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Interactions between α-Tocopherol and Rosmarinic Acid and Its Alkyl Esters in Emulsions: Synergistic, Additive, or Antagonistic Effect?

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ABSTRACT: Many antioxidants can interact to produce synergistic interactions that can more effectively inhibit lipid oxidation in foods. Esterification of rosmarinic acid produces a variety of compounds with different antioxidant activity due to differences in polarity and thus differences in partitioning in oil, water, and interfacial regions of oil-in-water emulsions (O/W). Therefore, rosmarinic acid and rosmarinate esters provide an interesting tool to study the ability of antioxidant to interact in O/W emulsions. In O/W emulsions, rosmarinic acid (R0) exhibited the strongest synergistic interaction with α-tocopherol while butyl (R4) and dodecyl (R12) rosmarinate esters exhibited small synergistic interaction and eicosyl rosmarinate esters (R20) exhibited slightly antagonistic interaction. Fluorescence quenching and electron paramagnetic resonance (EPR) studies showed that water-soluble rosmarinic acid (R0) exhibited more interactions with α-tocopherol than any of the tested esters (R4, R12, R20). This was also confirmed in O/W emulsions where R0 altered the formation of α-tocopherol quinone and α-tocopherol increased the formation of caffeic acid from R0. This formation of caffeic acid was proposed to be responsible for the synergistic activity of R0 and α-tocopherol since the formation of an additional antioxidant could further increase the oxidative stability of the emulsion.

KEYWORDS: *α-tocopherol, rosmarinic acid, ester, emulsions*

INTRODUCTION

Under current limitations of approved antioxidants for food applications, it is often challenging for food scientists to maintain the oxidative stability of processed foods. To try to solve this problem, several strategies have been attempted to improve antioxidant performance. One interesting strategy is to use combinations of antioxidants to produce synergistic interactions via free radical transfer mechanisms. For example, the regeneration of oxidized α-tocopherol by ascorbic acid, flavonoids, carotenoids, phospholipids, amino acids, and peptides has been reported.[1−7] These regeneration reactions have been postulated to produce synergistic antioxidant interactions.

Several models to study interactions between antioxidant combinations include the oxygen radical absorbance capacity (ORAC),[8,9] 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging,[10−14] ferric reducing antioxidant power (FRAP),[12] and homogeneous solutions of peroxidizing methyl linoleate.[15] Unfortunately, these models often produce inconsistent results. It was reported that the combinations between α-tocopherol and flavonoids exhibit synergistic, additive, and antagonistic effects in the ORAC model.[8,9] However, Hiramoto and co-workers[14] reported that synergistic antioxidant interactions were observed only when α-tocopherol was combined with ascorbic acid, but not with other water-soluble antioxidants in the DPPH model.

The free radical scavenging properties of antioxidants in homogeneous model systems (e.g., DPPH and ORAC) might not correlate to foods since foods are heterogeneous[16] and thus may have physical attributes not encountered in homogeneous systems that can impact antioxidant interactions. For example, the existence of both lipid and aqueous phases would affect antioxidant partitioning and thus interactions between oil and water-soluble antioxidants at the oil−water interface. Fukazawa and co-workers reported that interactions between α-tocopherol and ascorbic acid in liposomal membranes are influenced by physical barriers and surface charge of the membranes.[17] Currently, there are no systematic methods to be able to predict how combinations of antioxidants can inhibit oxidation in real food systems in a synergistic, additive, or antagonistic manner.

In this research, we hypothesized that antioxidants partitioning at different locations in oil-in-water (O/W) emulsions may influence the ability of antioxidants to interact. Rosmarinic acid and its alkyl esters are excellent tools to study interactions with α-tocopherol because their distributions and locations in O/W emulsions can be varied without impacting on their reactive hydroxyl groups.[18] In the current study, several methods were utilized to study the interactions between α-tocopherol and rosmarinic acid or its alkyl esters. Electrochemical properties of these phenolic compounds were investigated by cyclic voltammetry (CV) to explain thermodynamic reactions between antioxidants. Direct observations of interactions between rosmarinates, rosmarinic acid, and α-tocopherol were observed by a fluorescence quenching technique. The efficiency of tocopheroxyl radical regeneration by rosmarinic acid and rosmarinate esters was studied by

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electron paramagnetic resonance (EPR) in homogeneous (ethanol) and heterogeneous (Tween 20 micelles) systems. Finally, the sparing effects of antioxidant interactions between rosmarinate esters, rosmarinic acid, and α-tocopherol during the oxidation of O/W emulsions were investigated by determining the rates of antioxidant decomposition by high-performance liquid chromatography (HPLC). Through these studies, important mechanistic information can be obtained to better predict when synergistic, additive, or antagonistic antioxidant interactions can occur in O/W emulsions.

MATERIALS AND METHODS

Chemicals and Materials. Soybean oil was purchased from a local grocery market in Amherst, MA. Ethylenediaminetetraacetic acid (EDTA) disodium salt was purchased from Chempure Ultra (Houston, TX). Acetic acid, acetonitrile, methanol, and hydrochloric acid were obtained from Fisher Scientific (Pittsburgh, PA). Chelex 100, rosmarinic acid, caffeic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), $FeSO_4$, Tween 20 ($M_w \approx 1228$), $BaCl_2$, Bu_4NPF_6, phosphoric acid, sodium phosphate mono- and dibasic, and 2,2,6,6-tetramethylpiperidinoxyl (TEMPO) radical were purchased from Sigma-Aldrich (St. Louis, MO). α-Tocopherol quinone was purchased from Tokyo chemical industry (Tokyo, Japan). Double-distilled and deionized water was used for the preparation of all solutions.

Synthesis of Rosmarinate Esters. The chemoenzymatic esterification of rosmarinic acid to obtain rosmarinate esters was carried out following the procedure described by Lecomte and co-workers.[18] Briefly, the chemical esterification of rosmarinic acid (56 μmol) was conducted in sealed brown flasks each containing 5 mL of alcohol (methanol, 123.4 mmol; *n*-butanol, 54.6 mmol; *n*-octanol, 31.9 mmol; *n*-dodecanol, 22.5 mmol; *n*-hexadecanol, 17.0 mmol; *n*-octadecanol, 15.1 mmol; or *n*-eicosanol, 13.6 mmol). The reaction mixtures were stirred (orbital shaker, 250 rpm, 55−70 °C) prior to the addition of the catalyst, the strongly acidic sulfonic resin Amberlite IR-120H (5% w/w, total weight of both substrates) that had been previously dried at 110 °C for 48 h. The water generated during the reaction was removed by adding 3 Å, 4−8 mesh molecular sieves (40 mg/mL, Aldrich, St. Louis, MO) to the medium. Samples (20 μL) were regularly withdrawn from the reaction medium and then mixed with 980 μL of methanol, filtered (0.45 μm syringe filter Millex-FH, Millipore Corp., Bedford, MA), and analyzed by reverse phase HPLC with UV detection at 328 nm.[18] After complete (4−21 days) conversion of rosmarinic acid into the corresponding ester, the latter was purified in a two-step procedure. First, a liquid−liquid extraction using hexane and acetonitrile was performed to remove the excess alcohol. Then, the remaining traces of the alcohol and rosmarinic acid were eliminated by flash chromatography on a CombiFlash Companion system (Teledyne Isco Inc., Lincoln, NE). Separation was carried out on a silica column using an elution gradient of hexane and ether (20−100% in 35 min). The yield of purified esters, obtained as pale yellow to yellow amorphous powders, was calculated from calibration curves previously established with pure compounds. Pure esters and rosmarinic acid were then fully characterized by ESI-MS, ^{1}H NMR, and ^{13}C NMR as previously described by Lecomte et al.[18]

Emulsion Preparation. Stripped soybean oil was prepared according to the method of Waraho et al.[19] The effectiveness of stripping was monitored by measuring the removal of tocopherols by HPLC.[20] No tocopherol could be detected in the stripped oils. Oil-in-water (O/W) emulsions were prepared using 1.0% (wt) stripped soybean oil in a 10 mM phosphate buffer solution (pH 7.0). Tween 20 was used as an emulsifier at a 1:10 emulsifier/oil ratio. Stripped soybean oil, Tween 20, and phosphate buffer were added to a beaker, and a coarse emulsion was made by blending with a hand-held homogenizer (M133/1281-0, Biospec Products, Inc., Bartlesville, OK) for 2 min. The coarse emulsion was then homogenized with a microfluidizer (Microfluidics, Newton, MA) at a pressure of 9 kbar for three passes.

After the O/W emulsion was prepared, rosmarinic acid and its esters with various chain lengths (4, 8, 12, 18, and 20 carbons) in methanol were added to the emulsion at a final concentration of 30 μM and stirred for 1 h at room temperature. Samples with methanol but without antioxidant were used as control samples. The emulsions (0.5 mL) were transferred into 10 mL GC vials and sealed with (tetrafluoroethylene) butyl rubber septa, and then stored at 25 °C in the dark. Three vials of each treatment were taken every day to determine lipid hydroperoxides and hexanal formation.

In some experiments, when needed, emulsions were washed to remove aqueous phase surfactants as previously described by Faraji and co-workers[21] with some modifications. In short, emulsions were centrifuged at 38518*g* (17,000 rpm) for 1 h at 4 °C using a Fiberlite F40L-8×100 rotor with a high-speed centrifuge (Thermo Scientific WX Ultra 80, Asheville, NC). After the centrifugation, the bottom layer (phosphate buffer) was carefully removed using a needle and syringe, and then the same volume of the fresh phosphate buffer was used to redisperse the creamed emulsion droplet layer by vortexing. This washing procedure was performed a total of three times. The lipid content of the final washed emulsion was determined by the modified Bligh and Dyer method,[22] and then phosphate buffer was used to adjust the lipid content back to 1% (w/w). In some experiments, Tween 20 was added back into the washed emulsions (0, 0.1, 0.5, 1.0, and 2.5%; w/w) so that a known amount of surfactant would be in the continuous phase.

Measurements of Particle Size of Emulsions. The size of the emulsion droplets was measured by a dynamic light scattering (Zetasizer Nano-ZS, model ZEN3600, Malvern Instruments, Worchester, U.K.) and expressed as *z*-average mean diameter. Samples were diluted 50 times with the same buffer as the emulsion, mixed, and immediately measured by transferring the solution into 3 mL plastic cuvettes for determining the size. Measurements were performed on three replicates and repeated 3 times on each sample at room temperature. The emulsion droplet size ranged within 173.3 ± 11.7 nm, and there was no significant change in droplet size of each emulsion over the course of study (data not shown). In addition, there was no visual observation of creaming during storage in all treatments

Measurements of Lipid Hydroperoxides. Lipid hydroperoxide formation in emulsion solutions was determined according to the method described by Panya and co-workers[23] with some modifications. Emulsion solutions (0.2 mL) were mixed with 1.5 mL of isooctane/2-propanol (3:1 v/v) and vortexed (10 s, three times). After centrifugation at 1000*g* for 2 min, 30 μL of the organic solvent phase was mixed with 1.5 mL of methanol/1-butanol (2:1). Hydroperoxide detection was started by the addition of 7.5 μL of 3.94 M ammonium thiocyanate and 7.5 μL of ferrous iron solution (prepared by adding equal amounts of 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$). After 20 min of incubation at room temperature, the absorbance was measured at 510 nm using a UV−vis spectrophotometer (Genesys 20, Thermo Spectronic). Hydroperoxide concentrations were determined using a standard curve prepared from hydrogen peroxide.

Measurements of Hexanal. Headspace hexanal was determined according to the method described by Panya and co-workers[23] with some modification using a Shimadzu GC-2014 gas chromatograph (GC) equipped with an AOC-5000 autoinjector (Shimadzu, Tokyo, Japan). A 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/Carboxen/PDMS) stable flex solid phase microextraction (SPME) fiber (Supelco, Bellefonte, PA) was inserted through the vial septum and exposed to the sample headspace for 8 min at 55 °C. The SPME fiber was desorbed at 250 °C for 3 min in the GC detector at a split ratio of 1:7. The chromatographic separation of volatile aldehydes was performed on a fused-silica capillary column (30 m × 0.32 mm i.d. × 1 μm) coated with 100% poly(dimethylsiloxane) (Equity-1, Supelco). The temperatures of the oven, injector, and flame ionization detector were 65, 250, and 250 °C, respectively. Sample run time was 10 min. Concentrations were calculated by using a standard curve made from the above emulsions containing known hexanal concentrations and 200 μM EDTA.

Calculation of Antioxidant Interaction Indexes of Rosmarinate Esters and α-Tocopherol Combinations. Interaction indexes

of rosmarinate esters with α-tocopherol were calculated on the basis of the oxidation lag times of lipid hydroperoxides and hexanal formation. Lag times were determined as the first data point that was statistically ($p \leq 0.05$) greater than time zero. Briefly, the oxidation lag times of individual antioxidants were used to estimate the expected oxidation lag times of its combination. Interaction indexes were calculated from the ratio between the obtained oxidation lag times of the combination and the expected oxidation lag time of the combination with the following equation:

$$\text{interaction index} = (\text{observed lag time of the combination}) / (\text{expected lag time of the combination})$$

$$= [\text{lag time (control A + B)} - \text{lag time (A + B)}] / \{[\text{lag time (control A)} - \text{lag time (A)}] + [\text{lag time (control B)} - \text{lag time (B)}]\}$$

where A and B represent α-tocopherol and rosmarinates, respectively. Controls of A, B, and A + B represent the lag time of individuals and combinations without adding antioxidants. Interaction indexes were expressed as synergistic (>1), additive (≈1), and antagonistic (<1) antioxidant effects

Determination of Antioxidant Partitioning. Determination of the physical location of rosmarinic acid and its alkyl esters in the emulsions were performed according to the procedure described by Panya and co-workers.[23] EDTA (200 μM) was added to regular emulsions and washed O/W emulsions with added surfactants (0, 0.1, 0.5, 1.0, and 2.5%; w/w) to minimize oxidation during analysis. Rosmarinic acid and its alkyl esters in methanol were added to the emulsion at a final concentration of 100 μM followed by stirring at room temperature for 1 h. The emulsions were centrifuged at 162102*g* (46,000 rpm) for 1 h at 4 °C using a PTI F65L-6×13.5 rotor with a high-speed centrifuge (Thermo Scientific WX Ultra 80, Asheville, NC). The continuous phase was carefully collected with a pipet, and the amount of aqueous phase rosmarinic acid esters was determined directly by HPLC using a modified method described by Lecomte and co-workers.[18] Briefly, HPLC determination of rosmarinic acid and its alkyl esters was carried out with a Hypersil GOLD C18 reversed phase column (250 mm × 4.6 mm, 5 μm) equipped with a Hypersil gold guard column (10 mm × 4 mm, 5 μm) (Thermo Scientific, USA) using a LC-10ATvp HPLC system (Shimadzu, USA). Peak integration was performed using Shimadzu EZstart (version 7.2). Gradient elution was performed using methanol and 3 mM phosphoric acid at 1 mL/min at 40 °C (column temperature), in linear gradients from 0/100 (v/v) to 100/0 (v/v) for 5 min, then 100/0 (v/v) for 10 min, back to 0/100 (v/v) for 5 min, and held at 0/100 (v/v) for 5 min. Rosmarinic acid and its alkyl esters [(R4 (4 carbons) − R20 (20 carbons)] were detected with a photodiode array detector (SPD-M10Avp, Shimadzu, USA) at 328 nm. α-Tocopherol was detected at 295 nm. The concentrations of rosmarinic acid esters and α-tocopherol were calculated using a standard curve made from each antioxidant dissolved in methanol.

Determination of Antioxidant Decomposition during Oxidation Studies. Determination of the decomposition of α-tocopherol and rosmarinic acid and its alkyl esters in the emulsions during storage was performed according to the procedure described by Panya and co-workers.[23] A 200 μL sample of O/W emulsions was transferred into 1.5 mL eppendorf tubes containing 50 μL of 200 μM EDTA to inhibit further lipid oxidation. Samples were frozen at −80 °C until freezing drying. The freezing drying condition was operated at −10 °C for 16 h, and then the temperature was increased to 5 °C for 4 h. Dried emulsions were stored at −80 °C until analysis. Antioxidants in dried emulsions were extracted immediately prior to analysis by adding 200 μL of methanol. The mixtures were vortexed for 2 min, sonicated in an ultrasonic bath for 2 min, and then centrifuged at 1000*g* for 5 min.

The clear methanolic solutions were carefully collected with a pipet, and antioxidant concentrations were determined directly by HPLC using a modified method described by Fujimoto and Masuda.[24] Briefly, HPLC determination of rosmarinic acid and its alkyl esters was carried out with a Hypersil GOLD C18 reversed phase column (250 mm × 4.6 mm, 5 μm) equipped with a Hypersil Gold guard column (10 mm × 4 mm, 5 μm) (Thermo Scientific, USA) using a LC-10ATvp HPLC system (Shimadzu, USA). Peak integration was performed using Shimadzu EZstart (version 7.2). Gradient elution was performed using acetonitrile and 1% acetic acid at 1 mL/min in linear gradients from 5/95 (v/v) to 100/0 (v/v) for 40 min, then 100/0 (v/v) for 10 min, and then back to 5/95 (v/v) for 5 min. Rosmarinic acid and dodecyl rosmarinate ester (12 carbons) were detected with a photodiode array detector (SPD-M10Avp, Shimadzu, USA) at 328 nm. The oxidation products of rosmarinic acid and its dodecyl ester were detected at 280 and 328 nm. α-Tocopherol and α-tocopheryl quinone were detected at 295 and 265 nm, respectively. The concentrations of antioxidants (rosmarinic acid and its ester, α-tocopherol, and α-tocopheryl quinone) were calculated using a standard curve made from the standard antioxidants dissolved in methanol.

Front-Face Fluorescence Quenching Measurements. Front-face fluorescence quenching between α-tocopherol and rosmarinic acid and its alkyl esters in O/W emulsions was determined by steady-state emission measurements recorded with a PTI spectrofluorometer (PTI, London, Ontario, Canada). Stripped soybean oil (1%; w/w) emulsions were prepared with 0.1% (w/w) Tween 20 in 10 mM phosphate buffer (pH 7) with 200 μM EDTA to minimize oxidation. A final concentration of 100 μM of α-tocopherol was added to the O/W emulsions from the stock solution of α-tocopherol in methanol. The mixtures were stirred at room temperature for 1 h. Then, rosmarinate esters in methanol were transferred into the emulsions containing α-tocopherol at concentrations of 0, 5, 10, 25, 50, and 100 μM.

After vortexing for 2 min, the final emulsions (1.5 mL) were transferred into triangular Suprasil cuvettes. The samples were held at 30 °C and stirred with a 3 mm magnetic stirring bar (Fisher Scientific, USA). Emission was observed at 90° to the incident beam, that is, 22.5° with respect to the illuminated cell surface. The emission of α-tocopherol was measured at 320 nm using an excitation wavelength of 295 nm. Spectral bandwidth for both excitation and emission slits was 2.0 nm, integration time was 1 s, and the wavelength increment was 2.5 nm. The intensity of the spectra (I) of α-tocopherol after addition of the rosmarinate derivatives was determined as the emission signal intensity (counts per second) measured by means of a photomultiplier. The intensity ratio (I_o/I), where I_o is the fluorescence intensity of α-tocopherol, was plotted versus the concentrations of rosmarinate esters. The slope of this line was used to determine the quenching constant of the different rosmarinate esters.

Cyclic Voltammetry (CV) Measurements. Cyclic voltammetry was performed according to the method described by Wilson and co-workers.[25] Rosmarinic acid and its alkyl esters (2 mM) were freshly dissolved in acetonitrile and evaluated with a BASi model C-3 cell stand using a planar 1 mm diameter glassy carbon working electrode, Ag/AgCl reference electrode, and a Pt wire auxiliary electrode. Samples were scanned at 100 mV s^{-1} at 25 °C in acetonitrile with 0.5 M Bu_4NPF_6 as a salt bridge in 50 mM phosphate buffer, pH 7 or in 1% Tween 20 in 50 mM phosphate buffer, pH 7.

Measurement of α-Tocopherol Regeneration by Electron Paramagnetic Resonance Spectroscopy. The efficiency of rosmarinic acid and its alkyl esters to regenerate α-tocopherol from α-tocopheroxyl radicals was determined by electron paramagnetic resonance (EPR) spectroscopy in two different systems: a homogeneous ethanolic solution and a heterogeneous Tween 20 micelle solution. The experiment procedure was adapted from the method described by Pazos and co-workers[26] with some modifications.

For experiments in homogeneous environments, stock solutions of α-tocopherol (2 mM), DPPH radical (0.5 mM), and rosmarinates were prepared freshly with N_2 saturated ethanol. In heterogeneous environments, a stock solution of α-tocopherol (2 mM) was prepared in 100 mM Tween 20 in N_2 saturated 50 mM phosphate buffer solution at pH 7. 2 mL of the stock solution of α-tocopherol (ethanolic or Tween 20 solutions) was transferred into a 4 mL vial which was purged with N_2. Then, 50 μL of the DPPH solution was transferred into the stock solution of α-tocopherol, and mixed immediately at room temperature. After reacting for 20 s to form α-

tocopheroxyl radicals, 50 μL of the stock solutions of rosmarinic acid and its alkyl esters were added and mixed. The final concentrations of α-tocopherol and DPPH were 1.9 and 0.01 mM, respectively. The final concentrations of rosmarinic acid and rosmarinates ranged from 2.5 to 20 μM. All solutions were transferred into an EPR spectrometer via a 5 mL syringe. EPR spectra were recorded 1 min after the reaction with DPPH.

The experiments were performed using a Bruker ELEXSYS E-500 EPR spectrometer (Bruker, Germany) equipped with an X-band microwave bridge and an ER 4122-SHQE high sensitivity single cavity. Samples were injected into the cavity with Aqua-X flow-through cell. EPR parameters were at the following settings: microwave power, 10 dB; sweep width, 100 G; sweep time, 20.9 s; modulation amplitude, 3 G; time constant, 81.92 ms; receiver gain, 80 dB. All samples were handled under N_2 sealed environment at room temperature.

The Mn(II) marker attached with the Aqua-X flow-through cell was used to determine the relative signal intensity of the α-tocopheroxyl radical (the peak-to-peak ratio between α-tocopheroxyl radical and the marker). Concentrations of α-tocopheroxyl radical were quantitated by comparing the double-integrated areas of α-tocopheroxyl radical to known concentration of TEMPO radical. The integration of the signal was performed by using Bruker Xepr software. Efficiencies of the regeneration of α-tocopherol by each rosmarinate were estimated from the slopes of α-tocopheroxyl radical reduction at various concentrations of rosmarinates.

Statistical Analysis. All analyses were performed on triplicate samples. Oxidation lag phases were defined as the first data point significantly greater than the 0 time value. In all cases, comparisons of the means were performed using Duncan's multiple-range tests. A significance level of $p < 0.05$ was defined as being statistically different. All calculations were performed using SPSS17 (http://www.spss.com; SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Antioxidant Activity of Rosmarinic Acid and Its Esters and α-Tocopherol Combinations in Stripped Soybean O/W Emulsions. The ability of rosmarinic acid and rosmarinate esters and α-tocopherol to synergistically inhibit lipid oxidation in the O/W emulsions was tested with rosmarinic acid (R0) and its different esters (R4, R12, and R20). This system was used since the different forms of rosmarinate could primarily exist in the aqueous phase, the interfacial layer, or the emulsion droplet core and thus could interact differently with α-tocopherol (T) which would mainly associate with the oil–water interface and/or the emulsions droplet core. Lipid hydroperoxide and hexanal formation in the O/W emulsions were determined during storage at 25 °C in the dark. All forms of rosmarinic acid and its esters (30 μM) were able to inhibit the formation of lipid hydroperoxides and hexanal compared to the control (Figure 1). For example, the R4 was slightly better than R12 at increasing hexanal lag times, and R0 was slightly better than R20, which is the worst antioxidant. This antioxidant efficiency order (R4 > R12 ≫ R0 > R20) confirms the cutoff effect we already observed in O/W emulsion with rosmarinate derivatives[27,28] and chlorogenate alkyl esters.[29]

All the combinations of rosmarinic acid and its esters (30 μM) with α-tocopherol (30 μM) exhibited better antioxidant activity compared to the individual compounds. This was not entirely unexpected since the total antioxidant concentrations were higher. As shown in Figure 2, it was noted that the combinations of α-tocopherol and butyl rosmarinate ester (T-R4), and α-tocopherol and dodecyl rosmarinate ester (T-R12) exhibited similar increases in the lag phase of lipid hydroperoxides and hexanal formation as the sum of the individual antioxidants (Figure 1). Surprisingly, the combination of α-tocopherol and rosmarinic acid (T-R0) showed significant increases in the lag phase of lipid hydroperoxides and hexanal formation compared to the sum of antioxidants analyzed individually (synergistic effect). The lag times of hexanal formation and lipid hydroperoxides for the T-R0 combination ended up being similar to the lag times of the T-R4 and T-R12 combinations. In this case, the strong synergistic effect between α-tocopherol and R0 compensates for the much lower antioxidant activity of R0 taken individually compared to R4 and R12. Finally, it clearly appears from Figure 2 that the combination between R20 and α-tocopherol produces an antagonistic effect.

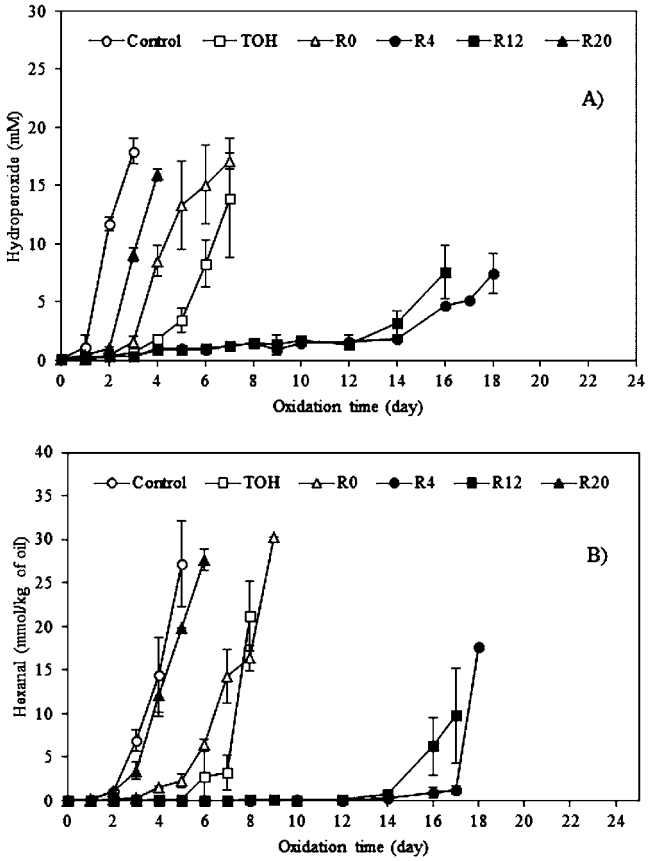

Figure 1. Lipid hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil–Tween 20 emulsions at 25 °C in the presence of individual of rosmarinic acid and its alkyl esters and α-tocopherol (TOH) (30 μM). Data points and error bars represent means ($n = 3$) ± standard deviations.

To quantitate the effects of the antioxidant combinations, the lag times for the formation of lipid hydroperoxides and hexanal for both individual and combined antioxidants were used to calculate the interaction index. As illustrated in Figure 3, R0 had the strongest antioxidant synergy with α-tocopherol producing interaction indexes for lipid hydroperoxides and hexanal formation of 4 to 5, while the interaction indexes of α-tocopherol and R4 or R12 were approximately 1.5 to 2, meaning that the synergistic effects were smaller. In contrast, R20 showed significant antagonistic effect with α-tocopherol exhibiting an interaction index of 0.3 to 0.6.

Partitioning of Rosmarinic Acid, Rosmarinate Esters, and α-Tocopherol in O/W Emulsions. A factor that could be important for the observed variations in synergistic antioxidant interactions would be differences in the physical

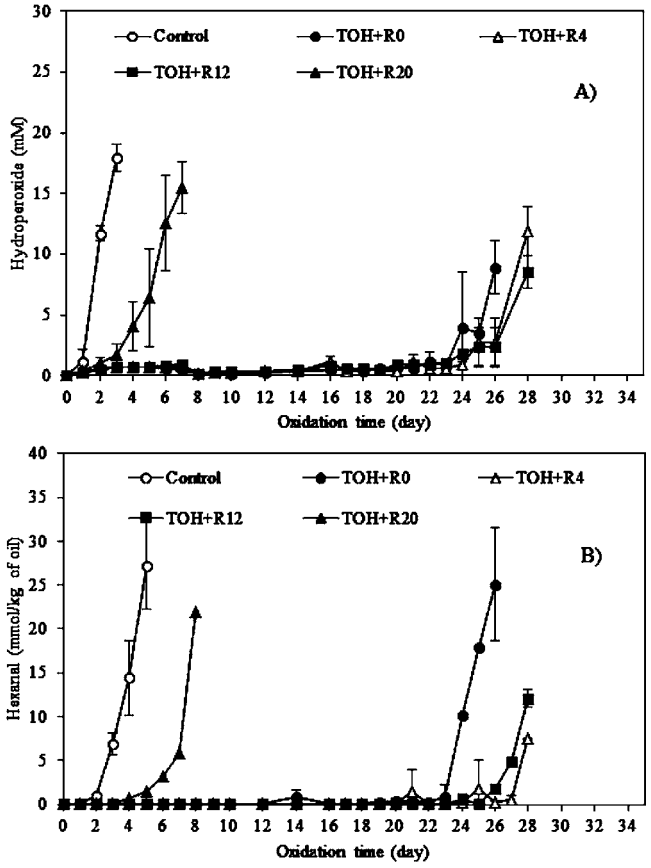

Figure 2. Lipid hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil–Tween 20 emulsions at 25 °C in the presence of combinations of rosmarinic acid and its alkyl esters with α-tocopherol (TOH) (30 μM + 30 μM). Data points and error bars represent means (n = 3) ± standard deviations.

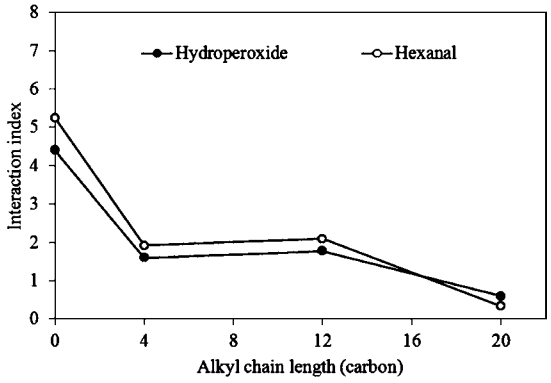

Figure 3. Interaction indexes of the combinations of rosmarinic acid and its esters with α-tocopherol in 1% stripped soybean oil–Tween 20 emulsions at 25 °C. Data were calculated from oxidation lag time obtained from lipid hydroperoxide and hexanal formation.

location of rosmarinic acid and rosmarinate esters which would impact their ability to interact with α-tocopherol. The aqueous phase of O/W emulsions was collected to determine whether the antioxidants partitioned into the aqueous or emulsion droplet phases (interface + droplet core). Results showed that rosmarinic acid (R0) had the lowest association with emulsion droplets, partitioning at approximately 90% in the aqueous phase, while butyl (R4) and dodecyl (R12) rosmarinate esters and α-tocopherol were highly associated with the emulsion droplets indicated by low concentrations (2–9%) in the aqueous phase (Figure 4). The antioxidant partitioning of R4,

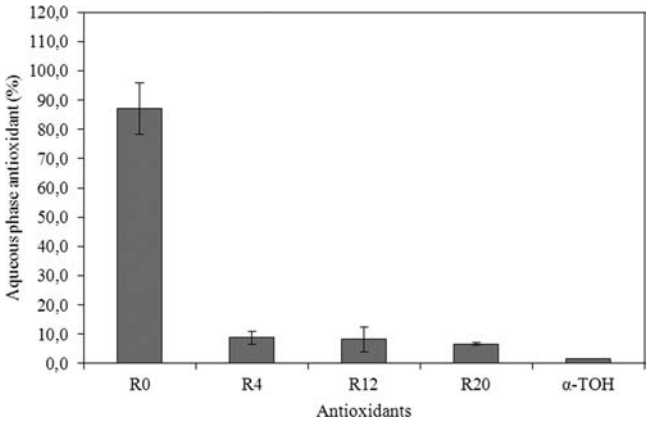

Figure 4. The antioxidant partitioning of rosmarinic acid (R0), butyl (R4), dodecyl (R12), and eicosyl (R20) rosmarinate esters and α-tocopherol (100 μM) into aqueous phase of O/W emulsions (not washed). Data points and error bars represent means (n = 3) ± standard deviations.

R12, and R20 was consistent with our previous report.[28] There was slight precipitation observed in the continuous phase of emulsions containing R20. According to our previous study, R20 may form poorly soluble self-assembled aggregates and/or comicelles with Tween 20 in the aqueous phase.[27,28]

Interactions between Antioxidants in O/W Emulsions as Determined by Front-Face Fluorescence Quenching Measurements. Just because antioxidants partition into the continuous phase or the emulsion droplet does not mean they will interact to produce synergistic or antagonist interactions. Direct observations of the rosmarinic acid or rosmarinate esters interacting with α-tocopherol in O/W emulsions can be determined by quenching of α-tocopherol fluorescence by the rosmarinates. Although the exact quenching mechanisms between these antioxidants have not been reported, one potential mechanism might be Förster resonance energy transfer (FRET) because the emission wavelength of α-tocopherol (325 nm) overlaps with the excitation wavelength (323 nm) of the rosmarinate esters and rosmarinic acid. Therefore, closer the proximity between α-tocopherol and the rosmarinates or rosmarinic acid would be expected to produce greater quenching.

In the O/W emulsions, the fluorescence intensity of α-tocopherol was decreased in the presence of butyl and dodecyl rosmarinates and rosmarinic acid. As illustrated in Figure 5, R0 was more effective at quenching the α-tocopherol fluorescence in O/W emulsion compared to the rosmarinate esters (R4 to R12) as shown by R0's higher quenching constant. Results suggest that the more water-soluble rosmarinic acid (R0) was able to interact with α-tocopherol on the emulsion droplet surface. The lower quenching constants of rosmarinate alkyl esters suggest that slow lateral diffusion on the droplet surface would limit their ability to interact with α-tocopherol. This slowing down of the rosmarinate diffusivity may be due to hydrophobic interactions between their alkyl chains and the aliphatic tails of surfactants and lipids at the interface and/or in the droplet interior.

When O/W emulsions are produced, excess surfactant that is not absorbed onto the emulsion droplet surface partitions into

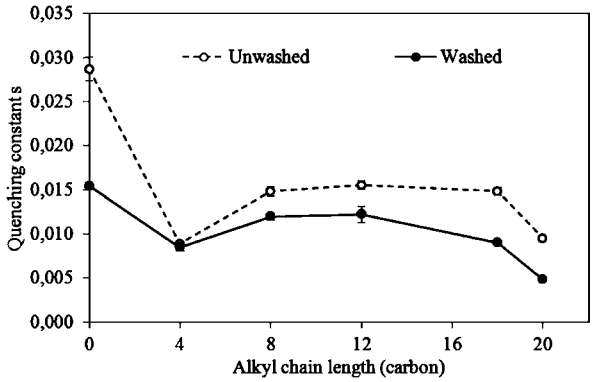

Figure 5. Fluorescence quenching of α-tocopherol (λ_{em} = 325 nm) by rosmarinic acid and rosmarinate esters in O/W emulsions with/without influences of surfactant micelles.

the aqueous phase and forms micelles. These micelles can alter the partitioning of antioxidants into the continuous phase by solubilizing the antioxidants into the micelles. To eliminate the influence of surfactant micelles, the excess surfactants in O/W emulsions were removed by a washing process.[28] Removal of the surfactant micelles decreased the ability of all the rosmarinic acid derivatives to quench the fluorescence of α-tocopherol with the exception of the R4 ester. This decrease was most dramatic for rosmarinic acid (R0) such that its quenching constant became similar to that of the rosmarinic acid esters (R8, R12). This suggests that the removal of the surfactant micelles decreased the partitioning of α-tocopherol into the aqueous phase which decreased the ability of α-tocopherol and water-soluble rosmarinic acid to interact. The α-tocopherol quenching constants of the other rosmarinate esters also decreased in washed emulsions, suggesting that the Tween 20 micelles also decreased their ability to interact with α-tocopherol. Since all of the antioxidants can partly partition into the interface, the decrease in interfacial area caused by the removal of the micelles would be expected to decrease the partitioning of the antioxidants in the interface of the emulsion droplets and micelles and thus decrease antioxidant interactions especially if some of the antioxidant was forced into the interior of the emulsion droplet.

Electrochemical Properties of Rosmarinic Acid, Rosmarinate Esters, and α-Tocopherol. One possible reason for the observed variations in antioxidant activity for combinations of rosmarinic acid esters and α-tocopherol could be due to the regeneration of one antioxidant by the other. The hierarchy of antioxidant regeneration by electron transfer can be estimated by their oxidation−reduction potentials. Generally, an antioxidant with lower reduction potential is thermodynamically preferred to give electrons to an antioxidant with higher reduction potential.[30] Cyclic voltammetry can be a useful method for studying the reduction potential of antioxidant compounds.[31−35] In this study, cyclic voltammetry was performed in order to measure the electrochemical potential of α-tocopherol and also to investigate the influence of esterification on the electrochemical properties of rosmarinic acid in various solutions.

Results showed that cyclic voltammograms of the rosmarinate esters and α-tocopherol exhibited one anodic and one cathodic peak (data not shown) in acetonitrile. This is also true for other phenolic compounds.[36,37] As shown in Table 1, all the rosmarinate esters showed a similar oxidation peak (E_{Pa}^{ox}) at

Table 1. Electrochemical Parameters of Tested Antioxidants in Acetonitrile, 50 mM Phosphate Buffer (pH 7), and 1% Tween 20 in 50 mM Phosphate Buffer (pH 7) Solutions at 25 °C Obtained from Cyclic Voltammetry (CV)

antioxidants	oxidation peak potential (mV vs Ag/AgCl)
R0 (ACN)	1115.1 ± 10.7
R4 (ACN)	1132.9 ± 7.1
R8 (ACN)	1126.0 ± 8.2
R12 (ACN)	1133.9 ± 5.6
R18 (ACN)	1135.3 ± 8.4
R20 (ACN)	1137.4 ± 1.6
α-TOH (ACN)	634.9 ± 0.85
R0 (PBS, pH 7)	359.0 ± 19.2
Trolox (PBS, pH 7)	275.0 ± 12.0
R0 (Tw20, pH7)	394.3 ± 16.9
α-TOH (Tw20, pH 7)	263.3 ± 2.3
Trolox (Tw20, pH 7)	298.7 ± 18.5

approximately 1115−1137 mV (vs Ag/AgCl) in acetonitrile solution, which was significantly higher than that of α-tocopherol (634.9 mV). Electrochemical parameters of the rosmarinate esters did not show the nonlinear behaviors that were observed when their activity was tested with the DPPH assay or in lipid oxidation studies.[27−29,38]

Oxidation peak potentials of rosmarinic acid and α-tocopherol were further studied in 50 mM phosphate buffer, pH 7.0 and 1% Tween 20 in 50 mM phosphate buffer solutions. Trolox was used instead of α-tocopherol in these experiments due to solubility limitations of α-tocopherol in aqueous solutions. Results showed that rosmarinic acid reduction potential decreased in the phosphate buffer, however, rosmarinic acid still had higher reduction potentials than Trolox. This was also true in the presence of Tween 20 micelles where the rosmarinic acid reduction potential decreased compared to acetonitrile but was still greater than that for α-tocopherol. These results suggested that α-tocopherol is thermodynamically preferred to donate electrons to rosmarinic acid.

Regeneration Efficiency of the Rosmarinic Acid Esters To Reduce α-Tocopheroxyl Radical in Homogeneous and Heterogeneous Systems. The ability of rosmarinic acid and its esters and α-tocopherol to regenerate each other's radicals can be determined by electron paramagnetic resonance (EPR) technique. To determine these interactions, antioxidant radicals were produced by exposing the antioxidants to DPPH radicals. However, rosmarinic acid and its esters were not able to produce stable radicals that could be observed by electron paramagnetic resonance (EPR) using this method, so the following studies focused on the fate of α-tocopherol radicals in the presence of rosmarinic acid and its esters. Experiments were performed in both ethanol and 100 mM Tween 20 solutions in order to observe the ability of the different alkyl chain lengths of rosmarinic acid to impact the efficiency of α-tocopherol regeneration in homogeneous and heterogeneous systems. Overall, the efficiency of rosmarinic acid and its esters to reduce α-tocopherol radical was very low as predicted by electron reduction potential (Table 1). The range of α-tocopherol radical regeneration efficiencies by rosmarinic acid and its esters ranged from 0.08 to 0.55 mol of α-tocopherol radicals reduced/mol of phenolics (Figure 6).

As shown in Figure 6, esterification of rosmarinic acid increased electron-donating ability toward α-tocopheroxyl

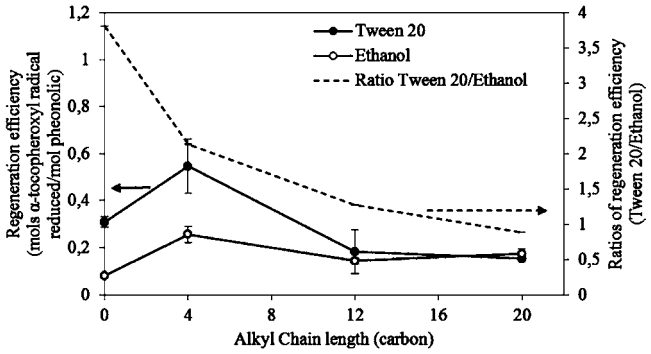

Figure 6. Regeneration efficiencies of the rosmarinic acid esters to reduced α-tocopheroxyl radicals in homogeneous (ethanol) and heterogeneous (Tween 20) micelle solutions. Dashed line indicates ratios of regeneration efficiency of Tween 20 and ethanol. Data points and error bars represent means (n = 5) ± standard deviations.

radicals compared to rosmarinic acid in ethanolic solutions. Esterification has also been observed to increase the DPPH scavenging activity of rosmarinic acid esters[18,28] and chlorogenic acid esters[39] in methanolic solution. Lecomte and co-workers[18] reported that dodecyl rosmarinate (R12) had the greatest DPPH scavenging activity of all the esters tested (4−20 carbons), while Lopez-Giraldo and co-workers (2009) showed that butyl and octyl chlorogenate esters had higher DPPH scavenging activity than chlorogenic acid itself and its esters with alkyl chains longer than 12 carbons. In this study, R4 had the highest ability to regenerate the α-tocopheroxyl radical scavenging efficiency compared to other esters in ethanol solution.

To investigate the influence of physical structures on α-tocopherol radical regeneration by the rosmarinates, measurements were also performed in 50 mM phosphate buffer solution with surfactant micelles produced from 100 mM Tween 20, pH 7.0. The ratio of scavenging regeneration efficiency in Tween 20 versus ethanol for R0 and R4 increased by 381 and 214%, respectively, while R12 and R20 were essentially the same in ethanol and Tween 20. The results for R0 are similar to those observed for R0 quenching of fluorescence, again suggesting that the high partitioning of R0 in the aqueous phase allowed it to interact with α-tocopherol radicals at the Tween 20 micelle interface. The fact that R4 was effective at interacting with α-tocopheroxyl radicals but did not alter α-tocopherol fluorescence could be due to the lack of emulsion droplets in the EPR study. The presence of emulsion droplets in the fluorescence study could result in R4 partitioning in the emulsion droplet in a manner where it did not readily interact with α-tocopherol whereas R4 would interact with α-tocopheroxyl radicals in surfactant micelles.

Depletion of α-Tocopherol, Rosmarinic Acid, and Its Alkyl Esters during Oxidation of O/W Emulsion. Studies on α-tocopherol fluorescence quenching indicated that, of all the tested polyphenols, R0 interacted with α-tocopherol more than the rosmarinate esters. This suggests that the ability of α-tocopherol to greatly increase the antioxidant activity of R0 could be due to their molecular interactions. To further investigate the potential interaction between the rosmarinates

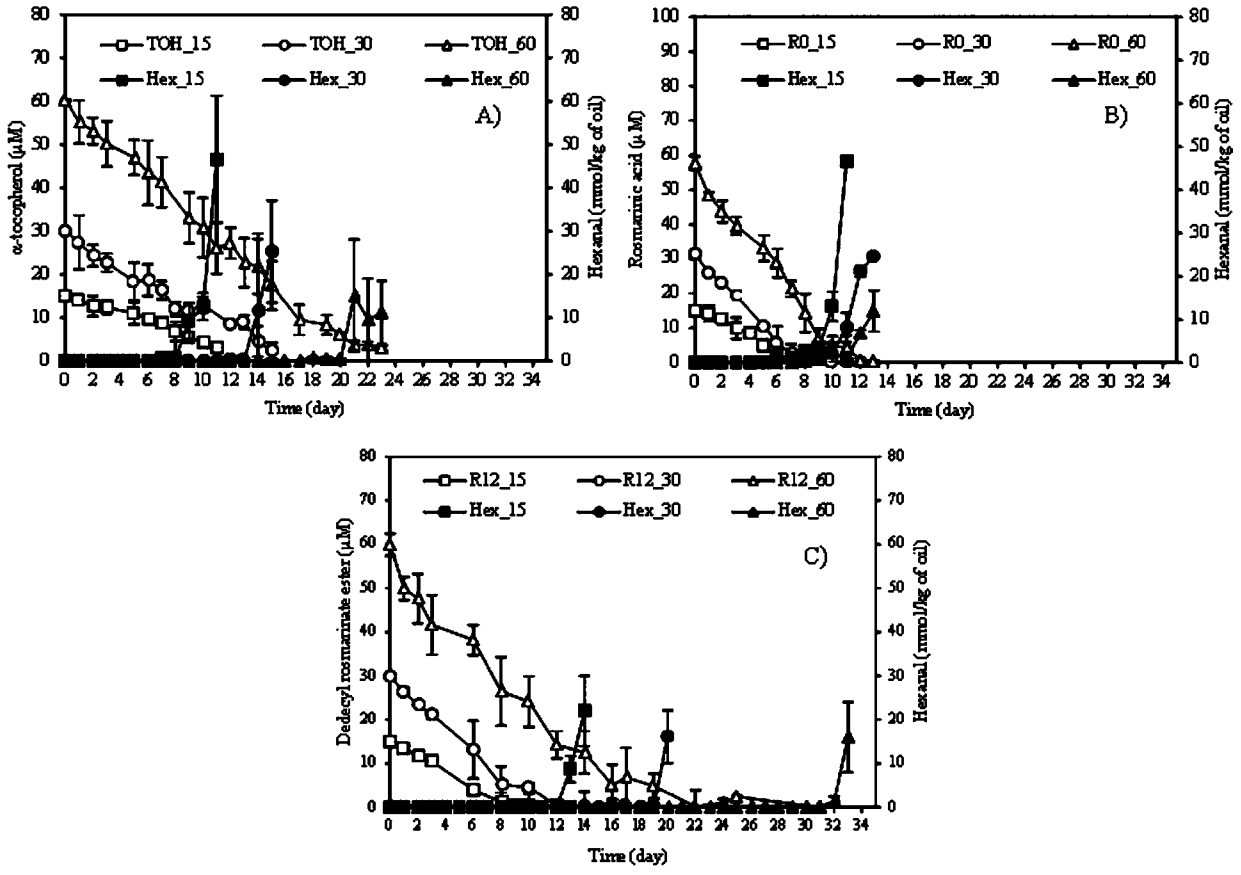

Figure 7. Depletion of α-tocopherol (A), R0 (B), and R12 (C) when added individually at different concentrations (15, 30, and 60 μM) and their effect on hexanal formation during the oxidation of O/W emulsions at 25 °C.

and α-tocopherol, decomposition of the antioxidants was determined during storage and compared to formation of the lipid oxidation product, hexanal, in the O/W emulsion.

The decomposition of R0, R12, and α-tocopherol analyzed individually in the O/W emulsion and subsequent hexanal formation are shown in Figure 7A–C. Results showed that α-tocopherol concentrations decreased in a linear fashion (Figure 7A). α-Tocopherol concentrations were approximately 8–12 μM when the lag phase of hexanal formation ended. The concentration of R0 and R12 also decreased in a linear fashion during storage of the O/W emulsions (Figure 7C). However, both R0 and R12 were completely depleted prior to formation of hexanal.

Depletion of R0, R12, and α-tocopherol during the storage of the O/W emulsions when the antioxidants were added in combination is shown in Figures 8 and 9. In this study, α-tocopherol concentration was constant at 30 μM while R0 and R12 concentrations ranged from 15 to 60 μM. As was previously observed in Figure 3, the combination of α-tocopherol and R0 produced synergistic antioxidant activity, while α-tocopherol and R12 showed an additive effect.

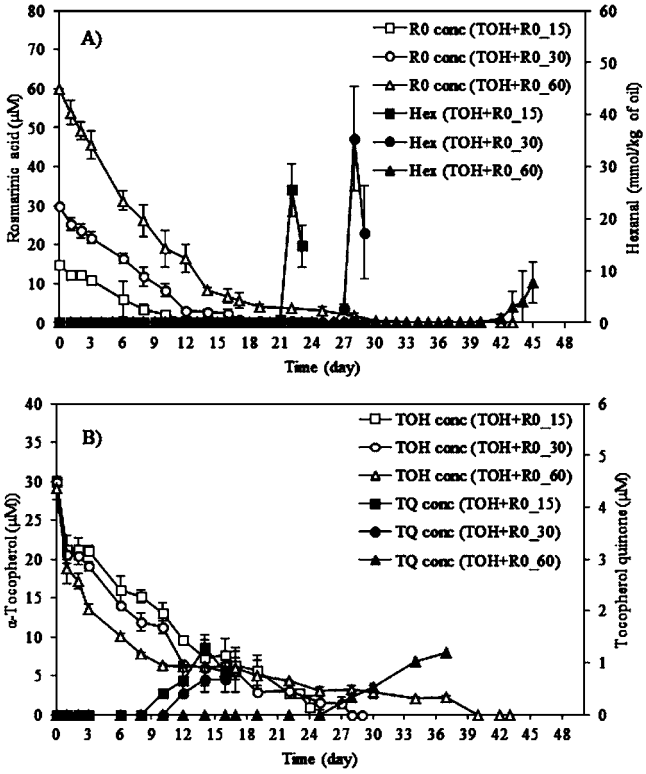

Figure 8. Depletion of rosmarinic acid (R0) (A) and α-tocopherol (TOH) (B) analyzed in combination and their effect on tocopherol quinone (TQ) and hexanal (Hex) formation in 1% stripped soybean oil–Tween 20 emulsions at 25 °C. Three concentrations of R0 were tested (15, 30, and 60 μM) in combination with 30 μM α-tocopherol. Data points and error bars represent means (n = 3) ± standard deviations.

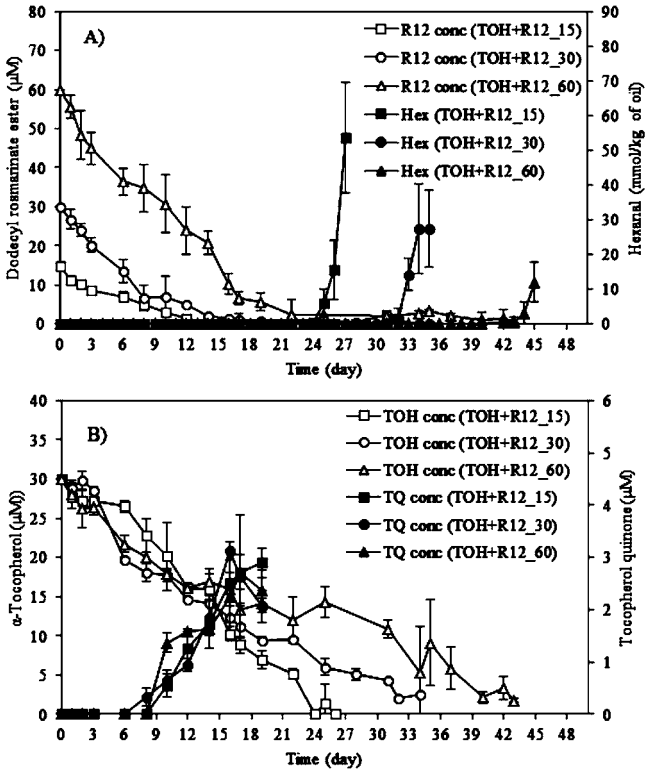

Figure 9. Depletion of rosmarinic acid (R12) (A) and α-tocopherol (TOH) (B) analyzed in combination and their effect on tocopherol quinone (TQ) and hexanal (Hex) formation in 1% stripped soybean oil–Tween 20 emulsions at 25 °C. Three concentrations of R12 were tested (15, 30, and 60 μM) in combination with 30 μM of α-tocopherol. Data points and error bars represent means (n = 3) ± standard deviations.

The decomposition disappearance of R0 and R12 was very similar in the presence of α-tocopherol (Figures 8A and 9 A). For example, the time at which approximately 50% of R0 and R12 was lost was about 9 days. The similarity of R0 and R12 depletion in the presence of α-tocopherol suggests that the increase in the antioxidant activity of R0 by α-tocopherol was not due to α-tocopherol regenerating R0 and keeping R0 concentrations higher. Conversely, α-tocopherol depletion was much faster in the presence of R0 than R12 (Figures 8B and 9B). For example at 30 days of storage, α-tocopherol concentrations were less than 5 μM in the presence of R0 (60 μM) compared to 11 μM in the presence of R12 (60 μM). In addition, the lag times of tocopheryl quinone (TQ) formation were different between R0 and R12 samples (Figures 8B and 9B). For example, tocopheryl quinone was detected in the R0 samples when R0 was almost depleted whereas tocopheryl quinone was detected much earlier during storage in the presence of R12 with the lag phase for TQ formation being independent of R12 concentration.

The observation that R0 and α-tocopherol samples increased the lag phase for TQ formation and improved their oxidative stability similar to R12 and α-tocopherol samples suggests that the two antioxidants were interacting. Even though the concentrations of α-tocopherol in the combination with R0 were lower than that observed in R12 and α-tocopherol samples, antioxidant activity was greater. This suggests that other antioxidative compounds might exist in the emulsions since it has been reported that some antioxidants can produce other antioxidative compounds via their oxidative degradation.[24,40]

The major oxidation products generated from R0 and R12 were analyzed to get a better understanding of the dynamics of the antioxidant mechanisms in the O/W emulsions. Only one major breakdown product of R0 was observed during storage

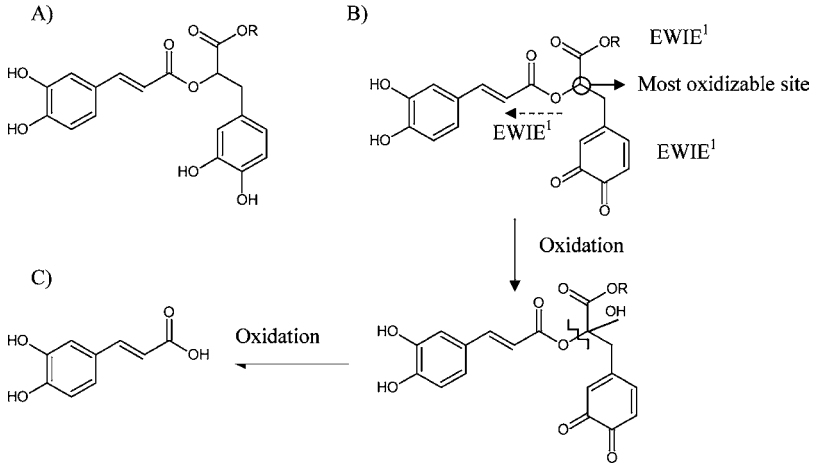

Figure 10. Structures of main antioxidant products observed during oxidation. (A) Reduced form of rosmarinic acid and dodecyl rosmarinate. (B) Quinone formed on the 2-oxyphenylpropanyl moiety of dodecyl rosmarinate (the quinone of rosmarinic acid was not detected). (C) Caffeic acid observed in the oxidation of rosmarinic acid and dodecyl rosmarinate. [1]EWIE: electron-withdrawing inductive effect.

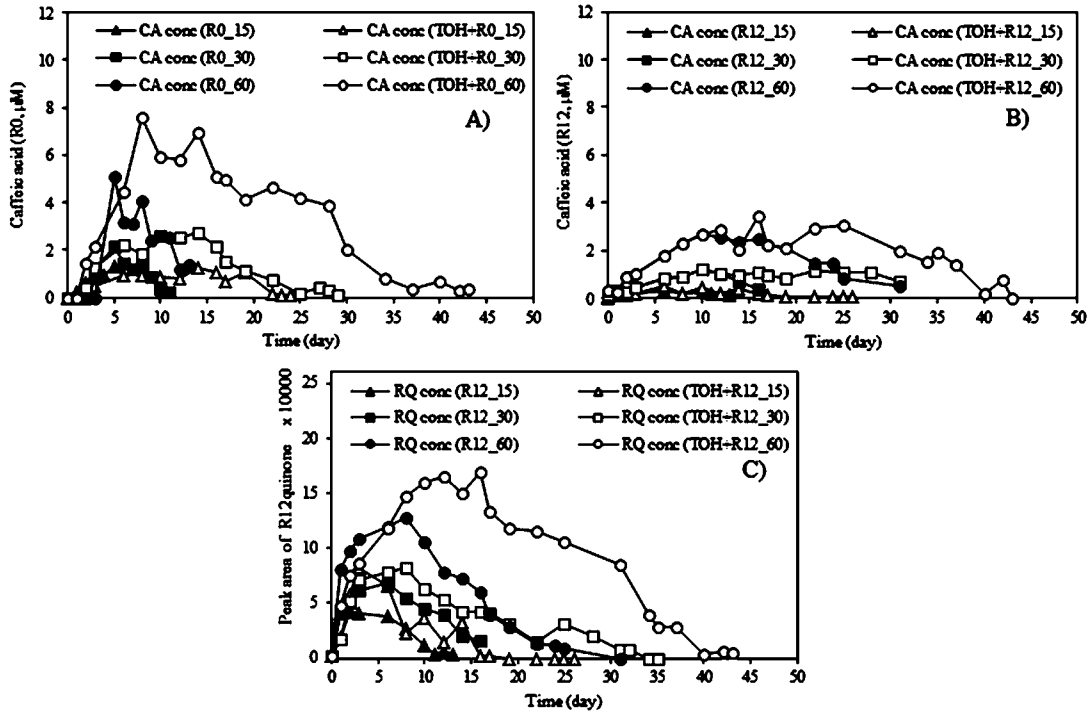

Figure 11. Accumulation (A_{330}) of caffeic acid (CA) in R0 (A) and R12 (B), and the quinone (RQ) in R12 (C) observed in the individual and the combinations with α-tocopherol at different concentrations during the oxidation in O/W emulsions.

while two major breakdown products were observed for R12. From LC−MS analysis, the products at HPLC retention times of 10.5 (in R0 and R12) and 35.9 (in R12) min are caffeic acid [m/z = 179.2 $(M - H)^-$] and a dodecyl rosmarinate quinone formed on the 2-oxyphenylpropanyl moiety [m/z = 525.06 $(M - H)^-$]. The structures of major antioxidant oxidation products are shown in Figure 10. The quinone was previously reported to be the major oxidation product of rosmarinic acid as determined in 2,2-azobis(isobutyronitrile) (AIBN)-induced oxidation of ethyl linoleate and DPPH model systems.[24]

However, unlike the above single phase model systems using ethyl linoleate and alcohols, our results indicated that the antioxidant product of R0 in O/W emulsions was mainly caffeic acid (Figure 11). This could be because the R0 quinone was unstable in O/W emulsions and was fragmented to caffeic acid. It was reported that rosmarinic acid can be decomposed into caffeic acid via a McLafferty rearrangement (γ-H rearrangement with β-cleavage in the electron ionization) in mass spectrometry analysis.[40] Caffeic acid has also been found to be one of the metabolites of rosmarinic acid in rats.[41] Hydrolysis of rosmarinic acid by esterases into caffeic acid and 3,4-dihydroxyphenyllactic acid in vitro was reported; however, the hydrolysis was not observed in a gastrointestinal model with lipase and pancreatic enzymes.[42]

In our conditions, however, hydrolysis is unlikely. Instead, an oxidation breakdown is possible on the tertiary carbon (circled carbon in Figure 10), which represents, by far, the most oxidizable carbon due to the three electron-withdrawing effects exerted by the ester, the acid, and the quinone groups. Such a

position could be oxidized twice, which would provoke the scission of the ester bond to produce caffeic acid (Figure 10).

In both the absence and presence of α-tocopherol, caffeic acid was produced from both R0 and R12 (Figure 11). More caffeic acid was produced from R0 in the presence of α-tocopherol (Figure 11A) suggesting that α-tocopherol could be involved in the formation of caffeic acid. The same was true with the case of R12; however, the production of caffeic acid by α-tocopherol was found to a much lower (Figure 11B).

In all treatments, caffeic acid was detected at the beginning of the oxidation process and then decreased at the end of the oxidation lag times (Figure 11A,B). Except for R0 alone, the hexanal lag times ended before all caffeic acid was depleted. Caffeic acid has the ability to scavenge free radicals,[46] and Chen and Ho[47] reported that caffeic acid had better antioxidant activity in O/W emulsions than rosmarinic acid and α-tocopherol. In this study, the degradation of caffeic acid prior to the end of the lag phase also suggests that it is being preferentially oxidized prior to the fatty acids and thus is acting as an antioxidant. Therefore, the formation of caffeic acid from R0 in the presence of α-tocopherol could explain why this combination had much better antioxidant activity than the individual antioxidants since the caffeic acid would provide an additional antioxidant to slow down oxidation (Figure 10A). In addition to the possibility that the formation of caffeic acid might be driven by the proximity between R0 and α-tocopherol, it might be enhanced by π−π aromatic interactions between phenolic compounds. It was suggested that the formation of a stable complex between antioxidants due to π−π stacking between the aromatic rings of phenolic compounds may influence the overall electron donating capacity, resulting synergistic effects.[43−45]

A quinone of dodecyl rosmarinate ester was observed with R12 but not R0, suggesting that the esterification of rosmarinic acid with an alkyl chain can increase the stability of the quinone (Figure 11C) and can thus disfavor the formation of caffeic acid. Steric hindrance exerted by the dodecyl chain of the R12 quinone may slow the rosmarinic acid oxidation reactions which produce caffeic acid. Therefore, unlike the extremely reactive R0 quinone which is rapidly converted into caffeic acid, the R12 quinone may exist long enough to be detected.

If we consider that α-tocopherol promotes the breakdown of the quinone into caffeic acid, the lower concentration of caffeic acid with R12 compared to R0 could also be due to R12 reacting less with α-tocopherol than R0 as shown by the fluorescence and EPR data (Figures 5 and 6), thus decreasing the conversion of the quinone to caffeic acid.

Fluorescence quenching results demonstrate that R12 interacts to a much lesser extent than R0 with α-tocopherol. In the absence of bimolecular complex, consequently, the R12 quinone may be too far from the hydroxyl radicals generated by α-tocopherol. In these conditions, α-tocopherol does not promote any conversion of R12 quinone into caffeic acid, which is the observation made in Figure 11B. Furthermore, this could explain why R12 does not affect the depletion rate of α-tocopherol during the first days (Figure 9B).

In conclusion, R0 was physically able to interact with α-tocopherol in surfactant micelles and O/W emulsions. We hypothesize that the observed synergistic antioxidant activity of the combination of R0 and α-tocopherol was not due to the regeneration of α-tocopherol by rosmarinic acid due to the thermodynamic infeasibility of this reaction and the fact that α-tocopherol degradation rates in O/W emulsions were not decreased by rosmarinic acid. In addition, the regeneration of the rosmarinate radical by α-tocopherol was also unlikely since this reaction was slow and α-tocopherol did not alter R0 degradation rates. Instead α-tocopherol and R0 interactions produced an increase in antioxidant activity by promoting the conversion of rosmarinic acid into caffeic acid thus providing a third molecule that could inhibit lipid oxidation and increased the oxidative stability of the O/W emulsion. In contrast, R12 in combination with α-tocopherol did not produce synergistic antioxidant activity and did not significantly increase its conversion into caffeic acid during lipid oxidation in O/W emulsions. This could be because R12 did not interact strongly with α-tocopherol as determined by fluorescence. Overall, physical interaction between α-tocopherol and rosmarinic acid or its alkyl esters seems to be an important factor in their ability to inhibit lipid oxidation. Further research is needed to determine if physical interactions are important in determining the antioxidant activity of combinations of other antioxidants.

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Notes

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