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Antioxidant Activity of 3-Dehydroshikimic Acid in Liposomes, Emulsions, and Bulk Oil

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The antioxidant activity of 3-dehydroshikimic acid (DHS), an intermediate in the biosynthesis of aromatic amino acids, was evaluated in three assay systems: bulk oil (lard), liposomes, and a 10% corn oil-in-water emulsion. Upon initiation of peroxidation in the liposome or emulsion systems, DHS exhibited weak antioxidant activity. In contrast, DHS displayed strong antioxidant activity in lard, suppressing peroxidation with activity comparable to that of *tert*-butylhydroquinone, propyl gallate, and gallic acid and superior to that of α-tocopherol. Two major DHS oxidation products, gallic acid and protocatechuic acid, were identified by gas chromatography/mass spectral analysis of lard extracts; both compounds are effective antioxidants in the bulk oil system. In the liposome system, DHS remained intact throughout the assay period. A small amount of gallic acid was observed in extracts of the emulsion; however, protocatechuic acid was not detected. A mechanism to explain the different activities of DHS in the three lipid systems is proposed.

KEYWORDS: Gallic acid; propyl gallate; BHA; BHT; TBHQ; α-tocopherol; protocatechuic acid; GC/MS analysis

INTRODUCTION

One of the primary factors limiting the shelf life of food products is development of undesirable flavor, aroma, and color compounds triggered by peroxidation of lipids. To extend food product shelf life and to improve food safety and quality, numerous antioxidant compounds, both synthetic and naturally derived, have been developed and utilized in the food industry. Synthetic compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate are effective and relatively inexpensive, but consumer concerns regarding the safety of these compounds have accelerated the search for safe, inexpensive, naturally occurring antioxidants. Moreover, there is an accumulating body of evidence to suggest that many naturally derived antioxidants provide additional health benefits as antimutagenic, anticarcinogenic, or antiinflammatory agents.

A naturally occurring antioxidant candidate is 3-dehydroshikimic acid (DHS) (**Figure 1**). DHS is an intermediate in the common pathway of aromatic amino acid biosynthesis as well as an intermediate in the catabolism of shikimic acid and quinic acid by way of the β-ketoadipate pathway (*1*−*3*). We have previously reported that DHS displays excellent antioxidant activity in a bulk oil system (*4*). Peroxidation in lard was strongly suppressed in the presence of 0.01 and 0.02% DHS; the antioxidant activity of DHS was equivalent to or superior to that of BHT, *tert*-butylhydroquinone (TBHQ), and other commercial antioxidants. These preliminary results suggested that DHS, derived from bioprocessing of plant starch (*5*), may be an effective, inexpensive antioxidant for food applications.

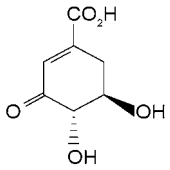

Figure 1. DHS.

The antioxidant activity of a compound is strongly influenced by numerous factors including the nature of the lipid substrate, the hydrophilic−lipophilic balance of the antioxidant, the physical and chemical environment of the lipids, and various other interfacial interactions (*6*, *7*). Thus, compounds that are effective antioxidants in one model system or food matrix may be unsuitable in other systems. The objectives of the present study were to evaluate the antioxidant activity of DHS using liposomes, corn oil-in-water emulsions, and lard as model food substrates. The results extend our previous studies by demonstrating that DHS is a highly effective antioxidant in bulk oil but is less effective in liposomes and emulsions, results consistent with the polar paradox theory of Porter (*6*). Further experiments were conducted to identify and to quantify the oxidation products of DHS in each experimental system. Results of these experiments suggest that the differences in antioxidant activity of DHS in different experimental systems may be

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explained in part by its ability to undergo dehydration to protocatechuic acid and oxidation to gallic acid in oil but not in liposomes or emulsions.

MATERIALS AND METHODS

Materials. DHS was synthesized according to methods previously reported (*5*). Gallic acid, protocatechuic acid, and $FeCl_2 \cdot 4H_2O$ were purchased from Aldrich Chemical Co. (Milwaukee, WI). BHT, α-tocopherol, propyl gallate, and tertiary Tween 20 were from Sigma Chemical Co. (St. Louis, MO). BHA and TBHQ were from Eastman Chemical Co. (Rochester, NY). All chemicals were used directly without further purification. For all aqueous systems, water was purified using a Barnstead NANOpure II system (Barnstead/Thermolyne Corp., Dubuque, IA). Prime steam lard, prepared without stabilization by exogenous antioxidants, was provided by Monfort of Colorado, Inc. (Greeley, CO). Stripped corn oil was purchased from Acros Organic (Pittsburgh, PA). The lipid used for liposomes, 1-stearoyl-2-linoleoyl-*sn*-glyceryl-3-phosphocholine (SLPC), was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The fluorescent probe 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid (DPH-PA) was from Molecular Probes, Inc. (Eugene, OR). 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer was obtained from Boehringer Mannheim Co. (Indianapolis, IN). 2,2-Azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Chemical USA Inc. (Richmond, VA). Silylation grade pyridine was purchased from Pierce (Rockford, IL) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylsilane (TMCS) was from Aldrich Chemical Co.

Determination of Antioxidant Activity in Liposomes. The antioxidant activity of DHS in liposomes was evaluated using the procedure described by Arora and Strasburg (*8*). Briefly, 200 nmol of SLPC and 0.6 nmol of fluorescent probe, DPH-PA, were suspended in 2 mL of buffer consisting of 100 mM NaCl and 50 mM HEPES buffer, pH 7.0, in the presence or absence of 10 μM antioxidant compounds. The reaction mixture was maintained at 23 ± 1 °C by circulating coolant from a water bath through the sample holder. Metal ion-induced peroxidation was initiated by the addition of $FeCl_2$ to a final concentration of 5 μM in the reaction mixture, and fluorescence was monitored over the course of 21 min. Alternatively, peroxidation was triggered by the addition of the hydrophilic free radical initiator AAPH to a final concentration of 5 mM; these experiments were conducted at 37 °C. Percent inhibition for the test compounds was calculated using eq 1:

$$\% \text{ inhibition} = (F_{rel})_{AO}/[(F_{rel})_{con} - (F_{rel})_{PO}] \times 100$$

where $(F_{rel})_{AO}$ = relative fluorescence of the sample containing antioxidant plus prooxidant Fe(II) or AAPH, after 21 min of reaction; $(F_{rel})_{con}$ = relative fluorescence for the control sample (no added prooxidant) after 21 min; and $(F_{rel})_{PO}$ = relative fluorescence for the Fe(II)- or AAPH-containing prooxidant sample with no added antioxidant, after 21 min of reaction.

Determination of Antioxidant Activity in Corn Oil Emulsions. Stripped corn oil (2.5 g) and water (25 mL) were emulsified at 23 °C with Tween 20 (0.25 g) by agitation for 5 min with a Polytron homogenizer (Kinematica Ag littau, Switzerland). The corn oil emulsions, with or without added antioxidants (0.02% w/v) and with or without added $FeCl_2$ (20 μM), were incubated at 60 ± 1 °C in darkness in a shaking water bath (New Brunswick Scientific Co. Inc., Edison, NJ). Aliquots (1 mL) of the emulsions were collected at the time intervals indicated in the figures, and each aliquot was extracted three times with hexane. The hexane extracts were combined, dried under nitrogen, and redissolved in 100 μL of high-performance liquid chromatography (HPLC) grade acetonitrile. The acetonitrile solutions were injected into an HPLC system (Waters Corp., Milford, MA) equipped with a model 486 tunable absorbance detector (Waters Corp.). Compounds were separated using a reverse phase Supelcosil LC-8 column (15.0 cm × 4.6 mm ID, Supelco, Inc., Bellefonte, PA) and isocratic elution at room temperature using 100% acetonitrile with a flow rate of 1.4 mL/min. The eluent was monitored at 234 nm to detect the presence of conjugated dienes, and the peak areas were integrated using Waters Baseline 810 Chromatography Workstation Software (Waters Corp.).

Determination of Antioxidant Activity in Bulk Oil. Oxidation of prime steam lard, with or without added antioxidants, was determined by the Schaal oven method (*9*). Samples were incubated at 60 ± 1 °C in the dark, and 5 g aliquots of the lard suspension were collected every 7 days for the determination of peroxide values (*9*).

Analysis of DHS Reaction Products. To define the fate of DHS in bulk oil during peroxidation, 1 g samples of lard were extracted three times with 1 mL of 10% water/methanol solution. The aqueous methanolic extracts were combined and extracted with hexane (1 mL) to remove excess lard. The extracts were then dried under nitrogen and derivatized with silylation grade pyridine (20 μL) and BSTFA containing 1% TMCS at room temperature in the dark overnight. The silylated mixtures were analyzed by gas chromatography/mass spectrometry (GC/MS) immediately after derivatization. To determine the fate of DHS in the corn oil emulsion and Fe^{2+}-oxidized liposome samples, aliquots of the lipid/antioxidant mixtures were dried under nitrogen and derivatized directly without extraction. GC/MS analysis was conducted using a HP 5890 Gas Chromatograph connected with a HP 5970 Series Mass Selective Detector (Hewlett-Packard, Avondale, PA). Capillary GC analysis was performed on a DB-5 capillary column (30 m × 0.25 μm, J & W Scientific, Folsom, CA) using helium as the carrier gas with a linear flow rate of 35 cm/min. The derivatized samples (1 μL) were applied to the column through a glass splitless injector. Analysis was completed using a temperature program of 150 °C for 40 min, followed by temperature elevation to 180 °C at 2 °C/min, and holding at 180 °C for another 25 min. Injector and transfer line temperatures were maintained at 250 and 220 °C, respectively. The standard solution concentrations of DHS, gallic acid, and protocatechuic acid for the calibration curves were 0.98, 1.95, 3.90, 7.81, 15.63, 31.25, 62.5, 125, and 250 ng/μL, respectively. Peak areas were used to generate the calibration curves using the following equation: $y = A + Bx$, where y = peak area for respective standards; A = intercept of calibration curve; B = slope of calibration curve; and x = the amount of respective standards.

RESULTS AND DISCUSSION

The efficacy of each antioxidant compound was evaluated in liposomes, as described by Arora and Strasburg (*8*). The large unilamellar vesicles prepared for this assay are composed of a defined phosphatidylcholine compound (SLPC), which is a major constituent of biological membranes, plus the fluorescent probe DPH-PA. Reaction of free radicals with the probe results in a time-dependent decrease in fluorescence, and antioxidants reduce the rate of fluorescence decrease.

All of the compounds tested in the liposomal system demonstrated antioxidant activity when Fe^{2+} was used to induce peroxidation (**Figure 2A**). However, in contrast to previous results in the bulk oil system, in which DHS was a superior antioxidant (*4*), DHS had the lowest activity of the compounds tested in the liposome system. To determine whether antioxidant activity might be different with a different prooxidant, the hydrophilic free radical initiator AAPH was used to initiate oxidation (**Figure 2B**). There were clear differences in activity of some of the antioxidant compounds between the two experiments. Most notably, protocatechuic acid was much more effective with AAPH as the source of free radicals than with Fe^{2+}, while conversely, the activities of BHT and BHA were lower with AAPH as the prooxidant. DHS had the lowest antioxidant activity of the compounds tested, and there was little difference between the two prooxidant systems. In both the Fe^{2+}-induced peroxidation and the AAPH-induced peroxidation, superior antioxidant activity was observed with gallic acid, TBHQ, and BHT. It should be noted that the antioxidant activity of gallic acid depends on the overall charge of the membrane. Our liposomes consist of zwitterionic, electrically neutral

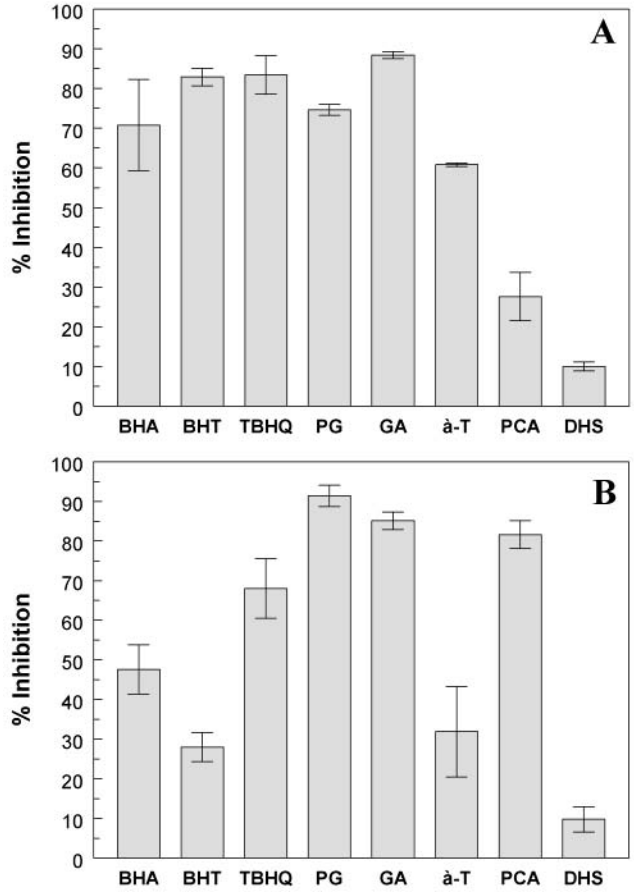

Figure 2. Antioxidant activity of commercial antioxidants, phenolic compounds, and DHS in liposomes. (**A**) Peroxidation was initiated by addition of Fe^{2+} as described in Materials and Methods. All test compounds were present at concentrations of 10 μM. (**B**) Peroxidation was initiated by addition of AAPH as described in Materials and Methods. Test compounds were present at concentrations of 10 μM. Each value represents the mean of three experiments; the error bars indicate the standard error of the mean. Additional abbreviations: PG, propyl gallate; GA, gallic acid; α-T, α-tocopherol; and PCA, protocatechuic acid.

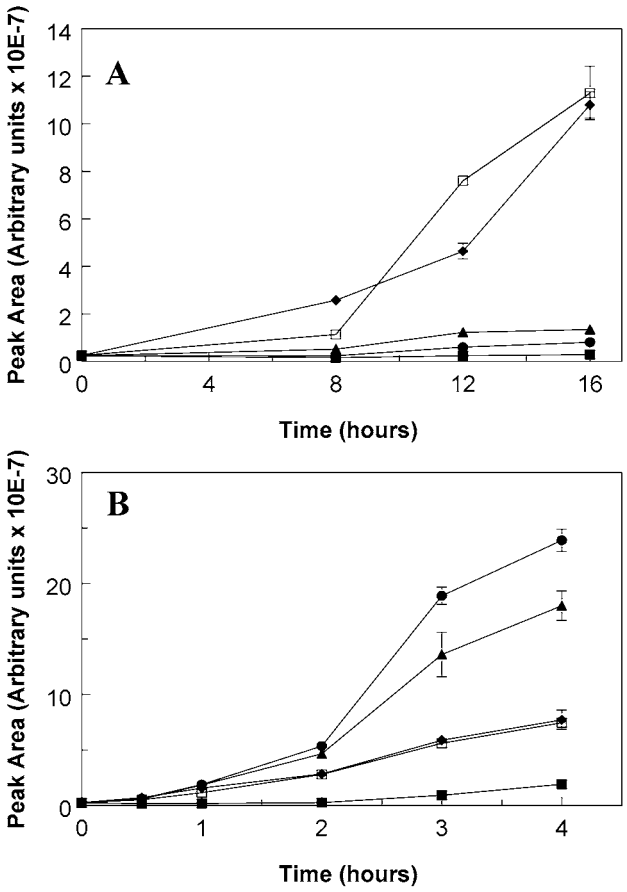

Figure 3. Antioxidant activity of test compounds in an oil-in-water emulsion. (**A**) A 10% corn oil-in-water emulsion was prepared, and peroxidation was initiated as described in Materials and Methods. These experiments were conducted in the absence of added Fe^{2+}. (**B**) A 10% corn oil-in-water emulsion was prepared, and peroxidation was initiated by 20 μM Fe^{2+} as described in Materials and Methods. Each value represents the mean of three experiments; the error bars indicate the standard error of the mean. ◆, Blank; □, DHS; ▲, gallic acid; ●, propyl gallate; ■, TBHQ.

phosphatidylcholine, whereas biological membranes carry a net negative charge due to the presence of additional anionic phosphatidyl molecules. With the latter lipid substrate, gallic acid is relatively ineffective as an antioxidant (*6*).

The antioxidant activities of these compounds were also evaluated using a corn oil-in-water emulsion with or without added ferrous ions. The antioxidant efficacy of a test compound was defined as its ability to inhibit the formation of conjugated dienes. In the absence of added ferrous ions, gallic acid, propyl gallate, and TBHQ effectively inhibited the formation of conjugated dienes in corn oil emulsions, whereas DHS showed limited antioxidant activity, or slight prooxidant activity depending on time point of the assay (**Figure 3A**). Upon addition of Fe^{2+} to a final concentration of 20 μM, DHS showed no activity, whereas only TBHQ was inhibitory. In this system, gallic acid and propyl gallate functioned as prooxidants (**Figure 3B**). The latter results are consistent with previous reports indicating that these compounds are capable of stimulating the formation of hydroxyl radicals by reduction of ferric ions, produced during lipid peroxidation, to ferrous ions by the *o*-hydroxy group of these phenolic compounds (*10*). The recycling of Fe^{3+} to Fe^{2+} in solution is likely responsible for the prooxidant effect of gallic acid and propyl gallate in the corn oil emulsion. In contrast to our results demonstrating the effectiveness of gallic acid as an antioxidant in the absence of ferrous ions, Porter et al. (*11*) have shown that gallic acid is relatively ineffective as an antioxidant in a soy lecithin-in-water emulsion system. Their lecithin source actually consisted of several phospholipids including phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidic acid; thus, their emulsions carried a substantial negative surface charge. Electrostatic repulsion by the negatively charged phospholipids would account for the low activity of gallic acid in this experimental system. Our corn oil-in-water emulsion consisted of neutral triglycerides; the choice of lipid substrates, therefore, likely accounts for the differences in antioxidant activity of gallic acid between this study and that of Porter et al. (*11*).

The effectiveness of DHS in bulk oil as compared to lack of efficacy in liposomes or emulsions is consistent with the polar paradox theory proposed by Porter (*6*). This theory is based on observations that polar antioxidants are more effective than nonpolar compounds in nonpolar systems such as bulk oils; conversely, nonpolar antioxidants are more effective in dispersions such as oil-in-water emulsions or liposomes than in bulk oils. Huang et al. (*12*) demonstrated that this paradox could be resolved on the basis of partitioning of antioxidants. Using this model, the hydrophilic DHS molecule would be concentrated at the air-oil interface in bulk oils, where oxidation is most likely to occur. In emulsions and suspensions of liposomes, DHS

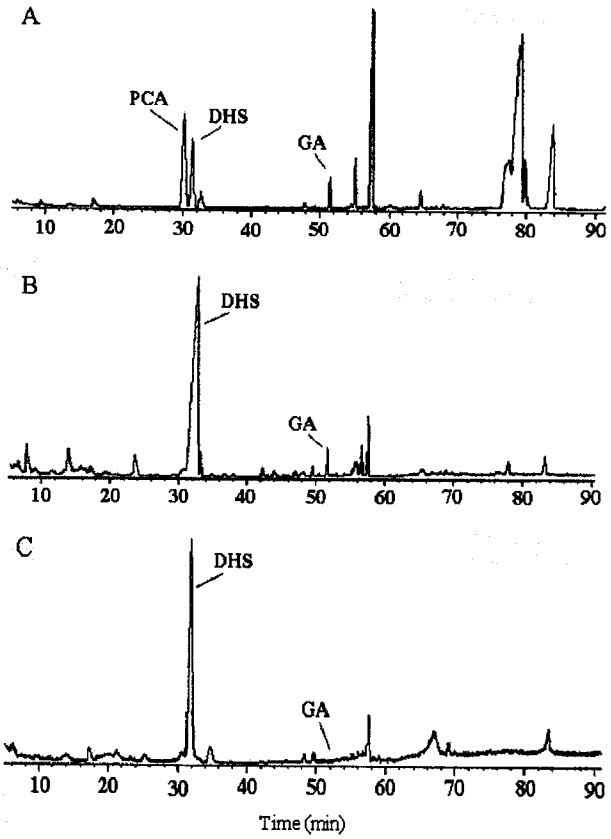

Figure 4. Gas chromatograms of extracts from DHS-treated samples. (**A**) Lard, (**B**) corn oil-in-water emulsion, and (C) liposomes.

would partition into the much larger aqueous volume where its activity would be diminished by dilution, which would reduce interaction with lipid radicals.

The dramatic differences in activity of DHS among the different model systems led us to speculate as to whether DHS was the primary antioxidant in oil, or whether it could convert to another, more active compound in oil but not in aqueous media. To address this hypothesis, the peroxidized samples of lard, liposomes, and emulsion systems containing added DHS were extracted and analyzed for DHS and DHS-derived components. Gas chromatograms of extracts of DHS-treated lard showed the presence of three new peaks (**Figure 4A**), which were not present in chromatograms of untreated lard (data not shown). The retention times of the new peaks were 29.9, 31.0, and 51.6 min. The compounds associated with these peaks were identified as protocatechuic acid, DHS, and gallic acid, respectively, based on comparison of retention times and mass spectra of authentic compounds.

In contrast to the results with lard, analysis of extracts of the liposome system indicated that DHS remained intact throughout the assay time. Likewise, in the emulsion system, DHS remained mostly unchanged with only a small amount of gallic acid formation (**Figure 4**). No protocatechuic acid was detected in either the peroxidizing liposome or the emulsion systems to which DHS had been added. These results suggest that the antioxidant activity of DHS in lard is related to its ability to form protocatechuic acid and gallic acid in bulk oils.

To establish that loss of DHS was proportional to formation of the new products, we analyzed DHS-spiked lard extracts at various time points during peroxidation (**Figure 5**). The results indicated that protocatechuic acid increased in concert with decreasing DHS. After 24 h of peroxidation, protocatechuic acid was the major component in lard, and the concentration

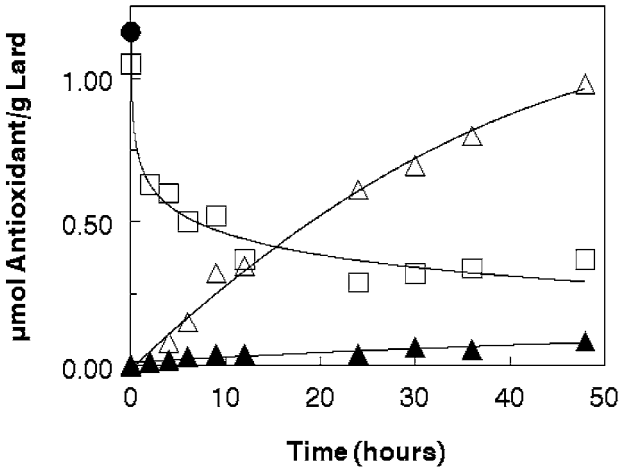

Figure 5. Analysis of DHS and its oxidation products in bulk oil using GC/MS. Samples from oxidizing lard were collected at the indicated time points and analyzed as described in Materials and Methods. △, Protocatechuic acid; □, DHS; ▲, gallic acid. The amount of DHS added at time zero, 0.02% (w/v), is indicated by the ● symbol.

increased slowly to 66% of the total antioxidants after 48 h. The concentration of gallic acid increased slowly over time; however, gallic acid remained less than 10% of the total antioxidants present in lard. At 48 h, only about 28% of the original DHS remained in lard. The results suggest that the superiority of DHS as an antioxidant in oil is the result of its ability to undergo conversion in this system to protocatechuic acid and gallic acid.

The antioxidant effectiveness of protocatechuic acid and gallic acid in lard was then compared with DHS and other widely used antioxidants: propyl gallate, α-tocopherol, and TBHQ (**Figure 6**). As was observed previously, the antioxidant activity of DHS was comparable to that of gallic acid and TBHQ (*4*). Moreover, although propyl gallate appeared to have the best antioxidant activity, the induction period for propyl gallate, gallic acid, TBHQ, and DHS was virtually the same; i.e., approximately 49 days. From a practical standpoint with respect to oil quality, the induction period is more important than numerical differences in peroxide value after the exponential rise in peroxidation has begun. Protocatechuic acid was only moderately effective as an antioxidant in bulk oil as compared to DHS or gallic acid (**Figure 6**). It is possible that the maximum antioxidant activity noted for DHS as compared to that of protocatechuic acid is the result of the particular combination of protocatechuic acid, gallic acid, and DHS and/or some as yet unidentified compound, which results in positive synergism.

Figure 7 illustrates the formation of antioxidant phenolics from DHS in the lipid systems of the current study. Tautomerization of DHS would lead to formation of a reactive enediol. The formation of protocatechuic acid would require the elimination of water from the enediol while oxidation of the enediol would lead to gallic acid. Abiotic formation of protocatechuic acid and gallic acid has been reported when aqueous solutions of DHS containing 1 M $Na_{1.5}H_{1.5}PO_4$ are reacted with air (*4*). However, the relevance of this reactivity to the solution matrix found in the bulk oil, oil-in-water emulsion, and liposomal systems is uncertain. Protocatechuic acid was observed in the bulk oil system where the water concentration was extremely low as compared to that of 10% oil-in-water emulsion and of the liposomal system. This suggests that the water content may be a critical factor for the formation of PCA through the elimination of one molecule of water from the enediol intermediate. Temperature may also be another critical factor for

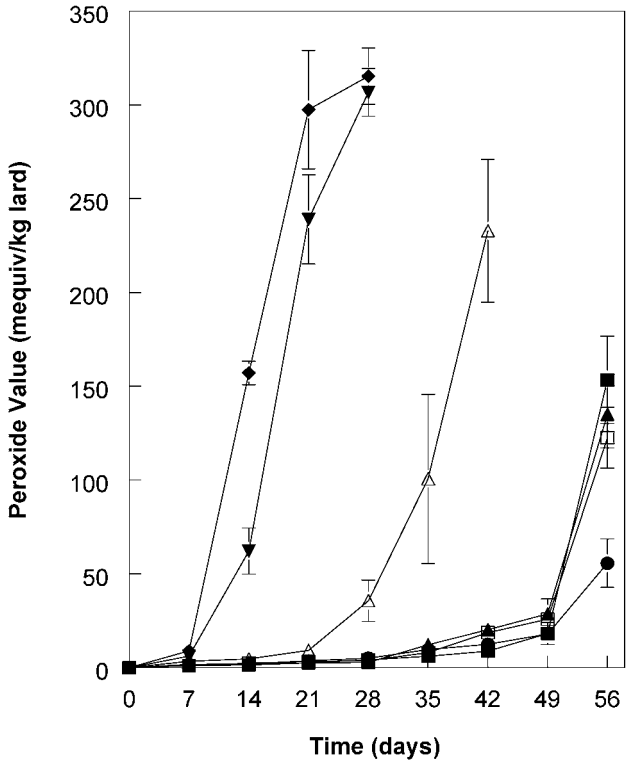

Figure 6. Antioxidant activity of test compounds in lard. Compounds were added to a final concentration of 0.02% (w/w) of lard. Peroxide values were determined by AOCS Official Method Cd8-53. ◆, Blank; ▼, α-tocopherol; △, protocatechuic acid; ■, TBHQ; ▲, gallic acid; □, DHS; ●, propyl gallate.

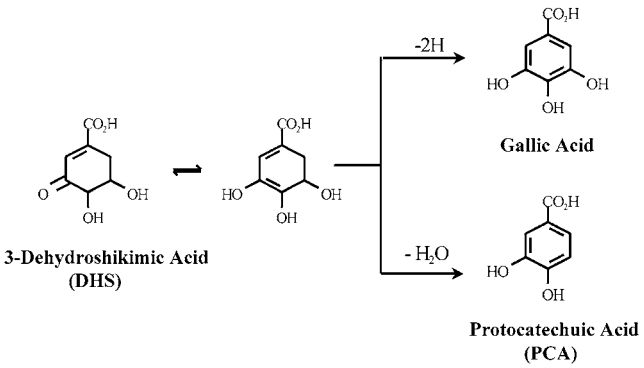

Figure 7. Proposed scheme for formation of oxidation products of DHS in oil and aqueous media.

the formation of oxidation products. A small amount of gallic acid was detected in the bulk oil and corn oil emulsion assays, which were conducted at 60 °C. However, gallic acid was not detected in the liposome system, which was maintained at 23 °C. The small amounts of gallic acid observed relative to protocatechuic acid (**Figure 5**) may indicate a higher rate of consumption of gallic acid by reactions associated with its antioxidant activity.

DHS, gallic acid, and protocatechuic acid are all natural products widely present in vegetables and fruits. Our results suggest that DHS would be an effective antioxidant in bulk oil systems because of its novel ability to form to additional phenolic compounds, which are effective as antioxidants. These results also suggest that DHS has the potential to be used as a naturally derived antioxidant for specialized food application in processed food products.

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