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Factors Influencing the Antioxidant and Pro-Oxidant Activity of Polyphenols in Oil-in-Water Emulsions

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ABSTRACT: The nonenzymatic oxidation of polyphenols bearing di- and trihydroxyphenol groups results in the generation of hydrogen peroxide (H_2O_2), a reactive oxygen species that can potentially compromise the oxidative stability of foods and beverages. An investigation of the factors that promote the oxidation of a model polyphenol, (−)-epigallocatechin-3-gallate (EGCG), was undertaken in a model lipid-based food system. Factors affecting oxidative stability, such as exogenous iron chelators (ethylenediaminetetraacetic acid; EDTA and 2,2-bipyridine; BPY) and pH (3 and 7) were evaluated in hexadecane and flaxseed oil-in-water (o/w) emulsions. At neutral pH, H_2O_2 levels were observed to rise rapidly in hexadecane emulsions except for EDTA-containing treatments. However, EDTA-containing samples showed the highest rate of EGCG oxidation, suggesting that H_2O_2 was rapidly reduced to hydroxyl radicals (HO•). Conversely, at pH 3, H_2O_2 concentrations were lower across all treatments. EDTA conferred the highest degree of EGCG stability, with no loss of the catechin over the course of the study. In order to assess whether or not the H_2O_2 production seen in oxidatively stable hexadecane emulsions translated to pro-oxidant activity in an oxidatively labile food lipid system, the effect of EGCG on the stability of flaxseed o/w emulsions was studied. EGCG displayed antioxidant activity at pH 7 throughout the study; however at pH 3, pro-oxidant activity was seen in EGCG-containing emulsions, with and without BPY. This study attempts to provide a mechanistic understanding of the conditions wherein polyphenols simultaneously exert pro-oxidant and antioxidant behavior in lipid dispersions.

KEYWORDS: *polyphenols, lipid oxidation, hydrogen peroxide, emulsions, hydroxyl radicals, electron paramagnetic resonance*

INTRODUCTION

Polyphenols represent a class of compounds commonly associated with high antioxidant activity,[1−5] yet their effect on the oxidative stability of lipid dispersions have been mixed with both antioxidant[6] and pro-oxidant activities reported.[7−9] A possible explanation for observed pro-oxidant activity may arise from the nonenzymatic, metal-catalyzed oxidation of polyphenols,[10] which results in hydrogen peroxide (H_2O_2) generation (Scheme 1). While H_2O_2 is not a particularly potent oxidant in many food systems, it is capable of undergoing metal-catalyzed reduction to form highly reactive hydroxyl radicals (HO•), a nonspecific oxidant capable of reacting with organic matter at diffusion limited rates.[11] We have previously examined the use of proteins to scavenge H_2O_2 in the aqueous phase of oil-in-water (o/w) emulsions to prevent HO• radical formation.[12] However, the addition of protein also led to decreases in polyphenol stability, which would not be considered desirable in polyphenol-fortified foods (i.e., functional foods). An alternative strategy is to limit polyphenol oxidation and its resulting reactive oxygen species (ROS) generation by controlling metal-catalyzed polyphenol oxidation reactions. Iron chelators are commonly added to foods to inhibit metal-catalyzed lipid oxidation reaction. Chelators with oxygen-based ligands preferentially bind ferric ions (Fe^{3+}), while chelators with nitrogen or sulfur ligands preferentially bind ferrous ions (Fe^{2+}). The stabilization of a specific transition metal oxidation state may promote or inhibit certain reactions (Scheme 1) when active cycling of iron is necessary for catalysis. For instance, ethylenediaminetetraacetic acid (EDTA), a ferric ion chelator, has been shown to stimulate the oxidation of ascorbic acid[13] as well as phenolic compounds in the presence of trace metals[14−16] due to the stabilization of the catalytic ferric ion necessary for the oxidation of the above compounds.

As multiphase systems, emulsions contain a number of HO• radical-reactive compounds in the aqueous phase, which may nonselectively scavenge HO• radicals before they are able to reach lipid droplets. The likelihood of HO• radicals initiating lipid oxidation in o/w emulsions is largely unknown, and is most likely dependent on site-specific reactions given the high reactivity of these species. Studies in dispersed lipid systems have shown that the physical location of iron catalysts strongly affects HO• radical-promoted lipid oxidation reactions (i.e., oxidation reactions increased in systems when iron was in close proximity to the interface).[17] However, the proximity of iron to interfaces is not the only factor that contributed to oxidative stability, as it has been shown that while ADP and ATP are capable of removing iron from liposome membranes, lipid oxidation reactions increased.[18] Such an effect was not seen in the presence of EDTA and citrate, suggesting that the reactivity of the iron chelate also plays a significant role. Transition metals are also important catalysts in propagating lipid oxidation reactions, as they are able to reduce lipid hydroperoxides to alkoxyl radicals.[19,20] Although EDTA may result in rapid polyphenol loss and potential HO• radical formation, it

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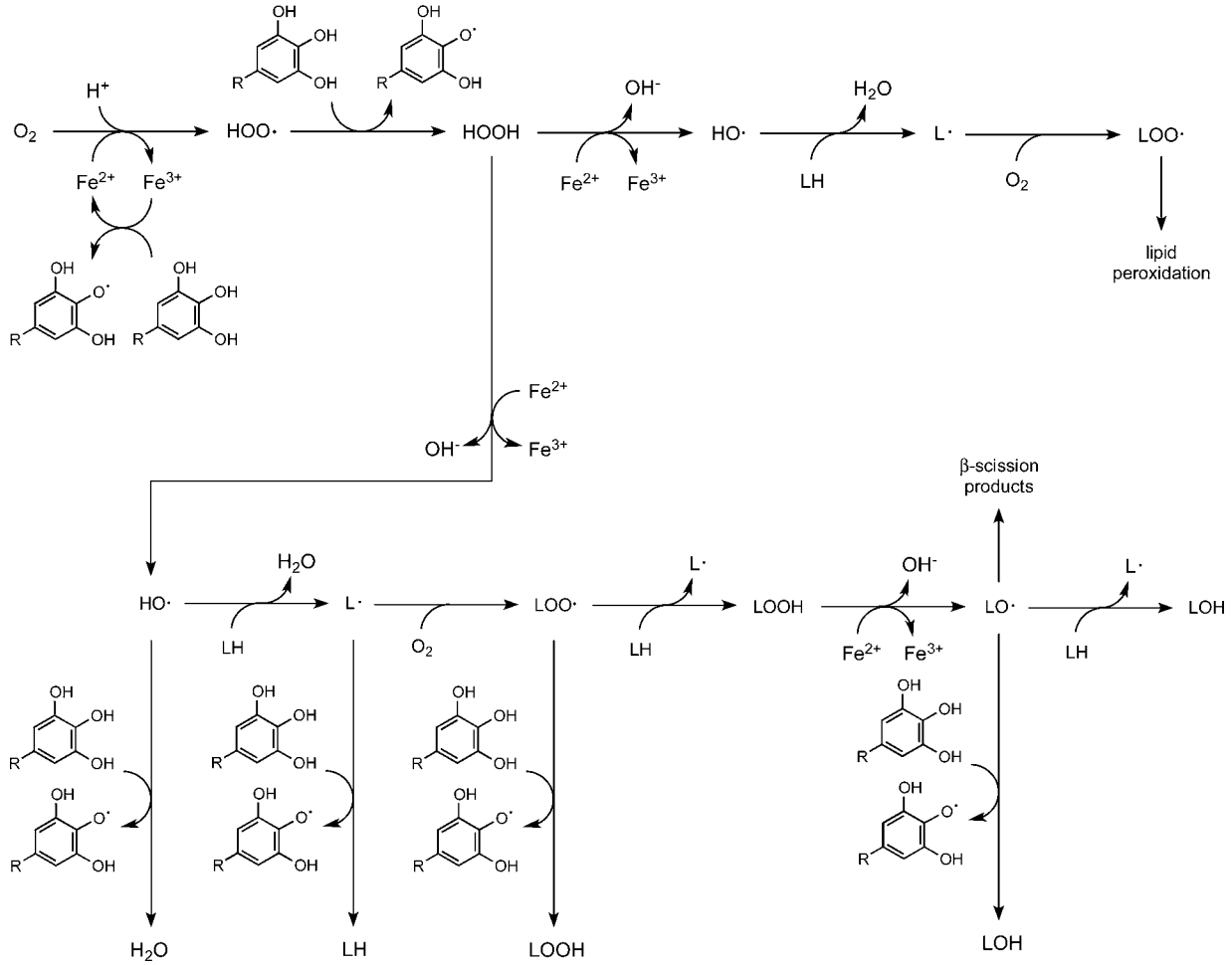
Scheme 1. Proposed Mixed Mechanism of Polyphenol-Mediated Lipid Oxidation and Polyphenol Radical Scavenging Antioxidant Activity[a]



[a]For simplicity, oxidation of a simple gallate group is depicted. Radical intermediates and selected products are abbreviated as follows: hydroperoxyl radical (HOO•), hydroxyl radical (HO•), reduced lipid (LH), lipid alkyl radical (L•), lipid hydroperoxyl radical (LOO•), lipid hydroperoxide (LOOH), lipid alkoxyl radical (LO•).

appears to exert a net antioxidant effect in lipid dispersions by directing iron away from interfaces,[21,22] where Fe^{2+} has been shown to initiate lipid oxidation by reacting with endogenous lipid hydroperoxides.[23]

The objective of this study was to investigate the role of a ferrous (2,2-bipyridine; BPY) and ferric (EDTA) chelator on epigallocatechin-3-gallate (EGCG) oxidation and H_2O_2 generation as a function of pH in an oxidatively stable hexadecane o/w emulsion system. As a saturated hydrocarbon, hexadecane will not readily undergo lipid oxidation reactions, thus limiting the confounding effect of polyphenol loss due to reaction with lipid-derived radicals. EGCG (Figure 1) was selected as a model polyphenol in the present study because it is commonly added to foods, both as an antioxidant in lipid systems, and as a bioactive ingredient. The oxidation of a flaxseed o/w emulsion was then studied under identical conditions to determine the net effect of EGCG oxidation on the oxidative stability of lipids more prone to oxidation due to high unsaturated fatty acid content. The elucidation of metal-catalyzed oxidation reactions of polyphenols may aid in preventing the loss of these bioactive polyphenols as well as preventing the potential pro-oxidant activity their oxidation may exert when introduced into lipid foods.

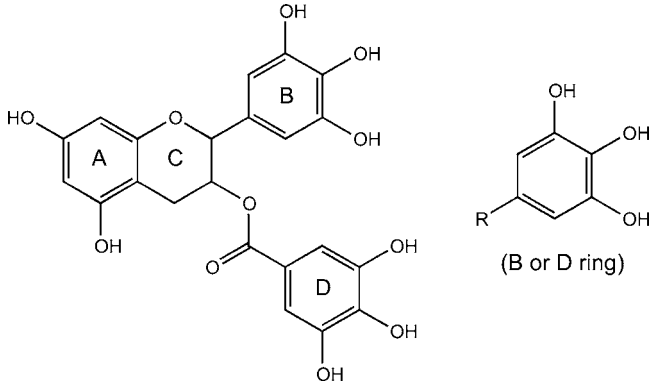


Figure 1. Structure of (−)-epigallocatechin-3-gallate (EGCG).

MATERIALS AND METHODS

Materials. Butylated hydroxytoluene (BHT), 2,2-bipyridine (BPY) (≥99%), ethylenediaminetetraacetic acid (EDTA), Tween 80, xylenol orange tetrasodium salt, and ferrous sulfate heptahydrate were purchased from Sigma-Aldrich (St. Louis, MI). H_2O_2 (30% w/v) and ammonium thiocyanate were purchased from EMD Chemicals (Gibbstown, NJ). *n*-

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Hexadecane (99% purity) was obtained from Acros Organics (Morris Plains, NJ). D-Sorbitol (98% purity) and cumene hydroperoxide were purchased from Alfa Aesar (Ward Hill, MA). 1,1,3,3-Tetraethoxypropane was acquired from TCI America (Portland, OR). Ferric chloride 6-hydrate and sodium azide were purchased from Mallinckrodt (Phillipsburg, NJ). α-Phenyl-*N*-tert butyl nitrone (PBN) was purchased from GeroNova Research (Carson City, NV). α-(4-Pyridyl 1-oxide)-*N*-*tert*-butylnitrone (POBN) was purchased from Alexis Biochemicals (Farmingdale, NY). Flaxseed oil was purchased from a local market and used as received. EGCG (93% purity) was purchased from Taiyo Green Power Company (Jiangsu, China). All other chemicals and solvents were purchased from Sigma, EMD, Alfa Aesar, and Mallinckrodt, and were of analytical or HPLC grade. Water was purified through a Millipore Q-Plus (Millipore Corp., Bedford, MA) purification rain.

Emulsion Preparation. Oil-in-water emulsions were prepared by dispersing 10 wt % hexadecane or 10 wt % flaxseed oil in water containing 2 wt % Tween 80, a nonionic surfactant. Coarse emulsions were prepared using an Ultra-Turrax T25 Basic high-speed blender (IKA, Wilmington, NC) on high speed for 0.5 min. Fine emulsions were prepared by passing coarse emulsions twice through a microfluidizer (Microfluidics M-110Y, Newton, MA) at 40 psi. All emulsions had a mean particle size (d_{32}) of 0.27 ± 0.01 μm, as determined by laser light scattering (Horiba LA 920, Irvine, CA). EGCG (0.4 mM final concentration) was added to 10 wt % hexadecane emulsions from a 10 mM stock solution prepared in 20 mM phosphate buffer (pH 3 or 7). Emulsions were diluted to a final concentration of 5 wt % with 20 mM phosphate buffer (pH 3 or 7). EDTA (50 μM) or BPY (100 μM) was added to 5 wt % emulsions from freshly prepared stock solutions in water. Ferric iron (25 μM) was subsequently added to all samples from a $FeCl_3$ solution prepared in water. Sodium azide (0.02 wt %) was added to emulsions in order to prevent microbiological growth. Emulsions were stored in the absence of light at 37 °C. Samples were analyzed over time to follow EGCG oxidation and H_2O_2 concentration. For lipid oxidation studies, a separate sample set was prepared for PBN analysis.

Hydrogen Peroxide Analysis. Hydrogen peroxide concentrations in emulsions were measured according to previously reported methods.[12] Briefly, emulsion samples were chemically destabilized by the addition of methylene chloride:methanol (2:1, v/v) followed by vortex mixing for 30 s and centrifugation at 800 × *g* for 2 min. The upper aqueous layer was collected and stored at −80 °C until analysis. A modified version of the concentrated ferrous oxidation-xylenol orange (FOX) assay[24] was used for H_2O_2 analysis. The FOX assay solution consisted of xylenol orange (1 mM), ferrous sulfate (2.5 mM), and sorbitol (1.0 M) in sulfuric acid solution (0.5 N), and was prepared daily from stock solutions. Peroxide analysis was performed by adding the assay solution (20 μL) to the aqueous extract (140 μL). To remain in the linear portion of the standard curve, samples were diluted as necessary using a 1:2 water:methanol mixture. Samples were mixed by vortex and absorbance values were read at 560 nm following incubation (30 min; ambient temperature) using an Agilent 8453 UV−vis diode array spectrophotometer (Agilent Technologies, Santa Clara, CA) blanked against aqueous extracts from unoxidized, freshly treated emulsions. Quantitation was performed using an external standard curve prepared using authentic H_2O_2, the concentration of which was validated using the peroxide's extinction coefficient ε_{240} = 40 M^{-1} cm^{-1}. A separate standard curve was prepared for H_2O_2 in the presence of EDTA, as ferric chelators are known to interfere with the FOX assay.

EGCG Analysis. EGCG was extracted from emulsions in the same manner as described above for H_2O_2 analysis. To prevent further EGCG oxidation during storage and analysis, a preservative solution (10 μL) consisting of ascorbic acid (20 wt %) and ethylenediaminetetraacetic acid (EDTA; 0.1 wt %) in phosphate buffer (pH 3.6; 0.4 M) was added to the aqueous extract (100 μL) prior to storage at −80 °C.[25] Preservative solutions were prepared daily. EGCG in extracts was measured by HPLC according to a modified method from Hu et al.[26] Chromatographic separation was achieved on a reverse phase Supelcosil LC-18 (4.6 × 150 mm, 5 μm; Supelco Inc., Bellefonte, PA) using a Shimadzu 10ADvp pump (Columbia, MD) with sample introduction by means of a Shimadzu 20ADvp temperature-controlled autosampler (4 °C). Samples were filtered over 0.45 μm PTFE syringe filters. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). EGCG was eluted by gradient according to the following program: 0−8.5 min from 25 to 45% B. The injection volume was 20 μL and the flow rate was held at 1 mL/min. EGCG was detected at 280 nm using a Shimadzu SPD-M10Avp photodiode array detector, with quantitation based on an external standard curve prepared from EGCG. Apparent first order rate constants were determined by plotting the natural log of EGCG concentrations versus time for the linear portion of the curve (i.e., initial 48 h).

Analysis of PBN-Lipid Radical Spin Adducts by EPR. EPR analysis was measured by the addition of the spin trap, PBN (30 mM), directly to the treated 5 wt % emulsions which were then stored at 37 °C for 8 days. EPR spectra were recorded using a Bruker e-Scan R (Bruker BioSpin, Rheinstetten, Germany) operating in X-band. The instrument settings used were as follows: center field, 3490 G; sweep width, 70 G; microwave power, 6 mW; microwave frequency, 9.78 GHz; modulation frequency, 86 kHz; modulation amplitude, 2.45 G; time constant, 164 ms; conversion time, 20.48 ms for a total of 3 scans per sample. Emulsion samples (50 μL) were transferred to borosilicate capillary tubes before they were introduced to the cavity of the EPR. Analysis was performed at room temperature. Radical concentration was determined by measuring the signal height of the EPR spectrum resulting from PBN-lipid radical adducts.

Lipid Hydroperoxide Analysis. Lipid hydroperoxide concentrations were measured according to the method described by Shantha and Decker.[27] Flaxseed o/w emulsions (0.15 mL) were mixed with 0.75 mL of isooctane/1-butanol (3:1, v/v) and vortexed 3 times for 10 s at 20 s intervals. Samples were subsequently centrifuged for 2 min at 3300 × *g*. The upper layer of the sample extraction (0.1 mL) was mixed with 1.4 mL of methanol/butanol (2:1, v/v). Sample extracts were diluted with water as needed. The ferrous iron solution was prepared by mixing an equal amount of a solution of 0.144 M $FeSO_4$ and 0.132 M $BaCl_2$. The iron solution was centrifuged for 3 min at 3300 × *g*, and an equal volume of the supernatant and 3.94 M ammonium thiocyanate was mixed to prepare the assay solution. A 15-μL portion of the assay solution was added to the samples and then analyzed at 520 nm after storage for 20 min. Lipid hydroperoxide concentrations were quantified using an external calibration curve prepared with cumene hydroperoxide.

TBARS Analysis. TBARS was determined using a method described by McDonald and Hultin.[28] Briefly, samples (0.5 mL) were mixed with 1.0 mL TBA reagent and heated in a boiling water bath for 15 min. Samples were cooled at room temperature for 10 min and centrifuged at 3200 × *g* for 15 min. Samples were then stored for 10 min and the aqueous layer analyzed at 532 nm. TBARS concentrations were quantified using an external calibration curve prepared with 1,1,3,3-tetraethoxypropane.

Analysis of POBN-1-Hydroxyethyl Radical Spin Adducts. HO• radicals derived from the metal-catalyzed reduction of H_2O_2 were measured indirectly by adding ethanol to emulsions. HO• radicals are known to oxidize ethanol to ethyl radical species (1-hydroxyethyl and 2-hydroxyethyl radicals, 85% and 15%, respectively), which eliminates the need to add high (i.e., molar) concentrations of spin trapping agents. The EPR spectra of POBN-1-hydroxyethyl (1-HER) spin adducts were obtained after the direct addition of the hydrophilic spin trap, POBN (50 mM), directly to 10 wt % hexadecane o/w emulsions. Emulsions were then diluted with 20 mM phosphate buffer (pH 3 or 7) and 10 mM EGCG prepared in 20 mM phosphate buffer (pH 3 or 7) to achieve a final EGCG concentration of 0.4 mM in a 5 wt % emulsion with 1 vol % ethanol and 25 μM Fe^{3+}. EDTA (50 μM) or BPY (100 μM) were quickly added to the emulsions to initiate the reaction. Samples were held at 37 °C.

The EPR spectra were recorded using a Bruker e-Scan R (Bruker BioSpin, Rheinstetten, Germany). The instrument settings used were as follows: center field, 3490 G; sweep width, 100 G; microwave power, 6 mW; microwave frequency, 9.78 GHz; modulation frequency, 86 kHz; modulation amplitude, 2.45 G; time constant, 40.96 ms; conversion time, 20.48 ms for a total of 5 scans per sample. Emulsion sample aliquots (50 μL) were transferred to borosilicate capillary tubes and analyzed at room temperature, as described above. Spin adduct intensities were measured according to the method described above.

Statistical Analysis. All experiments were performed in triplicate and results expressed as means and standard deviation. Two-way ANOVA with Bonferroni's post test was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, California, U.S.). Treatments were considered significantly different at $p < 0.05$.

RESULTS AND DISCUSSION

Influence of Metal Catalysis and pH on EGCG Stability in Hexadecane Emulsions. The oxidative stability of EGCG in 5 wt % hexadecane emulsions with ferric ions (25 μM) treated with EDTA (50 μM) or BPY (100 μM) was followed over the course of 8 days under both acidic (pH 3) and neutral (pH 7) conditions. At pH 7 (Figure 2a), the rate of EGCG oxidation, in order of highest to lowest, was as follows: EGCG + EDTA > EGCG control > EGCG + BPY with EGCG control not containing any added chelators. The rate of EGCG consumption during the initial phase (i.e., first 48 h) of the study appeared to follow first order kinetics. As such, first order rate constants for EGCG oxidation were calculated as 1.34 s^{-1}, 0.62 s^{-1}, and 0.43 s^{-1} for EGCG + EDTA, EGCG control, and EGCG + BPY, respectively ($R^2 > 0.99$ for all lines). At day 2, emulsion samples with EDTA retained only ca. 27 μM EGCG (7% of initial concentration), whereas the control emulsion and emulsions with BPY retained 119 μM (30%) and 171 μM (43%), respectively. By day 8, virtually all EGCG was lost with

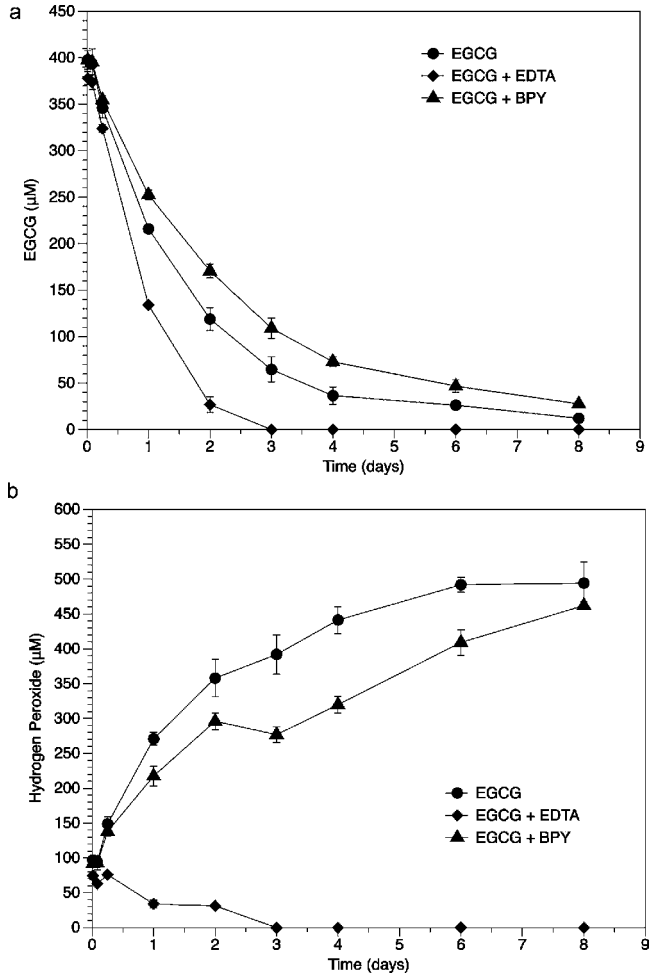

Figure 2. Changes in (a) EGCG and (b) H_2O_2 concentration resulting from the oxidation of 400 μM EGCG in Tween-stabilized 5 wt % hexadecane emulsions in 10 mM phosphate buffer (pH 7) treated with $FeCl_3$ (25 μM) and EDTA (50 μM) or BPY (100 μM).

no observed differences between any of the treatments with respect to EGCG concentration. The presence of the metal chelators EDTA and BPY profoundly affected EGCG oxidation rates, presumably due to their ability to stabilize different iron oxidation states. EDTA is a strong iron chelator, preferentially complexing ferric ions (1:1 ligand:metal ratio), and slightly increasing the reduction potential (E_h) of the ferric/ferrous couple from 0.110 V (for $Fe^{3+/2+}$) to 0.120 V (Fe-(EDTA)$^{3+/2+}$).[29] Though EDTA-iron complexes have slightly higher E_h, Fe(EDTA)$^{2+}$ has still been shown to exhibit higher reducing power as seen by faster Fe^{2+} auto-oxidation and concomitant reduction of dioxygen to superoxide radicals (Scheme 1),[30] as well as faster iron-mediated H_2O_2 reduction to HO•[30,31] compared to uncomplexed Fe^{2+}. Thus, ferric ion stabilization by EDTA promotes the oxidation of Fe^{2+}, which likely leads to the increased rate of EGCG oxidation observed due to an increase in redox cycling to the catalytic ferric state required for subsequent EGCG oxidation. Conversely, BPY preferentially chelates ferrous ions (3:1 ligand:metal ratio),[32] thereby increasing the E_h of iron from 0.110 to 1.074 V for the Fe(BPY)$_3^{3+/2+}$ couple.[29] The higher E_h translates to a decrease in the reducing power of Fe^{2+}, and should theoretically retard hydroperoxyl radical formation. An increase in the reduction potential for the BPY-iron complex also facilitates EGCG

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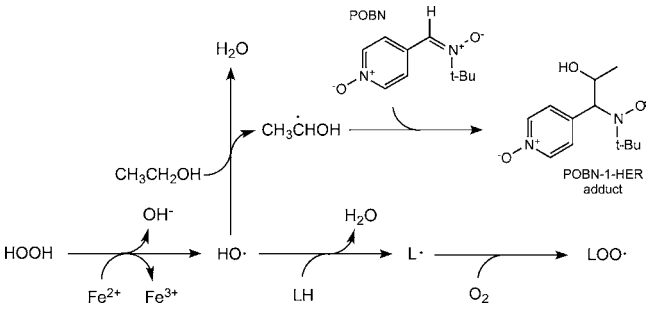
oxidation, the E_h of which is 0.43 V.[33] However, this increase in E_h also slows the redox cycling of ferrous ions to their catalytically active ferric state, which appears to be necessary for subsequent EGCG oxidation (Scheme 1). The E_h of the most effective metal catalysts typically fall in between those reduction potentials of the two species that will ultimately be oxidized or reduced by the metal (i.e., EGCG and molecular oxygen in the system described here).[15] The E_h of the $O_{2(aq)}/O_2^{\bullet-}$ couple is −0.16 V at pH 7[34] and 0.43 V for EGCG, suggesting that uncomplexed iron (0.110 V) and Fe(EDTA) complexes (0.120 V) should act as strong catalysts for EGCG oxidation and dioxygen reduction as opposed to the $Fe(BPY)_3^{3+/2+}$ couple (1.074 V). Furthermore, the Fe(EDTA) complex shows better catalytic activity due to the ease of Fe^{2+} oxidation and an increase in iron solubility at neutral pH.

The apparent rate of H_2O_2 production resulting from EGCG oxidation was highest for the EGCG control and BPY treatment yet, surprisingly, virtually no H_2O_2 was observed in hexadecane emulsions containing EDTA (Figure 2b). In the EGCG control and BPY treatment, H_2O_2 production rates corresponded well with EGCG oxidation rates, with the EGCG control showing faster EGCG oxidation and subsequent H_2O_2 production compared to the BPY treatment. By this measure alone, EDTA would appear to be the most effective treatment for preventing H_2O_2 generation, which would refute our proposed mechanistic interpretation based on EGCG oxidation. However, the low levels of H_2O_2 observed in the presence of EDTA does not appear to reflect lower peroxide generation rates, but rather is likely due to the rapid decomposition of H_2O_2 to HO• radicals via the Fenton reaction (Scheme 1). As discussed above, the reducing capacity of iron is increased when it is complexed to EDTA, which therefore should accelerate the reduction of peroxides. The ability of EDTA-iron complexes to rapidly generate HO• radicals from H_2O_2 is, in fact, commonly exploited in HO• radical scavenging studies.[35−37]

A spin trapping EPR technique was used to confirm that H_2O_2 was indeed being reduced to HO• radicals in EDTA-containing emulsions. Ethanol (1 vol %, final concentration) was added to emulsions and HO• radicals were measured indirectly as 1-hydroxyethyl radicals (as shown in Scheme 2).

Scheme 2. Indirect •HO Radical Measurement via POBN-1-HER Spin Adduct Formation Resulting from the Metal-Catalyzed Oxidation of Polyphenols



Under these conditions, HO• radicals oxidize ethanol to 1-hydroxyethyl radicals, which can be quenched by POBN to yield spin adducts with relatively long half-lives.[38] The time required for the formation of detectable levels of POBN-1-HER adducts (a representative spectrum is shown in Figure 3) was ca. 8 h for both the EGCG control and the BPY treatment (Figure 4); however, POBN-1-HER spin adducts were detected

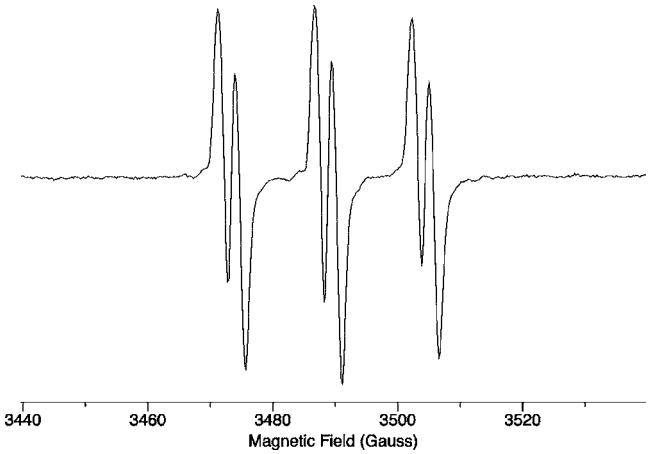


Figure 3. Representative spectra of POBN-1-HER spin adduct in 5 wt % hexadecane emulsion.

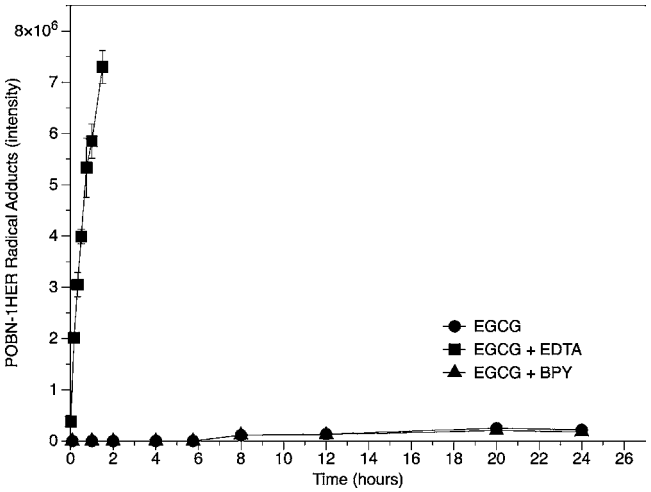


Figure 4. POBN-1-HER spin adduct generation resulting from the oxidation of 400 μM EGCG in 5 wt % hexadecane o/w emulsions at pH 7 treated with $FeCl_3$ (25 μM) and the metal chelators EDTA (50 μM) or BPY (100 μM).

within 1 min in EDTA-containing samples (Figure 4). The rapid formation of HO• radicals in the presence of EDTA accounts for the lack of observed H_2O_2 accumulation and accelerated EGCG oxidation at pH 7. Even after 24 h of storage, EGCG control and EGCG + BPY treatments showed intensities of ca. 2×10^5, while EDTA treatment reached an intensity of 7×10^6 within just 90 min.

The effect of EDTA and BPY on EGCG oxidation and H_2O_2 production was also investigated at pH 3 (Figure 5a,b, respectively). Nearly 90% of the EGCG was lost in control and BPY-containing emulsions within 8 days, whereas EGCG remained stable in the presence of EDTA over the course of the experiment. First order rate constants for EGCG oxidation were calculated as 0.3147 s^{-1} and 0.3111 s^{-1} for the EGCG control and BPY containing samples ($R^2 > 0.99$ for all lines), respectively, although no significant difference was observed between these two treatments. In comparison with results obtained at pH 7, no oxidation was observed in EDTA-containing samples at day 2, while both the EGCG control and BPY treatment retained ca. 200 μM EGCG (50%). As expected, EGCG showed greater stability under the acidic conditions employed here compared to the system at pH 7, which is

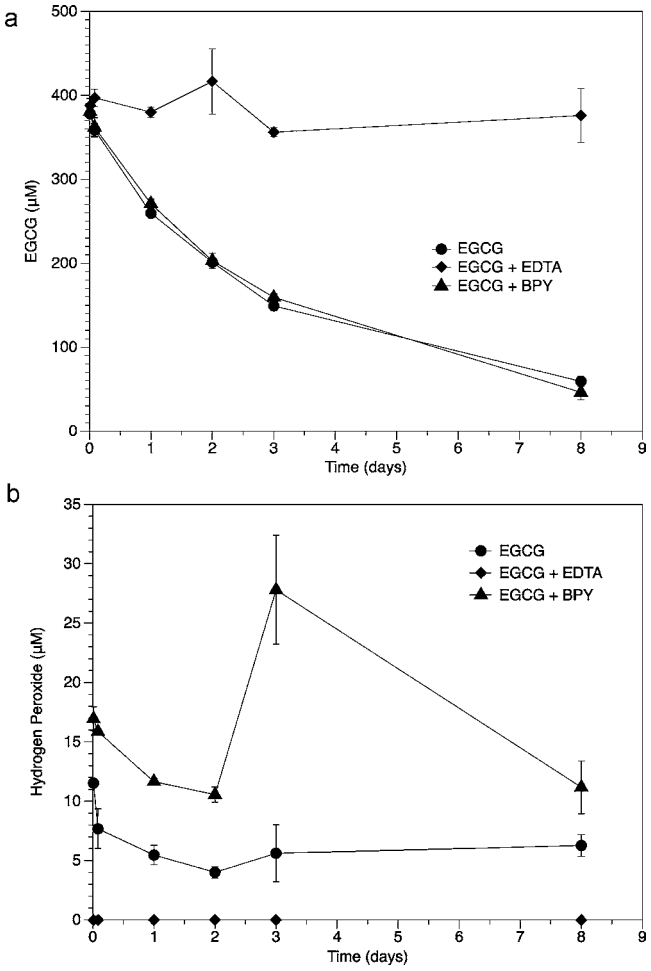


Figure 5. Changes in (a) EGCG and (b) H_2O_2 concentration resulting from the oxidation of 400 μM EGCG in Tween-stabilized 5 wt % hexadecane emulsions in 10 mM phosphate buffer (pH 3) treated with $FeCl_3$ (25 μM) and the metal chelators EDTA (50 μM) or BPY (100 μM).

consistent with previous reports.[39] The control showed greater increased EGCG stability at the acidic pH (0.3147 s^{-1} vs 0.6163 s^{-1}) as opposed to the BPY treatment (0.3111 s^{-1} vs 0.4328 s^{-1}). With decreasing pH, reduction potentials increase, thus increasing the reduction potential of the $Fe^{3+/2+}$ couple, which should lower the availability of catalytic Fe^{3+} required for EGCG oxidation. In contrast to what was observed at pH 7, the rate of EGCG oxidation was markedly faster in both the EGCG control and BPY treatment compared to the EDTA treatment. Under these acidic conditions, EDTA no longer accelerated EGCG oxidation as was observed at pH 7, but instead strongly inhibited EGCG oxidation. Consistent with the EGCG oxidation kinetics, the observed levels of H_2O_2 were significantly higher for the EGCG control and BPY treatment compared to the EDTA treatment (Figure 5b), though little H_2O_2 accumulation was observed in all treatments as compared to H_2O_2 levels at neutral pH.

EPR analysis of HO• radical formation at pH 3, as measured by POBN-1-HER spin adducts, revealed that H_2O_2 was quickly reduced to HO• radicals in both the EGCG control and BPY-containing emulsions, but that HO• radical formation was relatively slow in EDTA-containing emulsions (Figure 6). The time required for the formation of detectable levels of POBN-1-HER adducts was ca. 2 h for EDTA-containing emulsions;

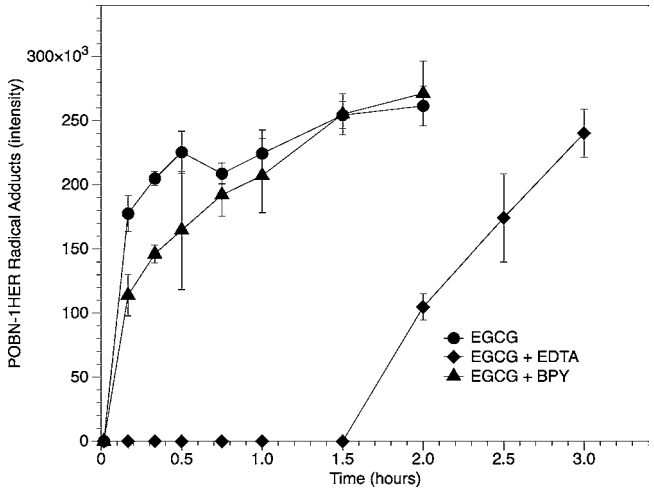


Figure 6. POBN-1-HER spin adduct generation resulting from the oxidation of 400 μM EGCG in 5 wt % hexadecane o/w emulsions at pH 3 treated with $FeCl_3$ (25 μM) and the metal chelators EDTA (50 μM) or BPY (100 μM).

however POBN-1-HER spin adducts were quickly detected within ca. 10 min in the EGCG control and BPY-containing emulsions. It is interesting to note that while their rate of formation was indeed slow, HO• radicals were observed in emulsions containing EDTA, despite the fact that EGCG was relatively stable and only a small quantity of H_2O_2 could be measured under the same conditions.

Effect of EGCG Oxidation and H_2O_2 Production on the Oxidative Stability of Flaxseed Emulsions. The consequences of EGCG oxidation and concomitant H_2O_2 and HO• radical generation on the stability of an oxidatively labile flaxseed emulsion were investigated. The objective of the experiments was to establish if, and under what conditions, EGCG-generated reactive oxygen species (i.e., HO• radicals, H_2O_2) could promote the oxidation of flaxseed o/w emulsions, thereby eclipsing the catechin's antioxidant capacity.

The rate of formation and final yield of lipid-derived radicals (e.g., lipid alkoxyl and hydroperoxyl radicals) in flaxseed oil emulsions was measured using a PBN spin trapping technique. At pH 7, emulsions containing EGCG + EDTA consistently gave high yields of PBN-lipid radical spin adducts over the 8-day study (Figure 7a). This is consistent with what was observed in hexadecane emulsions, wherein the EDTA treatment resulted in the highest rate of EGCG oxidation (Figure 2a) and HO• radical production (Figure 4). Overall, PBN-lipid radical adduct intensities for EGCG-containing samples at day 8 were highest for the EDTA treatment, followed by the EGCG control and finally the BPY treatment (Figure 7a). Unlike EGCG + EDTA treatments, at early time points EGCG and EGCG + BPY treatments showed significantly less PBN-lipid radicals compared to their corresponding controls, though by day 6, all EGCG treatments showed higher adduct intensities compared to their respective controls. This is consistent with HO• radical production where EDTA treatment generated HO• radicals immediately while the EGCG-control and BPY treatments showed a lag time prior to HO• radical formation (Figure 4). However, the increased levels of lipid-derived radicals observed in the presence of EGCG, as measured by PBN spin adducts, does not necessarily predict net pro-oxidant activity. In this system, PBN must be present at sufficiently high concentrations to effectively

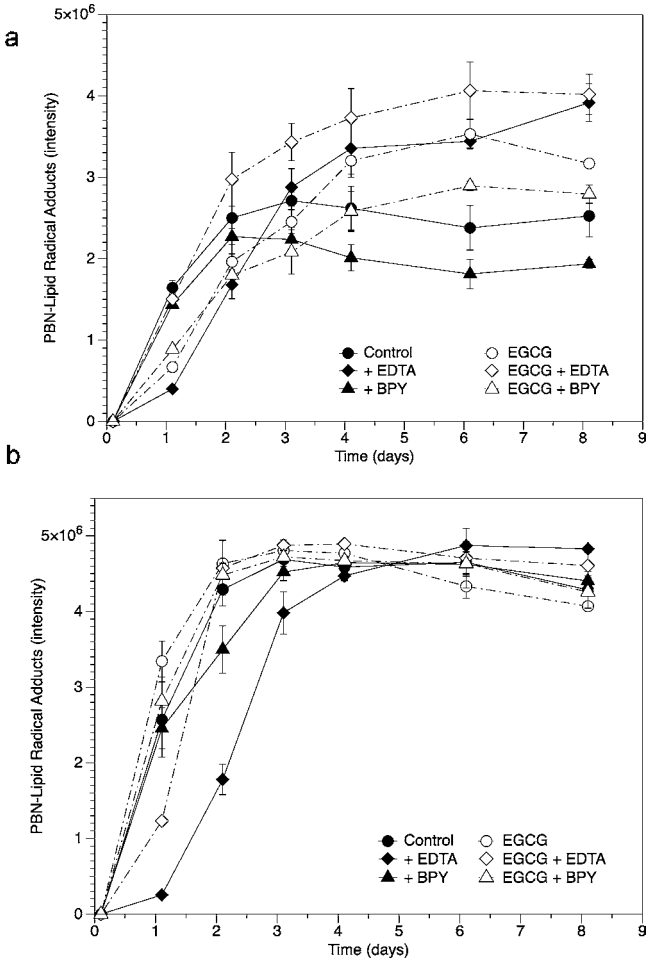


Figure 7. PBN-lipid derived spin adduct generation resulting from the oxidation of 5 wt % flaxseed o/w emulsions treated with $FeCl_3$ (25 μM) and the metal chelators EDTA (50 μM) or BPY (100 μM) in the presence of 400 μM EGCG at (a) pH 7 and (b) pH 3.

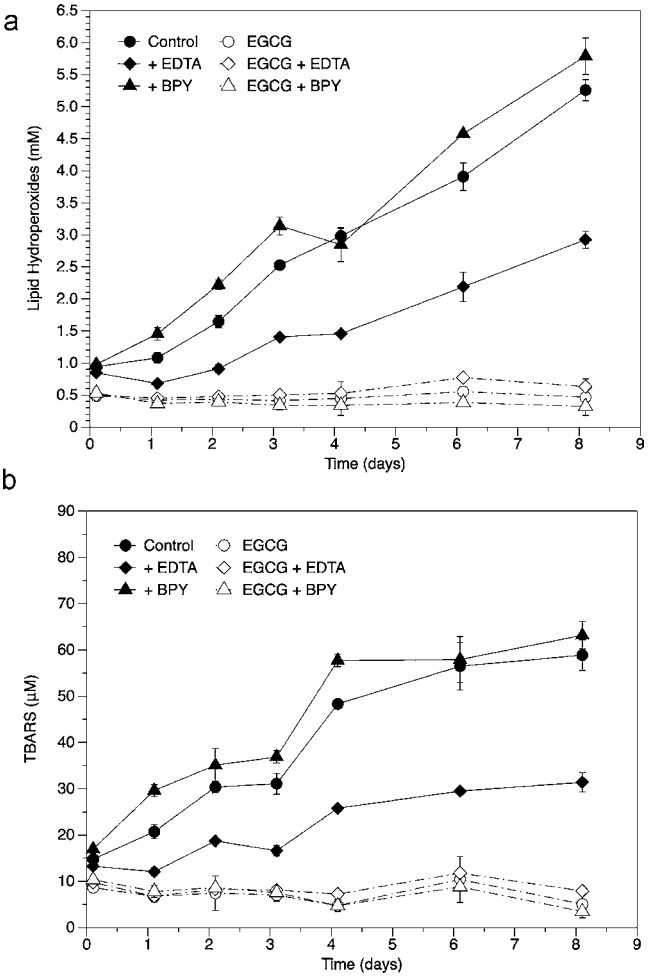


Figure 8. Changes in (a) lipid hydroperoxide concentrations and (b) TBARS resulting from the oxidation of 5 wt % flaxseed o/w emulsions in 10 mM phosphate buffer (pH 7) treated with $FeCl_3$ (25 μM) and the metal chelators EDTA (50 μM) or BPY (100 μM) in the presence of 400 μM EGCG.

compete with EGCG for lipid-derived radicals. In the same system without PBN, EGCG would be the sole lipid radical scavenger. Therefore, EGCG appears to promote lipid oxidation initiation reactions in emulsions by generating H_2O_2 and, eventually, HO• radicals; however, paradoxically, EGCG would also serve as an antioxidant downstream by quenching the same lipid-derived radicals it helped to promote (Scheme 1).

At pH 3, the initial rate of PBN-lipid radical formation was higher compared to those at pH 7, with a significantly higher yield of spin adducts observed (Figure 7b). Furthermore, EGCG-containing emulsions produced PBN-lipid radicals at a faster rate than their corresponding controls with the order of EGCG-control > EGCG + BPY > EGCG + EDTA. Unlike pH 7 emulsions, EGCG + EDTA samples showed markedly lower radical adducts compared to other EGCG treatments, as was expected based on the EGCG oxidation (Figure 5a) and HO• radical production (Figure 6) results in hexadecane emulsions reported above where the initial rate of POBN-1HER generation followed: EGCG-control > EGCG + BPY > EGCG + EDTA (Figure 7b).

On the basis of EPR spin trapping analysis alone, it would appear that EGCG is indeed pro-oxidative at pH 3 and 7. This, however, does not necessarily predict the net effect that EGCG will have on overall lipid stability, as was discussed above. Therefore, both lipid hydroperoxides and TBARS concentrations were followed for all treatments for 8 days at both pH 3 and 7 in flaxseed o/w emulsions. At pH 7, lipid hydroperoxide concentrations were markedly lower for all EGCG-containing emulsions, and showed a profound antioxidant effect (Figure 8a). There were no significant differences ($p > 0.05$) between EGCG treatments and no significant increases ($p > 0.05$) in lipid hydroperoxides in any of the EGCG treatments over the course of the study. In the absence of EGCG, EDTA inhibited lipid hydroperoxide formation compared to the control and the BPY treatment, which is consistent with previous studies.[40,41] The observed TBARS concentrations of oxidizing flaxseed oil emulsions followed a similar trend (Figure 8b), with EGCG inhibiting TBARS formation for all treatments. EDTA inhibited TBARS formation for EGCG-free emulsions, which again is consistent with lipid hydroperoxide results (Figure 8a).

The above results suggest that, at neutral pH, all EGCG-treated samples display antioxidant activity. At pH 7, EGCG oxidized and H_2O_2 was observed to accumulate in flaxseed o/w emulsions (data not shown) consistent with what was seen in 5 wt % hexadecane emulsions. The final H_2O_2 concentration after 8 days in flaxseed emulsions was 415 μM and 310 μM for the EGCG control and the BPY treatment, respectively, which is

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lower than that seen in 5 wt % hexadecane emulsions, likely due to the fact that EGCG was also consumed by lipid-derived radicals.The lack of pro-oxidant effect in EGCG + EDTA samples may suggest that HO• radicals generated in the aqueous phase are simply too reactive to reach the lipid droplet, or the antioxidant activity of EDTA resulting from iron chelation mitigated the pro-oxidant effect resulting from EGCG oxidation.

Lipid hydroperoxide and TBARS concentrations were followed in 5 wt % flaxseed oil emulsions at pH 3 (Figure 9a,b, respectively). Within the first 4 days, all EGCG-containing emulsions showed significantly less lipid hydroperoxides compared to their corresponding EGCG-free treatments. However, after day 4, emulsions containing EGCG only and EGCG + BPY showed marked increases in hydroperoxide production rates, resulting in significantly higher lipid peroxides than the EGCG + EDTA treatment, which showed no significant increase throughout the study. Analysis of TBARS production in flaxseed o/w emulsions at pH 3 were consistent with lipid hydroperoxide analysis for EGCG + EDTA samples and provided further evidence that this treatment inhibited lipid oxidation reactions (Figure 9b). However, a significant pro-oxidative effect was observed in EGCG only and EGCG + BPY emulsions compared to EGCG-free controls. The fact that lipid hydroperoxides were not observed to accumulate to any significant degree during the early stages of oxidation, yet high TBARS yields were observed, could be due to the fact that EGCG and iron promoted the reduction of hydroperoxides. The presence of BPY under these conditions is also expected to favor the speciation of iron to its reduced oxidation state (i.e., ferrous ions), which are important catalysts for the decomposition of lipid hydroperoxides to alkoxyl radicals.

Further examination of EGCG oxidation in flaxseed emulsions at pH 3 revealed a much more rapid loss of EGCG for the EGCG control and EGCG + BPY treatment (Figure 9c), consistent with our proposed scheme of EGCG loss due to lipid-derived radical quenching. Unlike in hexadecane emulsions (Figure 5a), the rate of EGCG oxidation from highest to lowest was in the order of EGCG + BPY > EGCG control > EGCG + EDTA (Figure 9c), with nearly all EGCG oxidized by day 2 in BPY containing samples and only 117 μM EGCG (29%) remaining in the EGCG control compared to the 50% EGCG remaining in hexadecane emulsions. EGCG was, thus, also depleted by reacting with lipid-derived radicals. This is in agreement with our results that show the EGCG + BPY treatment yield the most secondary lipid oxidation markers. Therefore, the pro-oxidant effect resulting from the fast reduction of lipid hydroperoxides and H_2O_2 may eclipse any antioxidant effect stemming from lipid radical scavenging by EGCG in this system.

Many studies have shown that antioxidant activity in food lipids is achieved when relatively high concentrations of phenolic compounds are used,[42] while some studies showed pro-oxidant activity when the same compounds were present at relatively low concentrations. In a study by Mei et al., low concentrations (5 μM) of galloyl derivatives (methyl gallate, gallamide, gallic acid) resulted in increased lipid hydroperoxide and TBARS concentrations in Brij-stabilized salmon o/w emulsions at pH 3.0, yet this pro-oxidant activity was not seen when high concentrations (500 μM) of the derivatives were used.[43] With the relatively high concentration of EGCG (400 μM) used in this study, the pro-oxidant effect contributed by H_2O_2 appears to be mitigated by the ability of EGCG to

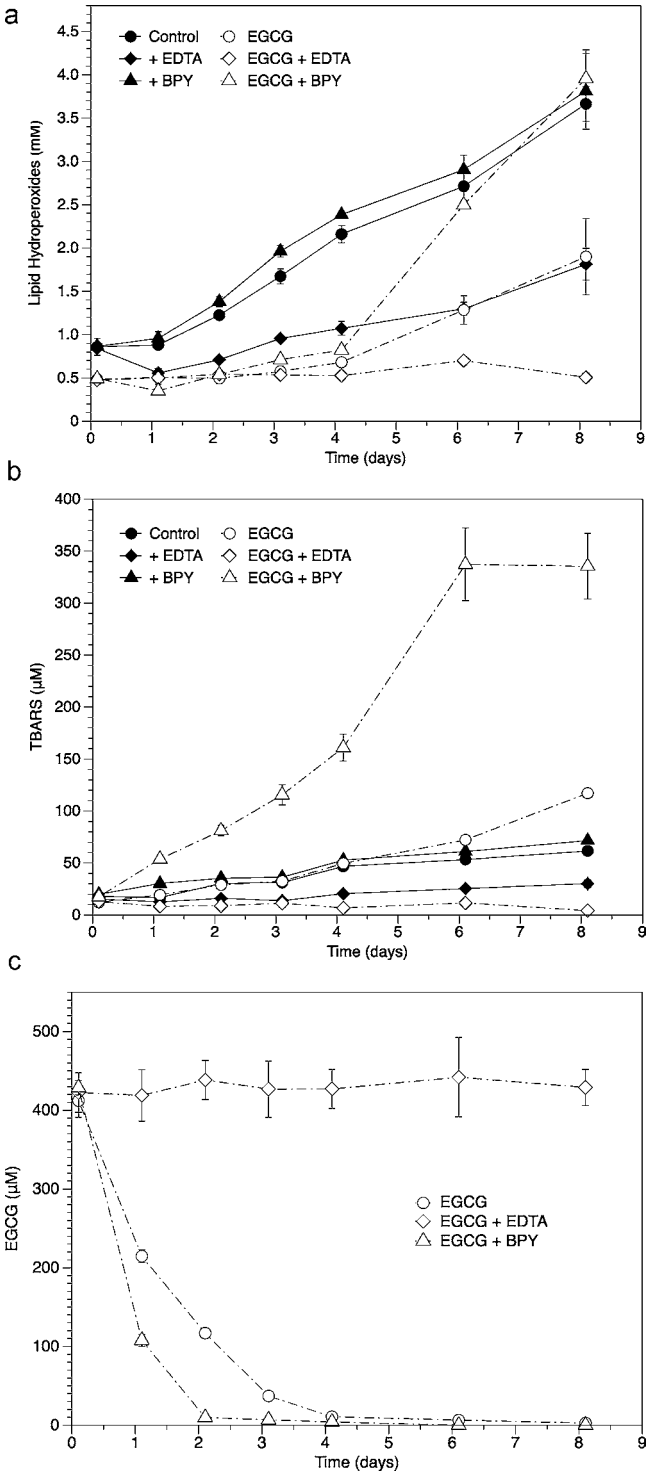

Figure 9. Changes in (a) lipid hydroperoxide concentrations, (b) TBARS, and (c) EGCG concentrations resulting from the oxidation of 5 wt % flaxseed o/w emulsions in 10 mM phosphate buffer (pH 3) treated with $FeCl_3$ (25 μM) and the metal chelators EDTA (50 μM) or BPY (100 μM) in the presence of 400 μM EGCG.

interfere with chain propagation of lipid oxidation reactions. The exception to this appears to be in low pH emulsions, especially when EGCG is added in the presence of a ferrous chelator (*e.g.*, BPY), which results in the rapid generation of TBARS. Other studies have also demonstrated the pro-oxidant activity of EGCG under acidic conditions. Huang and Frankel

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showed that tea catechins, including (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, and EGCG (5 and 20 μM) were pro-oxidative in low pH (3.0−3.5) corn o/w emulsions.[9] Pro-oxidant activity was also observed in sunflower o/w emulsions treated with 50 μM $FeCl_3$ and 100 μM tea polyphenols including myricetin, (−)-epicatechin gallate, and EGCG at pH 5.5.[8] In the same study by Mei et al., no pro-oxidant activity was observed with either 5 or 500 μM galloyl derivatives at pH 7.0,[43] suggesting the importance of pH on antioxidant activity. These results are in line with those observed in the present study, where EGCG exhibited strong antioxidant activity in flaxseed o/w emulsions at pH 7 regardless of chelator treatment while, at pH 3, pro-oxidant activity was observed in EGCG treated samples with the exception of EDTA treatment.

Factors such as pH and the presence and type of transition metal chelators have a profound effect on the stability of trihydroxylated phenolics like EGCG. Under some conditions, these compounds are rapidly oxidized while oxygen is reduced to H_2O_2 in the process. Rapid polyphenol oxidation and subsequent ROS generation did not directly correlate with the net anti/pro-oxidant effects in lipid dispersions. Instead, factors that increased ferrous iron availability such as ferrous chelators and acidic pH appeared to result in pro-oxidant activity. A more complete understanding of the factors that influence the net anti/pro-oxidant activity of phenolics will undoubtedly lead to improved technologies for the delivery of biologically significant levels of polyphenols in chemically stable lipid-based foods.

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Notes

The authors declare no competing financial interest.

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