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Effects of α-Tocopherol, β-Carotene, and Soy Isoflavones on Lipid Oxidation of Structured Lipid-Based Emulsions

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Structured lipids (SLs) are triacylglycerols that have been modified to change the fatty acid composition and/or positional distribution in the glycerol backbone by chemically and/or enzymatically catalyzed reactions and/or genetic engineering. Ten percent oil-in-water emulsions were formulated with a canola oil/caprylic acid SL and stabilized with 0.5% whey protein isolate (WPI) or sucrose fatty acid ester (SFE). The effects of α-tocopherol, β-carotene, genistein, and daidzein (added at 0.02 wt % of oil) on lipid oxidation were evaluated over a 15-day period in emulsion samples. Significantly ($p < 0.05$) less total oxidation (calculated from peroxide value and anisidine value measurements) occurred in the WPI emulsions compared to their SFE counterparts. In this study, α-tocopherol, β-carotene, and both soy isoflavones exhibited prooxidant activities in SFE emulsions. Because of their ability to exhibit prooxidant activity under certain conditions, manufacturers must experiment with these compounds before adding them to SL-based products as functional ingredients.

KEYWORDS: Anisidine value; β-carotene; daidzein; emulsions; genistein; lipid oxidation; peroxide value; structured lipids; sucrose fatty acid esters; α-tocopherol; TOTOX value; whey protein isolate

INTRODUCTION

Lipid oxidation in foods is a serious problem that leads to loss of shelf life, palatability, functionality, and nutritional quality (*1*). Antioxidants can be added to foods to delay the onset of oxidation or to slow the rate at which it proceeds (*1*). Interest in natural antioxidants found in plants has grown recently because of the worldwide trend toward the use of "natural" additives in foods (*2*). Additionally, many natural antioxidants are highly desirable to consumers because of their purported health benefits.

Tocopherols and carotenoids are two important commercial natural antioxidants that have been the subject of many experimental and epidemiological studies. Several plant phenolic compounds have also been identified as natural antioxidants. Studies on the antioxidant activity of phenolic compounds are often based on the in vitro oxidation of LDL to evaluate their potential health effects (*2*). However, fewer studies have evaluated the ability of phenolic compounds to protect against oxidation in food-relevant model systems. Such is the case with the group of bisphenolic compounds found in soybeans, known as isoflavonoid phytoestrogens. These compounds (mainly genistein and daidzein) have been shown to significantly prolong the lag time for LDL oxidation in vitro and in vivo (*3*, *4*). Studies on their efficacy as antioxidants in food systems are now needed.

Compounds with antioxidant activity can also exhibit prooxidant behavior under certain conditions (*5*). Conflicting results have been reported for the effects of α-tocopherol on lipid oxidation. The relative antioxidant activity of tocopherols depends on temperature, lipid composition, physical state (bulk phase or emulsion), and tocopherol concentration (*6*). α-Tocopherol's ability to have an antioxidant, neutral, or prooxidant effect is related to its complex function and chemical behavior (*7*). Increased levels of α-tocopherol can result in increased levels of α-tocopherol radicals, which can initiate processes of lipid peroxidation by themselves (*7*).

β-Carotene has also demonstrated both antioxidant and prooxidant activity in previous studies in a wide variety of lipid systems (*8*). The activity of β-carotene depends on the concentration used, the other antioxidants present, the oxidation model employed, and the oxygen tension (*9*). It is proposed that β-carotene traps peroxyl radicals under conditions of high oxygen tension or under atmospheric conditions by an addition mechanism (*10*). The carbon-centered β-carotene radical that is subsequently formed is readily autoxidized into a mixture of products with epoxy, hydroxy, and carbonyl groups (*9*). The autoxidation reactions begin to consume β-carotene without scavenging peroxyl radicals and might thus attenuate β-carotene antioxidant activity.

Flavonoids with a phenol-type substitution in their B ring, such as apigenin and naringenin, have been reported to result in a 30−50 times increase in the formation of reactive oxidant species (*7*). Chen et al. (*11*) reported that apigenin accelerated lipid oxidation of canola oil. The basic structural difference between the flavones and the isoflavones is the location of the B ring at the 3-position of the C ring, which removes the 3-OH

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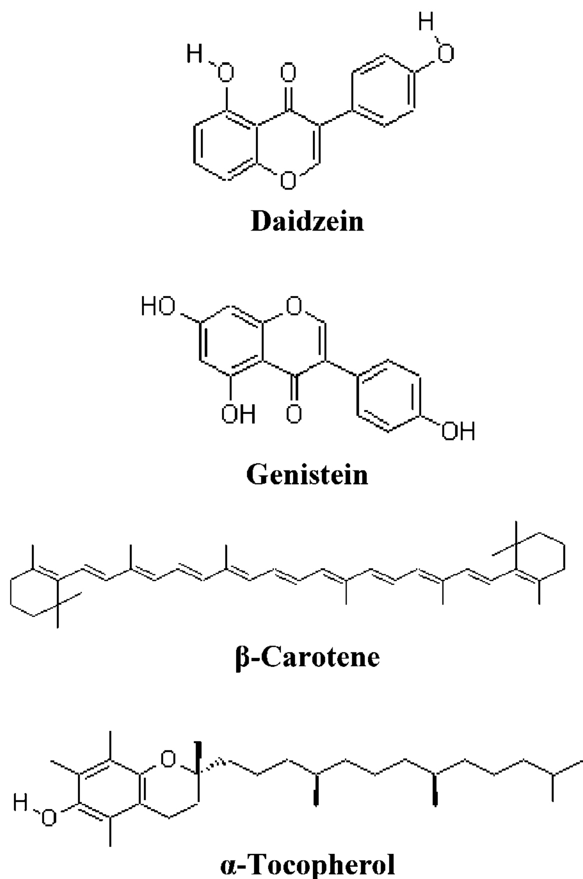

Figure 1. Chemical structure of antioxidants added to the canola oil/caprylic acid structured lipid-based emulsions.

from the structure. However, the monophenolic B ring, which is thought to be responsible for the prooxidant activity of apigenin and naringenin, remains intact in the isoflavones daidzein and genistein (*12*). Therefore, soy isoflavones should also be examined for prooxidant activity in food systems.

Despite their well-documented prooxidant activity, tocopherols, carotenoids, and phenolics continue to be hailed as excellent antioxidants. The objective of this paper was to illustrate the potential deleterious effects of α-tocopherol, *β*-carotene, and soy isoflavones (**Figure 1**) in structured lipid-based model emulsions stabilized by whey protein isolate or sucrose fatty acid esters. Structured lipids (SLs) are a new generation of fats that might provide the most effective means for delivering desired fatty acids for nutritive or therapeutic purposes and for targeting specific diseases and metabolic conditions (*13*). The potential health benefits of natural antioxidants and SLs make them ideal candidates for functional food formulations. Therefore, studies on the oxidation properties of emulsion systems containing both natural antioxidants and SLs are merited because of their likeliness of being found together in functional foods. The emulsifier variable was incorporated into the experimental design into determine the efficacy of natural antioxidants in the presence of both anionic and nonionic emulsifiers. Emulsifying agents are in direct contact with lipids on the surface of the emulsion droplet and are known to affect the rate of lipid oxidation (*14*−*17*).

MATERIALS AND METHODS

Materials. Canola oil was purchased from a local supermarket. Caprylic acid, α-tocopherol, *β*-carotene, genistein, and daidzein (all of purity > 98%) were purchased from Sigma Chemical Company (St. Louis, MO). An *sn*-1,3-specific immobilized lipase from *Rhizomucor miehei* (IM 60) was obtained from Novo Nordisk A/S (Bagsvaerd, Denmark). Whey protein isolate (WPI) (#27361) was provided by Land O'Lakes (St. Paul, MN). Ryoto sugar ester (S-1670) was supplied by Mitsubishi-Kasei Food Corporation (Tokyo, Japan). The sucrose fatty acid ester contained mainly stearic acid and consisted of approximately 75% monoester and 25% di-, tri-, or polyester. All other reagents were purchased from Fisher Scientific (Fair Lawn, NJ).

Structured Lipid Production. The SL was produced in a packed-bed bioreactor using optimal conditions previously reported (*18*) for reacting canola oil and caprylic acid. The product was purified using a KDL-4 short-path distillation unit (UIC Inc., Joliet, IL). 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 fatty acids (mol %) in the triacylglycerol: 37.3% C8:0, 1.8% C16:0, 1.7% C18:0, 47.3% C18:1, 8.9% C18:2, and 3.0% C18:3; and at the *sn*-2 position: 10.0% C8:0, 0.0% C16:0, 0.0% C18:0, 47.9% C18:1, 29.9% C18:2, and 12.2% C18:3 as determined by gas chromatography of methyl esters (*19*).

Emulsion Preparation. Antioxidants were added to the canola oil/caprylic acid SL at 0.02 wt % of the oil. Mixed antioxidant systems contained equal amounts (0.01 wt % of oil) of each antioxidant. α-Tocopherol was added directly to the SL. Genistein and daidzein were dissolved in methanol and then added to the oil. *β*-Carotene was dissolved in hexane before addition to the SL. Organic solvents were evaporated under N_2. Oil-in-water emulsions (100 g) were then prepared with 10% SL, 0.5% whey protein isolate (WPI) or sucrose fatty acid ester (SFE), and distilled water. The emulsions were passed through a high-pressure valve homogenizer (Emulsiflex, C5, Avestin, CA) 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. Particle size distribution was measured by integrated light scattering (Mastersizer S, Malvern Instruments, Malvern, U.K.) using standard optical parameters to ensure that similar droplet sizes were formed in the emulsions during homogenization. All samples had an apparent particle diameter ($D_{3,2}$ value) between 0.28 and 0.46 μm.

Oxidation Experiments. Emulsion samples (3 mL) were placed in 10-mL glass test tubes, covered with Parafilm, and allowed to oxidize in a 50 °C covered water bath for 30 days. The primary and secondary oxidation products were measured in the emulsion samples after 0, 1, 2, 4, 8, and 15 days of storage. Oil was extracted from the emulsions by adding isooctane/2-propanol (3:2, v/v), vortexing three times for 10 s each, and centrifuging for 5 min at 1000 rpm. The clear upper layer was collected, and the solvent was evaporated under nitrogen. Peroxide values (PVs) were determined using the International Dairy Foundation method described in detail by Shantha and Decker (*20*). Anisidine values (AVs) were determined according to AOCS Official Method Cd 18-90 (*21*). This method determines the amount of aldehyde (principally 2-alkenals and 2,4-alkadienals) present in the oil (*22*). The TOTOX value was calculated as TOTOX value = 2(PV) + AV (*22*). 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 (*22*).

Statistical Analysis. All experiments were performed on duplicate samples. Statistical analyses were conducted with the SAS (*23*) software package. Analyses of variance were performed by ANOVA procedures. Significant differences ($p < 0.05$) were determined by the least-squares difference method.

RESULTS AND DISCUSSION

Effect of Emulsifier. The amount of hydroperoxides present in the SL-based emulsions was significantly ($p < 0.05$) affected on all days of storage (excluding day 0) by the emulsifier variable. In the SFE control emulsions, hydroperoxides increased rapidly on the first day of storage (**Figure 2**A). However, WPI control emulsions showed a 4-day lag phase when little primary oxidation was occurring (**Figure 2A**). The emulsifier variable also significantly ($p < 0.05$) affected the AV on all days of the

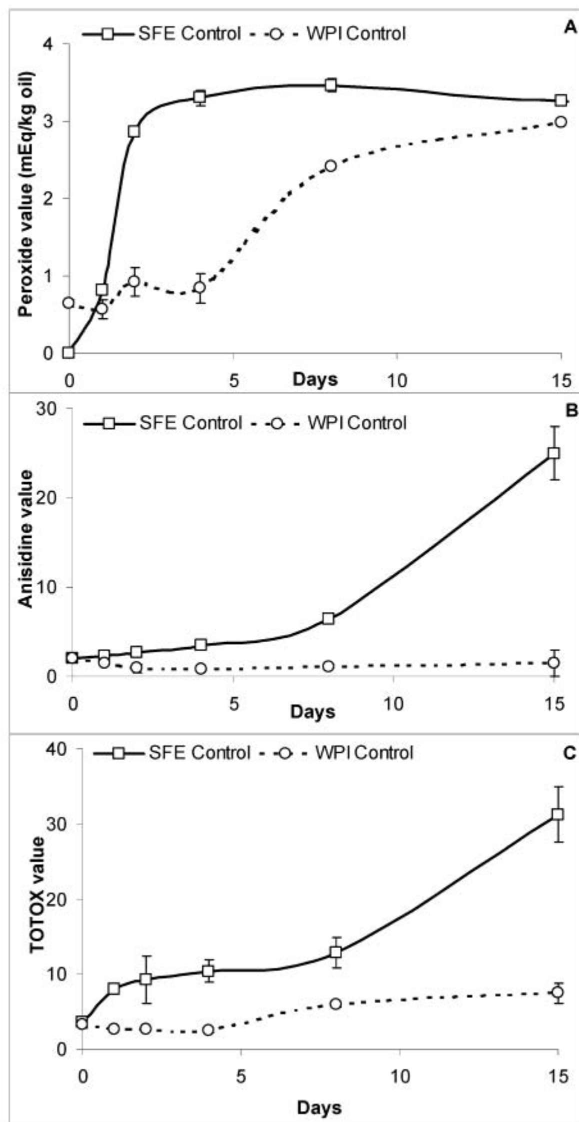

Figure 2. Effect of emulsifier (SFE, sucrose fatty acid ester; WPI, whey protein isolate) on (A) peroxide values (PVs), (B) anisidine values (AVs), and (C) total oxidation (TOTOX) values over time in structured lipid-based emulsions. Control emulsions contain no added antioxidants. Data shown are the averages of duplicate samples held at 50 °C. Error bars on chart represent standard deviations.

study (excluding day 0). **Figure 2B** illustrates the large increase in AV after day 8 in the SFE control emulsions compared to the WPI controls. The rise in AV demonstrates that the hydroperoxides are being decomposed into secondary oxidation products. Similarly to the AV results, TOTOX values were significantly ($p < 0.05$) lower in the WPI controls than in the SFE control emulsions (**Figure 2C**).

The antioxidant effect of WPI has been previously reported in SL-based emulsions (*14*). It was hypothesized that whey proteins inhibit lipid oxidation by inactivating peroxyl radicals (*24*). Exposure to heat increases the antioxidant activity of whey proteins by exposing more free-radical-scavenging sulfhydryl groups (*24*). This might account for the antioxidant activity of WPI observed during this study, because the emulsions were stored at 50 °C for 15 days.

Effect of Natural Antioxidants on Primary Oxidation. Day 15 primary oxidation results are presented for SFE and WPI emulsions in **Figures 3** and **4**, respectively. Emulsions containing β-carotene or a combination of α-tocopherol and β-carotene

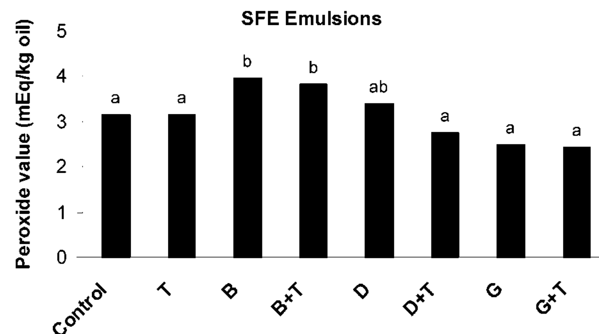

Figure 3. Peroxide values measured on day 15 for structured lipid-based emulsions stabilized by sucrose fatty acid esters (SFEs). T = α-tocopherol, B = β-carotene, D = daidzein, G = genistein. Data shown are the averages of duplicate samples held at 50 °C. Different lower-case letters indicate a significant difference ($p < 0.05$).

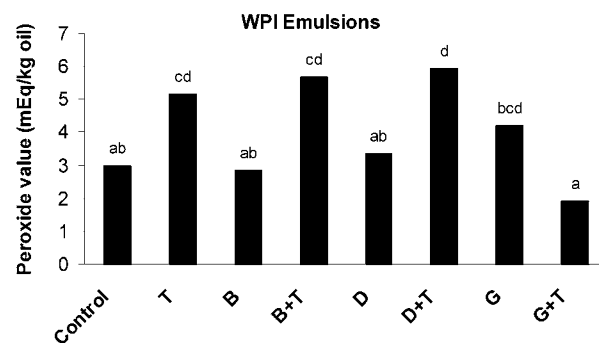

Figure 4. Peroxide values measured on day 15 for structured lipid-based emulsions stabilized by whey protein isolate (WPI). T = α-tocopherol, B = β-carotene, D = daidzein, G = genistein. Data shown are the averages of duplicate samples held at 50 °C. Different lower-case letters indicate a significant difference ($p < 0.05$).

had significantly ($p < 0.05$) higher peroxide values than the SFE control emulsions (**Figure 3**). Both soy isoflavones had no effect on the primary oxidation of SFE emulsions. In emulsions stabilized by WPI, α-tocopherol alone and in combination with β-carotene or daidzein had significant ($p < 0.05$) prooxidant effects after 15 days of storage (**Figure 4**).

Hydroperoxides are transitionary intermediates that decompose into various secondary products, including aldehydes, ketones, hydrocarbons, and alcohols. The PV quantifies the amount of hydroperoxides present in a sample at the time of measurement, which is affected by both formation and decomposition reactions. Therefore, it is difficult to determine an antioxidant's mechanism of action on the basis of the results of primary oxidation studies alone.

Effect of Natural Antioxidants on Secondary Oxidation. All of the natural compounds in this study (α-tocopherol, β-carotene, daidzein, and genistein) functioned as prooxidants with regard to secondary oxidation in SFE emulsions (**Figure 5**). The prooxidant function of α-tocopherol is likely a result of the molecule donating a hydrogen atom to a peroxyl radical and becoming a tocopheroxyl radical. The newly formed radical can then initiate processes of lipid peroxidation itself (*6*). After β-carotene traps peroxyl radicals, a carbon-centered β-carotene radical is subsequently formed and is readily autoxidized into a mixture of products with epoxy, hydroxy, and carbonyl groups (*9*). The autoxidation reactions begin to consume β-carotene without scavenging peroxyl radicals and might thus attenuate β-carotene antioxidant activity and allow the prooxidant activity of β-carotene to predominate. The prooxidant chemistry of isoflavones in model emulsions is not yet fully understood, but

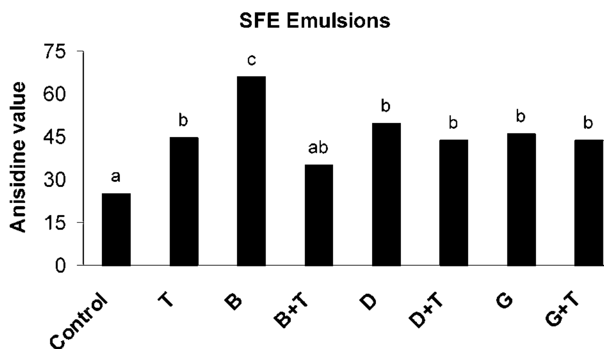

Figure 5. Anisidine values measured on day 15 for structured lipid-based emulsions stabilized by sucrose fatty acid esters (SFEs). T = α-tocopherol, B = β-carotene, D = daidzein, G = genistein. Data shown are the averages of duplicate samples held at 50 °C. Different lower-case letters indicate a significant difference ($p < 0.05$).

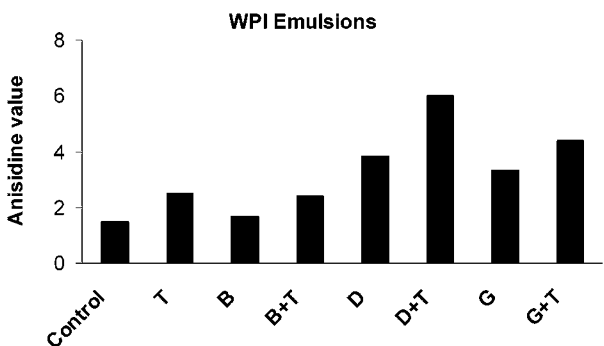

Figure 6. Anisidine values measured on day 15 for structured lipid-based emulsions stabilized by whey protein isolate (WPI). T = α-tocopherol, B = β-carotene, D = daidzein, G = genistein. Data shown are the averages of duplicate samples held at 50 °C. Significant differences ($p <$ 0.05) do not exist among emulsion samples.

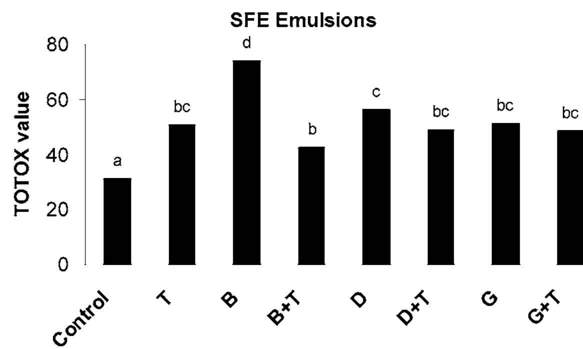

Figure 7. Totol oxidation (TOTOX value) calculated on day 15 for structured lipid-based emulsions stabilized by sucrose fatty acid esters (SFE). T = α-tocopherol, B = β-carotene, D = daidzein, G = genistein. Data shown are the averages of duplicate samples held at 50 °C. Different lower-case letters indicate a significant difference ($p < 0.05$).

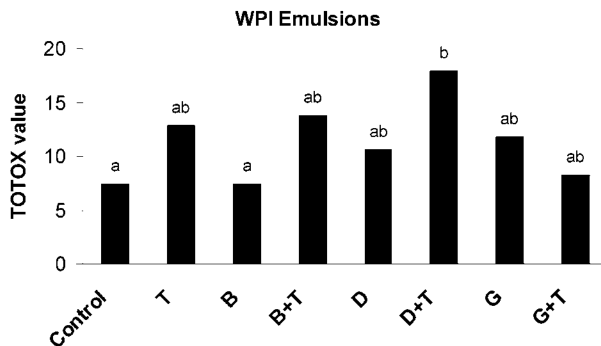

Figure 8. Total oxidation (TOTOX) value calculated on day 15 for structured lipid-based emulsions stabilized by whey protein isolate (WPI). T = α-tocopherol, B = β-carotene, D = daidzein, G = genistein. Data shown are the averages of duplicate samples held at 50 °C. Different lower-case letters indicate a significant difference ($p < 0.05$).

seems to partly correlate with the high one-electron oxidation potential of their corresponding phenoxyl radicals (*7*). Flavonoids have been reported to exert prooxidant chemistry, including the formation instead of scavenging of radicals, especially at low concentration (<500 μM) (*5*). Both genistein and daidzein were added to the model emulsions in our study at concentrations of 74 and 78 μM, respectively.

In WPI emulsions, none of the natural compounds affected secondary oxidation on day 15 (**Figure 6**). This might be due to the lack of hydroperoxide formation taking place in the WPI emulsions (days 0−4) due to the antioxidant effect of the emulsifier (**Figure 2A**). Thus, fewer free radicals are present, and less autoxidation of the natural compounds is taking place, which results in an overall neutral effect on oxidation.

Effect of Natural Antioxidants on Total Oxidation. TOTOX values were significantly ($p < 0.05$) influenced by the addition of natural compounds to SFE emulsions on day 15 (**Figure 7**). As for the AV results, β-carotene, α-tocopherol, daidzein, genistein, and their combinations increased the day 15 TOTOX values compared to the SFE control emulsions. This was becasue the PVs were relatively low compared to the AVs and, therefore, did not contribute substantially to the TOTOX values.

TOTOX values on day 15 for WPI emulsions containing natural compounds were statistically similar to those of the WPI control samples (**Figure 8**), except when α-tocopherol and daidzein were added in combination. This combination increased total oxidation after 15 days of storage in the WPI emulsions. It should be noted that the control WPI emulsions and those containing β-carotene alone or a combination of α-tocopherol and genistein did not reach TOTOX values above 10 during the study. This finding might be important for food manufacturers because sensory quality of vegetable oils is considered acceptable for TOTOX values below 10 (*25*).

The results of this study demonstrate that α-tocopherol, β-carotene, daidzein, and genistein exhibit prooxidant behavior on primary, secondary, and/or total oxidation of SL-based model emulsions stored at 50 °C for 15 days. Many factors affect oxidation of emulsified oils, and more studies are required to fully understand the prooxidant mechanisms behind these compounds. However, care should be exercised when these compounds are added to foods as functional ingredients because of their ability to promote lipid oxidation in some emulsions under certain conditions.

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