JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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New Insights into the Role of Iron in the Promotion of Lipid Oxidation in Bulk Oils Containing Reverse Micelles

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ABSTRACT: Formation of physical structures, known as association colloids, in bulk oils can promote lipid oxidation. However, the cause of this accelerated lipid oxidation is unknown. Therefore, the aim of this study was to investigate whether transition metals were important prooxidants in bulk oils containing reverse micelles produced from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and water. The Fe(III) chelator deferoxamine (DFO) increased the oxidative stability of stripped soybean oil (SSO) containing reverse micelles from 2 to 7 days. Because phosphatidylcholine (1,2-dibutyl-*sn*-glycero-3-phosphocholine) that does not form reverse micelles is not prooxidative, these results suggest that the prooxidant activity of DOPC reverse micelles could be due to their ability to concentrate both endogenous iron and lipid hydroperoxides at the water−lipid interface, thereby increasing the ability of iron to decompose lipid hydroperoxides. DFO was also able to improve the activity of α-tocopherol and Trolox in SSO containing DOPC reverse micelles increasing the lag phase from 2 to 11 and 13 days, respectively. DOPC reverse micelles decreased iron-promoted α-tocopherol and Trolox decomposition and decreased the ability of α-tocopherol and Trolox to decrease Fe(III) concentrations. Overall, these results suggest that iron is an important prooxidant in bulk oils containing reverse micelles; therefore, finding ways to control iron reactivity in association colloids could provide new technologies to increase the oxidative stability of oils.

KEYWORDS: *bulk oil, lipid oxidation, transition metal, antioxidants, chelators, rancidity*

■ INTRODUCTION

Lipid oxidation is one of the major factors limiting the shelf life of bulk oils, because it adversely affects flavor and quality by forming a very complex mixture of lipid hydroperoxides, fatty acid chain-cleavage products, and polymeric materials.[1,2] In addition, lipid oxidation presents food safety concerns as low concentrations of lipid oxidation products, such as 4-hydroxynonenal (4-HNE) and malonaldehyde (MDA), can promote inflammation, atherosclerosis, neurodegenerative diseases, and cancer.[3]

The current dietary trends to consume healthier oils, such as omega-3 fatty acids, is a major challenge for food scientists because these oils are extremely susceptible to lipid oxidation.[4] Therefore, new strategies to prevent or inhibit the oxidation of bulk oils are of major importance to consumers and the food industry. On the basis of the current knowledge of lipid oxidation mechanisms, some approaches have been developed to fulfill this goal. The application of antioxidants can retard lipid oxidation.[5] However, in many foods the currently approved antioxidants are not sufficient to prevent rancidity. Because very few new antioxidants are available to the food industry, new technologies are needed to enhance the effectiveness of currently available food-grade antioxidants.

Bulk oil is a complex food system; it contains numerous minor components, such as free fatty acid, sterols, antioxidants, phospholipids, monoacylglycerol, diacylglycerol, and water.[6,7] Recent studies from our group found that in bulk oils some of these minor components are able to form physical structures known as association colloids. These physical structures can increase lipid oxidation reactions and alter the effectiveness of antioxidants, such as α-tocopherol, Trolox, and chlorogenic acid esters.[8−10] For example, the more polar Trolox was able to partition into association colloids more than α-tocopherol. Because association colloids increase lipid oxidation rates, the association of Trolox with these association colloids could help to explain why it is a more effective antioxidant than α-tocopherol in bulk oils.[11]

The role of transition metals on lipid oxidation in food systems has been intensively studied.[12,13] Low-valence-state metal ions, such as ferrous and cuprous, can participate in the initiation and propagation steps of lipid oxidation by abstracting hydrogen to directly form free radicals and decomposing lipid hydroperoxides into free radicals such as the alkoxyl radical.[14] The prooxidative effect of high-valence-state metal ions is less clear, although there is some evidence that they can promote oxidation when they are reduced by food components to form the more prooxidative low valence state of the metal ions.[15,16] For example, ascorbic acid and gallic acid can promote lipid oxidation by reducing iron to its low valence state.[17,18]

Vegetable seed oils inevitably contain transition metals that originate from the seed and/or manufacturing equipment and ingredients, suggesting that metals could be important prooxidants in bulk oils. Tocopherols are important antioxidants in bulk oils, but they have been reported to have prooxidant activity in vegetable oils at high concentrations.[19,20] Although association colloids seem to act as important nanoreactors in bulk oils, it is unclear what promotes this oxidation. In addition, because molecules such as Trolox and tocopherols can also have prooxidative activity, it is possible

Received: January 11, 2012
Revised: March 9, 2012
Accepted: March 9, 2012
Published: March 10, 2012

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that association colloids could negatively affect lipid oxidation by enhancing the prooxidant activity of antioxidants in the presence of transition metals. Therefore, the present investigation was undertaken to explore if iron was an important prooxidant in bulk oil containing association colloids. In addition, the ability of high- or low-valence-state iron to interact with α-tocopherol and Trolox was also determined. Iron–antioxidant interactions were determined in a model system containing nonoxidizable medium-chain triacylglycerols (MCT) by measuring the depletion of antioxidants and the formation of antioxidant radicals.

■ MATERIALS AND METHODS

Materials. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dibutyryl-*sn*-glycero-3-phosphocholine (DC_4PC) were acquired from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Soybean oil was purchased from a local store and stored at 4 °C. MCT (Miglyol) were purchased from Sasol North America Inc. (Houston, TX, USA). Silicic acid, activated charcoal, calcein (CA), deferoxamine (DFO), methanol, hexane, α-tocopherol, Trolox, and *N-tert*-butylphenylnitrone (PBN) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Anhydrous ferric chloride and anhydrous ferrous chloride beads (99.998% purity) were purchased from Sigma-Aldrich Co. All other reagents were of HPLC grade or purer. Distilled and deionized water was used in all experiments.

Methods. *Sample Treatments. Formation of DOPC Association Colloids in Stripped Soybean Oil or MCT.* Stripped soybean oil (SSO) and stripped soybean oil with DOPC reverse micelles were prepared according to the method of Chen et al.[9] Thin layer chromatography (TLC) and HPLC analysis showed no tocopherol and phospholipids could be detected in SSO. Water content of the stripped oils decreased from ∼250 to <60 ppm as determined by Karl Fisher.[9] The formation of reverse micelles was accomplished by pipetting DOPC (1000 μM, final concentration) in chloroform into an empty beaker and then flushing with nitrogen until the chloroform was evaporated. The appropriate amount of MCT or SSO was then added followed by double-distilled water at a final concentration of 200 ppm. The oil samples were then stirred in a beaker at 1000 rpm in a 20 °C incubator room for 24 h.

Storage Conditions: (a) Lipid Oxidation Study. The antioxidants and DFO were dissolved in methanol, pipetted into beakers, and then flushed with nitrogen to evaporate the methanol. Bulk oil was then added to the beaker and stirred for 12 h. One milliliter samples (bulk oil with 0 and 1000 μM DOPC, 0 and 2 mM DFO, 0 and 100 μM α-tocopherols, or Trolox) were pipetted into the 10 mL headspace GC vials using an Eppendorf repetitive pipet with a 12.5 mL Plastibrand PD-TIP (Wertheim, Germany) and sealed with polytetrafluoroethylene (PTFE)/butyl rubber septa. For lipid oxidation studies, all vials were stored in a 55 °C incubator room in the dark for up to 50 days.

(b) Interaction between Antioxidants and Iron. Anhydrous $FeCl_3$ and $FeCl_2$ were dissolved in methanol, pipetted into beakers, and then flushed with nitrogen to evaporate the methanol. MCT was then added into the beaker and stirred for 12 h. Antioxidants were dissolved in MCT and mixed with the iron-containing MCT for 1 h. One milliliter samples (MCT with 0 and 1000 μM DOPC, 150 and 600 μM ferric and ferrous, 100 μM α-tocopherols, and Trolox) were pipetted into the 10 mL headspace GC vials using an Eppendorf repetitive pipet with a 12.5 mL Plastibrand PD-TIP and sealed with PTFE/butyl rubber septa. All vials were stored in a 55 °C incubator room in the dark for up to 48 h.

(c) EPR Study. Samples were stored for 24 h at 55 °C in an incubator room in the dark before analysis.

Measurement of Oxidation Parameters. Lipid hydroperoxides were measured as primary oxidation products using a method adapted from Shantha and Decker.[21] Oil samples were weighed and recorded before addition to a mixture of methanol/butanol (2.8 mL, 2:1, v/v) followed by the addition of 15 μL of 3.94 M thiocyanate and 15 μL of 0.072 M Fe^{2+}. The solution was vortexed, and after 20 min, the absorbance was measured at 510 nm using a Genesys 20 spectrophotometer (ThermoSpectronic, Waltham, MA). The concentration of hydroperoxides was calculated from a cumene hydroperoxide standard curve.

The secondary oxidation product, hexanal, was monitored using a GC-17A Shimadzu gas chromatograph equipped with an AOC-5000 autosampler (Shimadzu, Kyoto, Japan). Samples (1 mL) in 10 mL glass vials capped with aluminum caps with PTFE/silicone septa were heated at 55 °C for 15 min in an autosampler heating block before measurement. A 50/30 μm DVB/Carboxen/PDMS solid-phase microextraction (SPME) fiber needle from Supelco (Bellefonte, PA, USA) was injected into the vial for 2 min to absorb volatiles and was then transferred to the injector port (250 °C) for 3 min. The injection port was operated in split mode, and the split ratio was set at 1:5. Volatiles were separated on a Supleco 30 m × 0.32 mm Equity DB-1 column with a 1 μm film thickness at 65 °C for 10 min. The carrier gas was helium at 15.0 mL/min. A flame ionization detector was used at a temperature of 250 °C. Hexanal concentrations were determined from peak areas using a standard curve prepared from an authentic standard.

Determination of Trolox and α-Tocopherol Concentrations. For HPLC analysis, 100 μL of each sample was dissolved in 0.9 mL of methanol and then was passed through a 0.45 μm filter. A 20 μL aliquot of this sample solution was separated using a Shimadzu HPLC system equipped with a Shimadzu diode array detector (DAD), a Waters 474 scanning fluorescent detector, and a 250 mm × 4.6 mm i.d., 5 μm, Inertsil C18 analytical column. The mobile phase consisted of 4% purified water with 3 mM phosphoric acid at pH 2.6 (solvent A) and 96% methanol (solvent B) using isocratic gradient at a flow rate of 1 mL/min. Column temperature was set at 38 °C. Trolox was detected using DAD at a wavelength of 280 nm. Detection of α-tocopherol was conducted using both DAD at 295 nm and fluorescence at an excitation wavelength of 290 nm and an emission wavelength of 330 nm. Trolox and α-tocopherol in the samples were identified by comparing their relative retention times and UV spectra with authentic compounds.

Depletion of antioxidants was determined using $A_t/A_0 \times 100\%$, where A_0 and A_t were the peak area of measured antioxidant at time zero and t, respectively.

Determination of Ferric Iron Concentration in MCT. Ferric iron content was determined by UV–visible spectrophotometric method using the calcein (CA) dye method developed by Thomas et al.[22] Briefly, a calcein solution was prepared by adding 0.75 g of calcein into 400 g of MCT and stirred overnight to dissolve the dye. The solution was centrifuged at 10000 rpm for 10 min, and the upper clear orange color solution was collected and defined as the dye solution. The dye solution was added directly to an equal volume of bulk oil sample containing ferric iron, and the absorbance was measured at 352 nm using a Shimadzu UV-2101 PC UV–vis scanning spectrophotometer.

Remaining ferric iron in MCT was determined using $Ab_t/Ab_0 \times 100\%$, where Ab_0 and Ab_t were the absorbance at time zero and t, respectively.

EPR Spectroscopy. EPR measurements were carried out at room temperature using a Bruker EPR Elexsys-500 spectrometer operating at the X-band. The samples were placed in 707-SQ-250 M thin-wall quartz EPR sample tubes (Wilmad Glass Co., Buena, NJ, USA) and inserted into the ER 4122-SHQE high-sensitivity TE102 cylindrical mode single-cavity ($Q \sim 3000$) optical window of the EPR system. Instrument settings were as follows: center field, 3470 G; scan range, 100 G; gain, 60; time constant, 128 ms; modulation amplitude, 1 G; phase 0°; microwave power, 20 mW. Data collection was performed using the computerized program Xepr.

Statistical Analysis. Duplicate experiments were performed with freshly prepared samples. All data shown represent the mean value ± standard deviation of triplicate measurements. Statistical analysis of lipid oxidation kinetics was performed using a one-way analysis of variance. A significance level of $p < 0.05$ between groups was accepted as being statistically different. In all cases, comparisons of the means of the individual groups were performed using Duncan's multiple-range

tests. All calculations were performed using SPSS17 (http://www.spss.com; SPSS Inc., Chicago, IL, USA).

■ RESULTS AND DISCUSSION

Effects of the Metal Chelator Deferoxamine (DFO) on the Oxidative Stability of SSO. Transition metals are implicated in many lipid oxidation pathways as they are able to generate free radicals that initiate and propagate lipid oxidation in food systems.[23,24] The different redox states of iron have different pathways by which they can promote oxidation. For example, low-valence transition metals can promote the decomposition of lipid hydroperoxides into free radicals such as the alkoxyl radical (reaction 1). The high-valence state of transition metals can also decompose lipid hydroperoxide (reaction 2), but this pathway is very slow and may not be very important in foods.[25] However, compounds such as antioxidants can reduce metals, resulting in the formation of the highly prooxidative low-valence state, thus promoting oxidation (reaction 3).[6,26]

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO\bullet + ^{-}OH \quad (1)$$

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

$$Fe^{3+} + AH \rightarrow Fe^{2+} + A\bullet + H^{+} \quad (3)$$

Bulk oil naturally contains transition metals, which are variable due to the oil type, processing operations, and regional differences. Gaining a better understanding of the chemical role of transition metals in bulk oil oxidation is necessary to develop effective prevention strategies to extend the shelf life of oils. To gain a better understanding of the role of iron in lipid oxidation in bulk oils, we studied the effect of DFO, a specific Fe(III) chelator, on the oxidative stability of SSO in the absence and presence of phospholipid reverse micelles.

The formation of lipid hydroperoxides (LH) and headspace hexanal in SSO containing different levels of phospholipids (0 and 1000 μM DOPC) and DFO (0 and 2 mM) was measured during incubation at 55 °C (Figure 1). The lag phase for both lipid hydroperoxides and headspace hexanal for SSO lasted 4 days. The addition of DOPC reduced the lag phase to 2 days, which is in agreement with our previous study indicating that DOPC reverse micelles act as a prooxidant.[10] The incorporation of DFO had no significant effect on the lag phase of SSO ($p > 0.05$), but extended the lag phase of SSO containing DOPC from 2 to 7 days ($p < 0.05$).

To understand if iron played a role in lipid oxidation in the presence of free radical scavenging antioxidants, different combinations of DFO, α-tocopherol, and Trolox were added to the SSO in the presence and absence of phospholipid reverse micelles during incubation at 55 °C (Figures 2 and 3). The lag phase of LH and hexanal formation was 25 days for SSO containing 100 μM α-tocopherol (Figure 2). In the presence of DOPC reverse micelles and α-tocopherol, the lag phase decreased to 16 days for both LH and hexanal formation, showing that α-tocopherol was less effective in the presence of DOPC reverse micelles, which is in agreement with previous observations.[9] However, it should be noted that the combination of DOPC reverse micelles and α-tocopherol did inhibit lipid oxidation compared to the no-antioxidant control in Figure 1. DFO increased the lag phase of LH and hexanal formation in the presence of α-tocopherol both in the presence and in the absence of DOPC reverse micelles. In the absence of DOPC reverse micelles, the lag phase was increased by 5 days,

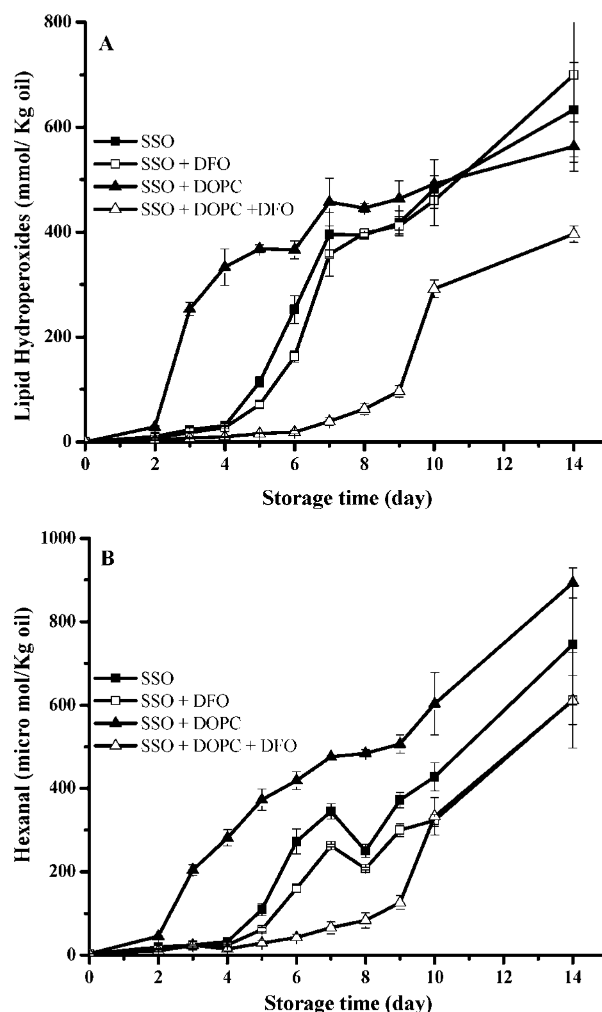

Figure 1. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil (SSO) containing 2 mM deferoxamine (DFO) and/or 1000 μM 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) during storage at 55 °C. Some of the error bars are within data points.

whereas in the presence of DOPC reverse micelles the lag phase was increased by 10−11 days.

The impact of DFO on the antioxidant activity of Trolox was also tested in the presence and absence of DOPC reverse micelles (Figure 3). The lag phase of LH and hexanal formation was 40 days for the SSO containing 100 μM Trolox, showing that the more polar Trolox was a more effective antioxidant than α-tocopherol as predicted by the antioxidant polar paradox.[27] However, unlike α-tocopherol, the incorporation of DFO did not increase the lag phase of LH and hexanal formation in the absence of DOPC micelles. As observed previously,[9] DOPC again diminished the effectiveness of Trolox, decreasing the lag phase to 21 days for both LH and hexanal formation. Even though DOPC decreased the activity of both α-tocopherol and Trolox, the more hydrophilic Trolox was still the more effective of the two. When DOPC reverse micelles, Trolox, and DFO were present in combination, DFO helped partially offset the prooxidative effect of DOPC by increasing the lag phase for LH and hexanal formation to 29 days.

DFO, a powerful iron chelator, has been successfully used in clinical settings to treat patients with acute and chronic iron overload syndromes.[28] DFO has a remarkably high affinity for

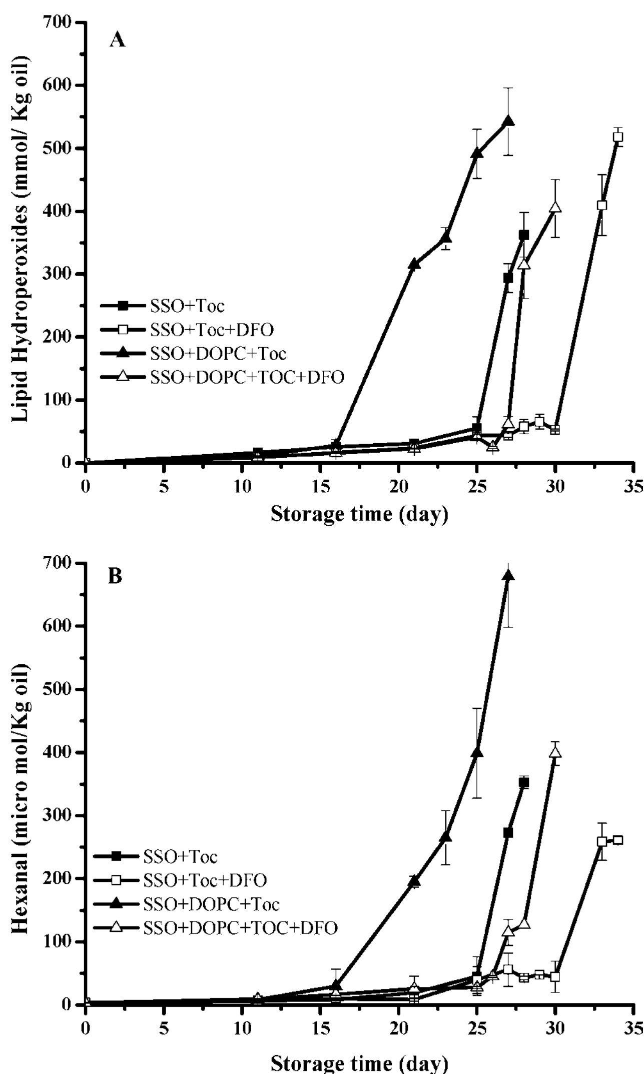

Figure 2. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil (sso) containing 100 μM α-tocopherol (Toc) , 2 mM deferoxamine (DFO), and/or 1000 μM 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) during storage at 55 °C. Some of the error bars are within data points.

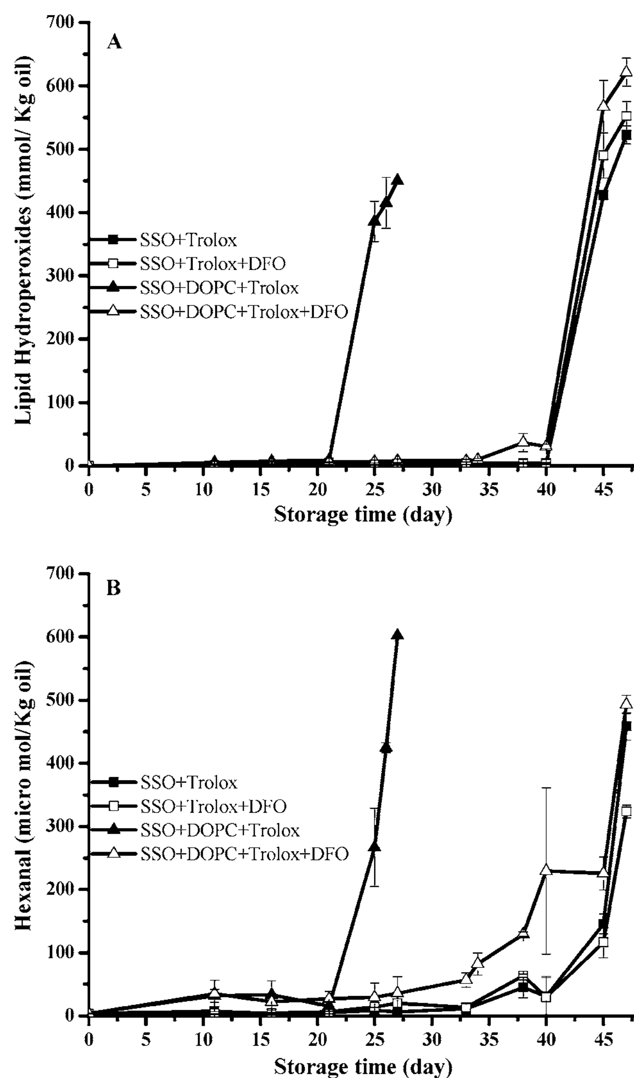

Figure 3. Formation of lipid hydroperoxides (A) and hexanal (B) in stripped soybean oil (SSO) containing 100 μM Trolox, 2 mM deferoxamine (DFO), and/or 1000 μM 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) during storage at 55 °C. Some of the error bars are within data points.

ferric ion, allowing it to inhibit the initiation and propagation of lipid oxidation promoted by ferric ion.[29] Therefore, it was anticipated that the oxidative stability of bulk oil would be improved by DFO if iron was an important prooxidant. In this study, DFO was not an effective antioxidant in soybean oil stripped of its minor components including endogenous antioxidants and phospholipids. However, DFO was effective in the presence of DOPC reverse micelle, indicating that the iron was an important prooxidant in the presence of reverse micelles. This could occur because phospholipids can bind iron[30] and thus could concentrate iron at the oil−water interface of reverse micelles. Lipid hydroperoxides are also surface active and could accumulate at the oil−water interface of the reverse micelles.[31] Thus, the prooxidant activity of DOPC could be due to their ability to concentrate both endogenous iron and lipid hydroperoxides at the oil−water interface of reverse micelles, thereby increasing the ability of iron to decompose lipid hydroperoxides.

The combination of the free radical scavengers, α-tocopherol and Trolox, and DOPC reverse micelles also affected the ability of DFO to inhibit lipid oxidation. In the absence of DOPC reverse micelles, DFO was a weak antioxidant in the presence of α-tocopherol (increased lag phase approximately 5 days) and did not inhibit oxidation in the presence of Trolox. However, in the presence of DOPC reverse micelles and α-tocopherol or Trolox, DFO was a strong antioxidant, increasing the oxidation lag phase 11 and 13 days for α-tocopherol and Trolox, respectively. This observation could be due to the ability of DFO to inhibit iron-promoted generation of free radicals that would deplete the antioxidants in the DOPC reverse micelle system. However, it is also possible that DFO could improve the activity of α-tocopherol or Trolox by inhibiting their direct degradation by iron.

Effects of Fe(III) and Fe(II) on the Depletion of α-Tocopherol and Trolox in MCT. To better understand interactions between high- and low-valence iron with α-tocopherol and Trolox, a MCT model bulk oil system was used. MCT was used in these experiments because it does not contain unsaturated fatty acids and thus there would be no fatty acid oxidation that could alter α-tocopherol or Trolox

concentrations. Figure 4A shows the depletion of α-tocopherol and Trolox during storage at 55 °C in the presence of high-valence Fe(III). The concentrations of both α-tocopherol and Trolox were relatively constant in the absence of Fe(III). Incubation of α-tocopherol or Trolox with 150 μM Fe(III) resulted in a similar trend in antioxidant depletion with 15 and 12% of α-tocopherol and Trolox consumed, respectively, after 1 h of storage. α-Tocopherol and Trolox concentrations in the presence of 150 μM Fe(III) reached equilibrium (∼50% consumed) after 12 h. When 600 μM Fe(III) was added, the pattern of antioxidant consumption was different, with α-tocopherol being completely consumed after 8 h of storage. Increasing the Fe(III) concentration to 600 μM had much less effect on Trolox consumption with a maximum of approximately 60% depletion of Trolox after 24 h. The observed consumption of α-tocopherol and Trolox in the presence of Fe(III) could be due to the ability of these antioxidants to donate an electron to iron, thus converting ferric to ferrous ions. Because Fe(III) was more reactive with α-tocopherol than with Trolox, this could help to explain why DFO was more effective at improving the antioxidant activity of α-tocopherol but not Trolox in the absence of DOPC reverse micelles (Figures 2 and 3). This improvement of the activity of α-tocopherol by DFO could be due to its ability to inhibit the α-tocopherol reduction of ferric ions to the more prooxidative ferrous ions and/or its ability to decrease Fe(III)-promoted α-tocopherol consumption, which would help maintain a higher concentrations of α-tocopherol, thus more effectively inhibiting lipid oxidation.

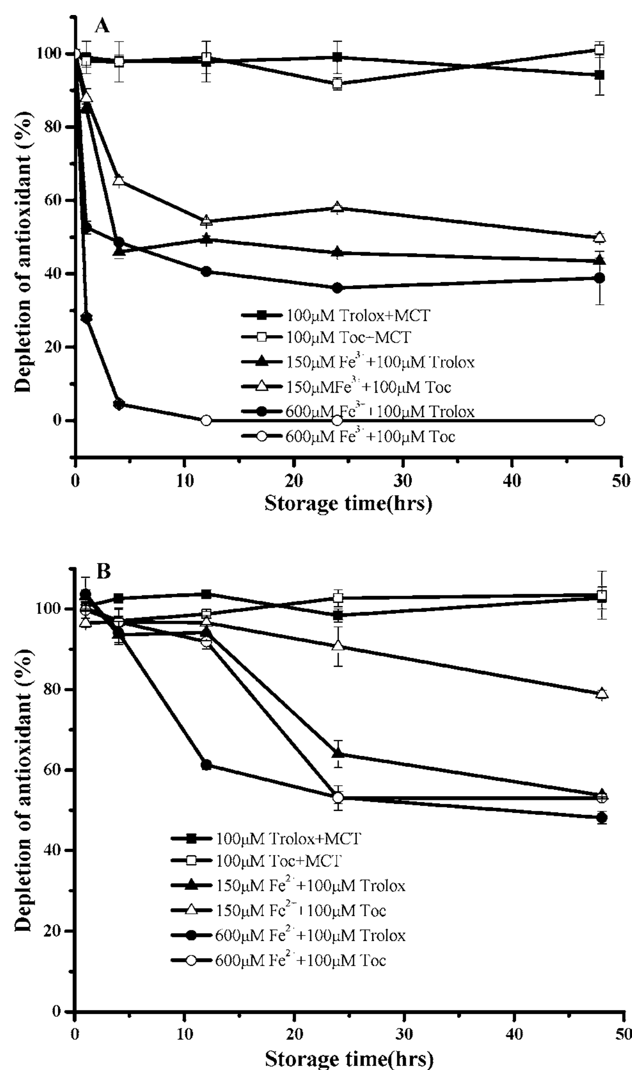

Figure 4. Depletion of α-tocopherol (Toc) and Trolox (100 μM) in medium-chain triacylglycerols (MCT) in the presence of (A) Fe(III) and (B) Fe(II).

α-Tocopherol and Trolox consumption reached an equilibrium and a further decrease in concentration was not observed during prolonged storage, suggesting that Fe(II) was not effective at decreasing the concentrations of the antioxidants. To test this hypothesis, Fe(II) was also added to the MCT model system (Figure 4B). Initial experiments were performed with reagent grade ferrous chloride powder, but rapid α-tocopherol and Trolox consumption were observed (data not shown). It was thought that this could be due to contaminating Fe(III), so experiments were repeated using a high-purity form of anhydrous ferrous supplied under vacuum. In these experiments, 150 μM Fe(II) did not significantly decrease α-tocopherol and Trolox for the first 12 h of storage compared to 42 and 50% decreases in α-tocopherol and Trolox, respectively, during the same time period in the presence of 150 μM Fe(III) (Figure 4A). In the presence of 600 μM Fe(II), Trolox consumption was more rapid than α-tocopherol. In all cases, α-tocopherol and Trolox consumption increased during prolonged storage, but the level of consumption was always significantly lower than with Fe(III) ($p < 0.05$, Figure 4A). The depletion of antioxidants during the later stages of incubation may be due to the oxidation of ferrous to ferric upon reaction with oxygen.

Effects of Fe(III) and Fe(II) on the Depletion of α-Tocopherol or Trolox in the Presence of DOPC Reverse Micelles. Previous results showed that DFO more effectively increased the antioxidant activity of α-tocopherol than of Trolox in the presence of DOPC micelles (Figures 2 and 3). This could be due to DFO's ability to decrease iron-promoted decomposition of lipid hydroperoxides into free radicals, a factor that would decrease α-tocopherol and Trolox concentrations with the subsequent reduced antioxidant concentration being less effective at scavenging free radicals. However, it is also possible that the improved activity of antioxidants by DFO in DOPC reverse micelles could be due to the ability of DFO to bind iron and inhibit the direct iron-promoted consumption of α-tocopherol and Trolox. This again would increase antioxidant effectiveness and would also decrease the reduction of ferric into the more prooxidative ferrous ions by antioxidants. To test this possibility, the depletion of α-tocopherol and Trolox by Fe(III) or Fe(II) in MCT in the presence of DOPC reverse micelles was determined (Figures 5 and 6). To determine the role of the choline headgroup in phospholipids in participating in the depletion of antioxidants, experiments were also conducted with DC_4PC, a phosphatidylcholine with butyric acid. DC_4PC does not form reverse micelles at the concentrations tested,[9] so experiments could be conducted with the same concentration of the choline headgroup in the absence of association colloids.

In the absence of Fe(III), neither DOPC nor DC_4PC promoted the consumption of α-tocopherol and Trolox, and α-tocopherol and Trolox were stable in the absence of added iron (data not shown). In the presence of 150 μM Fe(III) and DOPC reverse micelles, the consumption of both α-tocopherol and Trolox was less than in the absence of DOPC (Figure 5A).

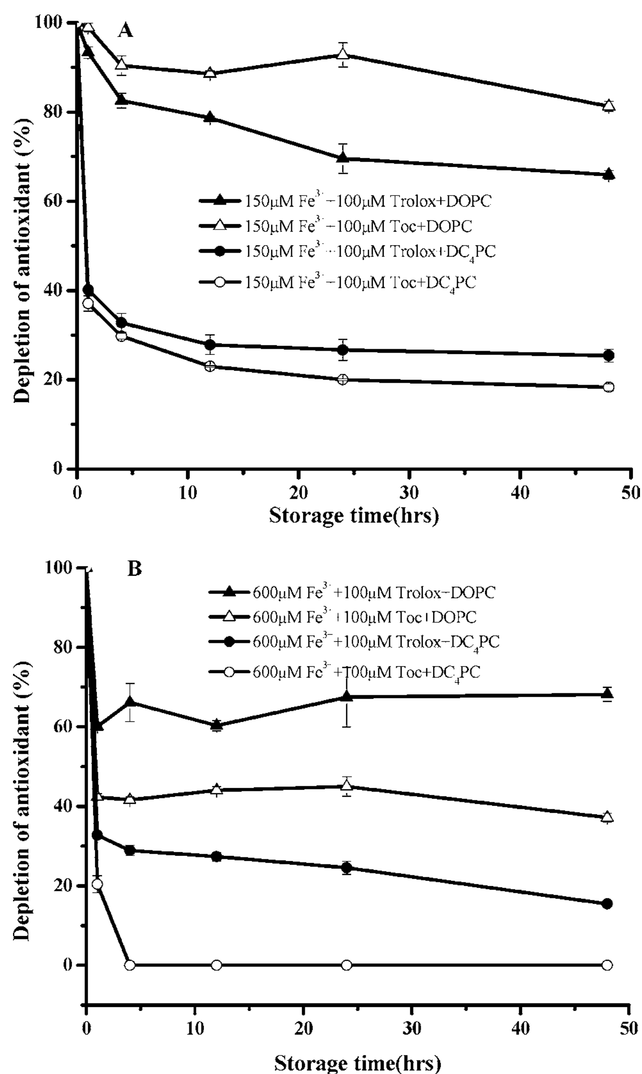

Figure 5. Depletion of α-tocopherol (Toc) and Trolox (100 μM) in medium-chain triacylglycerols (MCT) containing 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) or 1,2-dibutyryl-*sn*-glycero-3-phosphocholine (DC_4PC) in the presence of (A) 150 μM Fe(III) and (B) 600 μM Fe(III).

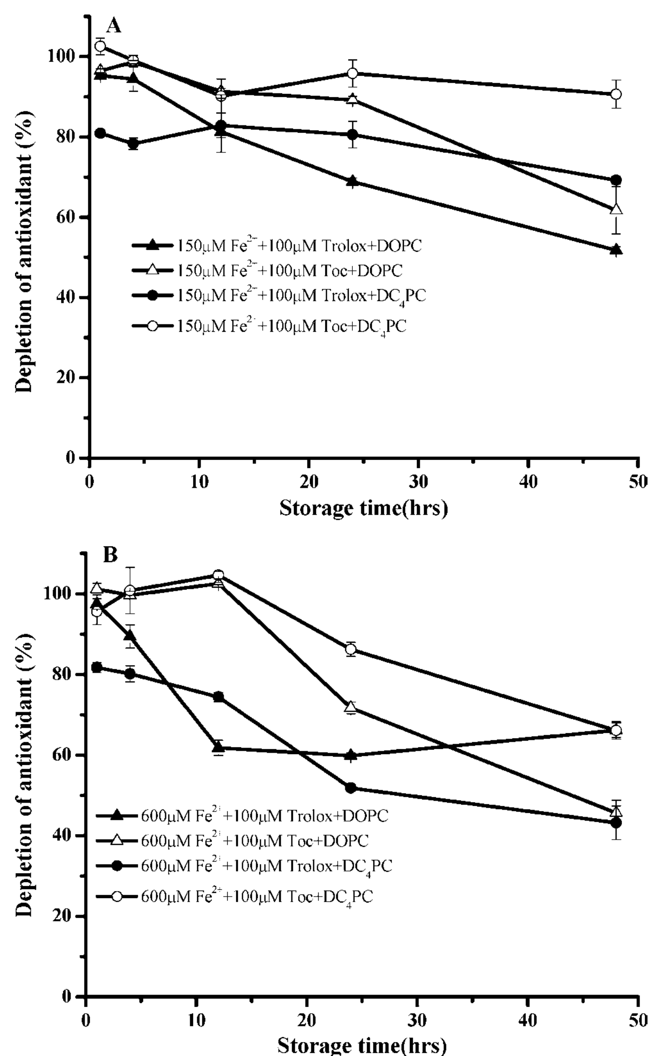

Figure 6. Depletion of α-tocopherol (Toc) and Trolox (100 μM) in medium-chain triacylglycerols (MCT) containing 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) or 1,2-dibutyryl-*sn*-glycero-3-phosphocholine (DC_4PC) in the presence of (A) 150 μM Fe(II) and (B) 600 μM Fe(II).

For example, after 24 h of incubation, α-tocopherol concentrations were 10% lower than at time 0 in the presence of DOPC reverse micelles compared to 40% lower in the absence of DOPC reverse micelles. In the presence of 600 μM Fe(III), Trolox was depleted more rapidly than α-tocopherol in the presence of DOPC reverse micelles, whereas the opposite was true in the absence of DOPC. Trolox has been found to be more highly associated than α-tocopherol with water in DOPC micelles.[10] This means that it could have greater contact with phosphatidylcholine bound iron at the water–lipid interface and thus could be more rapidly depleted.

In the presence of DC_4PC, which does not form reverse micelles, α-tocopherol and Trolox was rapidly consumed by Fe(III). At low (150 μM) and high (600 μM) Fe(III) concentrations, DC_4PC slightly increased both α-tocopherol and Trolox consumption compared to the absence of phospholipid (Figure 5). DC_4PC would be expected to bind iron as would any phosphatidylcholine.[32] The ability of DC_4PC to slightly promote the consumption of α-tocopherol and Trolox could be due to its ability to increase the solubility of iron in the oil by acting as a lipid-soluble metal chelator and thus making the iron more reactive if the iron chelated to DC_4PC would still be redox active. Because DC_4PC does not form reverse micelles at the concentrations tested, this suggests that the ability of DOPC to inhibit both α-tocopherol and Trolox depletion was not due to the phospholipid headgroup but instead was due to the presence of reverse micelles.

The interaction between Fe(II) and antioxidants in MCT was also investigated when DOPC reverse micelles or DC_4PC was present. Both 40 and 100 ppm Fe(II) had little impact on the depletion of α-tocopherol or Trolox after 12 h (Figure 6). This finding was in agreement with the previous results in the absence of DOPC reverse micelles, indicating Fe(II) cannot decompose α-tocopherol or Trolox in bulk oil. The later depletion of antioxidants after 12 h of storage might be due to the oxidation of ferrous to ferric iron with the resulting ferric iron causing the depletion of the antioxidants.

Effect of α-Tocopherol and Trolox on the Loss of Fe(III). Iron concentrations have been analyzed in bulk oils.[33−35] For example, Mendil and co-workers measured iron content in bulk oil with a flame atomic absorption spectrometer

(FAAS), and total iron was found to be 139, 127, 105, and 52 ppm (i.e., 200−800 μM) in olive oil, hazelnut oil, sunflower oil, and corn oil, respectively.[35] Although this kind of experiment is helpful to verify that iron exists in commercially refined oils, it does not provide any information on the redox state of the iron. Verifying the existence of an iron recycling pathway by measuring the redox form of iron has been extremely helpful in understanding the role of iron redox form in lipid oxidation in both in vivo systems and oil-in-water emulsions.[36,37] However, analytical techniques to measure iron redox state in bulk oil are limited. In this study we initially attempted to measure the concentration of added high-valence-state iron Fe(III) in MCT with calcein acetoxymethyl ester (calcein-AM), an Fe(III)-specific probe used successfully to measure the labile ferric in cells and biological fluids.[38] However, calcein-AM solubility in MCT was quite limited, so calcein was successfully used instead due to its higher solubility in hydrophobic solvents.[22] Preliminary studies showed that calcein had strong absorbance at 365 nm in MCT, which was quenched by ferric chloride. It also showed a good linear relationship ($r > 0.99$) with Fe(III) in MCT over the range of 0−600 μM Fe(III) (data not shown). Room temperature was chosen in these experiments because the 55 °C used in the previous experiments caused too rapid of a reaction between ferric ions and the antioxidants.

The ability of α-tocopherol and Trolox to reduce Fe(III) was determined at 600 μM Fe(III) and varrious concentrations of the antioxidants (Figure 7A). After 24 h of incubation, Fe(III) concentrations decreased only slightly (<5%) in the absence of added antioxidants. α-Tocopherol significantly decreased Fe(III) at concentrations as low as 20 μM. Maximal decrease in Fe(III) concentrations was observed at α-tocopherol concentrations of 60 μM. Trolox exhibited a similar trend on Fe(III) concentrations, although its maximal decrease in Fe(III) concentrations was obtained at a concentration of 40 μM. At 100 μM, the decrease in Fe(III) concentrations was slightly greater for Trolox than for α-tocopherol ($p < 0.05$).

DOPC reverse micelles caused a greater decrease in Fe(III) (Figure 7B) than in the absence of DOPC (Figure 7A), suggesting that the DOPC by itself could reduce Fe(III) (Figure 7B). DOPC reverse micelles decreased the ability of both α-tocopherol and Trolox to reduce iron as significant decreases in Fe(III) were not seen until α-tocopherol and Trolox concentrations were >40 μM. As in the absence of DOPC reverse micelles, at 100 μM, the decrease in Fe(III) concentrations was slightly greater for Trolox than for α-tocopherol ($p < 0.05$).

Overall, DC_4PC (Figure 7C) changed the concentration of Fe(III) in a similar manner to the absence of phospholipid (Figure 7A). For example, DC_4PC had very little impact on Fe(III) concentrations in the absence of antioxidants. In addition, both α-tocopherol and Trolox were able to significantly decrease Fe(III) at concentrations as low as 20 μM as seen in the absence of phospholipid. However, for DC_4PC the maximal decrease in Fe(III) concentrations by α-tocopherol was reduced from 60 to 40 μM.

Effects of Fe(III) and Fe(II) on the Formation of PBN Spin Adducts in MCT. Because both α-tocopherol and Trolox can scavenge free radicals generated in oils, one may argue that the depletion of antioxidants in this research could be due to the presence of free radicals. To clarify this potential pitfall and provide more evidence for the different roles of Fe(III) and Fe(II) in bulk oil, an EPR study was employed in an effort to

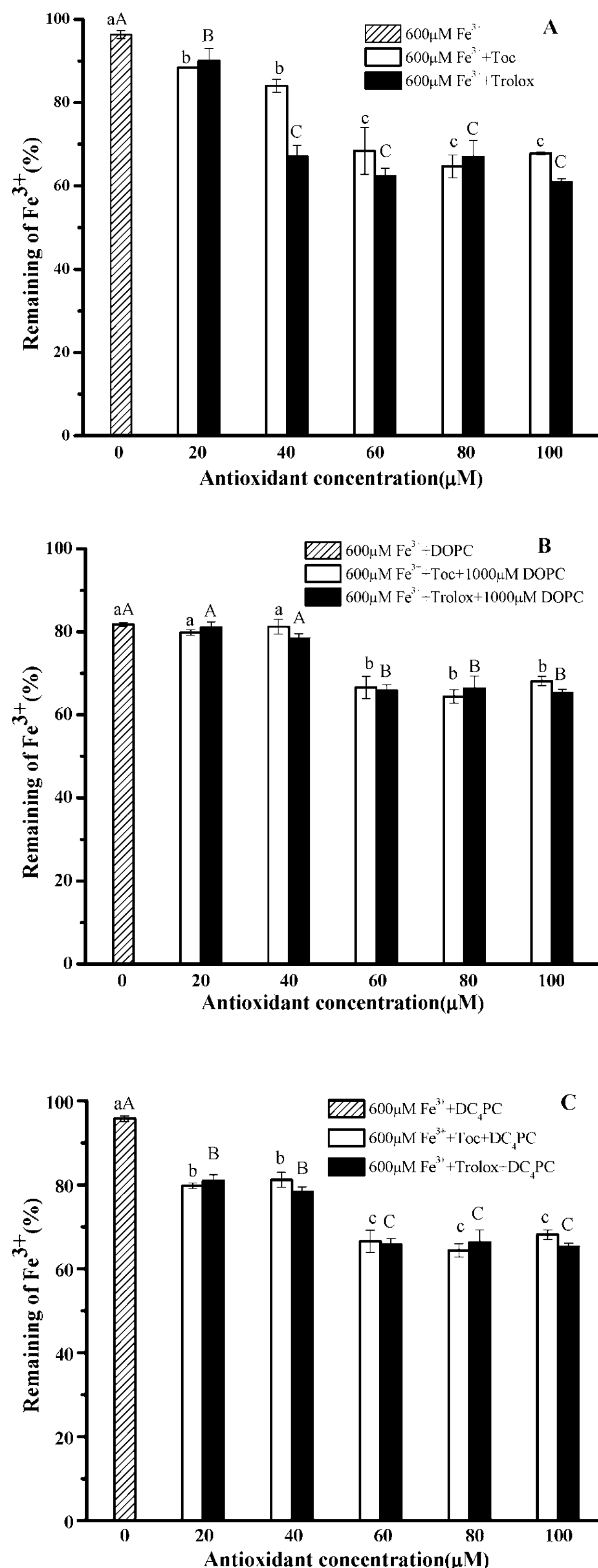

Figure 7. Fe(III) development in medium-chain triacylglycerols (MCT) containing (A) α-tocopherol (Toc) or Trolox, (B) 1000 μM 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and (C) 1,2-dibutyryl-*sn*-glycero-3-phosphocholine (DC_4PC) after 24 h of storage at room temperature. For each group, different letters on the top of columns represent significant differences ($p < 0.05$).

detect the formation of free radicals in our MCT model system under different conditions.

A comparison of the EPR signals of SSO (Figure 8A) and MCT (8B) after 24 h of storage at 55 °C showed that PBN-trapped free radicals were observed only in SSO, indicating that there were no detectable free radicals in our MCT model system. When antioxidants and iron were added to MCT, the formation of α-tocopheroxyl (Figure 8C) or Trolox (Figure 8D) radicals was not detected. This was true in the presence or absence of DOPC or DC_4PC (data not shown). To determine if iron was reactive in the MCT model, it was added in combination with cumene hydroperoxide. In this system, Fe(II) was observed to form PBN-trapped radicals (Figure 8E) but Fe(III) was not (Figure 8F). This confirms that Fe(II) could be an active prooxidant in bulk oils and that Fe(III) is much less reactive, as has been observed in oil-in-water emulsions.[37]

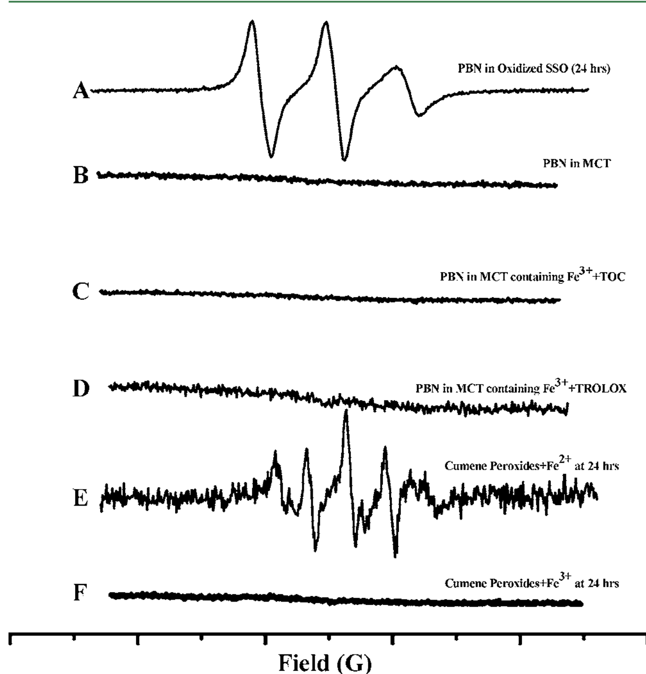

Figure 8. EPR detection of radical formations in the reaction consisting of (A) 0.1 M PBN in stripped soybean oil (SSO), (B) 0.1 M PBN in MCT, (C) 0.1 M PBN + 100 μM α-tocopherol (Toc) in MCT, (D) 0.1 M PBN + 100 μM Trolox in MCT, (E) 0.1 M PBN + 10 mM cumene hydroperoxides + 150 μM Fe(II) in MCT, and (F) 0.1 M PBN + cumene hydroperoxides + 150 μM Fe(III) in MCT after 24 h of storage at 55 °C.

DFO was able to inhibit lipid oxidation in SSO in the presence of DOPC reverse micelles, indicating that iron was an active prooxidant in the presence of association colloids. Because refined oils contain association colloids due to the presence of surface active minor components (e.g., phospholipids, free fatty acids, lipid hydroperoxides, etc.) and water,[7] this could help to explain why the metal chelator citric acid is an effective antioxidant in commercial oils. DFO was even more effective at increasing the oxidative stability of oils with DOPC reverse micelles in the presence of α-tocopherol and Trolox. This could be due to the decreased iron-promoted generation of free radicals or direct iron-promoted decomposition of antioxidants.

Many phenolic antioxidants, such as chlorogenic, caffeic, sinapic, and ferulic acid, can reduce Fe(III) to Fe(II) in aqueous environments, resulting in the consumption of the antioxidants. For example, 1 molecule of caffeic acid was reported to reduce 9 atoms of Fe(III).[39,40] Our result showed that α-tocopherol and Trolox can reduce Fe(III) in bulk oil as seen by both the ability of Fe(III) to decrease antioxidant concentrations (Figure 4) and the ability of antioxidants to decrease Fe(III) concentrations (Figure 7B). In this study, DOPC reverse micelles decreased interactions between iron and α-tocopherol or Trolox as seen by both a decrease in iron-promoted α-tocopherol and Trolox decomposition and a decrease in the ability of α-tocopherol and Trolox to decrease Fe(III) concentrations. This suggests that it is likely that the ability of DFO to enhance the activity of α-tocopherol and Trolox in DOPC reverse micelles was due to its ability to inhibit iron-promoted generation of free radicals through pathways such as hydroperoxide decomposition. This could occur if iron concentrated at the oil–water interface of the DOPC reverse micelles and increased its ability to decompose surface active lipid hydroperoxides sharing the same location. Decreased production of free radicals from decomposing lipid hydroperoxides by iron would decrease the consumption of the antioxidants and thus increase their effectiveness.

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Funding

This material is based upon work supported by the U.S. Department of Agriculture (Project 2007-02650) and a Thomas H. Smouse Memorial Fellowship Award from the American Oil Chemists' Society.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Paul Lahti for his help on EPR measurement.

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