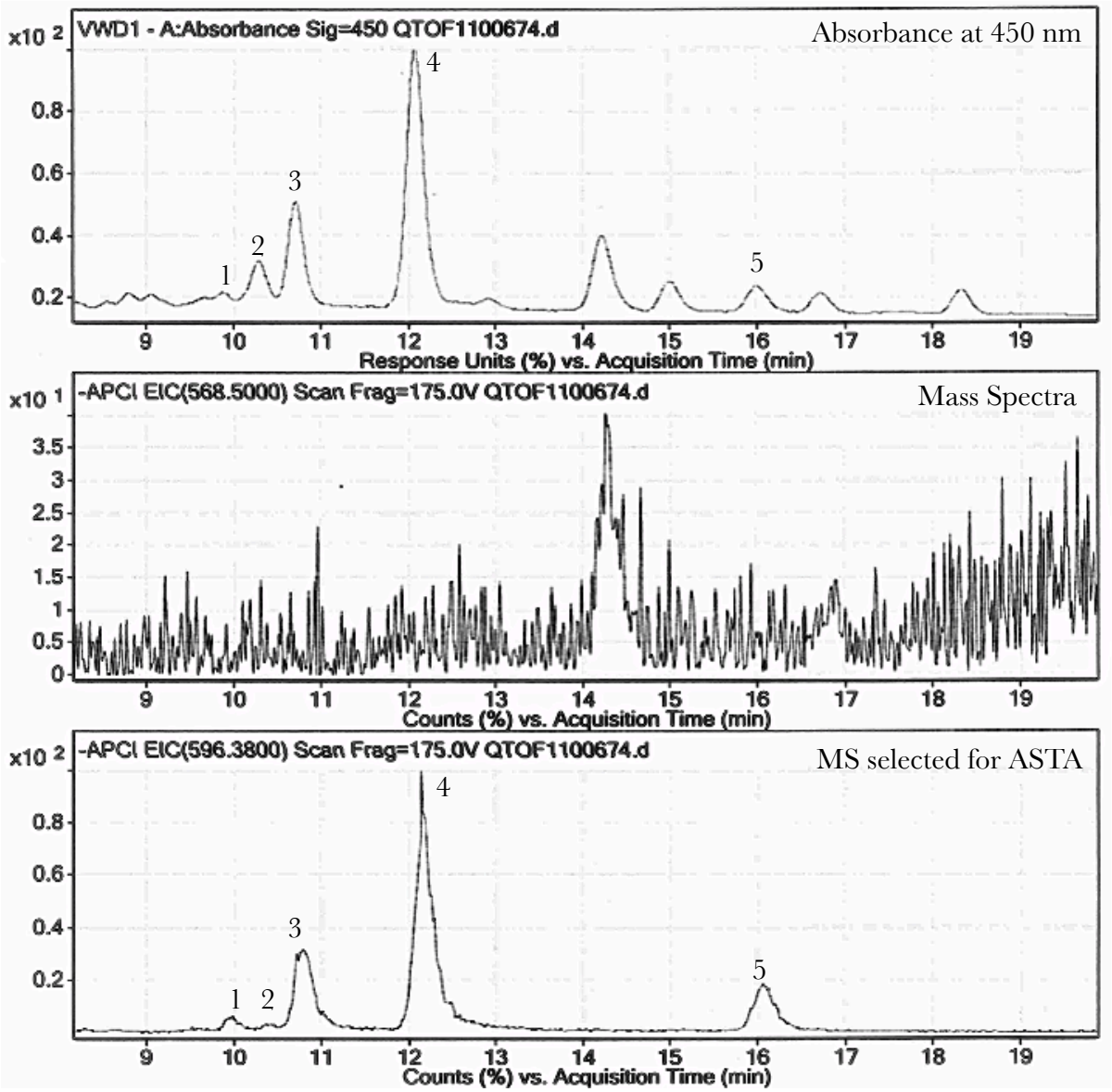


Figure 7. Liquid chromatography-mass spectra for Diet D egg sample after stabilization has occurred during the feeding study. Top three images are of the absorbance at 450 nm (same wavelength as the HPLC analysis), mass spectra, and the mass spectra selected for astaxanthin's molecular weight of 596. The five peaks are identified and their respective scans of each are shown with astaxanthin identified.

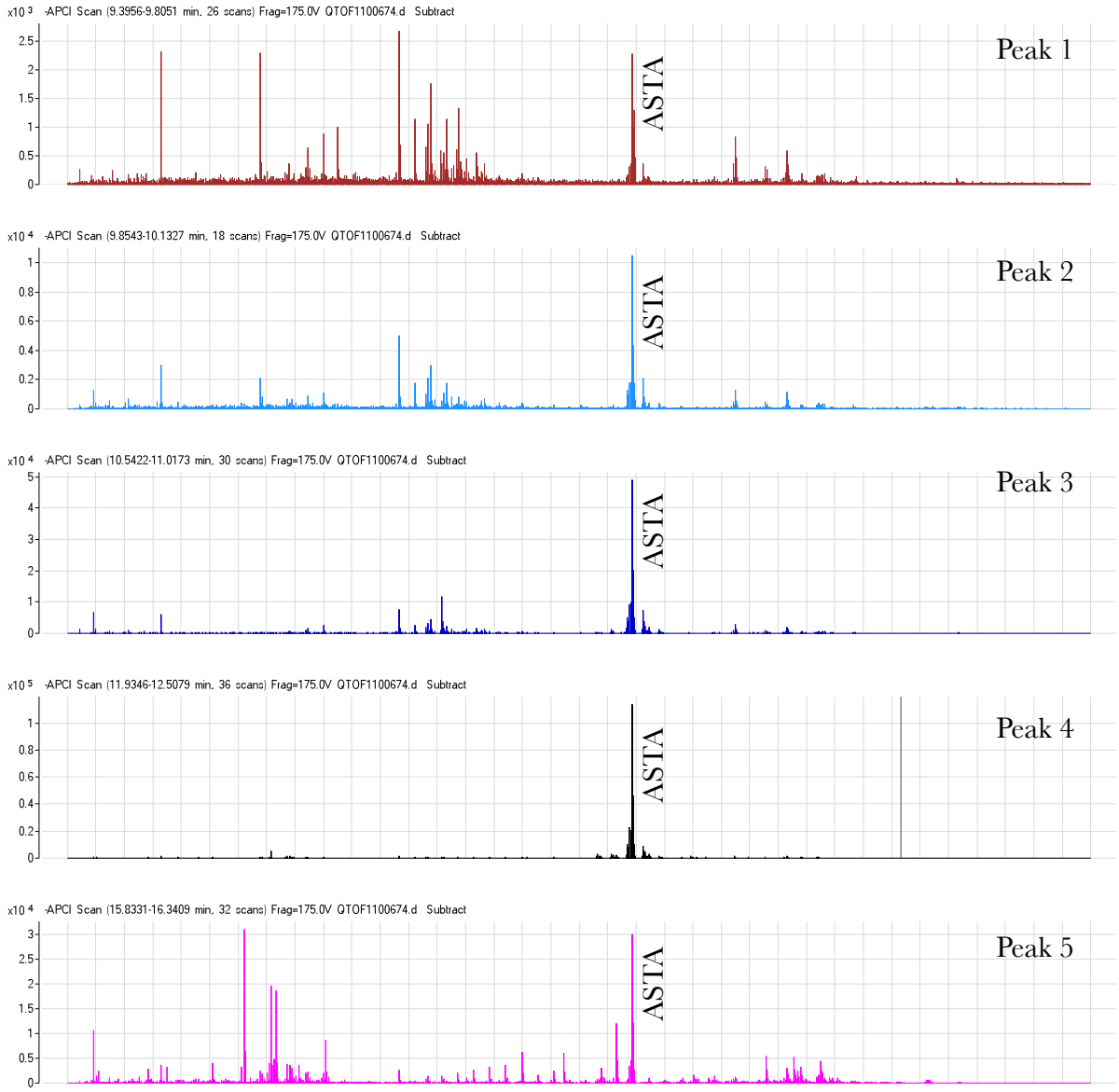


Figure 7. (continued).

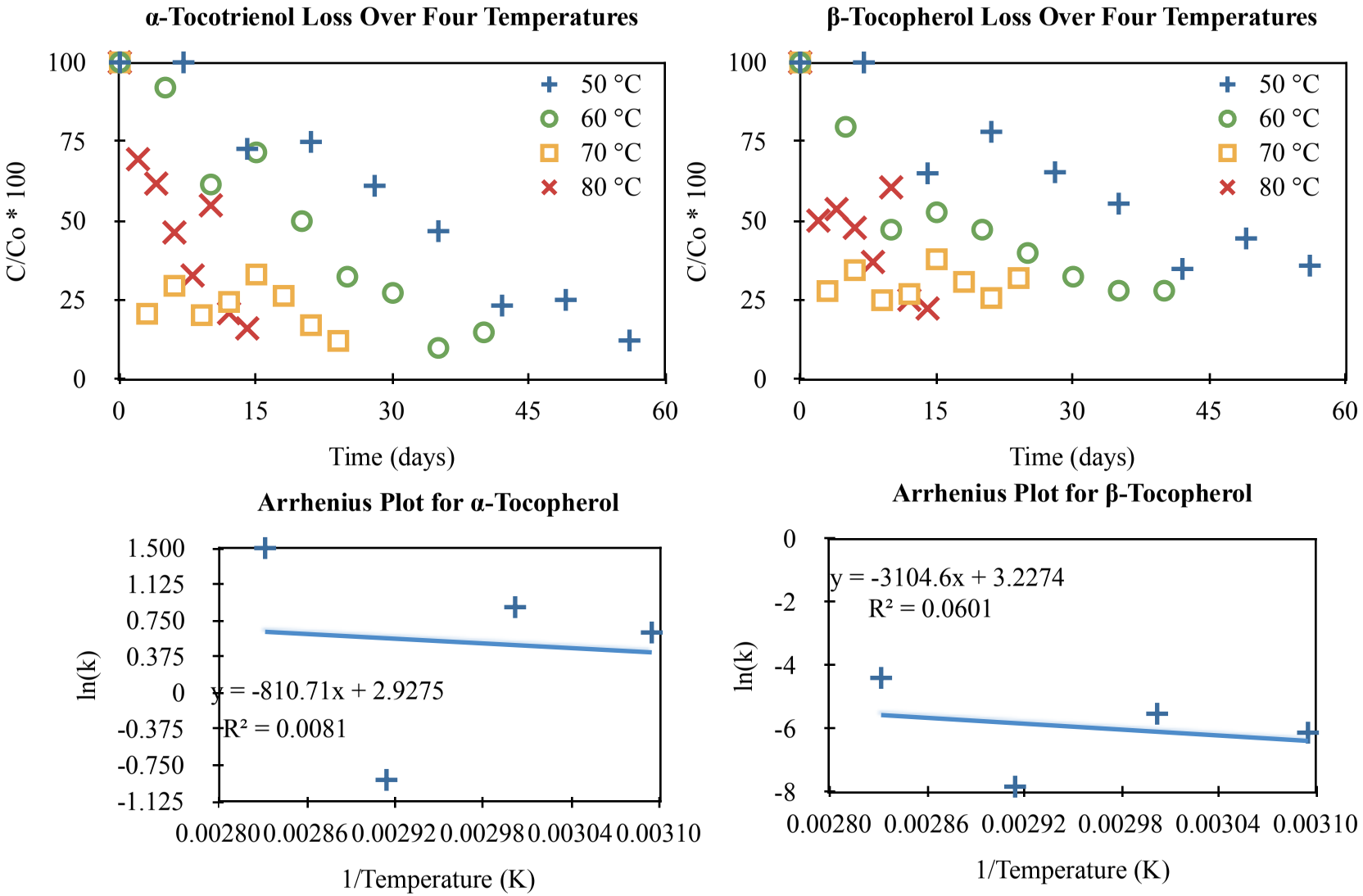
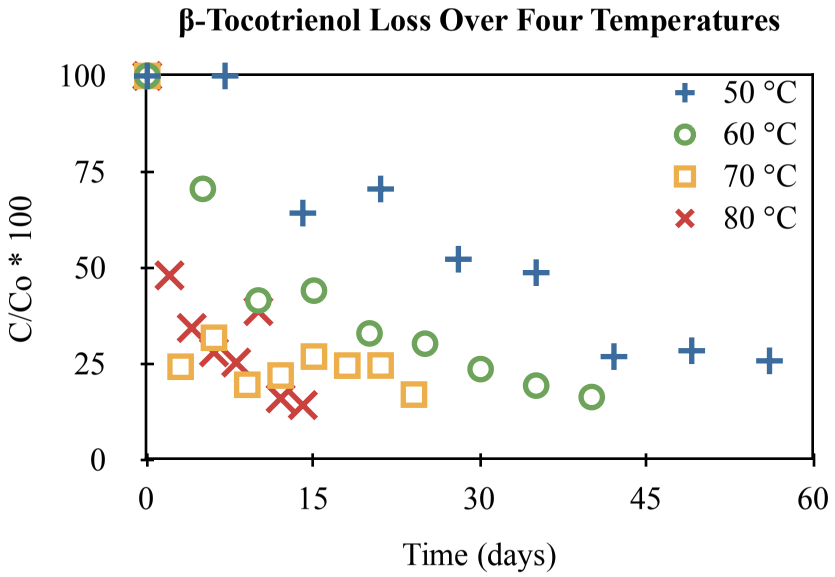
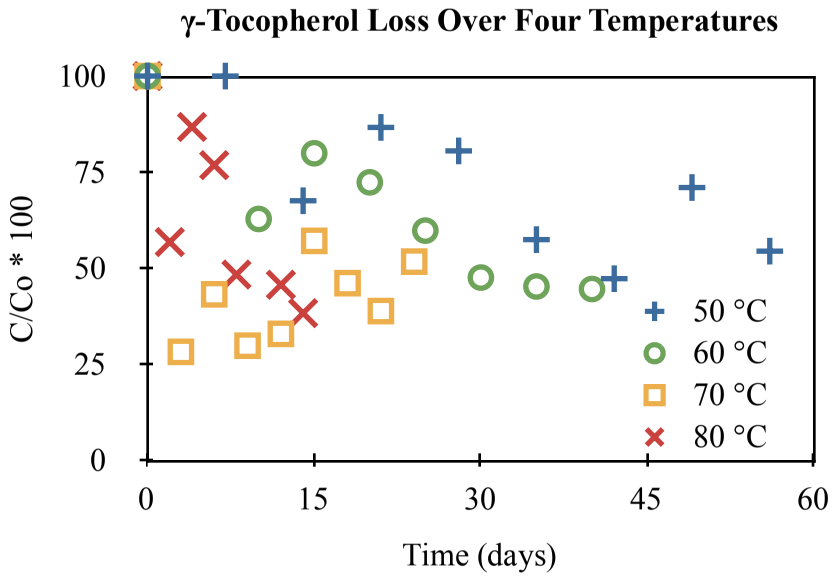
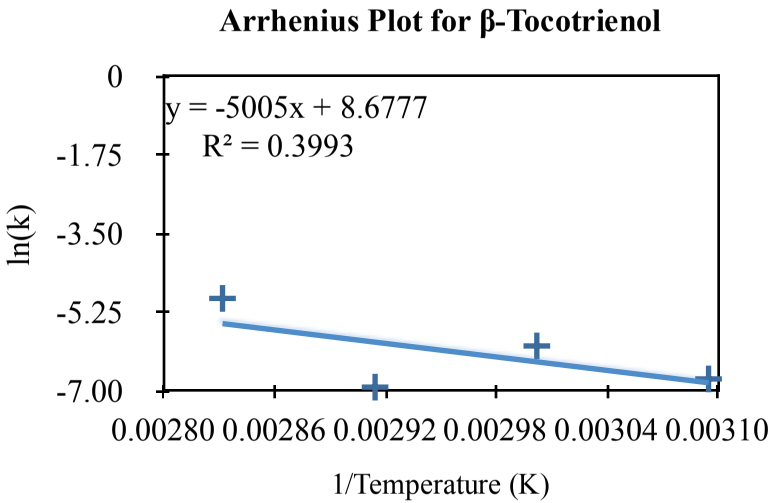


Figure 8. An example of the results from Arrhenius study of diet D (18,138 ppm) showing the loss of the additive over time at four different oven temperatures.







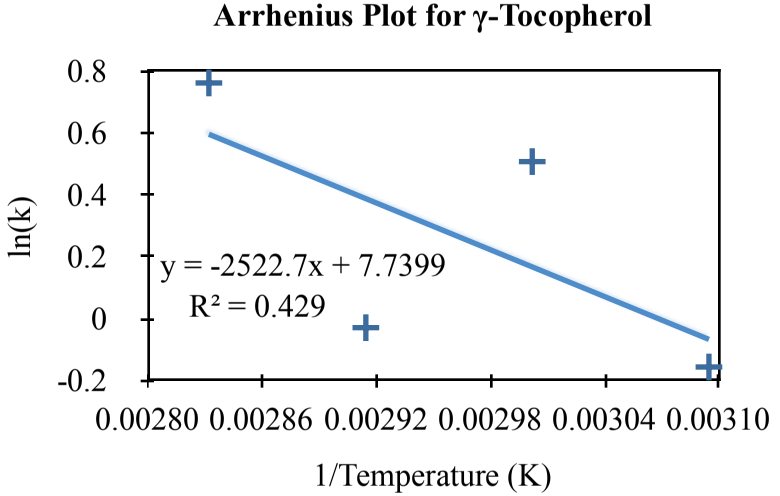
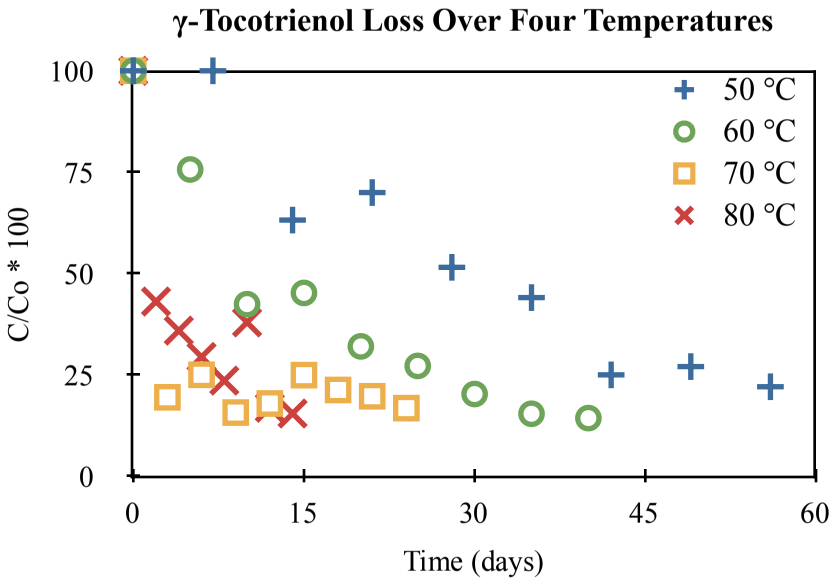
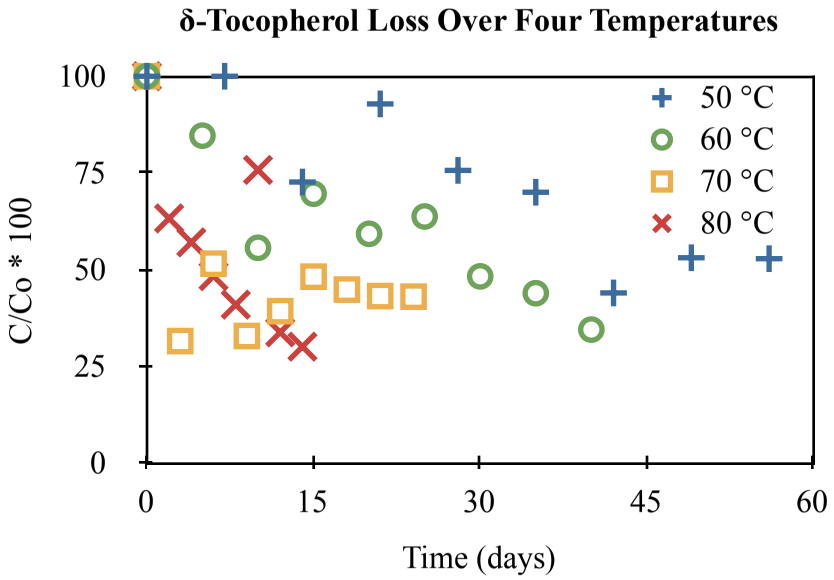
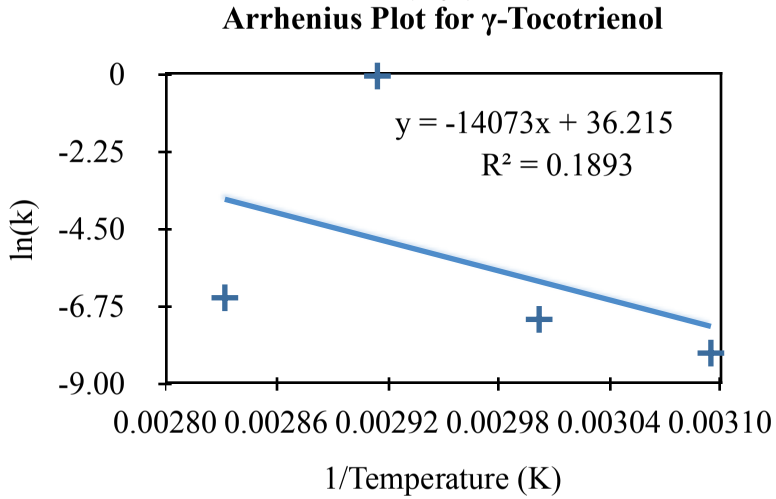
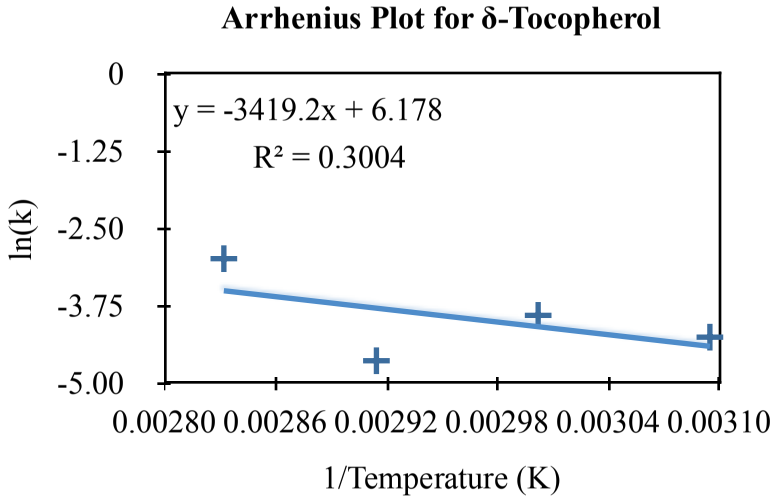


Figure 8. (continued).









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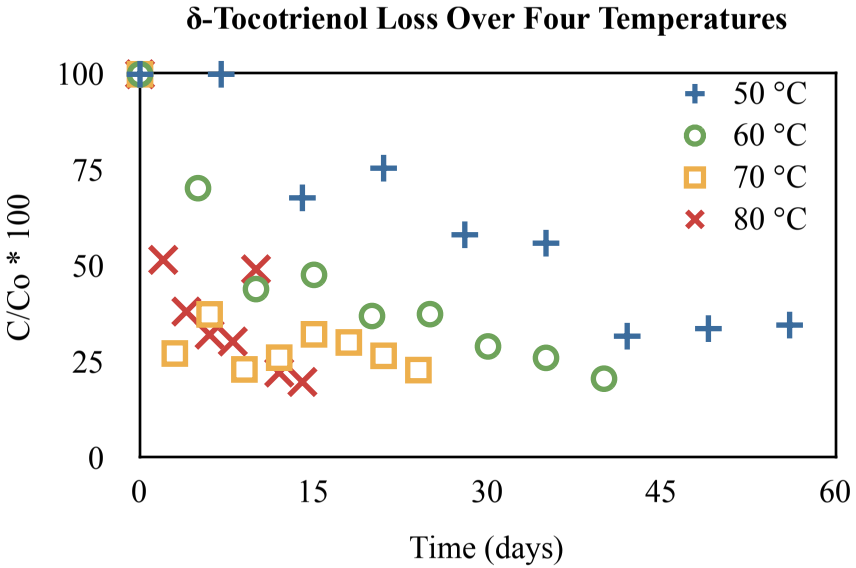

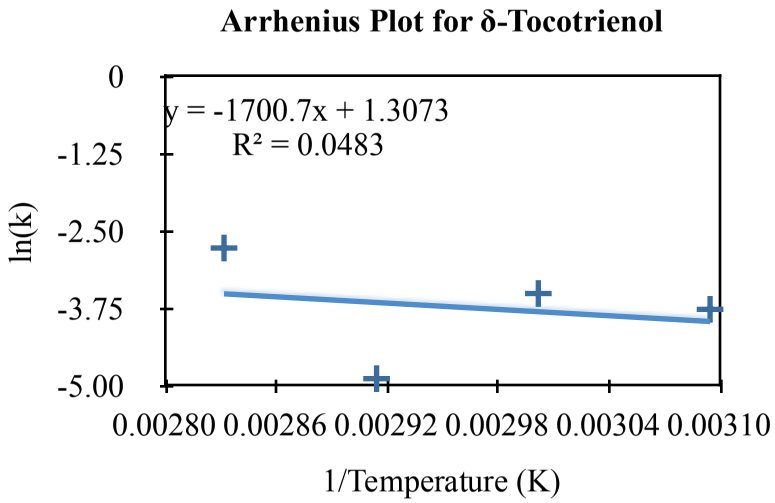

Figure 8. (continued).

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