Effects of Natural Antioxidants on Iron-Catalyzed Lipid Oxidation of Structured Lipid-Based Emulsions

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ABSTRACT: The effects of iron, pH, and natural antioxidants (α-tocopherol, gallic acid, and quercetin) on oxidation of structured lipid-based emulsions were evaluated. Ten percent oil-in-water emulsions were formulated with a canola oil/caprylic acid structured lipid and stabilized with 0.5% whey protein isolate. The PV, anisidine values, and Totox values of emulsions stored at 50°C were measured over a 15-d period. Iron significantly ($P < 0.05$) increased lipid oxidation rates compared to control emulsions. Greater iron-catalyzed lipid oxidation occurred in the pH 3.0 emulsions compared to their pH 7.0 counterparts. Quercetin and gallic acid exhibited significant ($P < 0.05$) prooxidant effects on total oxidation in the pH 3.0 emulsions. Emulsions at pH 7.0 were relatively stable to oxidation throughout the storage period. Because of the ability of some of these natural antioxidants to exhibit prooxidant activity, care should be exercised when adding them to food systems containing transition metals.

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KEY WORDS: Anisidine value, emulsions, gallic acid, iron-catalyzed lipid oxidation, peroxide value, quercetin, structured lipid, α-tocopherol, Totox value, whey protein isolate.

Much research has been devoted to the production, functionality, and potential health benefits of structured lipids (SL) in recent years. Among the FA considered for SL synthesis are medium-chain FA and PUFA. Medium-chain FA are readily metabolized in the body as a quick source of energy, whereas long-chain FA are required as sources of EFA (1). Unfortunately, PUFA are highly susceptible to oxidation in foods, which is of great concern to the food industry because oxidation leads to the development of undesirable "off-flavors" and potentially toxic reaction products (2).

Iron is the most common transition metal found in foods. It promotes lipid oxidation by catalyzing the breakdown of peroxides into free radicals (3). In oil-in-water emulsion systems, the charge status of emulsion droplets greatly influences iron-catalyzed lipid oxidation. Attractive/repulsive electrostatic interactions between charged emulsion droplets and charged prooxidants greatly affect the location and hence the activity of transition metals. Mei *et al.* (4) showed that Fe^{3+} and Fe^{2+} are strongly bound to negatively charged SDS-stabilized emulsion droplets, but not to positively charged or uncharged emulsion droplets. Consequently, lipid oxidation occurred much faster in the negatively charged emulsions when positively charged transition metal ions were present. SDS is not used as an emulsifier in foods. However, proteins can produce charged emulsion droplets in systems away from their isoelectric point and may have a similar impact on lipid oxidation rates. Because whey protein isolate (WPI) is used in a wide variety of foods for its valuable nutritional characteristics and ability to contribute unique and essential functional properties to the final products, a need exists for studies on the oxidation properties of WPI-stabilized SL-based emulsions at various pH levels.

The incorporation of antioxidants into foods that contain fats and oils is helpful in retarding the metal-catalyzed oxidation of lipids (5). Because of safety concerns, there is currently much interest in replacing synthetic antioxidants with natural ones. Tocopherols are the most important natural antioxidants in the food industry, and their antioxidant mechanism involves the donation of hydrogen to a peroxy radical (6).

Numerous naturally occurring phenolic antioxidants have also been identified from plant sources (6). However, their effectiveness is often difficult to predict because there are several different mechanisms by which phenolic compounds influence lipid oxidation rates (7). Hydroxybenzoic acids, including gallic acid, are phenolic compounds that can form metal complexes (6). However, the antioxidant activity of these compounds varies greatly and is dependent on the food system (6). Quercetin has gained attention as a potent antioxidant because of its ability to scavenge hydroxyl radicals and superoxide anions (6). Unfortunately, quercetin is autoxidized under certain conditions, which leads to an increase in hydroxyl radicals (8). Additionally, both quercetin and gallic acid can reduce Fe^{3+} to the more active Fe^{2+}, thereby increasing lipid oxidation (7).

In this study, the effects of pH and natural antioxidants on iron-catalyzed lipid oxidation of canola oil/caprylic acid SL-based emulsions were evaluated. Primary, secondary, and total oxidation were determined in the WPI-stabilized emulsions containing α-tocopherol, gallic acid, and quercetin over a 15-d storage period.

EXPERIMENTAL PROCEDURES

Materials. Canola oil was purchased from a local supermarket. Caprylic acid (purity >98%), gallic acid, quercetin, α-tocopherol, and ferrous sulfate were purchased from Sigma Chemical Company (St. Louis, MO). An *sn*-1,3-specific immobilized

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lipase from *Rhizomucor miehei* (IM 60) was obtained from Novo Nordisk A/S (Bagsvaerd, Denmark). WPI (#27361) was provided by Land O'Lakes (St. Paul, MN). All other reagents were purchased from Fisher Scientific (Fairlawn, NJ).

SL production. The SL was produced in a packed-bed bioreactor using the optimal conditions reported previously (9) for reacting canola oil and caprylic acid. The product was purified using a KDL-4 short-path distillation unit (UIC Inc.). The oil was passed through the distillation apparatus three times under the following conditions: holding temperature, 25°C; heating oil temperature, 185°C; cooling water temperature, 15°C; pressure, <0.01 Torr. The purified SL product contained the following FA (mol%): 37.3% 8:0, 1.8% 16:0, 1.7% 18:0, 47.3% 18:1, 8.9% 18:2, and 3.0% 18:3 as determined by GC of methyl esters according to a procedure described previously (10).

Emulsion preparation. Ten percent oil-in-water emulsions were prepared with the canola oil/caprylic acid SL, 10 mM phosphate buffer, and 0.5% WPI. α-Tocopherol was added directly to the SL. Quercetin was dissolved in ethanol and then added to the oil. The organic solvent was evaporated under N_2. Gallic acid was mixed into the phosphate buffer. Total antioxidant addition levels were 0.02% of the oil weight in each emulsion. The pH of the emulsions was adjusted to 3.0 or 7.0 by adding HCl (0.1 M) or NaOH (0.1 M). The emulsions were passed through a high-pressure valve homogenizer (Emulsiflex C5) six times at 10,000 psi. All samples were held on ice during processing. Sodium azide (1 mM) was added to the emulsions to slow microbial growth. Ferrous sulfate (50 or 100 μM) was added to the emulsions immediately prior to storage. Particle size distribution was measured by integrated light scattering (Mastersizer S, Malvern Instruments) using standard optical parameters to ensure that similar droplet sizes were formed in the emulsions during homogenization. The mean apparent particle diameter ($D_{3,2}$ value) was 0.82 ± 0.09 μm for emulsions in this study.

Oxidation experiments. Emulsion samples were allowed to oxidize in a 50°C covered water bath for 15 d. The primary and secondary oxidation products were measured in the emulsion samples after 0, 1, 2, 4, 8, and 15 d of storage. Oil was extracted from the emulsions by adding isooctane/isopropanol (3:2, vol/vol), vortexing three times for 10 s each, and centrifuging ($2150 \times g$, 5 min). The clear upper layer was collected and the solvent was evaporated under nitrogen. PV were determined by using the International Dairy Foundation method described by Shantha and Decker (11). Anisidine values (AV) were determined according to AOCS Official Method Cd 18-90 (12). This method determines the amount of aldehyde (principally 2-alkenals and 2,4-alkadienals) present in the oil (13). The Totox value was calculated as: Totox value = 2(PV) + AV (13). The Totox value combines evidence about the past history of an oil with its present state and is useful for estimating oxidative deterioration of food lipids (13).

Statistical analysis. All experiments were performed on duplicate samples. Statistical analyses were conducted with the SAS software package (14). ANOVA were performed. Significant differences ($P < 0.05$) were determined by the least squares difference method.

RESULTS AND DISCUSSION

Effect of iron. Control samples that did not contain added iron or antioxidants were included in this experiment. This was done to determine the effect of added iron on the rate of lipid oxidation of SL-based emulsions. Additionally, iron was added at two levels (50 and 100 μM) to determine the effect of concentration on the rate of oxidation.

PV were significantly ($P < 0.05$) greater in emulsions containing iron than in the corresponding control samples in all cases, except in the pH 3.0 emulsion that contained 50 μM iron (Fig. 1). Secondary oxidation was higher in the pH 3.0 emulsions containing iron than in their control counterparts (Fig. 2). However, at pH 7.0 significant ($P < 0.05$) differences between the AV of the Fe control and control samples did not exist (Fig. 2). Although the PV and AV results may seem contradictory, transition metals are known both to decompose peroxides and to produce free radicals; therefore, changes in peroxide concentrations in the presence of metals actually represent a balance between peroxide formation and decomposition (15). Thus, the similar PV between the Fe control (50 μM) and control samples at pH 3.0 do not necessarily correspond to equal amounts of primary oxidation because of the rapid decomposition that may also be occurring in the iron-catalyzed emulsion. When PV and AV results were combined into Totox values, the effect of iron became significant ($P < 0.05$) in all emulsions. At both pH values, greater total oxidation occurred in SL-based emulsions containing iron than in their control counterparts on the final day of the study (Fig. 3). The prooxidant effect of ferrous sulfate was expected because iron is known to stimulate lipid peroxidation by the Fenton reaction, and also to accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals. These radical species can then abstract hydrogen and further perpetuate the chain reaction of lipid peroxidation (5).

At pH 3.0, greater amounts of secondary and total oxidation occurred in the presence of higher concentrations of iron (100 vs. 50 μM) on the final day of the study (Figs. 2 and 3). However, at pH 7.0 no significant differences existed between the final AV or Totox values of emulsions containing 50 or 100 μM ferrous sulfate (Figs. 2 and 3). Increasing iron concentrations in the emulsion system would also increase the iron concentration in the aqueous phase. Iron that is not strongly associated with the emulsion droplets is not an important prooxidant in model emulsions (4). Iron is more soluble and in closer proximity to the droplet surface at pH 3.0 compared to pH 7.0 (3), which explains the increased prooxidant activity of iron at higher concentrations in low-pH systems.

Effect of pH. Emulsion pH significantly ($P < 0.05$) affected the PV on days 1, 4, and 8. However, the PV of emulsions at pH 3.0 and 7.0 were no longer different at day 15 (Fig. 1). Emulsion pH affected secondary (Fig. 2) and total oxidation (Fig. 3) to a greater extent. The influence of pH on secondary oxidation was significant ($P < 0.05$) on days 2–15 and on total oxidation on all days of the study (excluding day 0). Both secondary and total oxidation increased in absolute value when

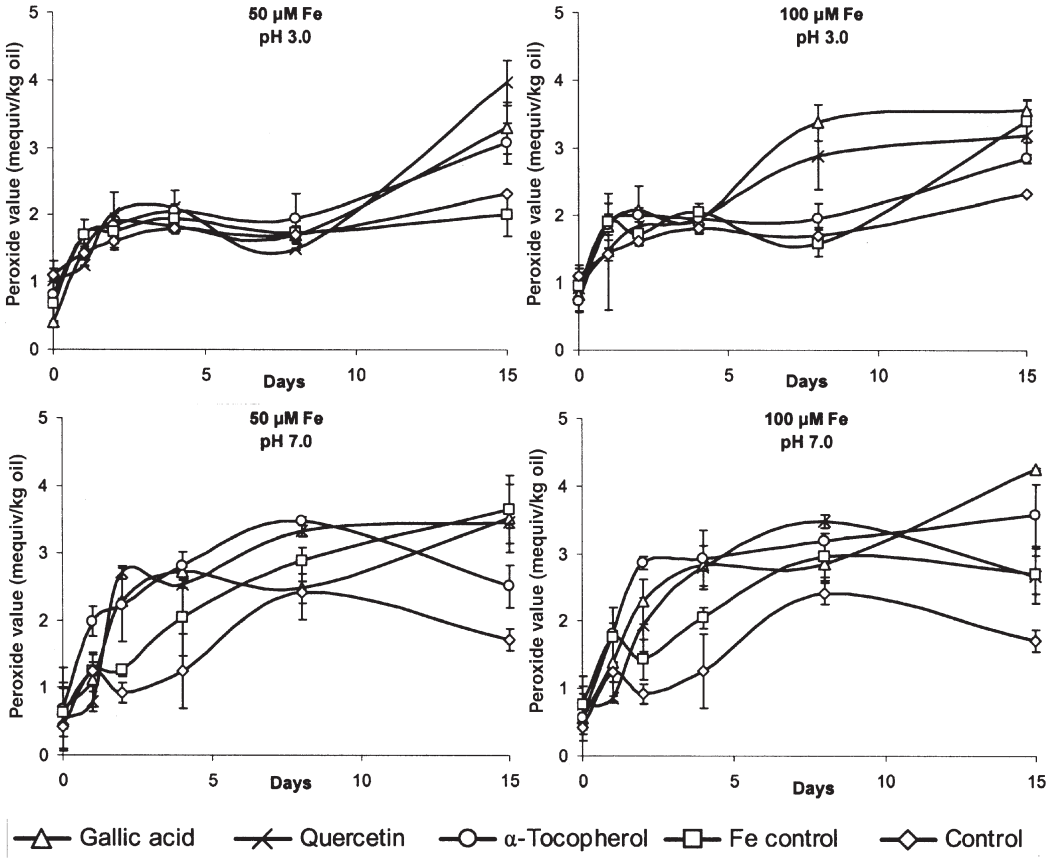


FIG. 1. Effect of iron concentration (50 and 100 µM), pH (3.0 and 7.0), and natural antioxidants (α-tocopherol, gallic acid, and quercetin) on PV over time in structured lipid-based emulsions stabilized by whey protein isolate. Control emulsions contained no added iron or antioxidants. Data shown are the average of duplicate samples held at 50°C. Error bars represent SE.

the pH was decreased from 7.0 to 3.0 in emulsions containing either 50 or 100 µM ferrous sulfate (Figs. 2 and 3). Similarly, low pH promoted oxidation in fish oil-enriched mayonnaise (16). Mei *et al.* (17) also reported that lipid oxidation increased with decreasing pH in corn oil emulsions prepared with the anionic surfactant SDS. These authors proposed that the increases in oxidation were due to increased iron solubility at lower pH.

Effect of natural antioxidants. The influence of natural antioxidants, α-tocopherol, gallic acid, and quercetin (Scheme 1), on iron-catalyzed lipid oxidation of SL-based emulsions at pH 3.0 and 7.0 were evaluated. The antioxidant variable had a significant ($P < 0.05$) effect on primary oxidation of the SL-based emulsions on days 1–8. In the pH 7.0 emulsions (50 µM Fe), α-tocopherol reduced the PV of the SL-based emulsion compared to the Fe control on day 15 (Fig. 1). In the pH 3.0 emulsions containing 50 µM iron, all the natural compounds exhibited a prooxidant effect on primary oxidation at the final day of storage (Fig. 1). In the emulsions containing higher concentrations of iron (100 µM), the PV for emulsions containing the natural compounds were not significantly different from the Fe control, except for gallic acid, which exhibited a prooxidant effect at pH 7.0 on day 15 (Fig. 1). Decomposition of hydroperoxides into secondary oxidation products is more closely related to flavor deterioration than hydroperoxide formation (18). Thus, when evaluating antioxidants, it is important to measure secondary and total oxidation before drawing conclusions on their efficacy and mechanisms of action.

Antioxidants significantly ($P < 0.05$) affected secondary oxidation on days 2–15 and total oxidation on all days (excluding day 0) of the study. Under all conditions, gallic acid and quercetin significantly ($P < 0.05$) increased AV compared to the Fe control samples on the final day of the study (Fig. 2). Fukumoto and Mazza (19) also reported that phenolic compounds (including quercetin and gallic acid) exhibited prooxidant behavior at low concentrations (<500 µM). The prooxidant activity of gallic acid may be related to the ability of flavonoids to undergo autoxidation catalyzed by transition metals to produce hydrogen peroxide and form hydroxyl radicals *via* Fenton chemistry (8). The prooxidant mechanism of flavonoids containing the catechol structural element, such as quercetin, has recently been demonstrated and described in detail by Rietjens *et al.* (20). The prooxidant activity is related to the formation of quinone-type metabolites from the B-ring of catechol flavonoids. The oxidation of catechols to quinones generates potent electrophiles that promote oxidation (20). At pH 3.0, emulsions containing α-tocopherol had AV similar to

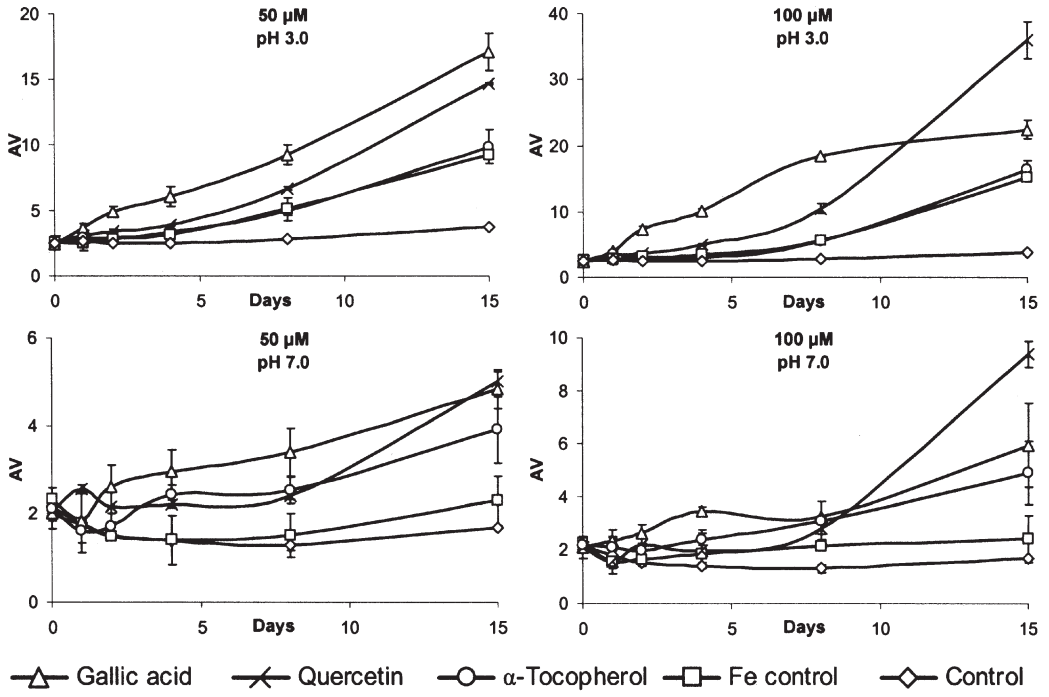


FIG. 2. Effect of iron concentration (50 and 100 µM), pH (3.0 and 7.0), and natural antioxidants (α-tocopherol, gallic acid, and quercetin) on anisidine values (AV) over time in structured lipid-based emulsions stabilized by whey protein isolate. Control emulsions contained no added iron or antioxidants. Data shown are the average of duplicate samples held at 50°C. Error bars represent SE.

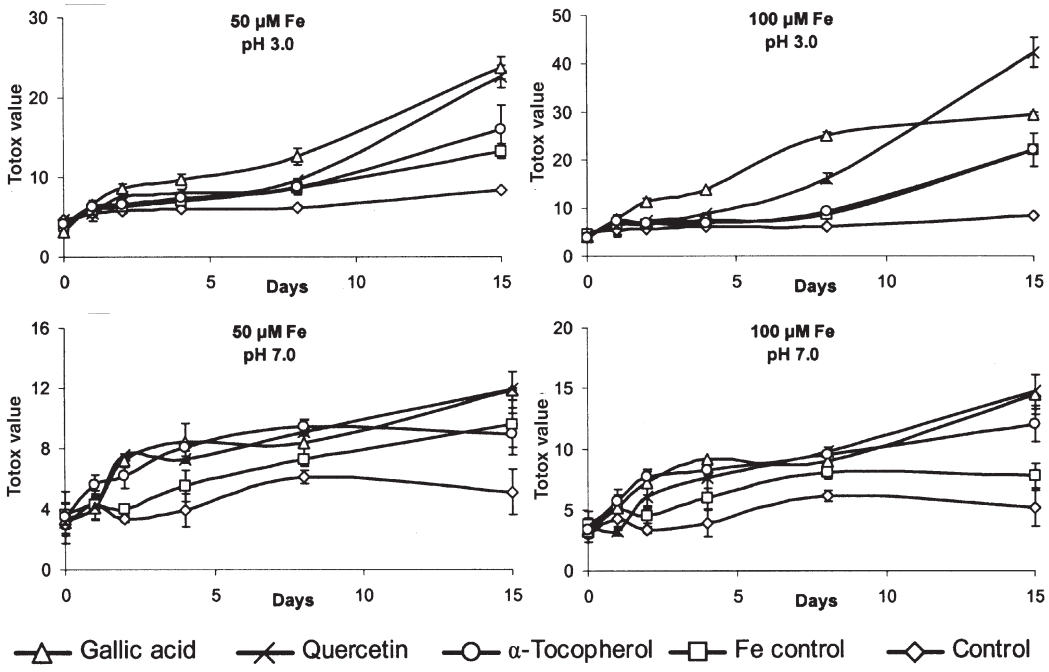
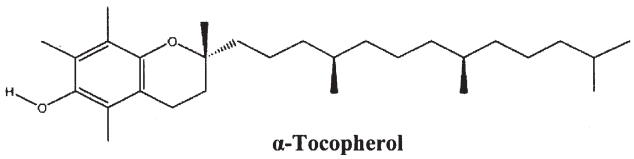
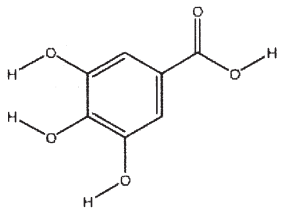


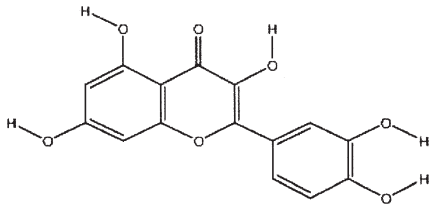
FIG. 3. Effect of iron concentration (50 and 100 µM), pH (3.0 and 7.0), and natural antioxidants (α-tocopherol, gallic acid, and quercetin) on total oxidation (Totox) over time in structured lipid-based emulsions stabilized by whey protein isolate. Control emulsions contained no added iron or antioxidants. Data shown are the average of duplicate samples held at 50°C. Error bars represent SE.



α-Tocopherol



Gallic acid



Quercetin

SCHEME 1

the Fe controls. However, α-tocopherol was a prooxidant in the pH 7.0 emulsions in this study (Fig. 2). α-Tocopherol is more stable at pH 3.0, less stable at pH 7.0 (21), and acts as an antioxidant or a prooxidant in different model conditions due to its ability both to scavenge oxy-radicals and to reduce iron (22).

Totox results (Fig. 3) indicated that quercetin and gallic acid were both prooxidants at pH 3.0 in the presence of iron (50 or 100 μM), whereas α-tocopherol did not affect Totox values under acidic conditions. The physical location of phenolics will affect their ability to influence lipid oxidation. In oil-in-water emulsions, nonpolar phenolics are retained in the lipid droplets and are more effective antioxidants than their more polar counterparts (7). Additionally, the polar antioxidants (quercetin and gallic acid) are better able to interact with aqueous-phase iron, which may explain their increased prooxidant activity, because the metal-reducing power of phenolics can increase oxidation reactions (7). In pH 7.0 emulsions, quercetin, gallic acid, and α-tocopherol did not significantly ($P < 0.05$) affect total oxidation compared to the Fe control sample in the presence of 50 μM iron. When higher concentrations of iron (100 μM) were incorporated into the emulsions, all three compounds exhibited prooxidant activity. The lack of full antioxidant activity exhibited by all three natural compounds in this study may be due to their inability to prevent the metal-catalyzed decomposition of peroxides. Jacobsen *et al.* (16) reported similar results for α-tocopherol in fish oil-enriched mayonnaise. However, it should be noted that all pH 7.0 emulsions were relatively stable to oxidation throughout storage at 50°C. Totox values of 10 correlate well with acceptable flavor scores for vegetable oils (23), and Totox values were less than 10 in all pH 7.0 emulsions at day 8 and remained below 15 on the final day of the current study.

Total oxidation was significantly ($P < 0.05$) higher in the pH 3.0 emulsions containing quercetin or gallic acid than in their pH 7.0 counterparts. Phenolic compounds from olive oil also exhibited a marked prooxidant effect in the presence of ferric ions at pH 3.5 (24). The increased prooxidant activity of quercetin and gallic acid at pH 3.0 may be due to their increased ability to reduce iron. Phenolic compounds can reduce up to 55-fold more iron per minute at pH 3.0 than at pH 7.0 (7). Therefore, food manufacturers must experiment carefully with gallic acid and quercetin before adding them to product formulations as antioxidants or functional ingredients because of their potential ability to exhibit prooxidant activity under certain conditions in the presence of transition metals.

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