JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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An Investigation of the Versatile Antioxidant Mechanisms of Action of Rosmarinate Alkyl Esters in Oil-in-Water Emulsions

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ABSTRACT: The antioxidant polar paradox postulates that nonpolar antioxidants are more effective in oil-in-water emulsions than polar antioxidants. However, this trend is often not observed with antioxidants esterified with acyl chains to vary their polarity. In this study, the nonpolar eicosyl rosmarinate (20 carbons, R20) was less effective at inhibiting lipid oxidation in oil-in-water emulsions than esters with shorter fatty acyl chains such as butyl (R4), octyl (R8), and dodecyl (R12) esters. Interestingly, in the presence of surfactant micelles, the antioxidant activity of R20 was significantly increased while the antioxidant activity of R4 and R12 was slightly decreased. The presence of surfactant micelles increased the concentration of R20 at the interface of the surfactant micelles and/or emulsion droplets as determined by partitioning studies, front-face fluorescence properties, and the ability of R20 to interact with the interfacial probe, 4-hexadecylbenzenediazonium. A possible explanation for why the antioxidant activity of R20 was so dramatically increased by surfactant micelles is that a portion of the nonpolar R20 localizes in the emulsion droplet core and the surfactant micelles are able to increase the interfacial concentrations of R20 and thus its ability to scavenge free radicals produced from the decomposition of interfacial lipid hydroperoxides.

KEYWORDS: *antioxidant, rosmarinate, oil-in-water emulsion*

INTRODUCTION

Lipid oxidation in food and biological systems has been a concern in various fields of science because it is related to both food quality deterioration and health complications such as cardiovascular diseases and cancers. In the food industry, the use of free radical scavenging antioxidants is one of the main strategies to delay the occurrence of rancidity by inhibiting the initiation and propagation steps of lipid oxidation. Because of the complexity of the lipid oxidation process, the selection of antioxidants for various applications based on their intrinsic chemical properties, including free radical scavenging rate and stoichiometry of electron transfer, has proven to be inefficient for predicting antioxidant activity in real food systems.[1]

Ideally, free radical scavengers should be located in the microenvironments where lipid radicals are generated for maximum effectiveness. With regard to this matter, the polar paradox hypothesis was developed in an attempt to predict the antioxidant activity of compounds based on their polarity in different lipid media (Porter et al., 1989[2]). Accordingly, nonpolar antioxidants are more effective than their polar homologues in oil-in-water emulsion. This hypothesis was later utilized by Frankel et al. (1994)[3] to explain how the physical location of free radical scavengers impacts their antioxidant activity in heterogeneous systems. Even though a number of studies seemingly confirmed the antioxidant polar paradox theory,[2,3] several recent publications have shown cases where the polar paradox theory does not accurately predict antioxidant behavior.

A series of recent papers examined the activity of antioxidants (chlorogenic acid, rosmarinic acid, hydroxytyrosol, dehydrocaffeic acid, and rutin) whose polarity was modified by esterification to alkyl chains of varying length (1−20 carbons).[4−7] In these studies, a nonlinear relationship of antioxidant effectiveness in oil-in-water emulsions as a function of polarity was observed presenting a challenge to the antioxidant polar paradox hypothesis. From a general perspective, as the alkyl chain length of the alkyl group was increased up to a medium chain length (typically 8−12 carbons), antioxidant activity increased in oil-in-water emulsions as would have been predicted by the polar paradox hypothesis. However, antioxidant activity then sharply declined with a further increase in the size of the alkyl chain even though this would have made the antioxidant even more nonpolar. One possible explanation of this cutoff effect is that the sudden decrease in the antioxidant activity with longer alkyl chains was due to their increased hydrophobicity which led to their partitioning into the oil phase rather than at the emulsion droplet interface. In addition, it has been suggested that the more nonpolar antioxidants might form mixed micelles with emulsifiers used to prepare the emulsions resulting in their migration away from the emulsion droplet.[4,5,8] Unfortunately, there is limited information on how the length of the alkyl chain of esterified phenolics impacts their partitioning inside emulsion droplets or in mixed micelles.

In this research, we aimed to study the influence of esterification of rosmarinic acid on its ability to inhibit lipid oxidation in oil−water emulsions in relation to its free radical

Received: November 25, 2011
Revised: February 9, 2012
Accepted: February 10, 2012
Published: February 10, 2012

scavenging capacity, antioxidant activity in emulsions, and interfacial partitioning behavior. Differences in stoichiometry of free radical scavenging of rosmarinic acid esters have been previously reported.[9] To avoid bias due to differences in the ability of rosmarinic acid esters to transfer electrons or hydrogen atom to free radicals, oxidation studies were performed at equal DPPH scavenging activity. Influence of excess surfactants on antioxidant partitioning and antioxidant activity in oil-in-water emulsions was also studied to obtain useful information on how the various rosmarinic acid alkyl esters partitioned in co-micelles and emulsion droplets. In addition, the microenvironments and distribution of rosmarinic acid esters on the emulsion droplets were evaluated by front-face fluorescence and by measuring interactions with hexadecylbenzenediazonium tetrafluoroborate (16-ArN_2BF_4).

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). Brij 35, acetonitrile, methanol, and hydrochloric acid were obtained from Fisher Scientific (Pittsburgh, PA). Rosmarinic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH•), $FeSO_4$, Tween 20, $BaCl_2$, phosphoric acid, sodium phosphate mono- and dibasic, and *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED) were purchased from Sigma-Aldrich (St. Louis, MO). 4-Hexadecylbenzenediazonium tetrafluoroborate (16-ArN_2BF_4) was prepared from commercial 4-hexadecylaniline (Aldrich, 97%) by diazotization according to refs 10 and 11. Miglyol 812 (medium chain triglycerides; MCT) was purchased from Sasol (Witten, Germany). 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.[9] Briefly, the chemical esterification of rosmarinic acid (56 μmol) was carried out 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), which 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 finally analyzed by reverse phase HPLC with UV detection at 328 nm.[9] 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.[9]

DPPH Scavenging Activity. The free radical scavenging activity of rosmarinate esters was determined using the modified DPPH• free radical method as previously described by Alamed and co-workers[1] with some modifications. Stock solutions (50 μL) of the test compounds in methanol were mixed with 1.5 mL of 50 μM methanolic DPPH• solution to make the final antioxidant concentrations of 10−100 μM. Loss of DPPH• after 1 h was measured at 515 nm using an Ultrospec 3000 pro UV/visible spectrophotometer (Biochrom Ltd., Cambridge, England). The exact DPPH• concentration at the completion of the reaction was determined using a DPPH• standard curve. The free radical scavenging activity of rosmarinate esters was compared with the activity of α-tocopherol in methanol.

Emulsion Preparation. Stripped soybean oil was prepared according to the method of Waraho et al.[12] The effectiveness of stripping was monitored by measuring the removal of tocopherols by HPLC.[13] 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 without addition of the antioxidant were used as control samples. The emulsions (0.5 mL) were transferred into 10 mL GC vials, 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 studies, emulsions were washed to remove aqueous phase surfactants as previously described by Faraji and co-workers[14] with some modifications. In short, emulsions were centrifuged at 38518*g* (17,000 rpm) for 1 h at 4 °C using a Fiberlite F40L-8x100 rotor with a high-speed centrifuge (Thermo Scientific WX Ultra 80, Asheville, NC). After the centrifugation, the bottom suspension (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,[15] and then phosphate buffer was used to adjust the lipid content back to 1% (w/w).

Measurement of Particle Size of Emulsions. The sizes of emulsions were measured by a dynamic light scattering measurements (Zetasizer Nano-ZS, model ZEN3600, Malvern Instruments, Worchester, U.K.), explained in terms of *z*-average mean diameter, respectively. Samples were appropriately diluted with the same buffer, mixed, and immediately measured by transferring the solution into 3 mL plastic curvettes for determining the size. Measurements were performed on three replicates and repeated 3 times on each sample at room temperature. Results showed that there was no visual observation of creaming during storage in all treatments. The emulsion droplets size were in the range 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).

Measurements of Lipid Hydroperoxides. Lipid hydroperoxide formation in emulsion solutions was determined according to the method described by Panya and co-workers[16] 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.

Measurement of Hexanal. Headspace hexanal was determined according to the method described by Panya and co-workers[16] 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.

Determination of Antioxidant Partitioning. Determination of the physical location of rosmarinic acid and its esters in the emulsions was performed according to the procedure described by Panya and co-workers.[16] Regular O/W emulsions and washed emulsions with added surfactants (0, 0.1, 0.5, 1.0, and 2.5%; w/w) were prepared with 10 mM phosphate buffer including 200 μM ETDA 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-6x13.5 rotor with a high-speed centrifuge (Thermo scientific WX Ultra 80, Asheville, NC). The aqueous phase was carefully collected with a pipet, and the amounts of rosmarinic acid esters in the aqueous phase were determined directly by HPLC using a modified method described by Lecomte and co-workers.[9]

Briefly, HPLC determination of rosmarinic acid and its 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) in 5 min, and hold at 0/100 (v/v) for 5 min. Rosmarinic acid and its alkyl esters [(R4 (4 carbons) to R20 (20 carbons)] were detected with a photodiode array detector (SPD-M10Avp, Shimadzu, USA) at 328 nm. The concentrations of rosmarinic acid esters were calculated using a standard curve made from each rosmarinic acid ester dissolved in methanol.

Front-Face Fluoresence Measurements. Front-face fluorescence of rosmarinic acid and its alkyl esters in O/W emulsions was determined by steady-state emission measurements recorded with a PTI spectrofluorometer (PTI, Ontario, Canada). Washed emulsions were prepared as above, but phosphate buffer including 200 μM EDTA was used in order to minimize oxidation, and Brij 35 was employed instead of Tween 20. Brij 35 was used because Tween 20 contained fluorescent components that interfered with the fluorescence signal of the rosmarinic acid derivatives. A 10% (w/w) Brij 35 solution in 10 mM phosphate buffer with 200 μM EDTA was added into the washed emulsions to obtain surfactant concentrations of 0, 0.1, 0.5, 1.0, and 2.5% (w/w). All antioxidants were used at the concentration of 30 μM in the final emulsions. Samples (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 emissions of the rosmarinic acid and its esters were scanned from 370 to 470 nm at the excitation wavelength of 323 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 was determined as the emission signal intensity (counts per second) measured by means of a photomultiplier.

Determination of Interfacial Rosmarinate Esters. The existence of rosmarinic acid esters in the interface of emulsion droplets and surfactant micelles was determined using 4-hexadecylbenzenediazonium ions, 16-ArN_2^+. Medium chain triglyceride (1% w/w) oil-in-water emulsions stabilized with 0.1% (w/w) Tween 20 were prepared as described above. Freshly prepared emulsion (1 mL) was transferred into test tubes, and 20 μL of the stock solution (30 mM) of rosmarinate esters in methanol (R4, R12, and R20) was added by vortexing for 1 min and placing in a sonicating water bath for 30 min at 25 °C. The reaction between rosmarinate esters and 16-ArN_2^+ was measured as described by Sánchez-Paz and co-workers (2008).[19] In brief, 10 μL of the 16-ArN_2^+ stock solution in acetonitrile (0.017 M) was added to rosmarinic acid containing emulsions at specific time intervals and then the reaction mixtures (40 μL) were transferred into 1 mL of a 0.01 M ethanolic solution of NED at 25 °C. Final concentrations of rosmarinate esters, 16-ArN_2^+, and NED were 30 μM, 170 μM and 0.01 M respectively. The reaction mixtures were incubated for 20 min. The NED azo dye formation was determined spectrophotometrically at 572 nm using an Ultrospec 3000 pro UV/visible spectrophotometer (Biochrom Ltd., Cambridge, England). Rate constants were obtained from the slope of the consumption of 16-ArN_2^+ during the first 5 min of the reaction in the presence of low surfactant (0%) concentrations and during the first 12 h in the presence of high surfactant (2.5%) concentrations. Results were presented as secondary rate constants.

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

Influence of Esterification on the DPPH Scavenging Activity of Rosmarinic Acid. DPPH scavenging activity of the rosmarinate esters was performed in this study so subsequent lipid oxidation studies in oil-in-water emulsions could be performed at antioxidant concentrations representing equal free radical scavenging activity (Figure 1). The

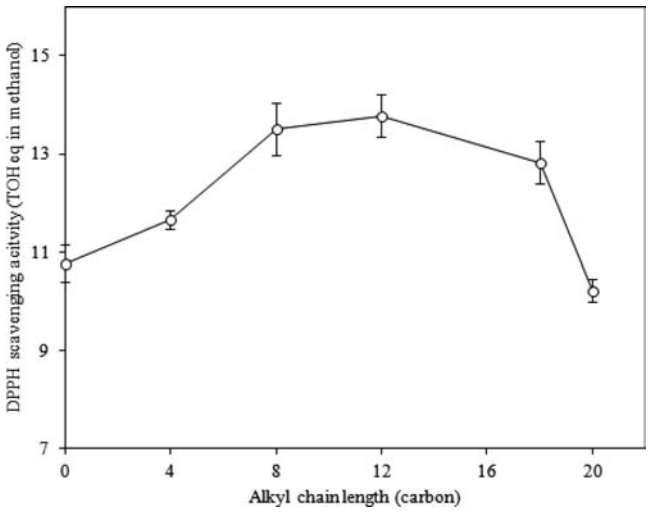

Figure 1. DPPH scavenging activity of the rosmarinic acid and its alkyl esters in methanol. Data points and error bars represent means ($n = 3$) ± standard deviations.

rosmarinate esters exhibited approximately 10−14 times more DPPH scavenging activity than α-tocopherol (data not shown). A nonlinear behavior of DPPH scavenging activity by rosmarinate esters was observed with maximum DPPH scavenging activity with the octyl and dodecyl rosmarinic acid esters (R8−R12). There was not a statistical difference ($p >$

0.05) between the octyl and dodecyl rosmarinic acid esters. This trend was also observed by Lecomte and co-workers,[9] who found that dodecyl rosmarinate had the greatest DPPH scavenging activity of all the esters tested (4−20 carbons). A similar nonlinear trend was also reported by Lopez-Giraldo and co-workers (2009),[17] who found 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.

Effects of Alkyl Chain Lengths of Rosmarinates on Oxidation Stability of Stripped Soybean Oil-in Water (O/W) Emulsions. Due to the observed nonlinear DPPH scavenging activity of the rosmarinic acid esters, the ability of the rosmarinic acid esters to inhibit lipid oxidation in oil-in-water emulsions was tested at both equal molar concentrations (Figure 2) and equal DPPH scavenging activity concentrations (Figure 3). For the equal DPPH scavenging activity experiments, the concentrations of the esters were normalized based on the DPPH scavenging activity of the R12 ester which had the highest level of activity. Hydroperoxide and hexanal formation in the O/W emulsions were investigated during storage at 25 °C in the dark. All forms of the rosmarinic acid were able to inhibit the formation of hydroperoxides and hexanal compared to the control (Figures 2 and 3). Results indicated there were no major differences in the trend of each ester to inhibit hydroperoxide or hexanal formation when determined on an equal molar or DPPH scavenging activity basis. Differences in the ability of the different esters to inhibit hexanal formation will be discussed below since volatile lipid oxidation products such as hexanal are more strongly related to rancidity development than lipid hydroperoxides. R4 was slightly better than R8 and R12 in increasing hexanal lag times at both equal molar (Figure 2B) and DPPH scavenging activity (Figure 3B). The R20 ester was consistently the worst of the antioxidants, and R18 tended to be slightly better than R0 (unesterified) rosmarinic acid. A similar decrease in antioxidant activity was observed when the alkyl chain lengths of the rosmarinate esters were increased above 8 carbon chain lengths in tung oil-in-water emulsions[5,16] while in chitosan coated liposomes the R4 ester exhibited the best antioxidant activity, although the octyl rosmarinate was not evaluated in this study.[16]

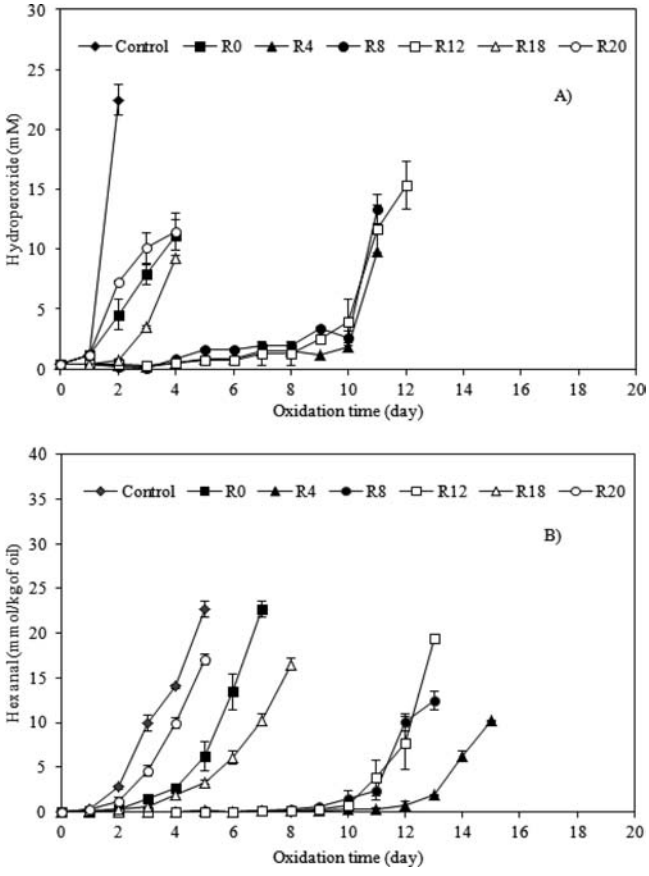

Figure 2. Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil−Tween 20 emulsions at 25 °C in the presence of rosmarinic acid and its alkyl esters at equivalent molarities (30 μM). Data points and error bars represent means (n = 3) ± standard deviations.

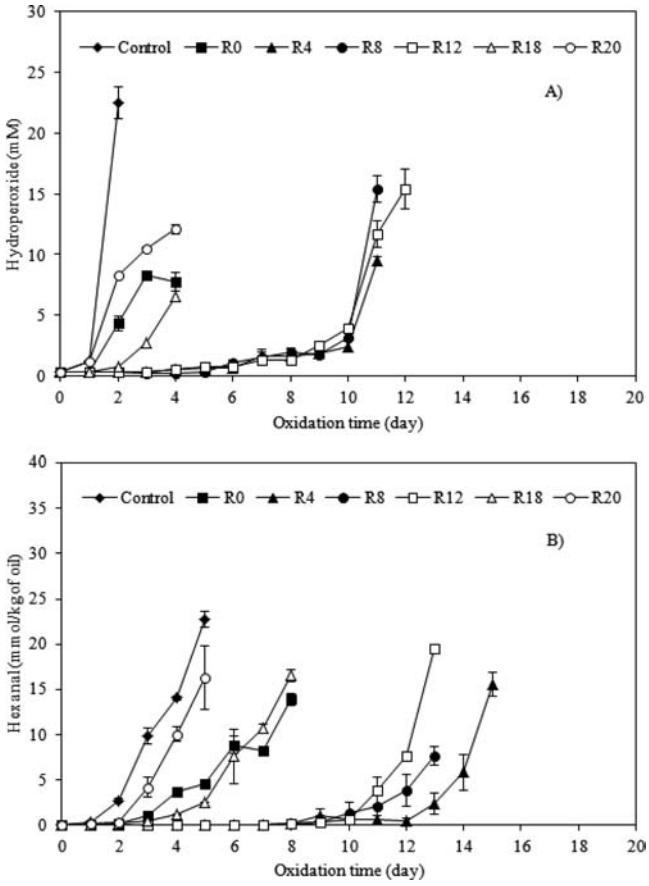

Figure 3. Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil−Tween 20 emulsions at 25 °C in the presence of rosmarinic acid and its alkyl esters at equivalent DPPH radical scavenging activity. Data points and error bars represent means (n = 3) ± standard deviations.

Effect of Tween 20 Micelles on the Physical Location and Antioxidant Activity of the Rosmarinic Acid Esters in Stripped Soybean O/W Emulsions. The antioxidant polar paradox hypothesis states that nonpolar antioxidants are more effective than polar antioxidants in oil-in-water emulsions presumably due to the greater retention of the nonpolar antioxidants in the interface.[3] However, the above results with the rosmarinic acid esters as well as the previous work of others[4−7,16] do not support the concept of the antioxidant polar paradox hypothesis for oil-in-water emulsions because the most nonpolar antioxidants (R18 and R20) had lower antioxidant

activity than their more polar homologues (R0, R4, R8, and R12). Our results thus confirm the cutoff hypothesis put forward by Laguerre et al.[4] One possible reason for this nonlinear influence of the alkyl chain length could be the ability of the esters to partition into the aqueous phase of the emulsion either by forming micelles by themselves or via mixed micelles with Tween 20 not absorbed at the emulsion droplet surface.[5] To better understand how the antioxidant activity and physical location of the rosmarinic acid esters is influenced by surfactant micelles, emulsions were first washed to remove Tween 20 not absorbed to the emulsion droplet interface. Surfactant micelles were then reintroduced into the emulsions by adding 0, 0.1, 0.5, 1.0, and 2.5% Tween 20 to the washed emulsions. Since Tween 20 has a low critical micelle concentration [<0.1 mM at 21 °C[18]], the majority of added Tween 20 would exist as surfactant micelles in the aqueous phase of the emulsion. In these experiments, R4, R12, and R20 esters were utilized to test antioxidant activity and partitioning. As illustrated in Figure 4A,B, increasing the surfactant and thus aqueous phase micelle

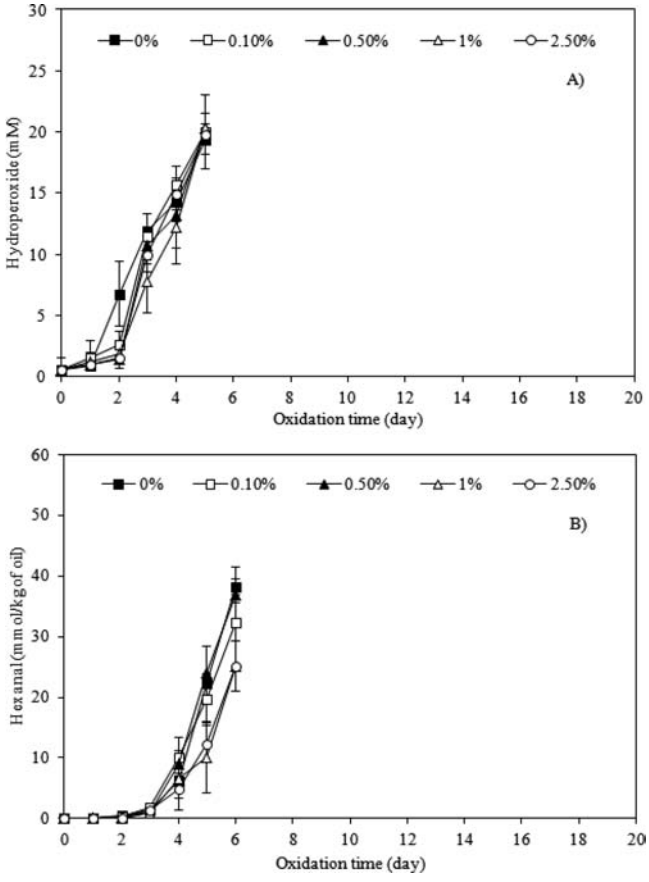

Figure 4. Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil–Tween 20 emulsions at 25 °C as a function of increasing Tween 20 concentrations (0.1, 0.5, 1.0, and 2.5% w/w). Data points and error bars represent means (n = 3) ± standard deviations.

concentrations did not impact the lag time of hydroperoxide and hexanal formation in the control (no added antioxidant) emulsions. This suggests that the additional Tween 20 did not impact oxidation chemistry directly through mechanisms such as free radical scavenging. In emulsions containing R4 (Figure 5A,B) and R12 (Figure 6A,B) increasing Tween 20 micelle concentrations had no effect or decreased the lag phase for

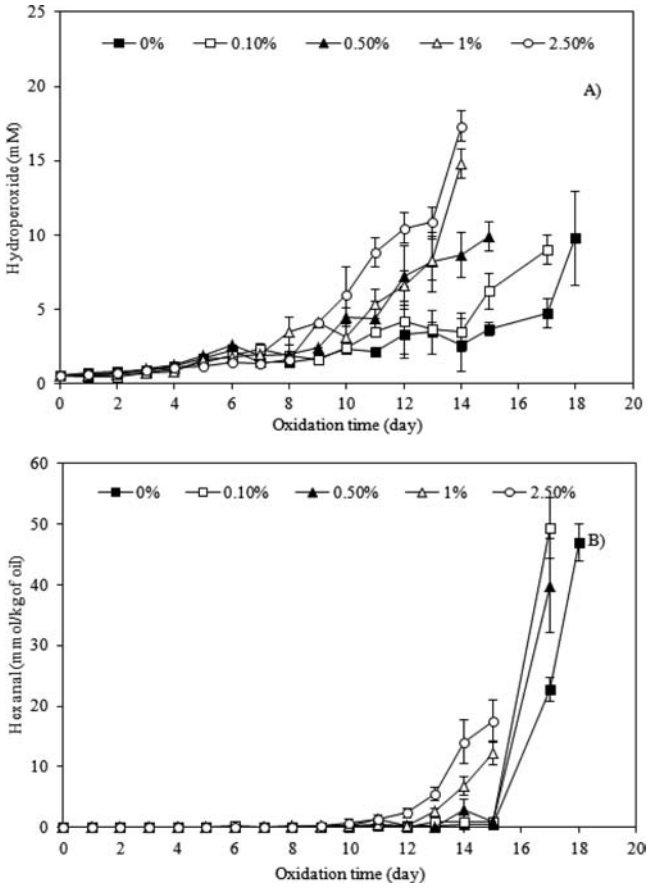

Figure 5. Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil–Tween 20 emulsions at 25 °C as a function of increasing Tween 20 concentrations (0.1, 0.5, 1.0, and 2.5% w/w) in the presence of butyl rosmarinate ester (R4). Data points and error bars represent means (n = 3) ± standard deviations.

both hydroperoxide and hexanal formation. Surprisingly, the antioxidant activity of R20 increased with increasing concentrations of Tween 20 micelles as determined by both lipid hydroperoxides and headspace hexanal (Figure 7A,B). The lag time of hexanal formation in the presence of R20 was improved from 4 to 10 days in emulsions containing 0% and 2.5% Tween 20, respectively. At 2.5% Tween 20, the differences among the lag times of all the rosmarinate esters had become similar with lag phases for hexanal formation of 11, 12, and 10 days for R4, R12, and R20, respectively.[4] Richards et al. (2002)[24] also reported that surfactant micelles from Brij 700 could increase the antioxidant activity of TBHQ in salmon oil-in-water emulsions.

To try to understand why the Tween 20 micelles had such varying effects on the antioxidant activity of the different rosmarinic acid alkyl esters, the impact of micelle concentration on antioxidant partitioning into the aqueous phase was determined (Figure 8). In emulsions with no added Tween 20, the amount of R4, R12, and R20 in the aqueous phase was similar, ranging from 4.2 to 8.3%. Increasing added Tween 20 to 0.1% resulted in an increase in the aqueous phase concentration of all the rosmarinic acid esters but most dramatically with R20, whose aqueous phase concentration increased over 7.5-fold. The equilibrium distributions of all the rosmarinate esters became saturated at 1% Tween 20. At all added Tween 20 concentrations, R12 exhibited the highest

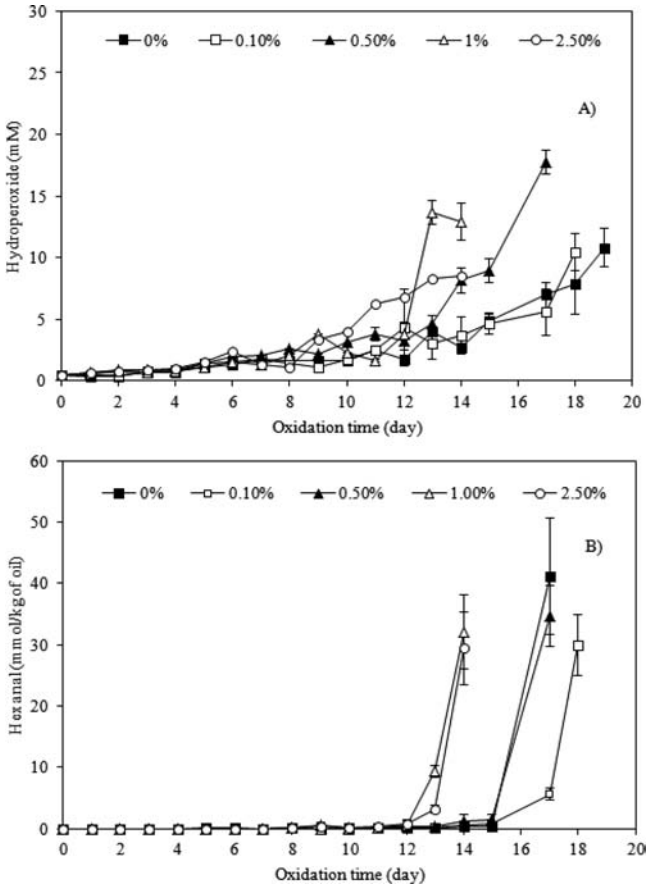

Figure 6. Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil–Tween 20 emulsions at 25 °C as a function of increasing Tween 20 concentrations (0.1, 0.5, 1.0, and 2.5% w/w) in the presence of dodecyl rosmarinate ester (R12). Data points and error bars represent means (n = 3) ± standard deviations.

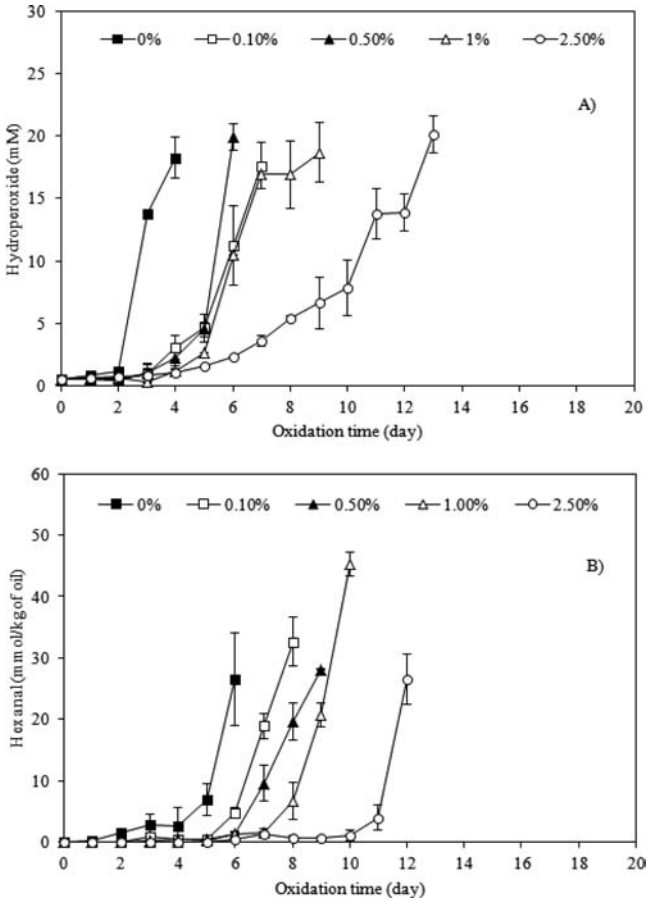

Figure 7. Hydroperoxide (A) and hexanal (B) formation in 1% stripped soybean oil–Tween 20 emulsions at 25 °C as a function of increasing Tween 20 concentrations (0.1, 0.5, 1.0, and 2.5% w/w) in the presence of eicosyl rosmarinate ester (R20). Data points and error bars represent means (n = 3) ± standard deviations.

association with the emulsion droplets followed by R4 and R20, with aqueous phase concentrations of 52, 64, and 75%, respectively. The partitioning patterns for R4 and R12 could help explain why Tween 20 micelles decreased their antioxidant activity (Figures 5–7) as increasing surfactant micelle concentration increased the partitioning of the antioxidants into the aqueous phase and thus presumably prevented them from scavenging lipid radicals in the emulsion droplets. Unexpectedly, while R20 was the most prone to be removed from the emulsion droplets by the surfactant micelles, its antioxidant activity improved. Effect of surfactants on the partitioning esterified antioxidants has also been reported in sunflower oil emulsions with Brij 35 as the emulsifier; however, the reason for this phenomenon was not discussed in detail.[4,5]

Measuring the Chemical Microenvironments of the Rosmarinate Esters in O/W Emulsions by Front-Face Fluorescence. In order to better understand the location of the rosmarinic acid alkyl esters in emulsions systems, their fluorescent properties were evaluated. As shown in Figure 9, the fluorescence emission spectra of R4 rosmarinate varied as a function of solvent polarities. For example, R4 exhibited relatively strong fluorescence intensity in isopropanol but had very low fluorescence emission in 10 mM phosphate buffer. In hexadecane there was no fluorescence peak observed for R4. The highest level of R4 fluorescence was observed in Brij 35 micelles (Brij 35 was used instead of Tween 20 since Tween 20 contained fluorescent compounds that interfered with the signal of R4). Similar trends were observed with the R12 and R20 esters (data not shown). These data suggested that the

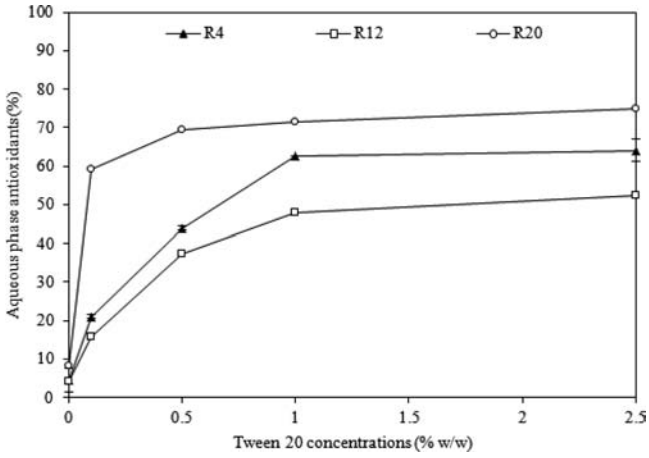

Figure 8. The effect of surfactant concentrations on the antioxidant partitioning of butyl (R4), dodecyl (R12), and eicosyl (R20) rosmarinate esters (100 μM) into aqueous phase of in oil-in-water emulsions. Data points and error bars represent means (n = 3) ± standard deviations. Error bars lie within data points.

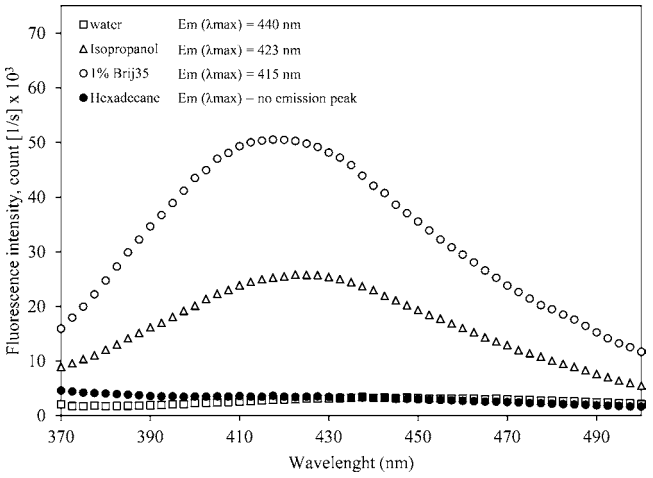

Figure 9. Fluorescence emission scans of butyl rosmarinate ester (30 μM) in various solvents with different polarities.

fluorescence properties of the rosmarinic acid esters could be used as a probe to help understand their physical location in emulsion and surfactant micelles.

The fluorescence intensity of the C4, C12, and C20 esters in washed oil-in-water emulsion to which excess Brij 35 was added is shown in Figure 10. R20 exhibited larger increases in

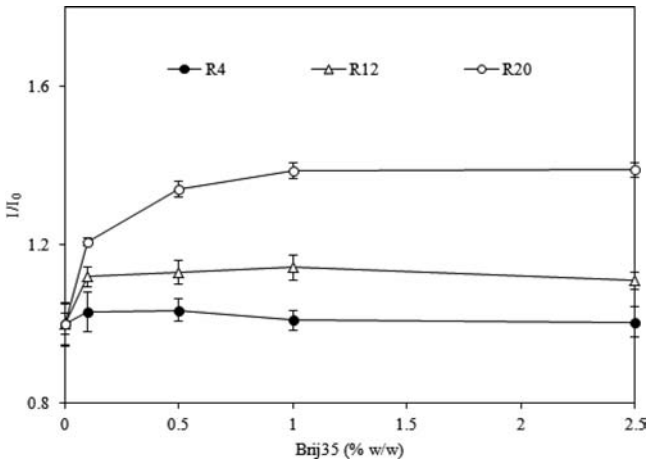

Figure 10. Front-face fluorescence measurement (λ_{ex} 323, λ_{em} 420) of rosmarinic acid and its butyl (R4), dodecyl (R12), and eicosyl (R20) esters in washed O/W emulsions with varying Brij concentrations. Data points and error bars represent means (n = 3) ± standard deviations.

fluorescence intensity than R4 and R12 as surfactant micelle concentrations were increased. The increase in R20 fluorescence could be due either to its greater association with the surfactant micelles or to the fact that R20 becomes more dilute in the emulsion system such that less self-quenching between R20 molecules was occurring. For example, if the R20 was concentrating in the lipid core of the emulsion droplet, its localized concentration would be high and thus self-quenching would be greater. When the R20 migrate into the surfactant micelles, they become more dilute in the system and less self-quenching occurs, thus a greater net fluorescence is observed. In either case, this data confirms the antioxidant partitioning data (Figure 8) where R20 partitioning into surfactant micelles in the aqueous phase of the emulsions was greater than R4 and R12.

Evidence of Rosmarinate Esters at Oil–Water Interfaces Using a Surface Active Probe. Increases in fluorescence intensity (Figure 10) suggest that R20 becomes more highly associated with surfactant micelles. However, as discussed above this also could be due to dilution of the R20 throughout the emulsion system resulting in less self-quenching. To gain further insights on whether the surfactant micelles increased the ability of rosmarinic acid esters to concentrate at the lipid–water interface, the ability of R4, R12, and R20 to interact with 4-hexadecylbenzenediazonium ions (16-ArN_2^+) was determined. These interactions are important because 16-ArN_2^+ is a surface active probe with a water-soluble cationic headgroup and a water insoluble hexadecyl tail that concentrates at the interface of oil-in-water emulsions and thus provides an indication of interfacial antioxidant concentrations.[19]

Reactions between rosmarinate esters and 16-ArN_2^+ were determined in a washed O/W emulsions with 1% (w/w) medium chain triglycerides and 0.1% (w/w) Tween 20 in a 3 mM HCl solution with and without an additional 2.5% (w/w) Tween 20. The rate constants between the rosmarinic acid esters and 16-ArN_2^+ are shown in Table 1. In the washed

Table 1. Initial Secondary Rate Constants of 16-ArN_2^+ (170 μM) Consumption in the Presence of Rosmarinate Esters (30 μM) in Washed Oil-in-Water Emulsions with and without 2.5% Tween 20

	k_{obs}[a] (M^{-1} s^{-1})	
rosmarinate esters	at 0% Tween 20	at 2.5% Tween 20
butyl rosmarinate (R4)	221.7 ± 13.2 a	0.12 ± 0.008 a
dodecyl rosmarinate (R12)	224.3 ± 21.3 a	0.11 ± 0.014 a
eicosyl rosmarinate (R20)	124.0 ± 8.6 b	0.12 ± 0.010 a

[a]Means (± SD) with different letters (a and b) in the same column indicate significant differences (P < 0.05).

emulsions, the rate constants for R4 and R12 were statistically similar. However, the rate constant for R20 was 45% lower than that for R4 and R12. These results indicate that the concentration of R20 at the interface of the oil-in-water emulsion was much less than that of R4 and R12. When 2.5% Tween 20 was added to the emulsions to increase surfactant micelle concentrations, interactions between the esters and 16-ArN_2^+ were much lower and all three esters became statistically similar. Interaction rates were most likely slower because the additional Tween 20 caused a dilution of both the 16-ArN_2^+ and the rosmarinic acid esters into the micelles. However, in the presence of Tween micelles the rate constant for R20 was similar to that for R4 and R12, unlike in the absence of Tween micelles, where R20 was much less reactive. This is possibly because the Tween micelles could increase the association of R20 with the interfacial layer at concentrations similar to those of R4 and R20. This could occur if the Tween 20 micelles were able to solubilize R20 out of the emulsion droplet core. These data again support the partitioning (Figure 8) and fluorescence (Figure 10) data, which also show that surfactant micelles increase the concentration of R20 at the water interface.

In summary, Figure 11 illustrates a possible mechanism for how Tween 20 micelles had such a different impact on the antioxidant activity of rosmarinic acid esters of varying polarity in oil-in-water emulsions. Fluorescence spectra suggest that the

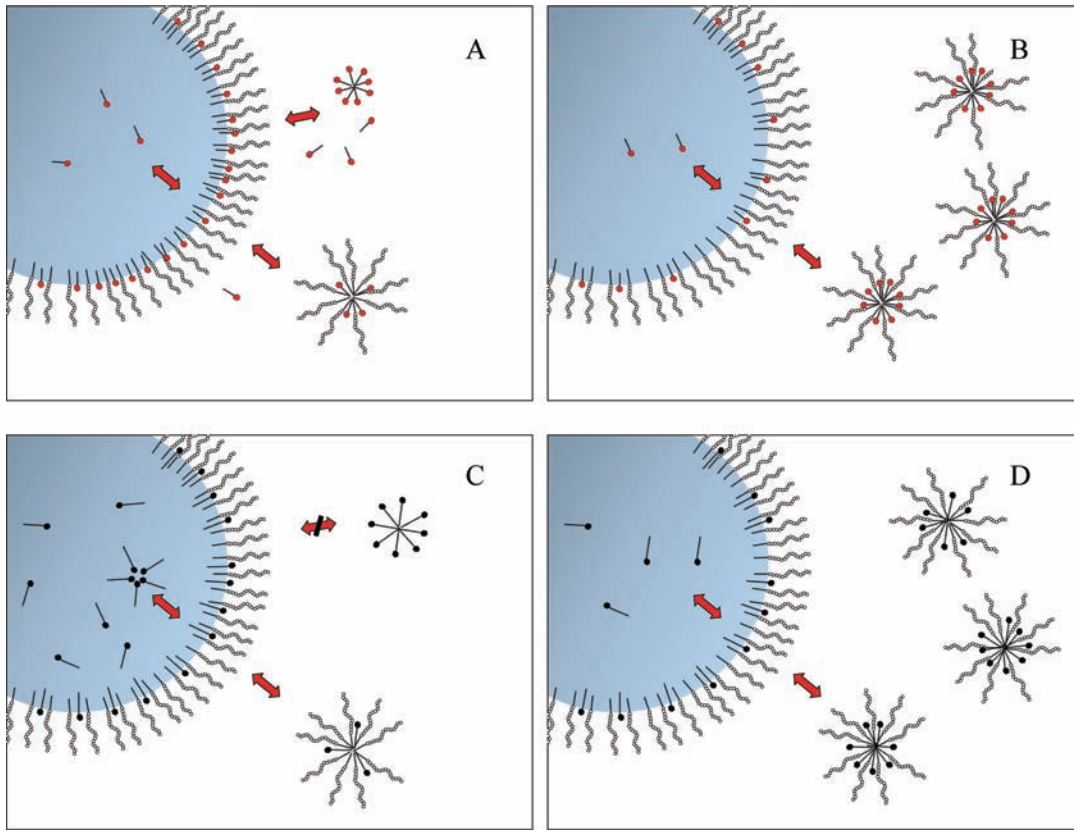

Figure 11. Schematic demonstration of the distribution of the rosmarinate esters in the interface region of O/W emulsions and in nonionized surfactant micelles. In the O/W emulsions the rosmarinate esters can exist as monomers, micelles (or reverse micelles), or co-micelles. A and B show medium chain rosmarinate esters (4−12 carbons) in low and high surfactant concentration. In this case, the rosmarinate esters are mainly at the oil−water interface of the emulsion droplet. C and D show long chain rosmarinate ester (20 carbons) in low and high surfactant concentration. In this case, a higher proportion of the rosmarinate ester is in the interior and/or aggregated outside of the emulsion droplet in the absence of excess surfactant micelles.

association of R4 and R12 with surfactant micelles was not increased by the presence of surfactant micelles (Figure 10). This suggests that these more polar rosmarinic acid esters will primarily localize at the emulsifier−water interface in washed emulsions with low concentrations of surfactant micelles as well as with emulsions with excess surfactant micelles. However, addition of surfactant micelles did remove some of the R4 and R12 from the emulsion droplets as shown by the increased aqueous phase R4 and R12 concentrations (Figure 8). Increased partitioning of R4 and R12 into the aqueous phase by the surfactant micelles could help explain why their antioxidant activity was decreased in the oil-in-water emulsions in the presence of excess surfactant micelles (Figures 5 and 6).

The R20 ester behaved very differently than R4 and R12. The concentration of R20 at the emulsion droplet interface in washed emulsions was lower than that of R4 and R12 as determined by the surface active probe, 16-ArN_2^+. However, R20 very readily associated with Tween 20 micelles as could be seen by low levels of Tween 20 increasing its aqueous phase concentration in the oil-in-water emulsions (Figure 8). This increase in aqueous phase concentrations is presumably due to its solubilization from the emulsion droplet into surfactant micelles as supported by its increasing fluorescence emission (Figure 10). Unlike R4 and R12, surfactant micelles increased the antioxidant activity of R20 in the oil-in-water emulsion (Figure 7).

Lipid oxidation chemistry in oil-in-water emulsions is thought to occur at the emulsion droplet interface since the oxidation substrate, lipid hydroperoxides, are surface active and thus can migrate to the emulsion droplet interface where they are decomposed into free radicals by prooxidants such as transition metals.[20,21] Therefore, one possible explanation for the differences in the antioxidant activity of the rosmarinic acid esters is that, in the absence of excess Tween 20 and thus surfactant micelles, a portion of the more nonpolar R20 localizes in the emulsion droplet core instead of the emulsion droplet interface. This would decrease its interfacial concentrations and thus its ability to scavenge free radicals produced from the decomposition of interfacial lipid hydroperoxides. Surfactant micelles could promote the migration of R20 out of the emulsion droplet core by forming Tween 20−R20 co-micelles. As aqueous phase surfactant micelles readily exchange with emulsion droplets, they could form a reservoir of R20 allowing it to replace R20 at the emulsion droplet interface after it is consumed by scavenging free radicals in the emulsion droplet.

This phenomenon may not be limited to rosmarinic acid alkyl esters. Tocopherols are also very nonpolar antioxidants that have very low water solubility and surface activity.[22] In studies by Cho et al. (2002), Nuchi et al. (2002), and Richards et al. (2002) it was found that surfactant micelles inhibited lipid oxidation to oil-in-water emulsions.[23−25] Unlike the present study these studies were conducted with nonstripped corn oil

dx.doi.org/10.1021/jf204848b | J. Agric. Food Chem. 2012, 60, 2692−2700

which would contain tocopherols (surfactant micelles did not alter lipid oxidation rates in this study which used tocopherol free oil, Figure 4). This suggests that it may be useful to determine if the ability of surfactant micelles to inhibit lipid oxidation in these studies was due to the ability of surfactant micelles to solubilize tocopherols out of the emulsion droplet core in a manner similar to what we have observed for the R20 ester of rosmarinic acid. This could help provide more insight into how nonpolar antioxidant such as tocopherol could be made more effective in oil-in-water emulsions.

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Professor Laurence S. Romsted, Department of Chemistry, Wright−Rieman Laboratories, Rutgers, The State University of New Jersey, New Brunswick, New Jersey (USA), for suggestions on the synthesis of 4-hexadecylbenzenediazonium tetrafluoroborate.

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