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Role of Iron and Hydroperoxides in the Degradation of Lycopene in Oil-in-Water Emulsions

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Lycopene has recently received interest as an antioxidant in human tissues. These antioxidant properties, however, present challenges to preventing oxidative degradation of lycopene within food products. In this research, oxidation of lycopene in a model emulsion system was examined. Lycopene loss was monitored using a spectrophotometer with an integrating sphere. Light was found to have little influence on the degradation of lycopene at pH 3, 5, or 7. The pH of the emulsion had a significant impact on the stability of lycopene, with most rapid degradation occurring in emulsions at pH 4 and below. Addition of EDTA significantly increased the stability of lycopene. Addition of TBHQ showed little impact on lycopene stability at pH 3, but exhibited a greater effect at pH 7. These results suggest that transition metal induced oxidation of lycopene may be the predominant mechanism of degradation at low pHs. At higher pHs, attack by free radicals was found to be a contributing mechanism to lycopene oxidation.

KEYWORDS: Emulsion; lycopene; carotenoid; iron; surfactants; hydroperoxides; hexadecane

INTRODUCTION

In recent years research on the benefits of carotenoids in the diet has sparked interest in incorporating compounds such as lycopene and lutein into functional food products. While incorporating these bioactive compounds into foods that do not naturally contain high amounts of these compounds may offer great health benefits and provide new opportunities for food, there are a number of stability issues that must be overcome before these products can come to market.

The conjugated polyene chain that is characteristic of carotenoids makes these compounds susceptible to degradation from a number of agents. Autoxidation of carotenoids is known to occur with relative ease at room temperature in certain solvents, producing a number of initial oxidation products including carbon-peroxyl triplet biradicals and epoxides (*1, 2*). Exposure to high acid environments can cause destruction of carotenoids due to production of ion-pairs, which can then dissociate to form a carotenoid carbocation (*3*). Light (*4*), heat (*5*), and singlet oxygen (*6*) are also known to degrade carotenoids.

Electron transfer has also been found to occur between transition metals like iron and carotenoids. If electron transfer occurs between a species like ferric iron and a carotenoid, the ferrous species of iron and a carotenoid radical cation can form. The resulting radical species formed can undergo further degradation reactions, potentially leading to further carotenoid loss (*7*−*9*).

If free radicals are already present in the environment, three types of reactions have been found to occur between carotenoids and these radicals. Carotenoids have the potential to undergo electron transfer reactions with radicals in the environment to form radical cations (*8, 9*). In addition, carotenoids may react with radicals through hydrogen abstraction (*10, 11*) or adduct formation reactions (*10, 12, 13*) that produce either carotenoid radicals or several types of radical adducts.

Once carotenoid degradation has been initiated by one of the mechanisms described above, a number of secondary reactions can occur leading to a variety of products including epoxides, endoperoxides, apo-carotenals, and apo-carotenones (*1, 2, 8, 11, 14*−*16*). It is this series of reactions that can lead to loss of both color and bioactivity of carotenoids in foods, leading to loss of product quality and consumer acceptance.

This study focuses on the mechanisms of lycopene degradation in an oil-in-water emulsion model system. Lycopene is the acyclic carotenoid responsible for the red color of tomatoes, watermelon, guava, and grapefruit (*17*). A number of studies have suggested that this compound may play a role in decreasing the risk of developing a number of health conditions including cancers of the prostate (*18*), cervix, colon, esophagus, stomach, and breast (*17*) as well as cardiovascular disease (*19*). It has been proposed that the benefits of lycopene are due to its ability to act as an antioxidant and to stimulate cell-to-cell communication (*20*).

Since carotenoids are lipid soluble, dispersing these compounds in the oil droplets of emulsions has great potential as a bioactive ingredient. Emulsion systems are easily incorporated

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into functional food products and can be designed to have several chemical protection hurdles that can increase stability of carotenoids. In previous work, emulsions were engineered to serve as a stable delivery system for omega-3 fatty acids in ice cream and yogurt, with little consumer detection of altered sensory attributes (*21*, *22*). The strategies that have been developed for increasing emulsion stability include engineering the oil/water interface, addition of antioxidants, and controlling the reactivity of prooxidant transition metals naturally present in foods through the use of metal chelators and product pH (*23, 24*).

In order to produce emulsion delivery systems for lycopene that exhibit optimum stability, it is critical to understand the predominant mechanisms of lycopene degradation. This work will examine the impact of factors known to play a role in carotenoid degradation (light, iron, free radicals, and pH), to determine the extent of damage these conditions create in a carotenoid-containing emulsion system. By identifying the mechanisms of lycopene loss, emulsions can be engineered to reduce interaction of carotenoids with prooxidative elements. Eventually, these strategies may make it possible to produce carotenoid delivery systems with adequate stability to be incorporated into functional food products.

MATERIALS AND METHODS

Materials. LycoVit Dispersion (11% lycopene) in sunflower oil was donated by BASF Corporation (Florham Park, NJ). Sodium dodecyl sulfate (SDS), imidazole, sodium phosphate monobasic, *tert*-butylhydroquinone (TBHQ), 5,6-diphenyl-3-(2-pyridyl)-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (ferrozine), ferric chloride, ferrous sulfate, and hexadecane were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium acetate, hydrochloric acid (HCl), methanol, and sodium hydroxide (NaOH) were purchased from Fisher Scientific (Fair Lawn, NJ). All chemicals were of analytical grade or purer.

Preparation of Oil Phase. The oil phase of the emulsions was prepared immediately before use by dispersing the LycoVit dispersion (11% lycopene in sunflower oil) into hexadecane at a final concentration of 0.33 mg of lycopene per gram of hexadecane. This amount of lycopene was chosen because it allows for a reasonable range for quantifying lycopene using an integrating sphere. In treatments using TBHQ, a methanol carrier was used to add TBHQ to the hexadecane. The methanol was evaporated with nitrogen before lycopene addition.

Preparation of Emulsions. Oil-in-water emulsions were prepared using 5% (w/w) oil phase in sodium acetate−imidazole−sodium phosphate buffer solution (10 mM each, pH 7.0) containing 30 mM SDS. An aqueous phase was prepared and stirred overnight to ensure complete dispersion of the surfactant. All emulsions were made at pH 7.0 by sonicating for 2 min, using 0.5 s pulses, at 70% amplitude using a Fisher Scientific Sonic Dismembrator 500. Emulsion preparation was conducted in the dark to prevent any potential degradation by light. All glassware used for emulsion preparation and sample storage was acid washed with 2.0 N HCl to remove residual metals. After sonication, the pH was adjusted to the appropriate treatment pH using 1.0 N HCl or NaOH. For EDTA treatments, EDTA was added to the aqueous phase prior to emulsification at a final concentration of 100 mM.

Sample Storage. Samples were stored in 23-G-20 capped glass fluorometer cells (Starna Cells, Inc., Atascadero, CA) placed on rocker plates, and stored at 15 °C or room temperature (22−25 °C). With the exception of experiments testing the effect of light, all samples were stored in the dark.

Influence of Light. To understand the ability of light to degrade lycopene, samples at various pH levels stored in light or dark were tested for lycopene degradation over time. Samples in the light treatment were stored on a rocker plate under a 60 Hz fluorescent lamp fixture. For comparison, a second set of samples were stored on rocker plates and covered to prevent light exposure. All samples were stored at 15 °C. All emulsion preparation procedures and lycopene concentration measurements were made in the dark.

Influence of Ferric and Ferrous Species of Iron. To determine the effect of iron species on the stability of lycopene in emulsions, ferric chloride or ferrous sulfate stock solutions were added to emulsion samples at a final concentration of 100 μM. For samples containing no iron, distilled, deionized water was added to the emulsions.

The effects of ferrous and ferric iron were also tested in bulk hexadecane containing 11 μM lycopene. For this work, a methanol carrier was used to add ferric chloride or ferrous sulfate to the hexadecane at a final concentration of 100 μM ferrous or ferric species. After stirring for five minutes to incorporate the lycopene into the hexadecane, lycopene concentration was determined.

Particle Size of Emulsions. Particle size distributions of the emulsion droplets were measured using a laser diffraction instrument (Malvern Mastersizer, Malvern Instruments, Worcestershire, U.K.). Particle size was determined on triplicate samples for each emulsion preparation. The average D_{43} of the emulsions was 0.4 ± 0.02 μm and the average D_{32} was 0.28 ± 0.02 μm. Particle sizes were stable throughout the storage studies.

Lycopene Concentration. The absorbance of emulsions was used to determine lycopene concentrations. Periodically, sample absorbances were measured using a Shimadzu UV-2101 PC UV−vis scanning spectrophotometer equipped with an ISR integrating sphere assembly (Shimadzu, Kyoto, Japan). Lycopene content was determined using a standard curve created using emulsions with varying amounts of added lycopene. Duplicate samples were tested for each treatment at each testing time.

To determine lycopene concentration for the bulk oil experiment in which ferric and ferrous iron were added, the absorbance of the hexadecane and lycopene mixture was read at 470 nm using a Shimadzu UV-2101 PC UV−vis scanning spectrophotometer. Triplicate samples were tested at each testing time. Lycopene content was determined from a standard curve prepared with lycopene and hexadecane.

Ferrous and Ferric Iron Content. Ferrous iron content was determined using ferrozine chelation, a method initially developed by Stookey (*25*). A ferrozine solution (3 mM final concentration) was added directly to emulsion samples and absorbance was measured at 562 nm using a Shimadzu UV-2101 PC UV−vis scanning spectrophotometer equipped with an ISR integrating sphere assembly (Shimadzu, Kyoto, Japan). Emulsion ferrous iron concentration was determined using standard curves prepared from ferrous sulfate.

Statistical Analysis. Statistical Analysis Systems Version 9.1 software (SAS Institute, Cary, NC, 2002) for analysis of variance procedures (PROC GLM combined with the LS MEANS, SLICE and PDIFF functions) were used to analyze results. In this analysis, pH, light, iron type, chelator addition, free radical scavenger addition, and storage time were considered fixed effects. Where significant interactions were found among the effects tested, a Bonferroni adjustment ($p \leq 0.05$/no. of comparisons) was used for declaring significance.

RESULTS AND DISCUSSION

The objective of this work was to gain a better understanding of the major mechanisms of lycopene degradation in oil-in-water emulsions to help to identify which food processing, formulation, and storage conditions might be important to consider for a lycopene-containing emulsion based functional food ingredient. Factors evaluated included light exposure, free radical scavengers and iron since these factors have been found in previous studies to decrease the stability of carotenoids in vitro.

Impact of Light. The influence of light on the stability of lycopene in emulsions was determined by splitting emulsion samples into two groups. One group was stored in the dark at 15 °C, and the other was stored under fluorescent lighting at the same temperature. Fluorescent lighting was chosen as it is common in many food markets. Emulsion samples at pH 3.0,

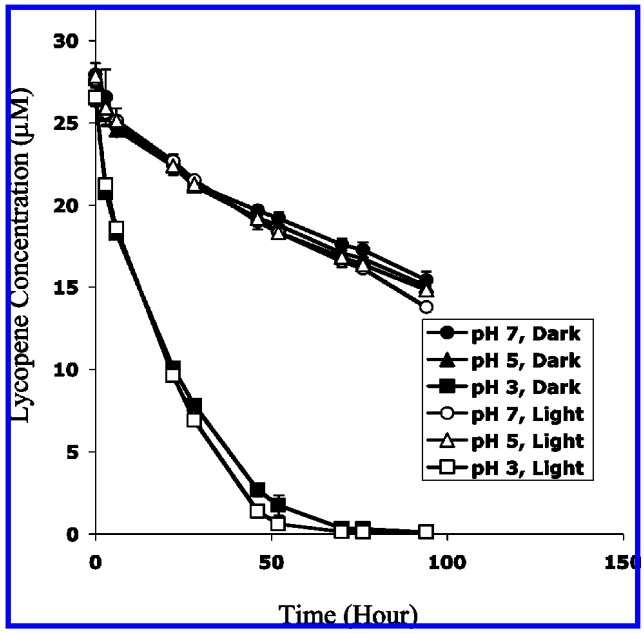

Figure 1. Lycopene concentration over storage time in SDS-stabilized emulsions at pH 3.0, 5.0, or 7.0 stored in light or dark conditions at 15 °C. Data points represent means ($n = 3$) ± standard deviations. Some error bars lie within the data points.

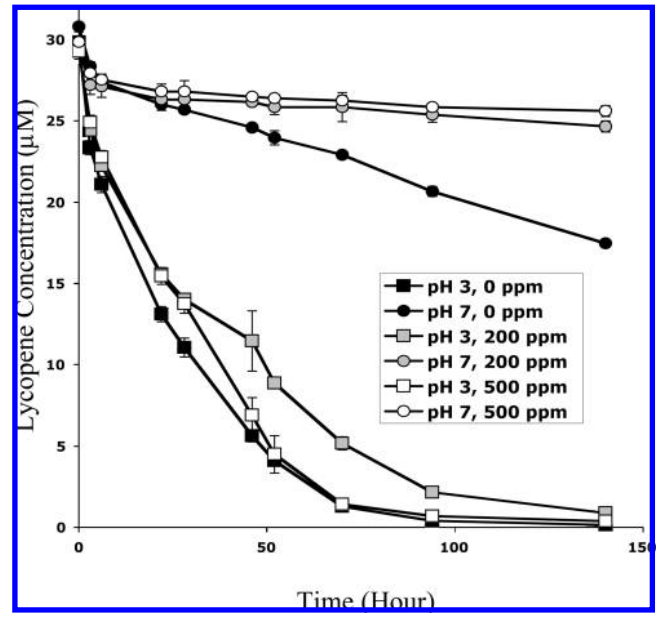

Figure 2. Lycopene concentration over storage time in SDS-stabilized emulsions at pH 3.0 or 7.0 stored 15 °C with 0, 200, or 500 ppm TBHQ. Data points represent means ($n = 3$) ± standard deviations. Some error bars lie within the data points.

5.0, and 7.0 that were stored in either the dark or light showed little difference in lycopene loss over storage (**Figure 1**). At pH 3, significant differences between samples stored in light or dark conditions were found only at the 28 and 46 h sampling intervals. In general, samples at pH 5.0 and 7.0, showed a greater stability than emulsion samples at pH 3.0. No significant differences were found between the samples stored in the dark or under fluorescent lighting at pH 5.0. Significant differences between samples stored in the light and dark were found at several testing times at pH 7.0, however, these differences were not consistently higher or lower, suggesting that there is little difference in lycopene stability in emulsions stored in the light and dark at pH 7.0.

Lack of significant lycopene degradation in the presence of light could be due to several reasons. First, the lipid droplets in oil-in-water emulsions will scatter light (*26*), which may result in limited penetration of the light into the sample. Unlike other studies which studied light degradation of carotenoids in optically clear solvents (*4*), the lack of light penetration would mean that most of the lycopene in the oil-in-water emulsion was not exposed to light thus decreasing its degradation. Second, light is very efficient in degrading carotenoids in the presence of singlet oxygen generators (*6*). However, since the oil-in-water emulsions used in this study did not contain singlet oxygen generators such as riboflavin or chlorophyll, this pathway is unlikely to be an important mechanism of lycopene degradation. This may not be the case if a lycopene-containing oil-in-water emulsion was added to foods containing singlet oxygen generators.

Impact of Free Radical Scavengers. Carotenoids can also degrade in the presence of free radicals (*8, 9*). Therefore, TBHQ, a free radical scavenger known to be effective in oil-in-water emulsions (*27*), was added to the emulsions using stock solutions made with methanol. Final concentrations of TBHQ emulsions were 200 or 500 ppm, while control samples were made by adding an equivalent volume of methanol without any TBHQ. TBHQ addition was found to have little impact on lycopene stability in emulsions at pH 3.0 stored at 15 °C (**Figure 2**) as all samples exhibited near complete loss of lycopene within 140 h of storage. At pH 7.0 TBHQ was more effective in decreasing lycopene loss than at pH 3.0. Within 52 h of storage, pH 7.0 samples without added TBHQ exhibited significantly greater lycopene loss than samples with either 200 or 500 ppm TBHQ. At the conclusion of the experiment, the samples without TBHQ exhibited a 44% lycopene loss, while those with TBHQ exhibited only a 14−17% loss.

Carotenoids can degrade through free radical mediated autoxidation reactions (*12*). The lycopene used in this study was suspended in sunflower oil (0.27% of the total lipids in the emulsion). While the concentration of sunflower oil in the emulsion is low, it is possible that its degradation could produce free radicals that could in turn degrade lycopene. The fact that TBHQ was effective at pH 7.0 but not 3.0 suggests that free radicals were the predominant mechanism for lycopene degradation at pH 7.0. The ineffectiveness of TBHQ at pH 3.0 could be due to lack of significant free radical production at lower pH values or could be due to the high reactively of other prooxidants that would rapidly degrade TBHQ making it ineffective.

Impact of pH. The previous experiments indicated that the pH of the emulsion appears to have a dramatic impact on lycopene stability. To further examine how the stability of lycopene in oil-in-water emulsions is influenced by pH, emulsions were prepared at pH 2.0−8.0 and stored at 15 °C (**Figure 3**). Emulsions at pH 2.0−4.0 exhibited near total loss of lycopene (96−99% loss) over the course of the 94 h experiment. Alternatively, the pH 5.0−8.0 emulsions exhibited a much slower rate of lycopene loss, resulting in a total lycopene loss between 47−54% after 94 h of incubation.

In strong acids like trifluoroacetic and sulfuric acid, carotenoids have been proposed to produce ion pairs, leading to destruction of carotenoids (*3, 7*). However, acid-catalyzed reactions may not be the predominant mechanism by which lycopene stability is decreased with decreasing pH. Also at play may be the solubility of iron. Iron has been shown to degrade several carotenoids in various solvent systems (*8, 9*). Since ferric iron increases in solubility from 1.6×10^{-18} M to 1.6 M as pH

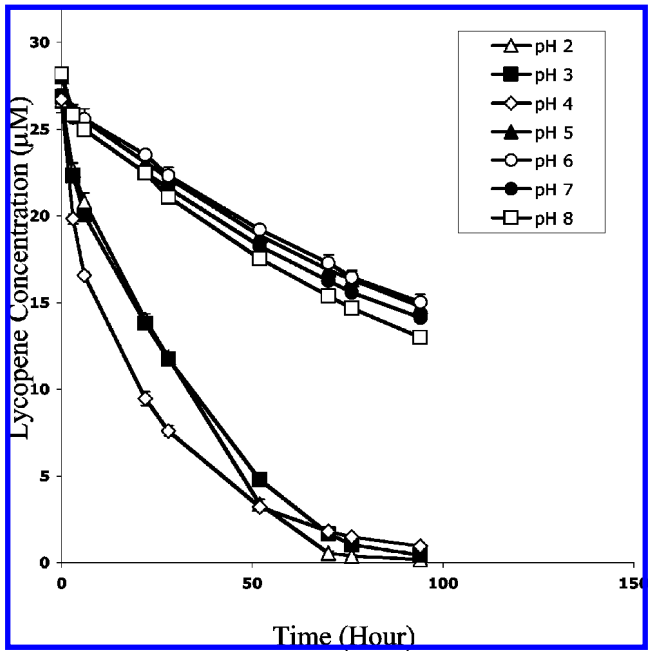

Figure 3. Lycopene concentration over storage time in SDS-stabilized emulsions at pH 2−8 stored at 15 °C. Data points represent means ($n = 3$) ± standard deviations. Some error bars lie within the data points.

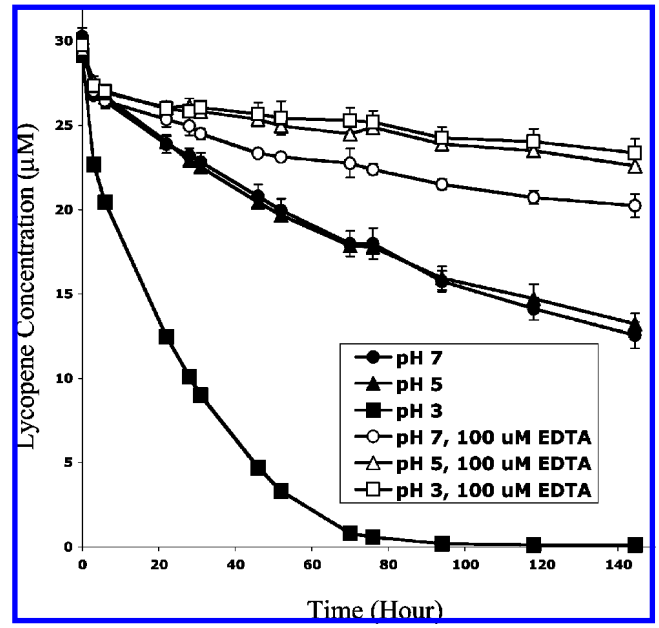

Figure 4. Lycopene concentration over storage time in SDS-stabilized emulsions at pH 3.0, 5.0, or 7.0 stored at 15 °C with 0 or 100 μM EDTA. Data points represent means ($n = 3$) ± standard deviations. Some error bars lie within the data points.

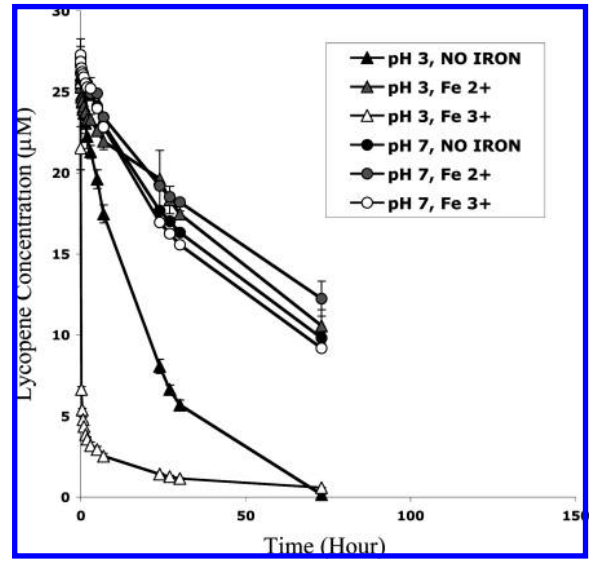

Figure 5. Lycopene concentration over storage time at room temperature in SDS-stabilized emulsions at pH 3.0 or 7.0, with 100 μM ferrous iron, 100 μM ferric iron, or no iron added. Data points represent means ($n = 3$) ± standard deviations. Some error bars lie within the data points.

decreases from pH 8.0 to pH 2.0 and ferrous iron increases in solubility from 3.7×10^{-3} to 3.7×10^{-9} M over the same range (*28*), it would be expected that iron-induced reactions would become more prominent at lower pHs. Increased prooxidant activity of iron with decreases in pH has previously been observed in the oxidation of fatty acids and fatty acid hydroperoxides in oil-in-water emulsions. For example, lipid hydroperoxides in SDS-stabilized emulsions and in Tween 20 micelles were proposed to experience a more rapid breakdown by iron at pH 3.0 than at pH 7.0 due to greater iron solubility at low pH (*29*). Low pH could not only impact iron solubility but could also impact the physical location of iron in emulsions. Mei and co-workers (1998) reported that the association of ferric iron with SDS-stabilized hexadecane-in-water emulsions increased with decreasing pH. Since binding of iron to the surface of emulsion droplets can increase oxidation rates by increasing metal−lipid interactions (*23*), this could also help explain why lycopene degradation increased with decreasing pH.

Impact of EDTA and Iron. To determine if metals were active at promoting lycopene degradation in the oil-in-water emulsions, 100 μM EDTA was added (**Figure 4**). Samples containing EDTA exhibited significantly greater stability than those without EDTA after 3 h for samples at pH 3.0 and 22 h for samples at pH 5.0 and 7.0. EDTA was more effective at protecting lycopene at pH 3.0 and 5.0 than pH 7.0, however, no significant differences in lycopene stability were observed in the presence of EDTA between pH 3.0 and 5.0 samples. This data provides strong evidence that transition metals such as iron and copper are highly involved in lycopene degradation in the oil-in-water emulsion especially at lower pH values.

Of the transition metals in foods, iron is an important prooxidant because it is typically found at greater concentrations than the other transition metals. To provide greater clarity to the question of the role of iron and specific iron species on lycopene degradation, ferrous or ferric ions were added to the emulsions. Ferrous sulfate (100 μM) increased the stability of lycopene in emulsions both at pH 3.0 and 7.0 (**Figure 5**), with ferrous-containing samples becoming significantly more stable than the other treatments within three hours at pH 3.0, and within 24 h at pH 7.0. The reason for the higher stability of lycopene in ferrous treated oil-in-water emulsions is not clear. Ferrous iron has been shown to act as an antioxidant at high concentrations in some systems. For instance, ferrous acetate (50 ppm) addition to an extrudate of corn starch and soybean oil was found to reduce lipid oxidation compared to a no iron added control (*30*).

In nonfood systems, some metal compounds have also been shown to act both as an inhibitor and as a promoter of oxidation reactions depending on their concentration. Betts and Uri first proposed that various cobalt compounds act to catalyze oxidation of peroxide-free methyl linoleate, docos-1-ene, hexadec-1-ene, and 2,6,10,14-tetramethylpentadecane at low concentrations, but

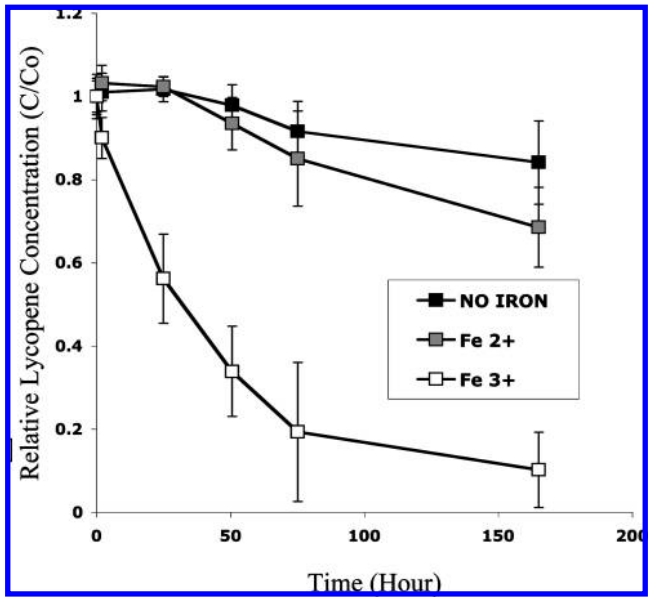

Figure 6. Lycopene concentration over storage time at room temperature in hexadecane with 11 μM lycopene and 100 μM ferrous iron, 100 μM ferric iron, or no iron added. Data points represent means ($n = 3$) $\pm$ standard deviations. Some error bars lie within the data points.

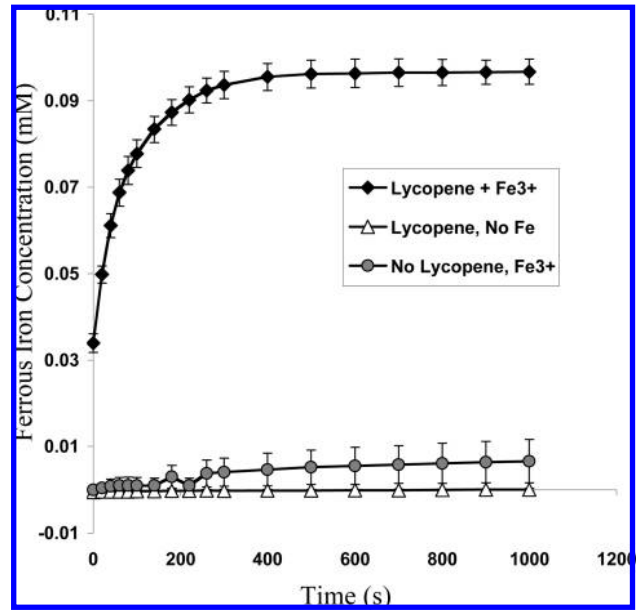

Figure 7. Ferrous iron development over time in SDS-stabilized emulsions with lycopene and 100 μM ferric iron added, with lycopene and no iron added, or with no lycopene and 100 μM ferric iron added. Data points represent means ($n = 3$) + standard deviations. Some error bars lie within the data points.

convert to inhibitors of oxidation at higher concentrations (*31*). It has been proposed that this antioxidant phenomenon occurs at high levels of transition metals because electrons from reduced metal ions can inactivate free radicals.

At pH 3.0, addition of ferric chloride (100 μM) resulted in a rapid loss of lycopene under room temperature storage (23−25 °C) (**Figure 5**). Within the first hour of storage, 83% of lycopene present in the emulsion was lost. Emulsions at pH 3.0 without added iron experienced the second fastest loss of lycopene, with a 69% decrease in lycopene concentrations after 24 h of storage. Ferric chloride addition did not accelerate lycopene degradation at pH 7.0. The rapid degradation of carotenoids upon exposure to ferric ions has been reported in studies using carotenoids and ferric chloride in dichloromethane (*8*, *9*) or sol−gels containing ferric ions in the aqueous fraction (*7*). The increased activity of ferric iron at pH 3.0 is likely due to higher solubility making interactions between aqueous phase iron and oil droplets more likely.

The ability of ferric and ferrous iron to degrade lycopene was also tested in bulk hexadecane containing lycopene (**Figure 6**). As in emulsion samples, ferric iron caused a rapid destruction of lycopene, with only 10% of lycopene remaining after 165 h of storage. The lycopene in treatments with no iron added or ferrous iron added exhibited greater stability, and no significant differences were found between these two treatments.

Ferric ions have been proposed to degrade carotenoids by the following mechanism:

$$Fe^{3+} + \text{carotenoid} \rightarrow Fe^{2+} + \text{carotenoid}^{\bullet+} \qquad (1)$$

To determine if ferric was converted to ferrous ions in the emulsion system used in this study, a ferrous ion indicator, ferrozine, was added to the samples. Ferric chloride (or distilled, deionized water, in the case of the no iron control) was then added to the emulsions at a final concentration of 100 μM, and formation of ferrous ions was monitored at room temperature for 1000 s at 562 nm. As seen in **Figure 7**, oil-in-water emulsions containing lycopene caused a rapid conversion of ferric to ferrous ions with over 90% of the ferric iron being lost within 300 s. When lycopene was not present in the emulsion, only minor amounts ferric ions were converted to ferrous ions during the 1000 s incubation period. These results strongly suggest that the mechanism described in eq 1 is responsible for the initiation of lycopene degradation at low pH levels.

It is clear from the work conducted in these experiments that lycopene in SDS-stabilized emulsions can undergo two degradation pathways, and it is possible that these two pathways can occur simultaneously. At low pH, where iron is more soluble, the predominant pathway of lycopene destruction is likely due to interactions with ferric ions. However, as the pH of emulsions is raised, iron may be less likely to interact with the lipid droplets due to loss of solubility and therefore ability to interact with emulsion droplets. When iron-induced degradation pathways are less frequent, free radical induced lycopene degradation becomes more dominant. In the hexadecane emulsion system, the free radicals are likely generated by autoxidation, from the minor amounts of sunflower oil present in the BASF lycopene ingredient, or from carotenoid radical cations produced by a limited number of iron−lycopene interactions that still take place at high pH.

This situation could change if a lycopene-containing emulsion was added to a real food system. For example, in lipid containing foods, iron is likely to react with both lycopene and naturally occurring lipid hydroperoxides. The reaction between iron and lipid hydroperoxides would result in generation of free radicals and ferric ions. Both the ferric iron and the free radicals could then attack lycopene, making both free radical and ferric ion-promoted degradation a concern especially in low pH foods. If the pH of a food product is higher, iron will be less soluble, in which case free radical reactions may become the more important pathway of degradation.

Therefore, when designing functional foods containing lycopene emulsions it would be important to add metal chelators and/or free radical scavengers. In high pH foods, free radical scavengers may be sufficient to inhibit lycopene degradation. In low pH foods, metal chelators would help stabilize the lycopene. In foods containing unsaturated fatty acids, it might

be advisable to add both chelators and free radical scavengers to inhibit interactions between iron and fatty acid hydroperoxides and inactivate any free radicals produced by hydroperoxide decomposition. This work also provides strong evidence that ferric ion concentrations should be keep as low as possible in lycopene-containing foods and supplements especially those with acidic pH values. It may also be possible to design lycopene delivery systems that have emulsion droplet interfaces engineered to inhibit metal−lycopene interactions (e.g., emulsion droplets with thick or cationic interfacial membranes that repel iron).

ABBREVIATIONS USED

SDS, sodium dodecyl sulfate; TBHQ, *tert*-butylhydroquinone; ferrozine, 5,6-diphenyl-3-(2-pyridyl)-1,2,4-triazine-4′,4″-disulfonic acid sodium salt; HCl, hydrochloric acid; NaOH, sodium hydroxide.

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