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Ability of Surface-Active Antioxidants To Inhibit Lipid Oxidation in Oil-in-Water Emulsion

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Lipid oxidation in dispersed lipids is prevalent at the oil–water interface where lipid hydroperoxides are decomposed into free radicals by transition metals. Free radical scavenging antioxidants are believed to be most effective in lipid dispersions when they accumulate at the oil–water interface. The surface activity of antioxidants could be increased by their conjugation to hydrocarbon chains. In this study, *p*-hydroxyphenylacetic acid (HPA) was conjugated with either a butyl or dodecyl group. The HPA conjugates were more effective at decreasing interfacial tension than unconjugated HPA, indicating that they were able to adsorb at lipid−water interfaces. However, free HPA was a more effective antioxidant than butyl and dodecyl conjugates in Menhaden oil-in-water emulsions as determined by both lipid hydroperoxides and thiobarbituric acid reactive substances. The increased antioxidant activity of free HPA could be due to its more effective free radcial scavenging activity and its higher concentration in the lipid phase of oil-in-water emulsions in the presence of surfactant micelles where it can act as a chain-breaking antioxidant.

KEYWORDS: Lipid oxidation; antioxidants; emulsions; surfactants; free radical scavenging

INTRODUCTION

In food products, natural and processed lipids are frequently used in the form of emulsions (*1*). Emulsions consist of two or more immiscible or partially miscible liquids (such as lipid and water) with one liquid being dispersed in the other in the form of droplets. While emulsions are thermodynamically unstable systems, they can be kinetically stabilized using emulsifiers. Emulsifiers are amphiphilic molecules that readily adsorb and accumulate at the interface between the dispersed phase (e.g., lipid) and the continuous phase (e.g., water) of an emulsion (e.g., oil-in-water). Emulsifiers decrease the interfacial tension and produce repulsive interacions between droplets to prevent droplet aggregation or coalescence (*2*).

The oxidative deterioration of lipids in emulsions negatively affects the quality of food emulsions by altering the product's flavor, odor, and nutritive value (*3, 4*). Lipid oxidation in emulsions is highly dependent on the ability of prooxidant metals to decompose lipid hydroperoxides into free radicals that can further promote oxidation. In addition, metal-promoted hydroperoxide decomposition can cause fatty acid scission, leading to the production of low molecular weight volatile compounds that are responsible for rancid aromas and flavors (*2, 5*). Lipid hydroperoxides are surface-active compounds and are thus able to accumulate at the lipid−water interface of emulsion droplets. The existence of transition metals (e.g., iron, copper) in the aqueous phase and lipid hydroperoxides at the emulsion droplet surface suggests that lipid oxidation in oil-in-water emulsions primarily occurs at the emulsion droplet interface (*2*).

Antioxidants are an effective tool to control lipid oxidation in oil-in-water emulsions. Free radical scavenging antioxidants interfere with the initiation or propagation steps of lipid oxidation reactions by scavenging lipid radicals and forming low-energy antioxidant radicals that do not readily promote oxidation of unsaturated fatty acids (*6*). The effectiveness of antioxidants in food emulsions depends on both their chemical and their physical properties. The importance of physical properties has been highlighted by the "antioxidant polar paradox" that states that nonpolar antioxidants are more effective in oil-in-water emulsions while polar antioxidants are more effective in bulk oils (*7–14*). In oil-in-water emulsions, nonpolar antioxidants are thought to be more effective because they are present in higher concentrations in the oil droplet. On the other hand, polar antioxidants readily partition into the aqueous phase, decreasing their concentration in the lipid phase and thus lowering their capability to prevent oxidation. However, the effectiveness of antioxidants in oil-in-water emulsions could also be dependent on their surface activity and ability to accumulate at the oil–water interface where oxidative reactions are most prevalent. The impact of surface activity on the effectiveness

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of antioxidants in oil-in-water emulsions can be seen with γ-tocopherol which is more surface active (*15*) and a more effective antioxidant than α-tocopherol in corn oil-in-water emulsions stabilized by Tween 20 (*16*).

The surface activity of antioxidants can be increased by their conjugation to fatty acid or fatty alcohol chains (*17*). Formation of such antioxidant surfactants could be an effective method to increase the concentration of antioxidants at the oil–water interface of emulsion droplets where oxidative reactions are prevalent. Therefore, the objective of this study was to determine if the activity of a phenolic antioxidant (*p*-hydroxyphenylacetic acid) could be increased by increasing its surface activity by conjugating it to hydrocarbon chains.

MATERIALS AND METHODS

Materials. Menhaden oil with no added antioxidants was donated by Omega Protein (Houston, TX). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), *p*-hydroxyphenylacetic acid (98%), 1-butanol (99.8%), 1-dodecanol (99.5%), molecular sieve (3 Å), acetonitrile, toluene, hexane, and ethyl acetate, analytical grade, were acquired from Aldrich Chemical Co., Inc. (Milwaukee, WI; Saint Quentin, France). Commercially immobilized lipase from *Candida antarctica* lipase B (Novozym 435) was purchased from Novozymes A/S (Denmark). Trichloroacetic acid and silica (granulometry 0.06–0.2 mm, pore diameter 0.6 nm) were purchased from Acros Organics (Morris Plains, NJ; Geel, Belgium). All other chemicals and solvents were of reagent or HPLC grade and were obtained from Fisher Scientific (Suwanee, GA) or Sigma Chemical Co. (St. Louis, MO).

Methods. *Enzymatic Synthesis of p-Hydroxyphenylacetic Acid Conjugates.* In 25 mL sealed flasks, *p*-hydroxyphenylacetic acid (65 mmol) was mixed with butanol or dodecanol (390 mmol) in acetonitrile (200 mL) and in the presence of 20 mg/mL molecular sieves. The medium was stirred with an orbital shaker (250 rpm) at 55 °C until substrates were completely solubilized. The reactions were then started by addition of a biocatalyst (*C. antarctica* lipase B) at a ratio of 10% (w/w, calculated from the total weight of both substrates) and stirred for 95 h at 55 °C. The reaction medium was then filtered to eliminate the biocatalyst and molecular sieves. Hexane (100 mL) and 400 mL of acetonitrile/water (3:1 v/v) were added to facilitate the liquid–liquid extraction. The hexane phase containing a majority of fatty alcohol was discarded, while the other phase was recovered and washed three times with hexane (3 × 100 mL). The remaining solvent phase was evaporated under vacuum, and the product was then purified in order to eliminate traces of residual fatty alcohol and phenolic acid by silica gel column chromatography using toluene/ethyl acetate (9:1 v/v) as eluant. The final product yield was 96%. The purified products were then identified by mass spectrometry. Mass spectrometric analysis was performed using a mass spectrometer with a quadripole ion trap mass analyzer, an external ion source API, and an integrated syringe pump (Finnigan LCQ Serie MS detector, San Jose, CA). The purified compounds were dissolved in methanol to a final concentration of 10 mg/L. Solutions were directly injected into the electrospray ion source (infusion mode) at a flow rate of 5 μL/min. ESI-MS data were acquired in zoom scan mode for the accurate determination of parent ion *m*/*z* in MS^n mode to obtain the fragment ion *m*/*z*. The MS operating conditions (negative ion) were a collision energy of 35%, an ionization voltage of 4.5 kV, and a capillary temperature of 250 °C.

Interfacial Tensiometry. The surface activity of antioxidants was determined by interfacial tensiometry using a drop shape analyzer (DSA10; Kruss USA, Charlotte, NC). Antioxidants were dissolved in hexadecane at 0−200 μM. Hexadecane was used as a nonoxidizable lipid to prevent oxidation of the antioxidants during analysis. The mixture was loaded in a syringe and ejected to form a droplet at the inverted tip of a hypodermic needle that was submerged in doubly distilled water. The tip of the needle was positioned on an optical bench between a light source and a high-speed charge couple device (CCD) camera. The CCD camera was connected to a video frame-grabber board to record the image onto the hard drive of a computer at a speed of one frame per second. The shape of pendent drops was determined through numerical analysis of the entire drop shape. The interfacial tension was calculated from the drop shape using the Young−Laplace equation of capillarity (*18*). The methodology requires accurate determination of solution densities, which were measured using a digital density meter (DMA 35N; Anton Paar USA, Ashland, VA). All interfacial tension measurements were carried out after 3 h of equilibrium.

Preparation and Characterization of Emulsions. Brij 35-stabilized Menhaden oil-in-water emulsions were used in all lipid oxidation studies. An emulsifier solution was prepared by dispersing 17 mM Brij 35 in phosphate-buffered saline (10 mM phosphate/0.15 M NaCl, pH 7.0) by stirring for 1 h at ambient temperature (22 °C). Menhaden oil was added to the emulsifier solution and homogenized at high speed for 2 min with a hand-held ultraturrax blender (Biospec Products Inc., Bartlesville, OK) to produce a coarse 2% (w/w) oil-in-water emulsion premix. Emulsion droplet size was then further reduced with an ultrasonicator (Fisher Sonic Dismembrator 500, Pittsburgh, PA) at 4 °C for 3 min at 70% power and a 0.5 duty cycle. Antioxidants including unconjugated HPA and its conjugates were dissolved in methanol and added to the emulsion at a final concentration of 0–200 μM. Control emulsion contained only methanol.

A laser light scattering instrument (Coulter LS-230, Miami, FL) was used to measure the particle size distribution. The instrument uses the Mie theory to calculate the particle size distribution from the intensitity of scattered light as a function of the scattering angle. The mean particle diameter d_{10} of emulsions ranged from 0.3 to 0.4 μm and did not change over the course of the experiments.

In select experiments, excess Brij 35 in the continuous phase of the oil-in-water emulsion was removed using the method of Elias and co-workers (*19*). To remove the excess Brij 35, emulsions were centrifuged at 15000 rpm for 40 min at 4 °C. After centrifugation, the aqueous phase (lower layer) was removed with a syringe and discarded, and fresh buffer was added by vortexing for 5 min to dilute the creamed emulsion back to its original volume. The procedure of centrifugation, removal of continuous phase, and reconstitution of emulsion droplets was repeated three times. Following the final wash, the total lipid content of the creamed emulsion was determined by a modified method of Floch and co-workers (*20*). In short, the creamed emulsion fraction (1.0 g) was extracted with 1 mL of methanol, 2 mL of chloroform, and 1 mL of water in preweighed glass test tubes. The samples were vortexed for 3 min and then centrifuged at 3300*g* for 15 min at ambient temperature. The upper layer (methanol/water) was discarded, and the remaining solvents were evaportated under nitrogen. The total lipid content was determined by subtracting the weight of the empty glass tube from the weight of the test tube containing the isolated lipid. After determination of the fat content of the emulsion, the lipid concentration of the creamed emulsions was adjusted to 2% (w/w) by diluting with fresh PBS buffer containing 0−10 mM Brij 35.

Lipid Oxidation Measurements. Emulsions (5 mL) were placed in capped test tubes (16 mm × 125 mm) and allowed to oxidize in the absence of light at 20 °C for up to 10 days. Lipid hydroperoxides were measured according to the method of Nuchi et al. (*21*) by mixing the emulsion (0.3 mL) with 1.5 mL of isooctane/1-butanol (2:1 v/v), followed by addition of 15 μL of 3.94 M ammonium thiocyanate and 15 μL of ferrous iron solution (prepared by adding an equal amount of 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$). After 20 min, the absorbance of the solution was measured at 510 nm using a UV–visible scanning spectrophotometer (Ultrospec 3000 Pro; Amersham Pharmacia Biotech, Cambridge, England). Hydroperoxide concentrations were determined using a standard curve prepared with cumene hydroperoxide.

The formation of secondary oxidation products was followed by measuring thiobarbituric acid reactive substances (TBARS) using the method of McDonald and Hultin (*22*). TBARS were determined by mixing between 0.1 and 1.0 mL (final volume adjusted to 1.0 mL with doubly distilled water) of emulsion with 2.0 mL of TBA reagent [15% (w/v) trichloroacetic acid and 0.375% (w/v) thiobarbituric acid in 0.25 M HCl] in test tubes, followed by heating in a boiling water bath for 15 min. The tubes were cooled to room temperature for 10 min and then centrifuged (1000*g*) for 15 min. After 10 min of incubation at

room temperature, the absorbance was measured at 532 nm. Concentrations of TBARS were determined using a standard curve prepared using 1,1,3,3-tetraethoxypropane.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The peroxyl radical scavenging activity of *p*-hydroxyphenylacetic acid and its conjugates was determined using a method adapted from Ou et al. (*23*). A 200 mM solution of 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) and a 50 nM solution of fluorescein in 75 mM potassium phosphate buffer at pH 7.0 was prepared for each experiment and kept on ice. For each run, fluorescein was held at 37 °C in a water bath for 15 min and then brought to a final concentration of 45 nM in a cuvette with 4.0 μM antioxidant, 0.1 mM EDTA, and 20 mM AAPH in phosphate buffer (pH 7.0). Analyses were performed on a Hitachi F-2000 (Tokyo, Japan) fluorometer set at an excitation wavelength of 493 nm and a emission wavelength of 515 nm with the temperature being maintained at 37 °C. Fluorescence was recorded every minute for 40 min, and the fluorescence relative to the fluorescence at time zero (F/F_0) was calculated from the fluorescence decay curve (*19*). The relative peroxyl radical absorbance capacity values of *p*-hydroxyphenylacetic acid and its conjugates were calculated and compared to control samples containing fluorescein alone.

Measurement of Antioxidant Components in Emulsion. To measure antioxidant concentration in the continuous phase of the emulsion, 2 mL of emulsions and 1 mL of PBS were put in a centrifuge tube (Sorvall, 75 mL, 15 mm × 80 mm, Kendro, CT) and centrifuged at 15000 rpm at 4 °C for 40 min. After centrifugation, 2 mL of the continuous phase (lower layer) of the emulsion was withdrawn with a syringe. This procedure of centrifugation and collection of the continuous phase of the emulsion was repeated three times. After the final centrifugation, the continuous phase was filtered through a 0.22 μm syringe filter (Millex-GS, Bedford, MA) to remove any residual emulsion droplets. The concentration of *p*-hydroxyphenylacetic acid and its conjugates in the continuous phase was measured at 285 nm using a UV–visible spectrophotometer (UV-2101PC; Shimazu, Kyoto, Japan). Concentrations of the antioxidants were determined using standard curves prepared with each antioxidant.

Statistical Analysis. All experiments were performed in triplicate. Statistical analysis was performed using Student's *t*-test (*24*).

RESULTS AND DISCUSSION

It has been proposed that antioxidants are more effective in oil-in-water emulsions if they are able to accumulate at the oil–water interface where oxidative reactions are thought to occur (*25*). Production of antioxidants conjugated to fatty acids or fatty alcohols to form surface-active antioxidants is a potential way to increase the ability of an antioxidant to concentrate at the oil–water interface of oil-in-water emulsions. The interfacial tension of antioxidants used in this study was measured at the hexadecane−water interface to determine if conjugation of antioxidants to a butyl or dodecyl hydrocarbon chain increased their surface activity (**Figure 1**). **Figure 1** shows that unconjugated HPA was not able to decrease the interfacial tension over a concentration range of 0−5 mM antioxidant/kg of oil. Conjugation to butyl or dodecyl hydrocarbon chains increased the surface activity of antioxidants. Conjugation of HPA to dodecyl significantly ($p < 0.05$) decreased the interfacial tension compared to the butyl conjugated HPA at a concentration ≥5 mM/kg of oil.

To determine if the antioxidant conjugates were more effective at inhibiting lipid oxidation than unconjugated HPA, they were added to a Menhaden oil-in-water emulsion at a concentration of 5 mM, and lipid oxidation was monitored by measuring formation of lipid hydroperoxides and TBARS. All antioxidants were able to inhibit lipid hydroperoxide and TBARS formation after 5 days of storage. Ability to inhibit lipid hydroperoxide formation was in the order of free HPA > dodecyl HPA > butyl HPA (**Figure 2A**). Unconjugated HPA and dodecyl HPA had similar abilities to inhibit the formation of TBARS, and both were more effective than butyl HPA (**Figure 2B**).

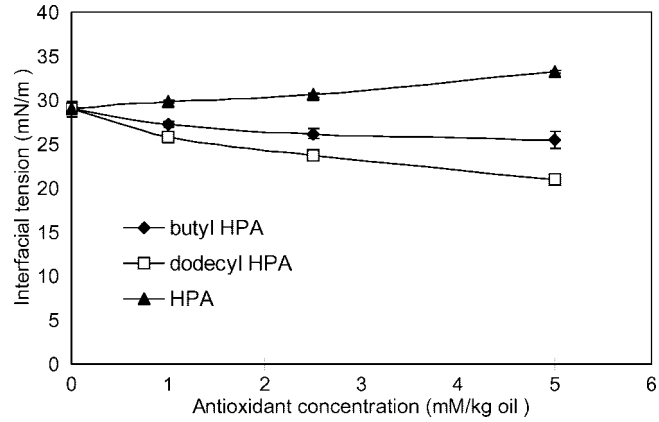

Figure 1. Interfacial tension measurement at a water−hexadecane interface in the presence of *p*-hydroxyphenylacetic acid (HPA), butyl HPA, or dodecyl HPA. Data points represent means ($n = 3$) ± standard deviations. Some error bars are within data points.

While the butyl and dodecyl HPA's were more surface active than unconjugated HPA, their ability to inhibit lipid oxidation was diminished by the addition of hydrocarbon chains. This decrease in antioxidant activity could be due to a decrease in their ability to scavenge free radicals or could be due to a change in their physical location in the emulsion. To determine if conjugation decreased free radical scavenging activity, the antioxidants were evaluated for their ability to scavenge peroxyl radicals using the oxygen radical absorbance capacity (ORAC) assay (**Figure 3**). Peroxyl radical scavenging activity by 4.0 μM antioxidant was in the order of free HPA > dodecyl HPA > butyl HPA. The decreased ability of the conjugates to scavenge free radicals could be due to the hydrocarbon chain increasing the required energy to dissociate the hydrogen on the hydroxyl group of HPA, making it therefore more difficult for the hydrogen to be donated to the peroxyl radical. Alternatively, the presence of the hydrocarbon chain could also decrease peroxyl radical scavenging by changing the physical orientation of the conjugates in solution. At low concentrations, surfactant molecules will exist in solution as indivual molecules. When concentrations are increased above the critical micelle concentration (CMC) of the surfactant, the surfactant will form micelles. It is possibile that formation of micelles could decrease peroxyl radical scavenging by decreasing the accessibility of the hydroxyl group of the HPA to the free radicals. However, the CMCs of surfactants tend to decrease as the hydrophobicity of the surfactant increases (e.g., increase in hydrocarbon chain length). This would mean that the dodecyl HPA should have a lower CMC than butyl HPA and thus would form micelles at lower concentrations. Since dodecyl HPA was a more effective peroxyl radical scavenger than butyl HPA, it seems unlikely that the formation of micelles was responsible for the observed decrease in peroxyl radical scavenging activity, since a larger proportion of the dodecyl HPA would need to exist as micelles compared to butyl HPA.

It is also possible that differences in antioxidant activity in the Menhaden oil-in-water emulsion could be due to differences in the physical location of the conjugated and unconjugated HPA in the emulsion system. The physical location of the antioxidants is dependent on their solubility in lipid and water. However, in oil-in-water emulsions, the location of antioxidants is also dependent on the emulsifier concentration (*26*). The ability of

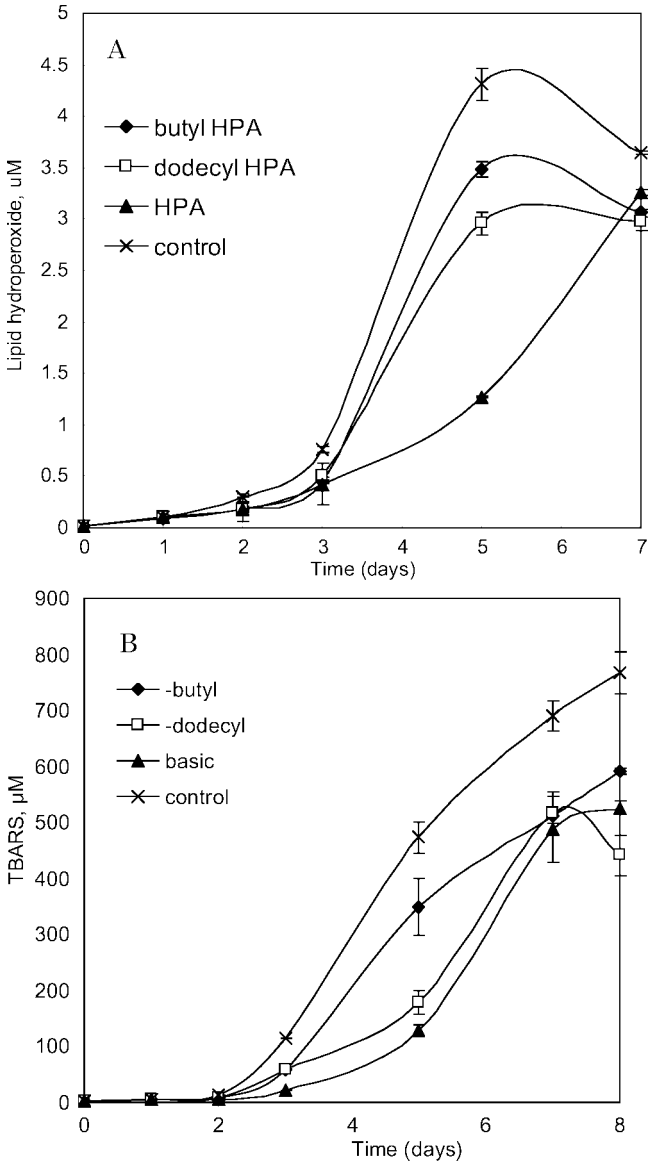

Figure 2. Ability of 5 mM *p*-hydroxyphenylacetic acid (HPA), butyl HPA, or dodecyl HPA to inhibit formation of (A) lipid hydroperoxides and (B) thiobarbituric acid reactive substances (TBARS) in 2% Menhaden oil-in-water emulsion at pH 7.0 and 20 °C. Data points represent means (n = 3) ± standard deviations. Some error bars are within data points.

emulsifiers to alter the physical location of antioxidants is due to their ability to form surfactant micelles. When an oil-in-water emulsion is produced, the emulsifier partitions between the lipid phase, lipid−water interface, and aqueous phase. If the aqueous phase emulsifier concentrations are above the CMC, surfactant micelles will form. These surfactant micelles have the ability to solubilize antioxidants and thus decrease the concentration of available antioxidants at the site of lipid oxidation with surfactant solubilization occurring to a greater extent with polar than nonpolar antioxidants (*26*). To determine if surfactant micelles could change the physical location of conjugated and free HPA, emulsions were first washed by repeated centrifugation, and the aqueous phase was replaced to remove excess surfactant from the continuous phase of the emulsion. Then antioxidants and/or excess surfactant was added back to the emulsion. In the washed emulsion which would not contain surfactant micelles, the concentrations of dodecyl and free HPA in the aqueous phase were similar, with less than 5% of the antioxidants being present in the continuous phase (**Figure 4**).

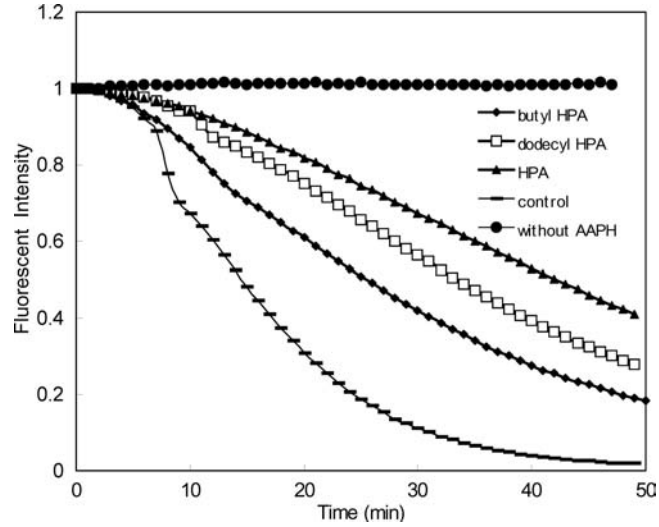

Figure 3. Ability of 4.0 μM *p*-hydroxyphenylacetic acid (HPA), butyl HPA, or dodecyl HPA to scavenge peroxyl radicals at 37 °C as determined by loss of relative fluorescein fluorescent intensity (ratio of fluorescence intensity F_x to fluorescence intensity at the initial time F_0). A control (blank) was prepared with AAPH and no antioxidants.

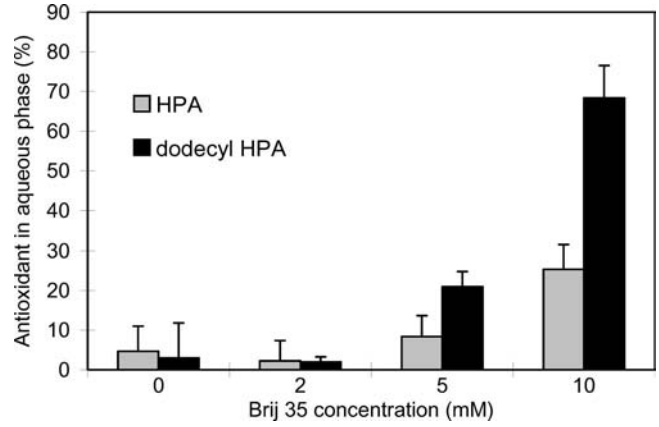

Figure 4. *p*-Hydroxyphenylacetic acid (HPA) or dodecyl HPA concentrations (% of total) in the continuous phase of a Brij 35-stabilized 2% Menhaden oil-in-water emulsion at pH 7.0 in the presence or absence of Brij 35 micelles. Data points represent means (n = 3) ± standard deviations. Some error bars are within data points.

When excess Brij 35 was added at concentrations ≥5 mM, both free and dodecyl HPA concentrations in the aqueous phase increased, with the dodecyl HPA being more readily solubilized than free HPA. These results are similar to those seen with Brij 700 which solubilized the more polar propyl gallate out of oil-in-water emulsion droplets more readily than the more nonpolar butylated hydroxytoluene (*26*). Our results therefore suggest that conjugation of HPA caused the antioxidant to be more readily solubilized by surfactant micelles, decreasing the effective concentration of antioxidant at the emulsion droplet interface. This may explain why conjugation to hydrocarbon chains did not increase the antioxidant activity of HPA in oil-in-water emulsions.

CONCLUSIONS

Conjugation of *p*-hydroxyphenylacetic acid (HPA) to butyl or dodecyl hydrocarbon chains increases their ability to accumulate at an oil–water interface. Despite the fact that the conjugated HPA's concentrate at the oil–water interfaces where lipid oxidation reactions are thought to be prevalent, free HPA

was a more effective antioxidant than the butyl and dodecyl conjugates in Menhaden oil-in-water emulsions. The increased antioxidant activity of free HPA could be due to its more effective free radical scavenging activity and/or to its higher concentration in the lipid phase of oil-in-water emulsions where it can act as a chain-breaking antioxidant. More research is needed to develop surface-active antioxidants that do not have diminished free radical scavenging activity and do not readily partition into the aqueous phase of oil-in-water emulsions, where they are unable to interact with lipid free radicals. The latter could be accomplished by increasing the hydrophobicity of the surface-active antioxidant by increasing the chain length of the hydrocarbon chain or by conjugation with multiple hydrocarbon chains.

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