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Increasing the Oxidative Stability of Liquid and Dried Tuna Oil-in-Water Emulsions with Electrostatic Layer-by-Layer Deposition Technology

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ω-3 Fatty acids have numerous health benefits, but their addition to foods is limited by oxidative rancidity. Engineering the interfacial membrane of oil-in-water emulsion droplets to produce a cationic and/or thick interface is an effective method to control lipid oxidation. Cationic and thick emulsion droplet interfacial membranes can be produced by an electrostatic layer-by-layer deposition technique resulting in droplets that are coated by multiple layers of emulsifiers. Tuna oil-in-water emulsion droplets coated by lecithin and chitosan produce cationic emulsion droplets that are more oxidatively stable than emulsions coated by lecithin alone. Ethylenediaminetetraacetic acid (EDTA) was able to increase the oxidative stability of emulsions stabilized with lecithin and chitosan more effectively than mixed tocopherols. The combination of EDTA and mixed tocopherols was not more effective than EDTA alone suggesting that control of prooxidant metals was the most important antioxidant technology. The production of emulsion droplets coated with lecithin and chitosan could be an excellent technology for stabilization of oxidatively unstable lipids for use in a variety of food products.

KEYWORDS: Tuna oil; ω-3 fatty acids; emulsion; chitosan; lecithin; lipid oxidation; antioxidants

INTRODUCTION

Fish oils and other marine oils such as squid oil contain high amounts of ω-3 fatty acids (ω-3 FA), especially eicosapentenoic (EPA) and docosahexenoic (DHA). EPA and DHA are considered beneficial for growth and development throughout the life cycle and may play an important role in the prevention and treatment of coronary artery disease (arteriosclerosis), hypertension, arthritis, and autoimmune disorders (*1*−*3*). Moreover, DHA is important in the development of the central nervous system of infants and it is considered to be a promising pharmaceutical because of its beneficial effects on the retina and brain (*4*). Because of their beneficial health properties, ω-3 FAs have great potential as functional food ingredients.

Utilization of oils high in ω-3 FAs in food is limited due to their high susceptibility to oxidation. Most functional foods would contain ω-3 FAs as dispersed lipids. Therefore, it is important to understand the mechanisms of oxidation of emulsified ω-3 FAs so that effective antioxidant technologies can be developed. The most commonly used method of retarding lipid oxidation in foods is by the addition of antioxidants (*5*). In emulsions, antioxidant behavior is different from the bulk oil system (*6*). The apparent activity of chain breaking antioxidants in multiphasic food systems such as emulsions is dependent on their effective concentrations in the physical location where lipid oxidation is most prevalent (e.g., lipid vs water). In bulk oils, hydrophilic antioxidants preferentially locate at the oil−air interfaces and reverse micelles where lipid oxidation rates are high. Therefore, hydrophilic antioxidants are more effective at protecting bulk lipids from oxidation than lipophilic antioxidants that are dispersed throughout the oil phase. In oil-in-water emulsions, lipophilic antioxidants would concentrate in the oil droplets or at the oil−water interfaces and inhibit lipid oxidation more effectively than hydrophilic antioxidants that can partition into the water phase.

In addition to chain breaking antioxidants, lipid oxidation in oil-in-water emulsions can be inhibited by many other mechanisms. Metal chelators such as ethylenediaminetetraacetic acid (EDTA) are effective at inhibiting lipid oxidation in oil-in-water emulsions when present at concentrations above the concentration of prooxidant metals (*7*, *8*). The oxidative stability of emulsifiled oil can also be increased by controlling emulsifier type, location, and concentration (*8*−*11*). For example, when oil-in-water emulsion droplets are surrounded by cationic emulsifiers, prooxidant metals are repelled and lipid oxidation rates decrease (*8*, *12*). An additional method to inhibit lipid oxidation in oil-in-water emulsions is to produce thick interfacial

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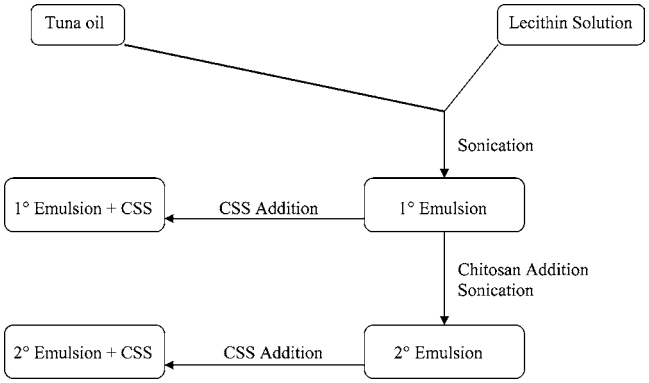

Figure 1. Preparation of primary and secondary emulsions in the presence and absence of CSS.

emulsion droplet membranes that hinder interactions between water soluble prooxidants and lipids inside the emulsion droplet (*13*).

Recent work in our laboratory has shown that oil-in-water emulsions with an improved stability to environmental stresses can be produced using an electrostatic layer-by-layer deposition technique that produces oil droplets that are coated by multiple layers of emulsifiers (*14*, *15*). The production of this emulsions system may prove to be an effective means of improving the oxidation stability of emulsified fish oil since both the emulsion droplet charge and the thickness can be controlled. Because ω-3 FAs could find utilization in functional foods as both liquid and dried ingredients, the objective of this study was to examine the oxidative stability of ω-3 FA in emulsions coated by lecithin alone or by lecithin−chitosan before and after drying. The ability of the antioxidants mixed tocopherol and EDTA on the stability of the emulsions was also examined.

MATERIALS AND METHODS

Materials. Powdered chitosan (medium molecular weight, deacetylation, 75−85%) was purchased from Aldrich Chemical Co. (St. Louis, MO). Powdered lecithin (moisture, 1 wt %; consist primarily of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol) was donated by ADM-Lecithin (Decatur, IL). Corn syrup solids (CSSs; total solids, 97.2 wt %; moisture, 2.8 wt %; ash, 0.2 wt %) were obtained from Roquette America. Inc. (Keokuk, IA). Degummed, bleached, and deodorized tuna oil was obtained from Maruha Co. [Utsunomiya, Japan; 16 wt % EPA; 14.1 wt % DHA; lipid hydroperoxides, 0.35 ± 0.01 mmol/kg oil; thiobarbituric acid reactive substances (TBARS), 0.12 ± 0.01 mmol/kg oil; no detectable tocopherols]. Mixed tocopherol homologues (Covi-ox T-70, 14% α-tocopherol, 2% β-tocopherol, 60% γ-tocopherol, and 24% δ-tocopherol) were obtained from Cognis Corp. (Cincinnati, OH). Disodium EDTA was purchased from the Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade or better. Distilled and deionized water were used for the preparation of all solutions.

Methods. *Solution Preparation*. A stock buffer solution was prepared by dispersing 2 mM sodium acetate and 98 mM acetic acid in water and then adjusting the pH to 3.0. An emulsifier solution was prepared by dispersing 3.5 wt % lecithin into the stock buffer solution. The emulsifier solution was sonicated for 1 min at a frequency of 20 kHz, an amplitude of 70%, and a duty cycle of 0.5 s (model 500, sonic disembrator, Fisher Scientific, Pittsburgh, PA) to disperse the emulsifier. The pH of the solution was adjusted to 3.0 using HCl and/or NaOH, and then, the solution was stirred for about 1 h to ensure complete dispersion of the emulsifier. A chitosan solution was prepared by dissolving 1.5 wt % powdered chitosan into the stock buffer solution. A CSS stock solution was prepared by dispersing 50 wt % CSS into the stock buffer solution.

Emulsion Preparation (**Figure 1**). A coarse tuna oil-in-water emulsion was made by blending 15 wt % tuna oil with 85 wt % emulsifier (lecithin) solution using a high-speed blender (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland). To produce the primary emulsion, the coarse emulsion was sonicated for 2 min at a frequency of 20 kHz, an amplitude of 70%, and a duty cycle of 0.5 (model 500, sonic disembrator, Fisher Scientific). This primary emulsion was mixed with the chitosan and buffer solution to form a secondary emulsion with a final concentration of 5 wt % tuna oil, 1 wt % lecithin, and 0.2 wt % chitosan. Any flocs formed in the secondary emulsion were disrupted by sonication for 2 min at a frequency of 20 kHz, an amplitude of 70%, and a duty cycle of 0.5 (*14*). The electrical charge on the droplets (determined by ζ-potential, ref *14*) changed from negative (−52 mV) for the primary emulsion to positive (+57 mV) for the secondary emulsion when the chitosan was present, which indicates that the cationic chitosan molecules adsorbed to the surface of the anionic lecithin-coated emulsion droplets (*14*, *16*). CSSs were added to the primary and secondary emulsions by mixing with the CSS stock solution to obtain a final concentration of 5 wt % tuna oil, 1 wt % lecithin, 0.2 wt % chitosan, and 20% CSSs. The pH of the final emulsions was adjusted to 3.0 using HCl or NaOH if necessary.

For antioxidant evaluation, four kinds of secondary (lecithin−chitosan-coated emulsion droplets) emulsions were prepared as follows: (i) control (without antioxidants); (ii) mixed tocopherols (100, 500, and 1000 ppm), (iii) EDTA (12, 60, or 120 M; 90, 448, or 896 ppm, respectively), and (iv) mixed tocopherols plus EDTA (500 ppm mixed tocopherol and 12, 60, or 120 M EDTA). In samples with mixed tocopherols, the antioxidant was added to the tuna oil prior to emulsion preparation whereas EDTA was added directly to the secondary emulsion after emulsion preparation.

Freeze Drying. The emulsion samples (1 mL) were transferred into microcentrifuge tubes, which were frozen by placing them overnight in a −80 °C freezer. A laboratory scale freeze-drying device (Virtis, the Virtis Company, Gardiner, NY) was used to dry the frozen emulsions at room temperature using a vacuum pressure of 1 atm for 48 h.

Lipid Oxidation Measurement. To monitor lipid oxidation during storage, emulsions (10 mL) were placed in lightly sealed screw-cap test tubes or microcentrifuge tubes and allowed to oxidize at 37 or 55 °C in the dark. Oxidative stability was evaluated by measuring lipid hydroperoxide and TBARS. Lipid hydroperoxide was measured by a modifiled method of Mancuso et al. (*8*). Lipids in 0.3 mL of emulsion were extracted by mixing with 1.5 mL of isooctane-2-propanal (3:1 v:v) and vortexing three times for 10 s each followed by centrifugation for 2 min at 3400*g* (Centrific Centrifuge, Fisher Scientific, Fairlawn, NJ). Next, the 0.2 mL of organic phase was added to 2.8 mL of methanol−butanol (2:1 v:v), followed by 15 L of thiocyanate solution (3.94 M) and 15 L of ferrous iron solution (prepared by mixing 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$ in acidic solution). The solution was vortexed, and the absorbance at 510 nm was measured after 20 min. Lipid hydroperoxide concentrations were determined using a cumene hydroperoxide standard curve.

TBARS were measured according to Mei et al. (*17*). A TBA solution was prepared by mixing 15 g of trichoroacetic acid, 0.375 g of TBA, 1.76 mL of 12 N HCl, and 82.9 mL of H_2O. One hundred milliliters of TBA solution was mixed with 3 mL of 2% butylated hydroxytoluene in ethanol, and 2 mL of this solution was mixed with 1 mL of emulsion diluted 30 times in water. The mixture was vortexed and heated in a boiling water bath for 15 min, cooled to room temperature, and centrifuged at 3400*g* for 25 min. Absorbance was measured at 532 nm. Concentrations of TBARS were determined from standard curves prepared using 1,1,3,3-tetraethoxypropane. For measurement of oxidative stability in freeze-dried emulsions, the dried emulsions were reconstituted with 1 mL of acetate buffer and then the lipid hydroperoxide and TBARS values were carried out as described above.

Statistics. All data represent the mean of six measurements of two different trials, and results are reported as the means and standard derivations of these measurements. The data were subjected to the analysis of variance (ANOVA) univariate method ($P \geq 0.05$). Comparison of means after the ANOVA test was performed using the Duncan's multiple range test.

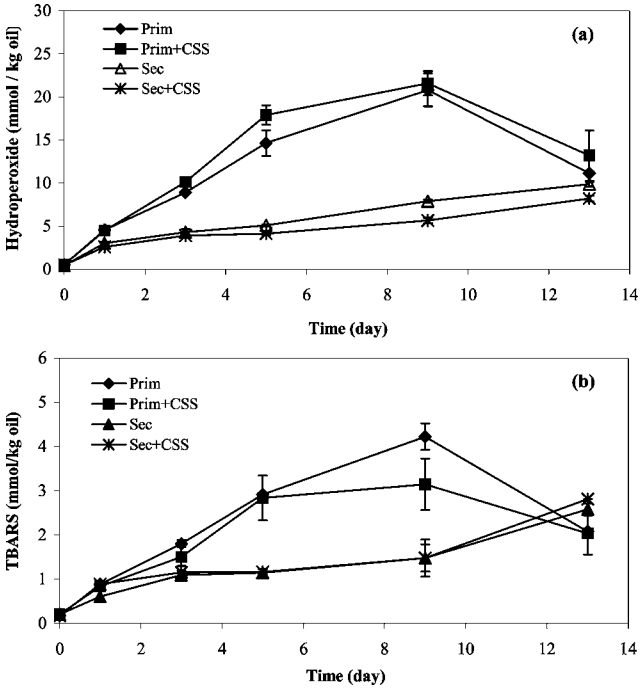

Figure 2. Formation of lipid hydroperoxides (**a**) and TBARS (**b**) in primary (lecithin alone) and secondary (lecithin + chitosan) emulsions in the absence and presence of 20 wt % CSSs during storage at 37 °C. Data markers represent means ($n = 6$) ± standard deviations.

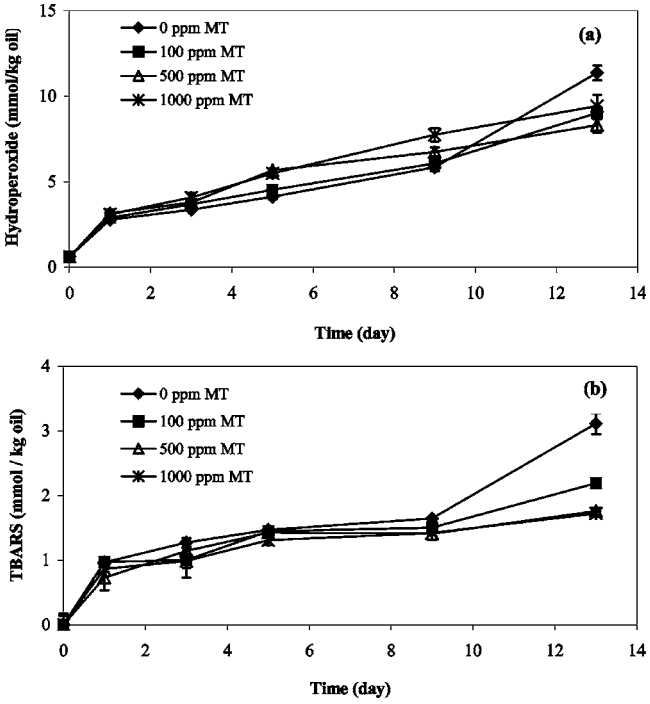

Figure 3. Formation of lipid hydroperoxides (**a**) and TBARS (**b**) in secondary emulsions (lecithin + chitosan) containing 0, 100, 500, and 1000 ppm mixed tocopherol during storage at 37 °C. Data markers represent means ($n = 6$) ± standard deviations.

RESULTS AND DISCUSSION

Oxidative Stability of ω-3 Fatty Acids in Oil-in-Water Emulsions with Multiple Interfacial Layers. Figure 2 shows a comparison of oxidation rates in tuna oil-in-water emulsions stabilized by lecithin alone (primary emulsion) or lechitin plus chitosan (secondary emulsion). Lipid oxidation markers (hydroperoxide and TBARS) were measured as a function of time (0−13 days) in order to monitor differences in oxidation kinetics. The oxidative stability of the primary tuna oil emulsion was less than the secondary emulsion as determined by both lipid hydroperoxide (at day 3; **Figure 2a**) and TBARS (at day 5; **Figure 2b**). Transition metals, in particular iron, are major prooxidatives in oil-in-water emulsions due to their ability to decompose lipid hydroperoxide into free radicals (*8*). However, the prooxidant activity of iron is related to its ability to interact with the droplet surface. The low amount of oxidation observed in secondary emulsions suggests that the positively charged emulsion droplets (+57 mV) inhibited iron−lipid interactions, presumably by decreasing the ability of iron to interact with the emulsion interface through electrostatic repulsive forces. Conversely, the greater oxidation rates in primary emulsion, negatively charged emulsion (−52 mV), could be due to increased interfacial iron concentrations because of attractive forces (*12*). Inhibition of metal-catalyzed lipid oxidation by positively charged and acceleration by negatively charge emulsion has also been observed in corn oil and salmon oil emulsions (*8*, *11*, *12*).

Emulsion droplets are surrounded by a membrane of emulsifier molecules. These membranes not only prevent the droplets from coalescing but also may protect lipids from oxidation by acting as a barrier to the penetration and diffusion of molecular species that promote lipid oxidation into the droplets (*18*). Therefore, lipid oxidation can also be inhibited by thick interfacial membranes as was reported by Silvestre et al. (*13*). The observed increased oxidative stability of the secondary emulsion as compared to the primary emulsion (**Figure 2**) could therefore be due to its thicker interfacial membrane that could decrease interactions between lipid and aqueous phase prooxidants.

Drying a lipid emulsion requires the presence of a bulking agent. These bulking agents are often carbohydrates such as CSSs. Sugars and polysaccharides have been reported to alter lipid oxidation rates in oil-in-water emulsions (*19*). Therefore, the impact of bulking agents on the rate of lipid oxidation in liquid primary and secondary emulsions, lipid hydroperoxides, and TBARS were determined in the presence and absence of CSSs. In both the primary and the secondary emulsions, CSSs had no major impact on lipid oxidation rates (**Figure 2**).

Influence of Mixed-Tocopherol Isomers on the Oxidation of the Secondary Emulsions. While lipid oxidation was slower in the secondary emulsions, lecithin−chitosan stabilized tuna oil-in-water emulsions as compared to the primary emulsions (**Figure 2**); oxidation markers were still observed to increase over time. Therefore, the ability of varying concentrations of the lipid soluble chain breaking antioxidant, mixed tocopherol (0−1000 ppm), to further decrease the lipid oxidation was determined. Mixed tocopherols were tested as the chain breaking antioxidant because lipophilic antioxidants have been reported to be more effective than hydrophilic antioxidants in oil-in-water emulsions (*6*) and because mixed tocopherols have been found to be more effective than α-tocopherol (*20*, *21*). Because CSSs have no impact on lipid oxidation (**Figure 2**), they were included in the emulsion since they will be used in later experiments on dried emulsions. None of the mixed tocopherol concentrations tested were able to inhibit the oxidation of the secondary tuna oil-in-water emulsion until 13 days of storage (**Figure 3a**,**b**). At day 13, all three tocopherol concentrations inhibited lipid hydroperoxide formation in a similar manner (**Figure 3a**) with inhibitions ranging from 17 to 27% as compared to the control at day 13. At day 13, differences were also seen in the effectiveness of mixed tocopherols when lipid oxidation was measured with TBARS with 500 and 1000 ppm

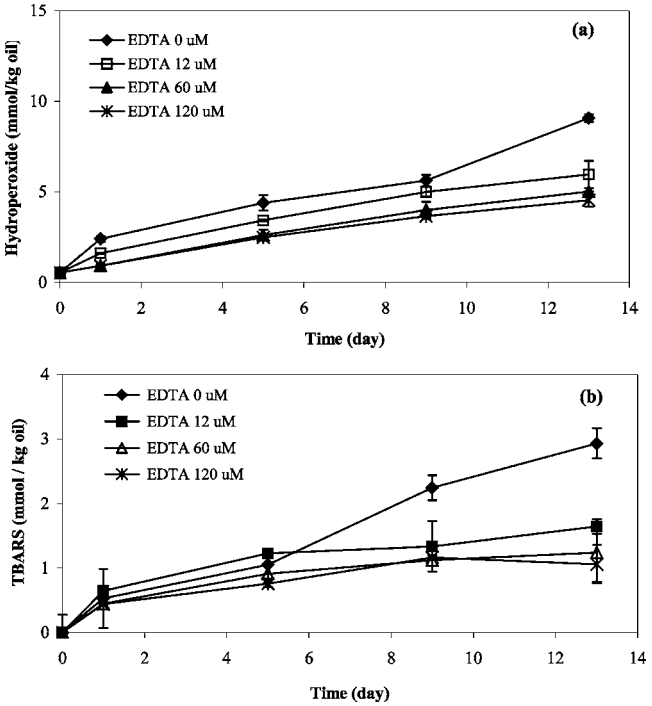

Figure 4. Formation of lipid hydroperoxides (**a**) and TBARS (**b**) in secondary emulsions (lecithin + chitosan) containing 0, 12, 60, and 120 M EDTA during storage at 37 °C. Data markers represent means ($n = 6$) ± standard deviations.

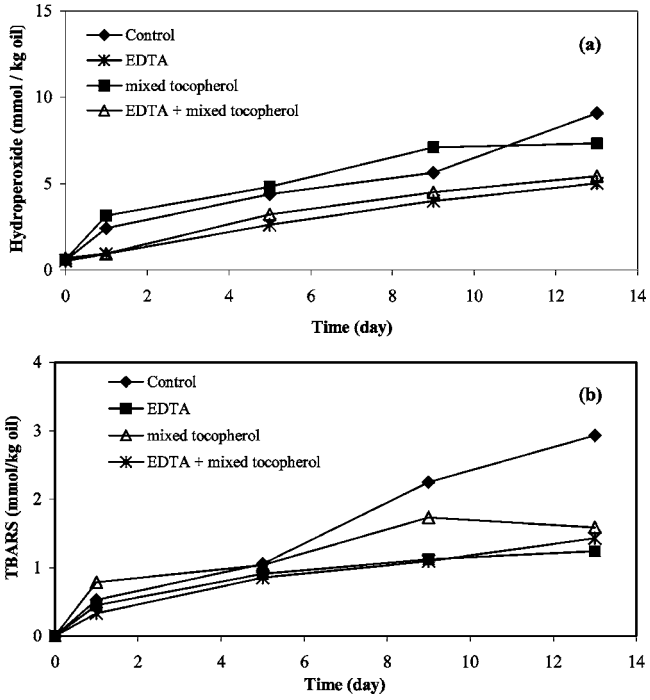

Figure 5. Formation of lipid hydroperoxides (**a**) and TBARS (**b**) in secondary emulsions (lecithin + chitosan) containing 500 ppm mixed tocopherol isomers and/or 60 M EDTA during storage at 37 °C. Data markers represent means ($n = 6$) ± standard deviations.

mixed tocopherols inhibiting lipid oxidation more effectively ($p < 0.05$) than 100 ppm (**Figure 3b**). No difference in effectiveness in the ability of 500 and 1000 ppm mixed tocopherols to inhibit TBARS formation was observed with TBARS being 43−45% lower than the control at day 13.

Influence of EDTA on the Oxidation of the Secondary Emulsions. EDTA has been shown to be a very effective inhibitor of lipid oxidation in oil-in-water emulsions stabilized by cationic emulsifiers (*22*). Therefore, the effect of varying concentrations of EDTA (0−120 M) on lipid oxidation in secondary tuna oil-in-water emulsions stabilized by lecithin and chitosan was evaluated at 37 °C for 13 days in the dark. All concentrations of EDTA were able to decrease lipid hydroperoxide concentrations after 1 day of storage ($p < 0.05$; **Figure 4a**). EDTA at 60 and 120 μM was more effective than 12 μM EDTA. No differences in lipid hydroperoxide concentrations were observed between the samples containing 60 and 120 μM EDTA ($p > 0.05$). All EDTA concentrations decreased TBARS after 9 days of storage (**Figure 4b**). EDTA at 60 and 120 M was more effective in retarding TBARS formation than 12 M EDTA after 13 days of storage ($p < 0.05$). There was no significant difference ($P \geq 0.05$) in TBARS concentrations in emulsions containing 60 and 120 M EDTA. At day 13, lipid hydroperoxides (**Figure 4a**) and TBARS concentrations (**Figure 4b**) were 34−50% and 44−64% lower in EDTA-treated emulsions, respectively. In general, EDTA inhibited lipid oxidation in the secondary tuna oil-in-water emulsions more effectively than mixed tocopherols as indicated by both hydroperoxide and TBARS concentrations (**Figures 4** and **5**). These results are similar to those reported by Jacobsen and co-workers (*23*, *24*) and Djordjevic et al. (*20*) who reported that EDTA, but not tocopherols, was able to inhibit lipid oxidation in oil-in-water emulsions. These results suggest that endogenous transition metals that are naturally present in the oil, surfactant, and/or water are important prooxidants in the secondary tuna-oil-in-water emulsions used in this study.

Influence of the Combination of Mixed Tocopherol and EDTA on the Oxidation of the Secondary Emulsions. The combination of a metal chelator and lipid soluble chain breaking antioxidant can be very effective in controlling lipid oxidation in oil-in-water emulsions (*20*). To determine if the combination of chelator and lipid soluble antioxidant was effective in the lecithin−chitosan-stabilized tuna oil-in-water emulsions, mixed tocopherol (500 ppm) and EDTA (60 M) were added alone and in combination and the emulsions were incubated at 37 °C for 13 days in the dark. As seen previously (**Figure 3**), mixed tocopherols were only able to decrease lipid hydroperoxides at the end of the incubation period (**Figure 5**). EDTA was again more effective than mixed tocopherols. In the presence of EDTA alone, the formation of lipid hydroperoxide was decreased after 1 day of storage while the inhibition of TBARS formation was not observed until 9 days of storage. No differences in lipid hydroperoxides or TBARS were observed in emulsions containing EDTA alone or EDTA + mixed tocopherols. These results suggest that control of the reactivity of prooxidant transition metals is more effective than inactivating free radicals in the emulsion droplet.

Influence of Freeze Drying EDTA on the Oxidation of the Secondary Emulsions. To determine if the properties of the emulsion droplet interfacial membrane also influence lipid oxidation in dried emulsions, both primary (lecithin) and secondary (lecithin−chitosan) tuna oil-in-water emulsions were freeze-dried and lipid hydroperoxides and TBARS were measured for 14 days during storage in the dark. As seen with the liquid emulsion, both lipid hydroperoxides and TBARS were much lower in the secondary emulsion (**Figure 6a**,**b**). The ability of mixed tocopherol (500 ppm) and/or EDTA (12 M) to further increase the oxidative stability of the freeze-dried secondary tuna oil-in-water emulsion was also evaluated (**Figure 7**). Because the previous study showed that lipid hydroperoxides and TBARS concentrations were very low in the freeze-dried secondary emulsions without antioxidant stored at 37 °C (**Figure**

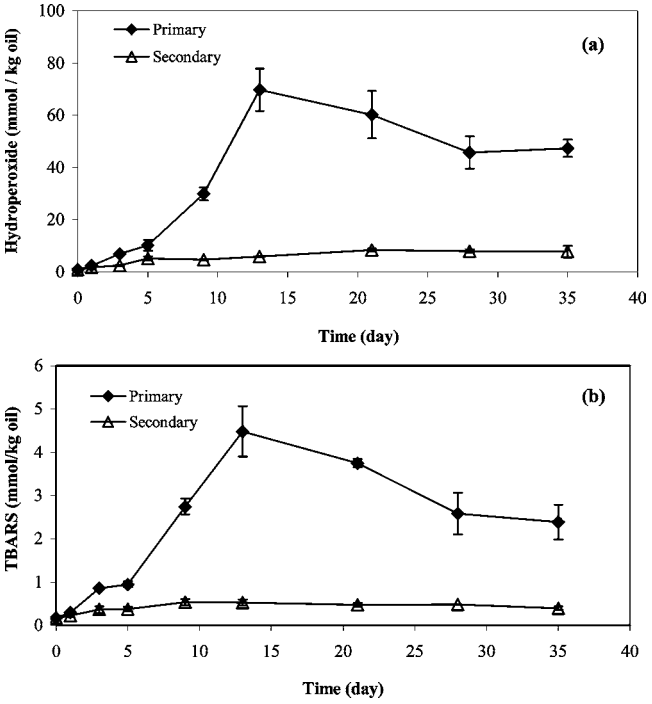

Figure 6. Formation of lipid hydroperoxides (**a**) and TBARS (**b**) in freeze-dried primary (lecithin alone) and secondary (lecithin + chitosan) emulsions during storage at 37 °C. Data markers represent means ($n = 6$) ± standard deviations.

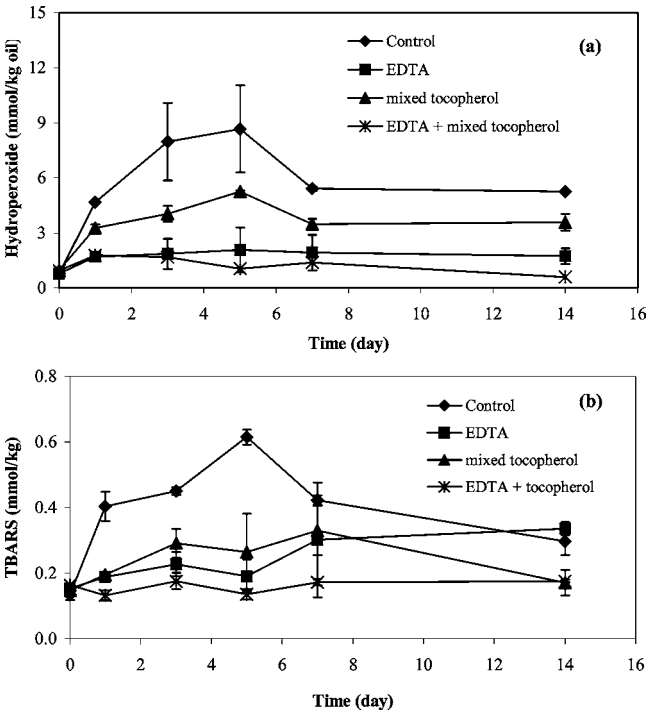

Figure 7. Formation of lipid hydroperoxides (**a**) and TBARS (**b**) in freeze-dried secondary emulsions (lecithin + chitosan) containing 500 ppm mixed tocopherols and/or 12 M EDTA during storage at 55 °C. Data markers represent means ($n = 6$) ± standard deviations.

5), incubation of freeze-dried emulsions containing antioxidants was performed at 55 °C. As compared to control freeze-dried emulsions (no antioxidant), addition of mixed tocopherols or EDTA, alone, caused a significant ($p < 0.05$) decrease in lipid hydroperoxide concentrations ranging from 29.8 to 49.6% and 63.7 to 76.1% for mixed tocopherols or EDTA, respectively, during the first 5 days of storage (**Figure 7a**). EDTA was able to decrease lipid hydroperoxides better than mixed tocopherols throughout storage ($p < 0.5$). Mixed tocopherols or EDTA also decreased TBARS concentrations in the freeze-dried emulsions from 1 to 5 days of storage (**Figure 7b**). No statistical difference was observed in TBARS concentrations in samples containing mixed tocopherols or EDTA during the first 5 days of storage. The combination of EDTA and mixed tocopherols hydroperoxide was more effective than mixed tocopherol alone in inhibiting lipid hydroperoxide formation during the entire incubation period ($p < 0.05$; **Figure 7a**). No differences in lipid hydroperoxide concentrations were observed between the EDTA alone and the EDTA/mixed tocopherol combination until 14 days of storage ($p < 0.05$). The combination of mixed tocopherols and EDTA tended to have lower TBARS concentrations than the individual antioxidants; however, these differences were not significant (**Figure 7b**).

In conclusion, this study has shown that the oxidative stability of both liquid and dried tuna oil-in-water emulsion droplets coated by a lecithin−chitosan multilayer system is higher than emulsion droplets coated with only lecithin. The improved oxidative stability of the emulsion droplets is likely due to its cationic nature that can repel prooxidative metals and possibly form a thicker interfacial region that could decrease interactions between lipids and water soluble prooxidants. EDTA was the most effective of the antioxidants tested at increasing the oxidative stability of both the liquid and the freeze-dried emulsions stabilized with lecithin and chitosan suggesting that control of prooxidant metals was the most effective method to prevent lipid oxidation. These data suggest that tuna oil-in-water emulsions stabilized by lecithin−chitosan membranes may be used to produce oxidatively and physically stable ω-3 FAs in functional foods.

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