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Maxima in Antioxidant Distributions and Efficiencies with Increasing Hydrophobicity of Gallic Acid and Its Alkyl Esters. The Pseudophase Model Interpretation of the "Cutoff Effect"

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Supporting Information

ABSTRACT: Antioxidant (AO) efficiencies are reported to go through maxima with increasing chain length (hydrophobicity) in emulsions. The so-called "cutoff" after the maxima, indicating a decrease in efficiency, remains unexplained. This paper shows, for gallic acid (GA) and propyl, octyl, and lauryl gallates (PG, OG, and LG, respectively), that at any given volume fraction of emulsifier, the concentrations of antioxidants in the interfacial region of stripped corn oil emulsions and their efficiency order follow PG > GA > OG > LG. These results provide clear evidence that an AO's efficiency correlates with its fraction in the interfacial region. AO distributions were obtained in intact emulsions by using the pseudophase kinetic model to interpret changes in observed rate constants of the AOs with a chemical probe, and their efficiencies were measured by employing the Schaal oven test. The model provides a natural explanation for the maxima with increasing AO hydrophobicity.

KEYWORDS: *polar paradox, cutoff effect, antioxidant distributions, antioxidant efficiency, emulsions, arenediazonium ions*

INTRODUCTION

Oxidation of polyunsaturated lipids by reactive oxygen species is a serious and continuing problem for the food industry. Peroxidation produces rancid odors and off-flavors, decreases shelf life, alters food texture and appearance, and may reduce nutritional quality and food safety.[1−7] Progress has been made in minimizing lipid oxidation by improved processing and packaging.[6,7] However, for practical, nutritional, and economical reasons, currently the most effective method for minimizing lipid oxidation is by adding natural or synthetic antioxidants (AOs).[1−4,6,7] AOs inhibit the actions of free radicals, prooxidants, and oxidative intermediates by interfering at various stages of oxidation.[1−4,6,7]

In bulk solution, the rate of peroxidation, the formation of peroxides from unsaturated lipids and molecular oxygen, is characterized by the measured, observed, or overall rate constant, k_{obs}, that depends on the concentrations of the reactants in solution. For simple bimolecular reactions, the treatment of the kinetic data is straightforward, but for multistepped, free radical, chain reactions such as peroxidation treatments the kinetics may become more complex. In addition, in food systems the reactions occur in organized media such as micelles, microemulsions, or emulsions or in vivo, in which the reactants, oil, AO, and molecular oxygen are distributed between oil, surfactant, and aqueous regions. Shahidi and Zhong pointed out in a recent review[8] that AO efficiency is affected by AO and polyunsaturated lipid structure, temperature, and a number of other experimental variables. In emulsions, the distribution and properties of the reactants and the volumes of the oil, emulsifier, and water and other added components, for example, salts and acid, also affect AO efficiency. Understanding the contributions of these multiple variables to peroxidation is extremely difficult.

In 1980, Porter formulated the polar paradox.[9,10] He brought into sharp focus the apparent contradiction repeatedly reported in the literature that polar AOs are more efficient than nonpolar AOs in bulk oils but that nonpolar antioxidants are more efficient in aqueous emulsions. Our focus here is on the emulsion half of the paradox. Recent research is aimed at understanding the relationship between an AO's efficiency and its hydrophobicity in emulsions, with clear indications that more hydrophobic AOs are more efficient than hydrophilic AOs, which are less efficient because they are primarily located in the aqueous region and the oils are compartmentalized in the interiors of the emulsion drops.[11−14] However, this does not mean that the AO efficiency has gone to zero. The reactants are in dynamic equilibrium in emulsions as they are in association colloids (see below), and whatever fraction of the antioxidant that dissolves in the interfacial region will slow oxidation to some extent, and its interfacial concentration will be replenished by the fractions of AO in the oil and aqueous regions.

A variety of problems with the polar paradox have been reported in recent years, and they were reviewed recently by Shahidi and Zhong.[8] One of the most serious is the apparent violation of the expectation that as the hydrophobicity of a series of homologous AOs increases, their efficiency in

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emulsions would increase because a larger fraction of the AO would be associated with the emulsion droplets. Thus, for strongly hydrophobic AOs the antioxidant efficiency should plateau once all of the AO is associated with the droplets. However, recent work shows that increasing the alkyl chain length of an AO produces a maximum in the efficiency at intermediate chain lengths, the so-called "cutoff effect",[8,15−20] so that further increases in AO chain length produce a significant decrease in their efficiency. A decrease in AO efficiency with increasing hydrophobicity is inconsistent with the expectations of the polar paradox, and it has been observed for a variety of AOs with different headgroups. However, although the polar paradox contains some perceptive observations, it is not a theory and does not predict the change in antioxidant efficiency with AO chain length. Similarly, a "cutoff" effect has been reported with increasing AO hydrophobicity of cell culture studies for a range of biological activities such as antimicrobial, cytotoxic, and anesthetic, but it remains unexplained.[8]

The reasons are several. First, kinetic methods for monitoring the peroxidation of polyunsaturated oils in emulsions report the weighted average change in the observed rate or rate constant, and no model or method is available to assess the contributions to the observed rate from potentially simultaneous reactions in the oil, interfacial, and aqueous regions.[7,21] Second, numerous attempts have been made to determine the distribution of AOs in emulsions. However, most methods involve separation and analysis of the oil and water phases and, by themselves, fail because the fraction of AO in the interfacial region of the emulsion droplets cannot be determined independently using this approach.[7,8,13,18,22−25] Uncharged phenolic AOs are generally polar organic molecules with substantial oil and water solubility. They are often soluble in food oils,[7,26,27] and functionalizing them with alkyl side chains will increase both their hydrophobicity and oil solubility. Consequently, determining the most efficient AO for a particular emulsion system remains an unsolved problem.

We have taken a very different approach by adapting pseudophase kinetic models to determine AO distributions in emulsions.[28−33] We have demonstrated that chemical reactivity in stirred, fluid, metastable emulsions can be treated using the same kinetic model as that used for microemulsions.[28−33] This approach works because the diffusivities of reactive components in emulsions and microemulsions are much faster than the observed rate of thermal chemical reactions[34] and because the distributions of reactive components in both microemulsions and stirred, fluid, emulsions are in dynamic equilibrium. Put differently, transport across apparent boundaries in both systems, for example, oil to interface and interface to water, is not rate limiting.

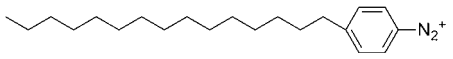
A substantial conceptual simplification is possible when the dynamic equilibrium assumption holds. In organized media, both microemulsions and emulsions, the observed rate of a bimolecular reaction is equal to the sums of the rates in each region, oil, interfacial, and water (eq 1):

$$\text{observed rate} = \text{rate in oil} + \text{rate in interface} + \text{rate in water} \quad (1)$$

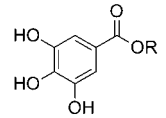
$$\begin{aligned} k_{obs}[16\text{-}ArN_2^+{}_T] &= k_2[16\text{-}ArN_2^+{}_T][AO_T] \\ &= k_O(16\text{-}ArN_2^+{}_O)(AO_O)\Phi_O \\ &\quad + k_I(16\text{-}ArN_2^+{}_I)(AO_I)\Phi_I \\ &\quad + k_W(16\text{-}ArN_2^+{}_W)(AO_W)\Phi_W \end{aligned} \quad (2)$$

where k_2 is a second-order rate constant; square brackets [ ] denote the concentration in moles per liter of total emulsion volume; parentheses ( ) indicate concentration in moles per liter of the volume of a particular region; subscript T stands for the stoichiometric or total amount; subscripts O, I, and W indicate the oil, interfacial and aqueous regions, respectively; and Φ is the volume fraction of a region, defined as $\Phi = V_{region}/V_{emulsion}$. The assumption holds because the volumes of these regions are much larger than the size of the reactants and, as noted above, the diffusivities of the reactants between regions is much faster than the observed rate of reaction. Thus, in oil-in-water microemulsions and emulsions, the totality of the aqueous, interfacial, and oil regions is equal to the sums of the volumes of those regions for all of the droplets (e.g., emulsion droplets, micelles, or other coexisting structures) in solution. This assumption is obviously true for the aqueous region because it is continuous.

Our aim is two-fold: First, we determine the distributions of a homologous series of gallic acid and gallate ester AOs of increasing chain length (Figure 1) in stripped corn and olive oil



4-hexadecylbenzenediazonium ion (16-ArN_2^+)



R = H, Gallic acid, GA
R = $CH_3(CH_2)_2$-, Propyl gallate, PG
R = $CH_3(CH_2)_7$-, Octyl gallate, OG
R = $CH_3(CH_2)_{11}$-, Lauryl gallate, LG

Figure 1. Structures of 16-ArN_2^+, gallic acid, and gallic acid esters.

emulsions and compare their distributions with their AO efficiencies. The distributions are characterized by two partition constants, one between the oil and interfacial regions and one between the water and interfacial regions. AO efficiencies were determined by using the Schaal oven test. Second, we evaluate the effects of emulsifier concentration and oil type on AO distributions. The overall results show that our approach provides a natural explanation for the maximum observed in antioxidant activity with increasing chain length, sometimes called the "cutoff effect." Gallic acid and its esters were selected for study because of their antibacterial and antivirus activities and their importance to the food industry as approved antioxidants, which are believed to act as highly effective chain-breaking AOs.[35,36]

Determining AO Distributions in Emulsions (or Microemulsions). To determine AO distributions from reaction rates, we chose the reduction of 4-hexadecylbenzenediazonium ion (16-ArN_2^+), prepared as its BF_4^- salt (Figure 1), by AOs that can be monitored either electrochemically or by a sampling method in fluid, opaque, emulsions.[33,37,38] Figure 2

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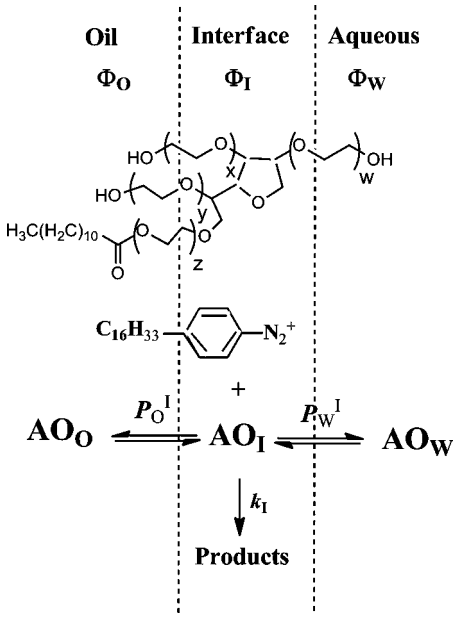

Figure 2. Cartoon representation of a small section of a microemulsion or emulsion droplet showing the oil, water, and interfacial regions, subscripts, O, W, and I, respectively, the emulsifier Tween 20, the partitioning of an AO between the regions of the emulsion, and the location of the reactive group of the hydrophobic arenediazonium ion that reacts with the AO only in the interfacial region.

summarizes the logic of the pseudophase model that is equally applicable to a stirred emulsion or a microemulsion.[28−32] The approach is robust, versatile, and applicable to a variety of antioxidants, oils, and emulsifiers under different experimental conditions.[28−30,32,37] Further details can be found in the Supporting Information (S1, S2) and references cited therein.

The totality of the emulsion system is represented by the oil, aqueous, and the small section of the interfacial region of a microemulsion or emulsion droplet (Figure 2), which also shows the important parameters needed to determine the distribution of the AO between the oil, interfacial, and aqueous regions. The distribution of an AO depends on a number of variables including its structure, which in turn also determines it relative solubilities in the oil, interfacial, and water regions of the emulsion (or microemulsion), and also on emulsifier and oil types and experimental conditions such as acidity, temperature, salt concentration, and type.[10,12] Two partition constants are needed to describe AO distribution, one between the oil and interfacial regions, P_O^I, and the other between the aqueous and interfacial regions, P_W^I (Figure 2).[29,31−33] Once values for P_O^I and P_W^I are known, the percentage of AO in each region can be calculated at any emulsifier, oil, and water concentrations. In addition, the second-order rate constant for the reaction in the interfacial region of the emulsion, k_I, is obtained from the same kinetic data. Some comments on the chemistry and mathematical models employed and some additional details are in the Supporting Information and other published papers.[29,31−33]

Although consensus is absent on how to interpret the effects of experimental variables on antioxidant distributions and efficiencies, we believe the pseudophase kinetic model provides the most promising approach to understanding their contributions.[29,31−33] Over the past few years, we have demonstrated that the pseudophase model provides reasonable estimates of AO partition constants in nonionic emulsions and a more realistic picture of AO distributions.[28−30,37] For instance, we recently demonstrated that the main factor controlling distributions of gallic acid and α-tocopherol in a model food emulsion is the emulsifier concentration, whereas changes in the oil to water ratio and in the temperature have only minor effects.[30,32]

MATERIALS AND METHODS

Gallic acid (GA, Riedel de Haën), propyl gallate (PG, Fluka), octyl gallate (OG, Fluka), and lauryl gallate (LG, Fluka) and the emulsifier Tween 20 (Fluka) were of the highest purity available and used as received. Hydrochloric acid (HCl) was from Riedel de Haën (37%), and its concentration was determined by potentiometric titration. Stripped corn oil (Acros Organics) was used as received. Olive oil was stripped of natural tocopherols and phenols from commercial extra virgin olive oil by washing with 0.5 M NaOH solution and passing the oil twice through an aluminum oxide column. Complete removal of tocopherols was confirmed by HPLC according to IUPAC method 2.432. Details can be found elsewhere.[39] Both olive and corn oil were kept at low temperature in the dark to minimize lipid peroxidation.

4-Hexadecylbenzenediazonium tetrafluoroborate (16-ArN_2BF_4) was prepared in high yield and purity under nonaqueous conditions from commercial 4-hexadecylaniline (Aldrich, 97%) by diazotization following a published method[29] and stored in the dark at low temperature to minimize its decomposition.

All aqueous solutions were prepared with Milli-Q grade water. The acidity of aqueous phase in the emulsions was controlled using acetic acid/acetate buffer (0.04 M). Solutions of the coupling reagent *N*-(1-naphthyl)ethylenediamine (NED, Aldrich) were prepared in a 50:50 (v/v) BuOH/EtOH mixture to give [NED] = 0.02 M.

Emulsion Preparation. Emulsions of different oil to water ratios were prepared by mixing together the necessary amounts of stripped corn oil or olive oil, acidic water, and a weighed amount of the nonionic surfactant Tween 20. The volume fraction of surfactant, Φ_I, defined hereafter as $\Phi_I = V_{surf}/V_{emulsion}$, was varied from $\Phi_I = 0.005$ to $\Phi_I = 0.04$. The required amounts of the antioxidants GA and PG were dissolved in the bulk water, and those of OG and LG were dissolved in the oil so that final concentrations of the antioxidants in the emulsions were [AO] $\sim 4 \times 10^{-3}$ M. The mixture was stirred with a high-speed rotor (Polytron PT 1600 E) for 1 min and transferred to a continuously stirred, thermostated, cell. Emulsion stability was checked visually. No phase separation was observed within 12−15 h, a much longer time period than required for complete reaction (>10 half-lives) between AO and 16-ArN_2^+ ions.

DPPH Radical Scavenging Activity.[20,25,39]

The DPPH chromogen radical is a long-lived, commercially available, *N*-centered radical that is deep purple in color. DPPH is reduced by AOs to the pale yellow hydrazine product. For each antioxidant, the decrease of the absorbance at 515 nm with time was recorded at different [AO]/[DPPH] ratios and kinetics plots were prepared. From these graphs, the amount of DPPH radical remaining, $[DPPH]_r$, was determined at selected times by using a previously obtained calibration curve. The values were transferred onto another graph showing the percentage of residual DPPH as a function of the [AO]/[DPPH] ratio. The fraction of unconsumed DPPH was determined from the equation $\%DPPH_r = 100[DPPH]_r/[DPPH]_{t=0}$. The antioxidant efficiency is defined as the EC_{50} value, the concentration of the AO required to decrease the DPPH concentration by 50%.[40,41] Analyses were run in triplicate, and the average EC_{50} values were <10%.

Schaal Oven Test. Oil-in-water emulsions (30 mL) were prepared as described before from olive or corn oil (final [AO] $\approx 3.3 \times 10^{-4}$ M) and were placed in stoppered 50 mL erlenmeyer flasks in the dark at constant $T = 45$ °C. Samples were vortexed every 24 h for 1 min to maintain emulsion physical integrity throughout the total reaction time. After each homogenization, a 3 mL aliquot of the emulsion was removed and added to 5 mL of a saturated NaCl solution to improve phase separation. The mixture was frozen and then thawed slowly to room temperature and centrifuged at 2500 rpm for 5 min. After

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centrifugation, 0.2 g of the oil phase was diluted to 10 mL using hexane. One milliliter of this solution was further diluted to 10 mL of hexane, and the conjugated content (CD, AOCS official method Ti 1a-64) was determined at λ = 233 nm with a UV−vis spectrometer (Thermo Scientific Evolution Array). The *p*-anisidine value (AV; AOCS official method Cd 18-90) was determined by mixing 5 mL of the oil−hexane solution with 1 mL of an aqueous acid (acetic acid, 0.02 M) solution of *p*-anisidine. After homogeneization, the absorbance at λ = 350 nm was recorded.

Treatment of Equilibrium and Kinetic Data. *Determining the Partition Constant P_W^O of Gallic Acid Derivatives between Stripped Oils and Water in the Absence of Emulsifier.* Calculations of P_W^I and P_O^I from the kinetic data require values of their bulk phase partition constants between the oil and water phases in the absence of emulsifier, P_W^O, for GA and each gallic acid ester. Values of P_W^O were determined in a 1:9 (v/v) oil to water mixture, in the absence of emulsifier, by employing a modified shake-flask method. GA and PG were dissolved in the water phase, and OG and LG were dissolved in the oil phase. The oil/water mixture was stirred with a high-speed rotor for 1 min and allowed stand for 30 min to permit phase separation and thermal equilibrium at ambient temperature. The phases were separated by centrifugation, and the AO concentrations in the aqueous phase were determined by UV spectrometry with the aid of previously prepared calibration curves. Values of P_W^O were calculated from eq 3, the standard definition of the partition constant,

$$P_W^O = \frac{(AO_O)}{(AO_W)} = \frac{(\%AO_O)}{(\%AO_W)}\frac{V_W}{V_O} = \frac{P_W^I}{P_O^I} \quad (3)$$

where V_W and V_O are the aqueous and oil region volumes, respectively, and concentrations in them are represented with parentheses and the partition constants P_W^I and P_O^I are defined by eqs 4 and 5, respectively.

$$P_W^I = \frac{(AO_I)}{(AO_W)} \quad (4)$$

$$P_O^I = \frac{(AO_I)}{(AO_O)} \quad (5)$$

Results were obtained in duplicate or triplicate with standard deviations of <5%. The last equality in eq 3 shows that the ratio of the partition constants for the distribution of the AO between the oil and interfacial region and the aqueous and interfacial region is equal to the partition constant in the absence of surfactant. Put differently, this ratio is true at the limiting condition of $\Phi_I = 0$.

Both OG and LG have very low solubilities in water (<0.001 g/L for OG).[42,43] Thus, values of (AO_W) in the oil/water mixture will also be very small and, small errors in the denominator of eq 3 may have a large effect on the value of P_W^O. Because the values of P_W^O for OG and LG are not reliable, they are not reported.

Conversely, GA is essentially oil-insoluble, and an average value (six runs) of P_W^O = 0.03 at T = 25 °C was determined. This value is similar to that found by Huang et al.[12] in unbuffered 1:9 corn oil/water binary mixtures, P_W^O = 0.02. The P_W^O value indicates that >99% of GA is in the aqueous phase, in keeping with the hydrophilic nature of this antioxidant. PG is both oil- and water-soluble, and an average value (four runs) of P_W^O = 0.84 at T = 25 °C was obtained, in good agreement with that of P_W^O = 0.85 estimated by Huang et al.[12]

Determining k_{obs} Values for the Reaction between 16-ArN_2^+ and the Antioxidants in Stripped Oil/Tween20/Acidic Water Emulsions. Derivatization Method: Azo Dye Formation. The reaction between the antioxidant and 16-ArN_2^+ was initiated by adding an aliquot (16 μL) of a 0.17 M stock solution in acetonitrile to a thermostated emulsion. Kinetic data were obtained by employing our derivatization method (azo dye formation) as described in detail elsewhere.[37] This methodology exploits the rapid reaction of ArN_2^+ ions with a suitable coupling agent such as NED to yield an stable azo dye (Figure 3) that is diluted with a 50:50 (v/v) BuOH/EtOH mixture to yield an optically transparent, homogeneous solution of which the absorbance is measured spectrometrically.[30,32,37] Control experiments showed that

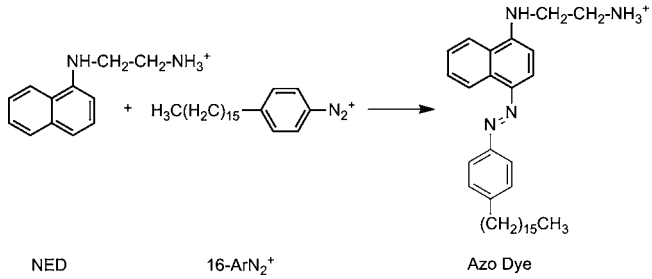

Figure 3. Coupling of 16-ArN_2^+ with *N*-(1-naphthyl)ethylenediamine (NED) to yield a stable azo dye of which the concentration is determined spectrometrically at λ = 572 nm.

the absorbance of the azo dye at λ = 572 nm follows Beer's law and is a linear function of the concentration of 16-ArN_2^+. Thus, the decrease in the absorbance of the azo dye with time can be used to determine k_{obs}.

The reaction between 16-ArN_2^+ and NED was monitored for at least 2−3$t_{1/2}$, with typical correlation coefficients >0.995. See the Supporting Information (S1) for a typical kinetic run.

Relationships between the Partition and the Observed Rate Constants (k_{obs}) in Emulsions: Pseudophase Kinetic Model. As noted in the Introduction, the observed rate, v, is the sum of the rates in each region of the macroemulsion.[28−30,37] Because 16-ArN_2^+ is insoluble in both water and oil, the interfacial and total concentrations of 16-ArN_2^+ are equal, k_{obs} is directly proportional to the concentration of the antioxidant in the interfacial region (eq 2).

The distribution of PG, which is both oil- and water-soluble (P_W^O = 0.84, see above), is described by two partition constants, P_W^I and P_O^I, because significant amounts of PG are in all three regions, oil, interface, and water (Figure 2), but for very hydrophobic (water insoluble) or very hydrophilic (oil insoluble) antioxidants, the description of their distribution can be simplified. For instance, the concentration of the very hydrophilic antioxidant GA (P_W^O = 0.03, see above) is essentially zero in the oil region. Thus, only P_W^I is needed to describe its distribution. However, for very hydrophobic antioxidants such as α-tocopherol, OG, or LG, the concentrations of which are effectively zero in water, only P_O^I is needed to describe their distributions. Details on these approximations can be found elsewhere.[29,30,32]

Equations 6−8 describe the dependence of k_{obs} on the surfactant, water, and oil volume fractions and the medium effect, k_I, of the micellar interface for antioxidants that are very hydrophilic and partition primarily between water and the interfacial regions[32] (eq 6), very hydrophobic and partition primarily between the oil and interfacial regions[30] (eq 7), and for antioxidants of intermediate hydrophobicity (or hydrophilicity)[28] (eq 8) and partition between all three regions. Additional details are in the Supporting Information (S2).

$$k_{obs} = \frac{k_I[AO]_T P_W^I}{\Phi_I P_W^I + \Phi_W} \quad (6)$$

$$k_{obs} = \frac{k_I[AO]_T P_O^I}{\Phi_I P_O^I + \Phi_O} \quad (7)$$

$$k_{obs} = \frac{k_I[AO]_T P_W^I P_O^I}{\Phi_O P_W^I + \Phi_I P_W^I P_O^I + \Phi_W P_O^I} \quad (8)$$

The values of the partition constants are obtained from the reciprocal forms of eqs 6−8 (not shown), that is, from plots of $1/k_{obs}$ versus Φ_I that should be linear with positive intercepts. The reciprocal forms were used to determine the values of P_W^I, P_O^I, and k_I for GA, PG, OG, and LG. Equations are provided as Supporting Information.[29,30,32,37]

Once the partition constants are known, determining the percentage of the antioxidant in the interfacial region of the emulsion is straightforward. Equations 9−11, which were derived previously,

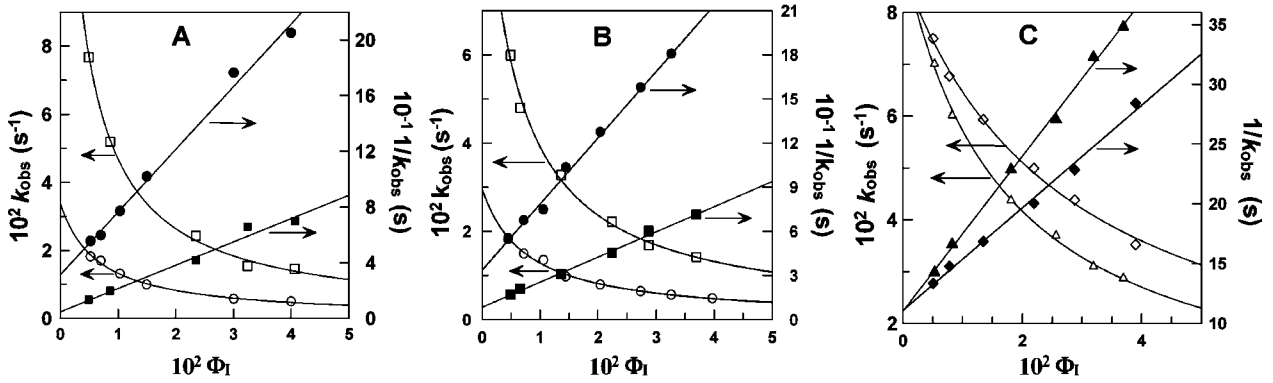

Figure 4. Change in k_{obs} (open symbols) and 1/ k_{obs} (solid symbols) with Φ_I for (A) GA (circles) and PG (squares) in 3:7 olive oil/water emulsions; (B) GA (circles) and PG (squares) in 1:9 corn oil/water emulsions; (C) OG (triangles) and LG (diamonds) in 1:1 corn oil/water emulsions. All emulsions were prepared by using oils stripped of their natural antioxidants and phenols, buffered acidic water (acetic acid/acetate buffer, 0.04 M, pH 3.7), and Tween 20 as nonionic emulsifier. [AO] ~ 4.17×10^{-3} M, [16-ArN$_2$BF$_4$] ~ 2.83×10^{-4} M, T = 25 °C.

were used to calculate the percentage of the antioxidants in the interfacial region of the emulsions. Similar expressions were employed to calculate the percentage of the antioxidant in the aqueous and oil regions.[28−30] Details on their derivatization from the partition constant are given in the Supporting Information (S3).

$$\%AO_I = \frac{100\Phi_I P_W^I}{\Phi_I P_W^I + \Phi_W} \quad (9)$$

$$\%AO_I = \frac{100\Phi_I P_O^I}{\Phi_I P_O^I + \Phi_O} \quad (10)$$

$$\%AO_I = \frac{100\Phi_I P_W^I P_O^I}{\Phi_O P_W^I + \Phi_I P_W^I P_O^I + \Phi_W P_O^I} \quad (11)$$

RESULTS

Determining the Antioxidant Partition Constants between the Oil, Aqueous, and Interfacial Regions of the Emulsions. Figure 4 illustrates the variations of k_{obs} with Φ_I for GA, PG, OG, and LG in olive and corn oil emulsions of different oil to water ratios. In all of the kinetics experiments, k_{obs} decreases asymptotically with increasing Φ_I. The excellent fits of eqs 6−8 to the k_{obs} versus Φ_I plots and their reciprocals in Figure 4 show that the assumptions employed in the derivation of eqs 6−8 are met. The trend in the k_{obs} values observed in corn oil emulsions, at the lowest Φ_I value, is k_{obs} (LG) ~ k_{obs} (OG) > k_{obs} (PG) ≫ k_{obs} (GA) (Figure 4). For all antioxidants, the values of k_{obs} decrease by factors ranging from 2 to 8 on going from Φ_I = 0.005 to Φ_I = 0.04.

Figure 5 shows the effects of the O/W ratio on k_{obs}. Very similar k_{obs} values (variations <7%) were obtained in 1:4 and 1:9 (GA) and 1:1 and 3:7 (OG, LG) corn oil emulsions. For PG, larger variations (>15−20%) were detected when employing 1:4 and 1:9 emulsions at two given Φ_I values (results not shown). Because a change in the O/W ratio has only a modest effect on k_{obs} (Figure 5), comparison of the results in panels A and B of Figure 4 shows that a change in the nature of the food oil employed in the preparation of these emulsions does not change k_{obs} values significantly at a given Φ_I.

Plots of 1/ k_{obs} versus Φ_I (reciprocals of eqs 6−8, see the Supporting Information) are the straight lines in Figures 4 and 5 and were used to calculate the corresponding P_W^I, P_O^I, and k_I for GA, PG, OG, and LG, Table 1. The average P_W^I value obtained for GA in corn oil emulsions, P_W^I ~ 125 (Table 1), is very similar to that previously reported for 1:9 corn oil

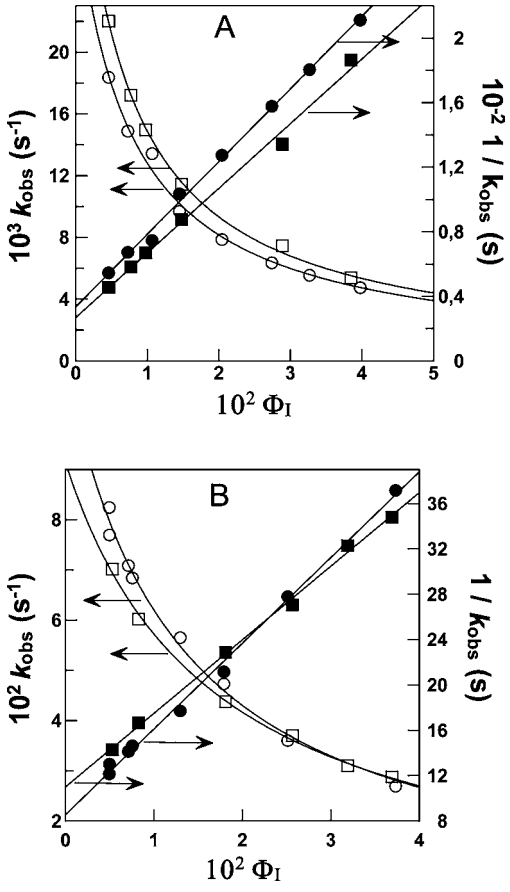

Figure 5. Variation of k_{obs} (open symbols) and 1/k_{obs} (solid symbols) with Φ_I for (A) GA in 1:4 (squares) and 1:9 (circles) corn oil emulsions and (B) OG in 1:1 (squares) and 3:7 (circles) corn oil emulsions.

emulsions at the same pH (3.7)[32] and is similar to that obtained for olive oil emulsions, P_W^I = 101 at this pH. At the measured acidities of these experiments, pH 3.7, approximately 25% of the carboxylic acid groups of GA in the aqueous phase are deprotonated (pK_a ≈ 4.3), which means that 25% of the GA molecules are in their anionic forms. However, PG has no carboxylic groups (Figure 1), and it is essentially completely protonated because the working pH value is nearly 10^5 times lower than the pK_a value of the phenolic group in the 4-position

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Table 1. Values of P_W^I, P_O^I, and k_I for GA, PG, OG, and LG Determined from the Slopes and Intercepts of the Straight Lines in Figures 4 and 5 as Described Elsewhere[29,30,32]

AO	emulsion	oil/water	P_W^I	P_O^I	$10^2\ k_I$ ($M^{-1}\ s^{-1}$)
GA	corn	1:4	131		5.59
GA	corn	1:9	119		5.42
GA	olive	3:7	101		5.30
PG	corn	1:9	204	242	14.0
PG	olive	3:7	328	449	14.2
OG	corn	1:1		29.8	36.6
OG	corn	3:7		26.5	31.6
LG	corn	1:1		19.4	58.0
LG	corn	3:7		16.5	56.0
LG	olive	3:7		17.2	59.0

of the pyrogallol moiety, $pK_a \approx 8.1$.[44] Thus, PG is more hydrophobic and less soluble in water than GA, and this is reflected in the calculated P_W^I value (Table 1), which is higher by a factor of almost 3 than that of GA.

Table 1 also shows that the P_W^I value for PG in olive oil emulsions is significantly higher than that in corn oil emulsions. However, for both olive and corn oil emulsions, $P_O^I > P_W^I$. The value of the ratio (eq 3), $P_W^I/P_O^I = P_W^O = 0.83$ (corn oil), compares very well with that obtained in binary corn oil/water mixtures in the absence of emulsifier and with literature data ($P_W^O = 0.85$).[12] The P_W^O value for olive oil/water mixtures from Table 1 is $P_W^O = 0.73$.

The P_O^I values for OG and LG are similar (Table 1), and both are much lower than that estimated for PG. This means that their $(AO_I)/(AO_O)$ ratios (eq 5) are lower and concentrations in the oil region are higher than that of PG. Because OG and LG are more hydrophobic than PG, they are more soluble in oil and less soluble in the interfacial region than PG. Solubility data for OG and PG in oils are not available in the literature, but solutions of these antioxidants ranging from 10^{-2} to 0.5 M have been prepared in corn and sunflower oils,[25,45] and they dissolved readily in the corn and soybean oils when the emulsions in these experiments were prepared.

The trends in partition constants with alkyl chain length of the esters illustrate the crucial conclusion of this paper: antioxidant efficiency depends to a significant extent on the concentration of antioxidant in the interfacial region, and the concentration is at a maximum for the antioxidant with the largest P_O^I and P_W^I values. PG fits this requirement best for this series of homologous AOs (see Table 1). Also note that the P_W^I values for GA are larger than the P_O^I values for OG and LG. As we will show, this difference correlates well with the lower

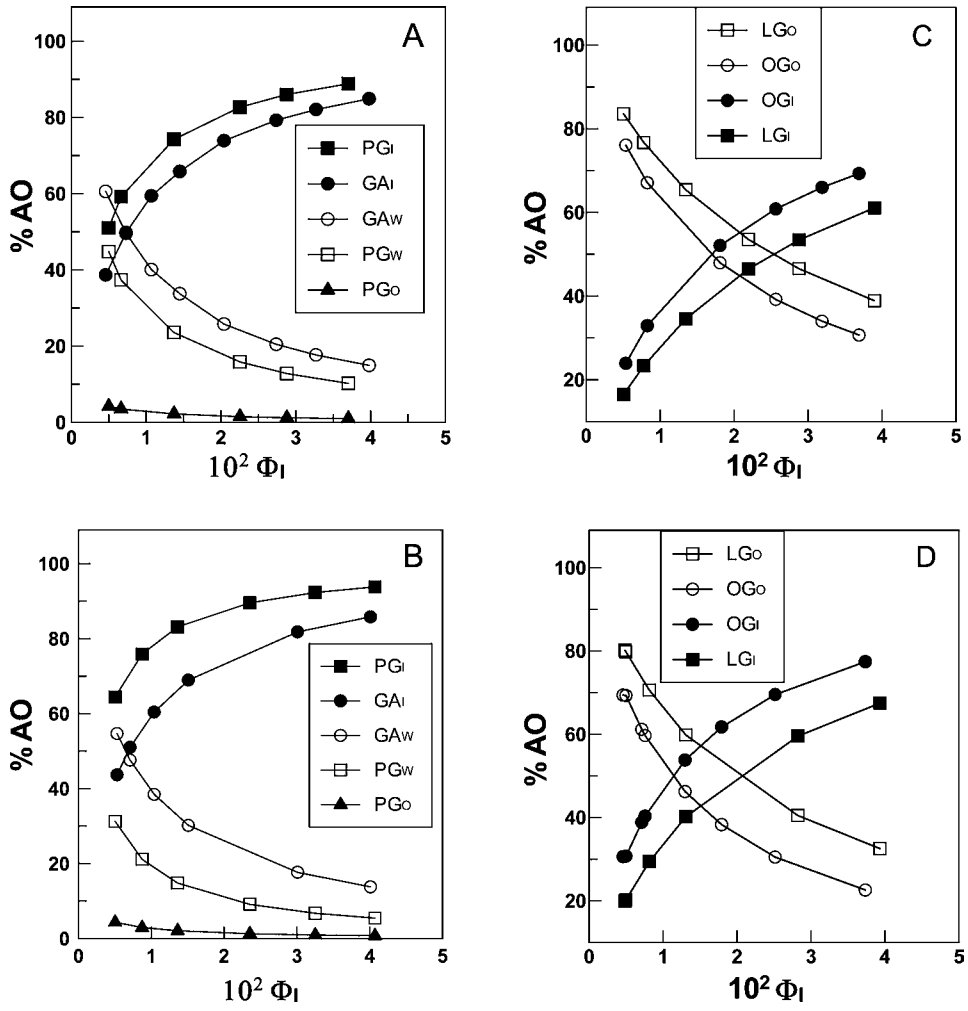

Figure 6. Distributions of GA, PG, OG, and LG between the oil (O), interfacial (I), and aqueous (W) regions of emulsions of different oil to water (pH 3.7) ratio emulsions: (A) 1:9 (v/v) corn oil; (B) 3:7 (v/v) olive oil; (C) 1:1 (v/v) corn oil; (D) 3:7 (v/v) olive oil in Tween 20 at 25 °C.

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antioxidant efficiencies of OG and LG compared to GA (see Discussion).

The pseudophase model was also used to obtain the value of k_I from the kinetic data in Figures 4 and 5 (see Table 1). A value for k_I is not needed to assess antioxidant distributions (eqs 9−11). Values of k_I reflect the properties, for example, polarity of the reaction medium, that is, the interfacial region of the emulsion. As in all pseudophase models, k_I is, in principle, independent of the distribution of antioxidants between the oil, interfacial, and aqueous regions, and comparisons of k_I values for different antioxidants might lead to a scale of antioxidant activity that is independent of antioxidant distributions.[29,30] Similarly, we have used the pseudophase model to determine, for the first time, k_I as a function of temperature and from them calculated the activation parameters for the reaction between 16-ArN_2^+ ions and gallic acid in the interfacial region of corn oil emulsions.[33]

The results in Table 1 show that the k_I value for GA is equal to that previously reported under very similar experimental conditions, $k_I = 5.4 \times 10^{-2}$ M^{-1} s^{-1},[32] but it is lower, by a factor of 10, than k_I for LG in Table 1. Differences in k_I values can be explained, at least in part, in terms of differences in the pK_a values for the 4-OH group of the pyrogallol moiety because kinetics studies on the reactions of ArN_2^+ ions with antioxidants[44,46,47] demonstrate that the rate of the reaction depends on the concentration of the monoanionic form of the antioxidant but that the rate constant is not significantly affected by the alkyl chain length.[48]

Distribution of the Antioxidants between the Oil, Water, and Interfacial Regions of Corn and Olive Oil Emulsions. Figures 6 shows the distributions of the antioxidants in the emulsions with increasing volume fractions of Tween 20. The percentages of the antioxidants in the aqueous, interfacial, and oil regions of the emulsions were obtained using eqs 9−11 and the calculated values of P_O^I and P_W^I listed in Table 1 for different emulsions.

Figure 6A shows the distribution of GA and PG in 1:9 corn oil emulsions. Both antioxidants are distributed primarily between the aqueous and interfacial regions of the emulsion because the fraction of PG in the oil region is quite low, <5%, at any Φ_I. The percentages of GA and PG in the interfacial region are similar to those in the aqueous region (%GA = 38 and %PG = 50 at Φ_I = 0.005). However, their percentages in the interfacial region increase rapidly upon increasing Tween 20 so that %GA = 85% and %PG = 90% at Φ_I = 0.04. Note that at any given Φ_I, $\%PG_I > \%GA_I$, in keeping with the higher hydrophobicity of PG compared to GA.

The distribution of GA in 3:7 olive oil emulsions is quite similar to that in corn oil emulsions. Comparison of the results in panels A and B of Figure 6 shows that a change in the nature of the oil (corn to olive) or in the oil to water ratio (1:9 to 3:7) has a limited impact on the distribution of GA because it is essentially insoluble in the oil (P_W^O < 0.03, see above).

The increase in %PG with increasing Φ_I in Figure 6A,B shows that at any Φ_I, $\%PG_I$ (3:7 olive oil) > $\%PG_I$ (1:9 corn oil). In principle, direct comparison of olive oil and corn oil emulsions is incorrect because oil to water ratios are different. However, Figures 6 A−B show that in 3:7 corn oil, $\%PG_I$ = 64% at Φ_I = 0.005 and increases to $\%PG_I$ = 94% at Φ_I = 0.04, whereas in %PG in 1:9 olive oil emulsions, $\%PG_I$ = 50% at Φ_I = 0.005 and $\%PG_I$ = 92% at Φ_I = 0.04. This modest decrease in $\%PG_I$ with increasing Φ_W is consistent with eq 11 and shows that the distribution of AOs depends more on oil/water ratios than the degree of unsaturation of the oil. Neither OG nor LG is water-soluble, and they distribute primarily between the oil and interfacial regions of the emulsions. The large differences in the measured P_O^I values between PG and OG and LG (Table 1) are reflected in their remarkable difference in their distributions. Results in Figure 6C show that large fractions of the hydrophobic antioxidants are located in the oil region, %LG = 84% and %OG = 76% at Φ_I = 0.004, and decrease to %LG = 39% and %OG = 30% at Φ_I = 0.04. As noted above, both OG and LG are oil-soluble and insoluble in water, consistent with these observations. Similar results were found for the distribution of OG and LG in olive oil emulsions (Figure 6D), although the fraction of antioxidants in the interfacial region of the olive oil emulsions seems to be slightly higher than that in corn oil emulsions at the same Φ_I value. Our results are in keeping with those found for rosmarinate alkyl esters by Panya et al., who hypothesized, but did not demonstrate, that a fraction of the long-chain (20 carbon atoms) rosmarinate antioxidants may be located in the oil region.[16]

In summary, our distribution results clearly show that (i) the fraction of antioxidants in the interfacial region of the emulsions does not correlate directly with the hydrophobicity of the antioxidant, as we demonstrated previously,[31] which supports the expectations of some researchers;[8,17,18] and (ii) the fraction of AO in the interfacial region reaches a maximum for AO's of intermediate polarity or intermediate alkyl chain length.

Antioxidant Efficiency. Lipid oxidation in O/W emulsions generally occurs in the proximity of the emulsion droplet interface.[7,49,50] Thus, a change in the fraction of AO in the interfacial region should affect the efficiency of lipid oxidation and should correlate with the maximum in AO distribution as a function of alkyl chain length. To probe this hypothesis, we investigated the antioxidant efficiency of the gallic acid derivatives both in bulk ethanolic solution (where all the antioxidants are soluble) by the DPPH method (see Materials and Methods) and in emulsified oils. The measured EC_{50} values listed in Table 2, for GA, PG, OG, and LG, are essentially constant and almost independent of time for up to 70 min, consistent with published results.[51,52]

Table 2. EC_{50} Values, Defined as Moles of Antioxidant per Mole of DPPH, Obtained at 5, 30, and 70 min

	t = 5 min	t = 30 min	t = 70 min
GA	0.105	0.08	0.07
PG	0.096	0.09	0.08
OG	0.103	0.09	0.09
LG	0.107	0.10	0.09

The results in Table 2 show that scavenging abilities of GA and its alkyl gallates are higher than that of monohydroxide or dihydroxide phenols such as α-tocopherol ($EC_{50} \approx 0.25$), Trolox ($EC_{50} \approx 0.25$), or hydroxytyrosol ($EC_{50} \approx 0.19$).[39,52] Note that the EC_{50} value is the concentration of AO required to reduce the DPPH concentration by 50%, and it values are inversely related to the antioxidant capacity of an AO.[40,41] The scavenging ability of AOs is also related to the number and position of aromatic −OH groups, and AOs bearing only one −OH group are less efficient that those with two or more.[53]

The oxidative stability of 1:9 olive oil emulsions was determined by measuring the formation of the primary oxidation product (conjugated dienes) on the basis of the

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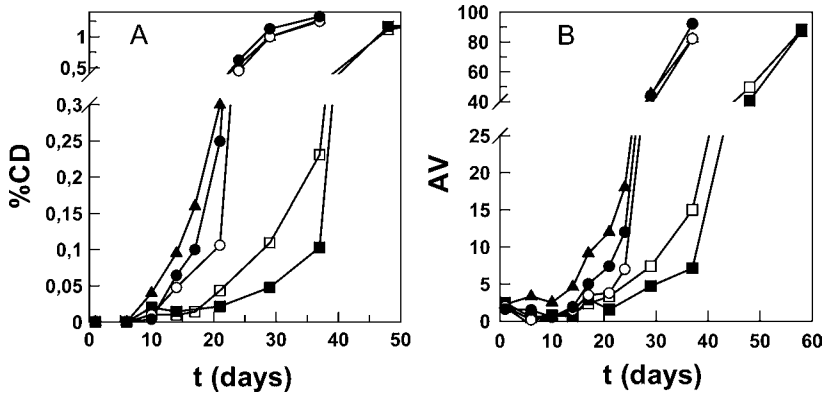

Figure 7. Oxidative stability of 3:7 olive oil emulsions as determined by the time required for the formation of 1% conjugated dienes (%CD) and the *p*-anisidine value (AV): (▲) control; (●) LG; (○) OG; (■) PG; (□) GA. [AO] ≈ 3.3×10^{-4} M, T = 45 °C.

time to reach a conjugated diene content of 1% (Figure 7A) and by the increase in the secondary oxidation products, the *p*-anisidine (AV) values (Figure 7B), as described under Materials and Methods. The order of increasing efficiency in Figure 7 is LG < OG ≪ GA < PG, and it is the same for both methods. GA and PG show similar antioxidant efficiencies in homogeneous solution, but PG is more efficient than GA in emulsions. These results are consistent with literature results for GA and PG in emulsified oils.[54] Results in Figure 7 also show that PG is much more efficient that OG and LG, in keeping with the results of Stöckman et al.,[25] who reported a lower activity of OG compared to that of PG in lecithin-based emulsions. Our results are also consistent with those of Yuji et al.,[55] who found that *p*-hydroxyphenyl acetate (HPA) was a better antioxidant than more nonpolar compounds such as HPA butyrate or HPA laurate in menhaden oil-in-water emulsions.

The efficiency order in emulsified systems in Figure 7 is quite different from that in ethanolic solution.[7,8,15,56] The antioxidant distributions in Figure 6 correlate directly with the antioxidant efficiency order in Figure 7 because (i) the partition constant values were determined in the intact emulsions, that is, they are not distorted by separating the emulsions into oil and water layers prior to measurement;[13,14,22,23,57] (ii) the P_O^I values of OG and LG are much lower than the P_W^I values for GA and PG (Table 1), and this means that, for the same antioxidant concentration, the fraction of OG and PG in the oil region is much higher than that of GA or PG in the water region as shown in Figure 6; (iii) the length and nature of the acyl side chain does not seem to play an important role in the antioxidant activity in ethanolic solution, as expected for homogeneous solution and in keeping with results found for other antioxidants;[58] and (iv) the differences in their antioxidant efficiencies are primarily due to the differential concentrations of the antioxidants in the interfacial region, that is, their partitioning. The similar values of k_I in the interfacial region for the pyrogallol moieties of these antioxidants show that they are located in a similar environment, and this suggests that the rate constants for reaction of antioxidants with the radicals in the interfacial region should also be similar because the length of the alkyl chain has a negligible effect on the antioxidant efficiency against DPPH in homogeneous solution. Finally, lipid oxidation is proposed to start in the interfacial region.[7]

To illustrate the relationship between the measured antioxidant activity order and the distribution of antioxidants, we plotted the fraction of AOs in the interfacial region of olive oil emulsions as a function of Φ_I values for the four AOs (Figure 8). The results show that significant fractions of all four

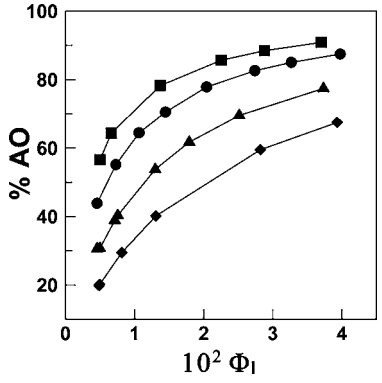

Figure 8. Percent antioxidant in the interfacial region of a 3:7 olive oil/Tween20/acidic water (pH 3.7) emulsion at 25 °C: (■) PG; (●) GA; (▲) OG (◆) LG. Data were extracted from Figure 6.

AOs are located in the interfacial region independent of the HLB of the antioxidant. For a given Φ_I, the percentage of antioxidant in the interfacial region follows the order PG > GA > OG > LG, matching the AO efficiency order found by the Schaal oven test. The results are consistent with those for hydroxytyrosol and hydroxytyrosol acetate antioxidants,[33] the efficiencies of which correlate with their distributions for both AOs in oil-in-water emulsions.

Taken together, our results support a model in which antioxidant efficiency correlates with the fraction of antioxidant in the interfacial region of the emulsion. These results suggest that AOs with intermediate HLBs have the highest concentrations in the interfacial regions of emulsions and should be the most efficient. In addition, within a single family, gallates, ascorbates, etc., the concentrations of the AOs in the interfacial region and their efficiencies should plateau at high emulsifier concentrations.

■ DISCUSSION

Pseudophase kinetic models were originally developed to treat chemical reactivity in homogeneous surfactant solutions such as micelles, microemulsions, and some vesicles. The great simplification of this approach is that because the molecular diffusivities of components are orders of magnitude faster than the observed reaction rates, the totality of the oil, interfacial,

and aqueous regions in solutions of self-assembled surfactants could be treated as separate single-reaction regions. Thus, both thermodynamically stable solutions of microemulsions and kinetically stable emulsions contain the same three reaction regions of oil, water, and surfactant. Reactive components are in dynamic equilibrium between them, such that Figure 2 is equally applicable to both microemulsions and emulsions. The crucial assumption in emulsions is that the distribution of reactants between the totalities of the interfacial and oil regions can be treated as single regions. Consequently, the overall or observed rate of reaction in such systems does not depend on droplet size, but on the total volumes of each region, and the reactant distributions are proportional to the volumes of added oil, surfactant, and water and to their relative solubilities in oil, in water, and in the interfacial regions. To a first approximation, the distribution of an AO in an emulsion depends on its HLB. Assigning HLB values to molecules is only approximate, but it leads naturally to the conclusion that for a series of homologous AOs, the more hydrophilic headgroups require more lipophilic tails to have an intermediate HLB.

In the reactions studied here, 16-ArN_2^+ is located only in the interfacial region. In a homologous series of AOs, the one with the highest concentration or percentage of AO in the interfacial region should be the most efficient because its rate of reaction will be the fastest, other things being equal. More lipophilic AOs and more hydrophilic AOs will have lower fractions in the interfacial region and react more slowly with 16-ArN_2^+. The same logic should hold for the efficiency of an AO in inhibiting peroxidation, and in both cases the measured antioxidant efficiency should go through a maximum with increasing AO hydrophobicity. Maxima have now been observed for a variety of homologous series of AOs.[8,17−20] This interpretation follows naturally from the application of pseudophase models to reactions in emulsions based on dynamic equilibria of components between the oil, interfacial, and aqueous regions in the emulsions, the relative solubilities of the AOs in each region, and the relative volume of the region.

Our results support several important observations. (1) Even at low Φ_I (0.01) values, a significant fraction of GA and PG, >40%, is located in the interfacial region of stripped olive or corn oils, but the amount of hydrophobic antioxidants OG and PG is substantially lower than more water-soluble antioxidants. (2) The %AO in the interfacial region increases with increasing Φ_I such that 60% or more of the AOs are located in the interfacial region at $\Phi_I = 0.04$ and they follow the order %PG > %GA > %OG > %LG. (3) The most efficient antioxidant with the highest fraction in the interfacial region is one of intermediate polarity in which the antioxidant is equally distributed between the oil and water regions. (4) A positive correlation between the antioxidant efficiency order and the fraction of the antioxidants in the interfacial region of the emulsions can be established, in keeping with previous results.[33]

Antioxidants have been added to emulsified foods for years to suppress lipid oxidation, but their effectiveness has remained poorly understood because multiple factors contribute to antioxidant efficiency, for example, oil and antioxidant polarity, emulsifier structure, and the presence of other additives.[7,8] Multiple attempts to correlate an antioxidant's properties with its efficiency have failed for the lack of suitable methods for determining its distribution within the emulsion. The primary difficulty with many attempts is that they depend on the physical separation of the oil and water phases of the emulsion and that this separation destroyed the interfacial region and prevented determination of the concentration of antioxidant in that region.[13,22−25,59]

The application of pseudophase kinetic models based on the distributions of antioxidants as defined by partition constants, to the contrary, permits determining the amount of antioxidant in all three regions. The method also provides an estimate of the second-order rate constant for the reaction in emulsions, and comparison of these rate constants provides an estimate of antioxidant efficiency. Currently, no other method provides reliable estimates of these parameters.[33]

Several researchers investigated the effects of lipophilization on the antioxidant efficiencies of hydroxytyrosol, chlorogenic acid, and rosmarinate antioxidants in different emulsified systems.[17,18,20,58] Each found that the antioxidant efficiency increased to a maximum upon increasing the antioxidant alkyl chain length and then decreased at longer chain lengths. This effect is called "cutoff", which was first coined by Laguerre et al.[17] Several hypotheses were proposed to explain it, but no definitive conclusions were reached because no convincing evidence was found to support one hypothesis or the other. Moreover, the reason for cutoff at different chain lengths for hydroxytyrosol,[20] chlorogenic,[17] rosmarinic,[18] series of homologous antioxidants remains unknown.[8]

All of these hypotheses highlight the importance of the interfacial region in the oxidation process, and each provides a potential explanation on how the length of the hydrocarbon chain affects the antioxidant efficiency and their distribution between the different regions of the emulsion.[8] For example, Lucas et al.[60] hypothesized that efficient antioxidants for oil-in-water emulsions should be, at the same time, good antioxidants and effective surfactants. The polarity and geometry of the polar AO headgroup[60] and its orientation in the interfacial layer may contribute to their effectiveness.[8,17,18,60]

Finally, we stress that our chemical probe method is robust and nondestructive and permits exploration of the parameters affecting antioxidant distribution, for example, the nature of the oil and of the emulsifier, the hydrophilic−lipophilic balance (HLB) of the antioxidant, and factors such as acidity and temperature. Extension of our method to determining the distributions of other antioxidant series should enhance current understanding of how antioxidant structure and physical location within food systems affects its ability to delay and slow lipid oxidation, and the results should provide basic information on the factors controlling antioxidant distributions and efficiencies and permit a more rational selection of antioxidants and emulsifiers in food stabilization.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Four sections covering example kinetic data and the calculation of k_{obs}, relationships between k_{obs} and the partition constants, and equations used to determine the distributions of the AOs in the emulsions, along with pertinent references. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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