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Distribution of Hydroxytyrosol and Hydroxytyrosol Acetate in Olive Oil Emulsions and Their Antioxidant Efficiency

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ABSTRACT: We employed a kinetic method to determine the distributions of the antioxidants hydroxytyrosol (HT) and hydroxytyrosol acetate (HTA) between the oil, aqueous, and interfacial regions of a model food emulsion composed of stripped olive oil, acidic water, and a blend of Tween 80 and Span 80 [hydrophilic–lipophilic balance (HLB) = 8.05] as an emulsifier. HT is oil-insoluble, but HTA is both oil- and water-soluble (partition constant $P_O^W = 0.61$). Results indicate that, at a given emulsifier volume fraction Φ_I, the fraction of HTA in the interfacial region is higher than that of HT. The percentage of both antioxidants increases with an increasing Φ_I, so that % HT > 40% at $\Phi_I = 0.005$ and % HT > 80% at $\Phi_I = 0.04$. HTA appears to be a better antioxidant than HT, as shown by an accelerated oxidative test (Schaal oven method). A correlation between their distribution in the emulsion and their efficiency was established.

KEYWORDS: *Antioxidants, food emulsions, hydroxytyrosol, hydroxytyrosol acetate, pseudo-phase model*

INTRODUCTION

Emulsions form the basis for many kinds of traditional foods, e.g., milk, cream, beverages, dressings, dips, sauces, butters, and deserts.[1−3] They are increasingly being used in nutritional beverages designed to deliver nutrients to infants, the elderly, athletes, or the sick.[3] Lipid oxidation in emulsions is a major food problem because oxidative damage in food causes unpleasant quality changes, such as off-odor and unpalatable flavor development, nutrient degradation, and color changes.[4,5]

The oxidative stability of food emulsions is normally lower than the stability of the corresponding edible bulk oils, giving to these foods a shorter shelf life.[5,6] Food emulsions may possess several native antioxidants for coping with oxidative stresses, but these compounds can be removed or inactivated during food processing operations; therefore, exogenous antioxidants are often added to foods during processing to extend product shelf life.

Establishing reliable criteria for selecting the most efficient antioxidant for a particular application is a major unsolved problem in food emulsions and dispersions[5,7,8] and one of general importance in nutrition and health.[9,10] Selecting the best antioxidant or set of antioxidants for a particular emulsified food application is difficult because multiple factors affect their antioxidant activity, including the types of antioxidant, emulsifier, and oil phase and the distributions of antioxidants within the food emulsions.

Part of the problem to fully understand the behavior of antioxidants in emulsified oils arises from the difficulty in determining their distribution between the different regions of the emulsion.[7,11−20] Component distribution in binary oil–water systems, in the absence of an emulsifier, is usually performed by measuring the concentrations of the antioxidant (or any other analyte) of interest in both the oil and water phases by employing a variety of analytical techniques,[21−23] and it is usually assessed by means of the partition constant between the oil and water phases P_O^W. This methodology, however, cannot be employed in emulsified systems because the emulsifier creates an interfacial region, which is physically impossible to isolate from the oil or aqueous regions. Moreover, in emulsified systems, two partition constants are needed to define the distribution of an antioxidant, one between the oil and interfacial region, P_O^I, and another between the aqueous and interfacial regions, P_W^I (Figure 1).[24,25]

A number of methods were proposed for determining the distributions of antioxidants between the aqueous and oil regions of emulsions based on separation and analysis of each phase, e.g., by centrifugation or ultrafiltration, followed by high-performance liquid chromatography (HPLC) analysis of antioxidant concentrations in each phase. Nevertheless, these methods cannot provide estimates of interfacial concentrations.[15,26−28] Stöckmann and Schwarz used a combination of ultrafiltration and dialysis techniques and a mathematical model to estimate the partitioning of low-molecular-weight phenol derivatives between the oil, interfacial, and aqueous regions of emulsions.[15] However, the ultracentrifugation technique allows for distinction between the aqueous and interfacial regions but not between the interfacial and oil regions.[19,29] Therefore, any determination of the partitioning of the antioxidants needs to be performed in the emulsion itself.

To overcome this problem, we have developed a new approach for determining the distributions of antioxidants in emulsions.[24,25,30−32] Rather than using analytical tools to measure the antioxidant concentrations in each region of the

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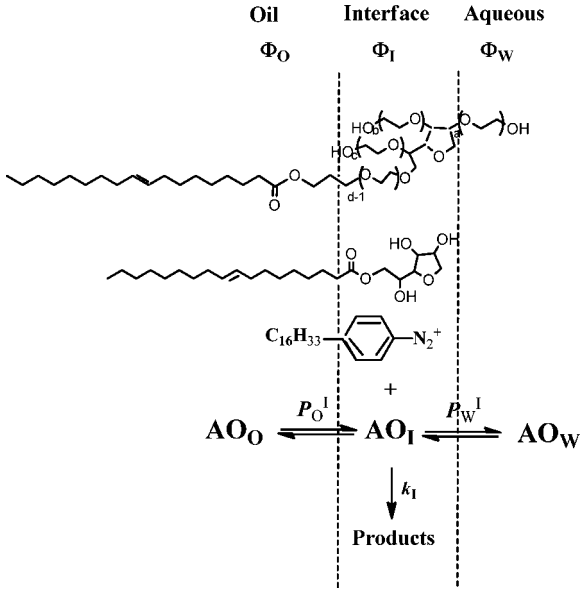

Figure 1. Basic representation of an emulsion showing the aqueous, oil, and interfacial regions, the emulsifiers, the hydrophobic ArN_2^+ ions, and the distribution of an antioxidant (AO). Subscripts O, I, and W indicate the oil, interfacial, and aqueous regions, respectively, and Φ_I, Φ_O, and Φ_W are the surfactant, oil, and water volume fractions, respectively.

emulsion, we focused on developing methods to determine the partition constants between the oil/interfacial, P_O^I, and aqueous/interfacial, P_W^I, regions of the emulsified system. The P_O^I and P_W^I partition constants are determined by employing a kinetic method, which exploits the reaction between a hydrophobic arenediazonium ion with antioxidants.[24,25] The observed rate constant, k_{obs}, values for this reaction depend upon the antioxidant distribution and medium effects, and their relative contributions can be determined by employing the pseudo-phase kinetic model, as shown previously.[24,25] As we will see, once the partition constants are known, determining the distribution of the antioxidant is straightforward.

Here, we have applied this kinetic methodology to determine the distribution of two important olive oil polyphenolic compounds, hydroxytyrosol (HT) and hydroxytyrosol acetate (HTA), in a model food emulsion composed of stripped olive oil, acidic (buffered) water, and a blend of Tween 80 and Span 80 [hydrophilic−lipophilic balance (HLB) = 8.05] emulsifiers. The chemical structures of the antioxidants and surfactants are shown in Figure 2. We also aim to establish the relationships between the antioxidant distribution and their efficiencies, and for this purpose, the emulsions were subjected to an accelerated oxidation test, the Schaal oven test, under standardized conditions. A suitable end-point was chosen to determine appropriate levels of oxidative deterioration.[16]

The Schaal oven test uses relatively mild temperatures; samples are heated at 50−70 °C until they become rancid.[33] The results of this test correlate best with the actual shelf life of the emulsion because, within this range of temperatures, the rate of oxidation is usually exponentially related to the temperature and independent of the oxygen concentration, and therefore, side reactions not relevant to normal storage temperatures, such as polymerization and cyclization, do not occur to a great extent.[34] The assessment of the level of oxidation determined by the peroxide value, conjugated diene content, or carbonyl compound content is therefore more meaningful with oil systems heated at 70 °C or below.[5,33]

Phenolic derivatives constitute an important group of antioxidants that are widely employed because of their biological relevance and their antioxidant activity in food matrices.[35,36] HT is one of the most potent antioxidants found in olives and olive oil, and it is very efficient in preventing oxidation in bulk and emulsified oils.[37−40] HT also shows very interesting biological properties, such as anti-inflammatory and anticancer properties, and it has also been reported to inhibit human low-density lipoprotein oxidation, a critical step in atherosclerosis.[41]

HT is a hydrophilic antioxidant,[33,36,38] and this makes it difficult to incorporate into fats and oils. Lipophilic derivatives of HT can be prepared to explore their antioxidant activity in different oil matrices, including emulsified oils.[37,42,43] The increase in the degree of lipophilicity of the antioxidant might change its partitioning in the emulsified system and, therefore, might have an effect on the oxidative stability of the system by either accumulating at the interfacial or in the oil region. Similar effects have been reported upon lipophilization of several antioxidants.[17,36,37,44]

As we will demonstrate, the percentage of HTA in the interfacial region of the emulsion is higher than that of HT, and this may be related with the fact that HTA is a better antioxidant than HT, as shown by the oxidative experiments.

MATERIALS AND METHODS

HT and HTA were obtained by following a published procedure as described elsewhere.[33] All chemicals 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. Solutions of the coupling agent *N*-(1-

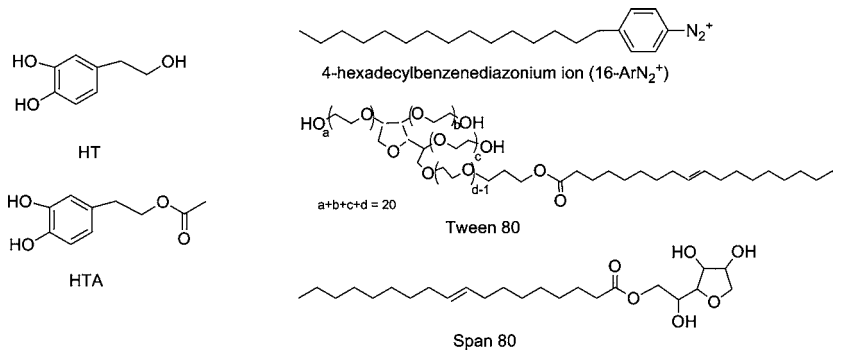

Figure 2. Chemical structures of the antioxidants, probe molecule 16-ArN_2^+, and emulsifiers employed in this work.

naphthyl)ethylenediamine (NED, Aldrich) were prepared in a 50:50 (v/v) BuOH/EtOH mixture to give [NED] = 0.02 M. The acidity of the aqueous phase was controlled using acetic/acetate buffer (0.04 M, pH 3.6). All solutions were prepared with Milli-Q-grade water. 4-Hexadecylbenzenediazonium tetrafluoroborate (16-ArN_2BF_4) was prepared in high yield and purity from commercial 4-hexadecylaniline (Aldrich, 97%) by diazotization following a published method.[25,45]

Olive oil stripped of natural tocopherols and phenols was prepared from commercial virgin olive oil by washing with 0.5 M NaOH solution and passing twice through an aluminum oxide column.[33] Complete removal of tocopherols was confirmed by HPLC according to the International Union of Pure and Applied Chemistry (IUPAC) method 2.432. Details can be found elsewhere.[33]

Stripping bulk or emulsified oils from their antioxidants prior to analyzing their distribution or their activity is a common practice to minimize the effect of endogenous antioxidants. A number of reports indicate that, upon stripping a variety of oils, their fatty acid composition does not change significantly.[46,47] Thus, we do not expect significant changes in the fatty acid composition of the olive oil employed in our experiments.

Emulsion Preparation. Emulsions of different oil/water ratios were prepared by employing stripped olive oil, acidic water (0.04 M acetate buffer, pH 3.6), and a blend of Tween 80 and Span 80 (HLB = 8.05) as an emulsifier. The mixture was stirred at high speed at room temperature with the aid of a Polytronic PT-100 homogeneizer, and the resulting emulsions were visually stable for at least 12−15 h, a time much longer than that required to complete the chemical reaction between 16-ArN_2^+ ions and the antioxidants.

Oxidation Experiments. Oil-in-water emulsions (30%, 33 g) were prepared as above in 100 mL Erlenmeyer flasks. Emulsions were allowed to spontaneously oxidize at 60 °C in the dark. Samples were vortexed every 12 h for 1 min to maintain emulsion physical integrity during the study. Progress of oxidation was monitored by determination of the conjugated dienes (CDs) [American Oil Chemists' Society (AOCS) Official Method Ti 1a-64] and the *p*-anisidine value (AV) (AOCS Official Method Cd 18-90). Isolation of oil from emulsions for analysis was by freezing, thawing, and centrifugation.

For the sake of comparisons, an accelerated method was also conducted. Oxidation was initiated by adding 1.0 mL of an aqueous solution of the radical initiator 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) to 10 mL of oil-in-water emulsions, prepared as above, placed in screw-capped vials (25 mL volume).[33,48] Samples were allowed to oxidize at 60 °C and vortexed every 12 h for 1 min to maintain emulsion physical integrity during the study. The level of oxidation of the emulsion was determined by monitoring the formation of CDs.[16] After homogenization, 0.1 mL of each emulsion was diluted to 10 mL with ethanol and the absorbance at 233 nm was determined with an ultraviolet−visible (UV−vis) spectrometer.

Determination of the Partition Constant of HT and HTA in Binary Stripped Olive Oil−Water Mixtures, P_W^O, in the Absence of the Emulsifier. The partition constants of HT and HTA between stripped olive oil and water, P_W^O, were determined, in the absence of the emulsifier, by employing a modified shake-flask method.[22] For the purpose, a number of 1:1 binary mixtures were prepared by mixing 1 mL of stripped olive oil and 1 mL of a buffered aqueous solution (0.04 M citrate buffer, pH 3.57) containing HT or HTA (4.40 mM), gently shaken and stirred for at least 1 h, and allowed to equilibrate. Phases were then separated at room temperature by centrifugation. An aliquot of the aqueous phase was then analyzed by HPLC, and the concentration of the antioxidant was determined by means of a previously determined calibration curve. The partition constant P_W^O was determined as the ratio of the concentrations of HT and HTA in the oil and water regions by employing eq 1, where $P_W^I = (AO_I)/(AO_W)$ and $P_O^I = (AO_I)/(AO_O)$ and V_W and V_O are the aqueous and oil region volumes, respectively. Parentheses, ( ), indicate concentration in mol/L of the volume of a particular region.[21,25]

$$P_W^O = \frac{P_W^I}{P_O^I} = \frac{(AO_O)}{(AO_W)} = \frac{\%\,AO_O}{\%\,AO_W}\frac{V_W}{V_O} \quad (1)$$

Results show that P_W^O for HT is <0.02, indicating that HT is sparingly soluble in olive oil and that more than 99% of HT is located in the aqueous phase, in keeping with the hydrophilic nature of this antioxidant. The result is very similar to literature data reporting P_W^O = 0.03 at pH 5.5.[33] HTA is both oil- and water-soluble, and a partition constant P_W^O = 0.61 was estimated. Therefore, HTA is less polar than HT and can be considered to be of moderate hydrophobicity.

Determination of k_{obs} Values in Emulsions by Employing a Derivatization Method: Azo Dye Formation. The reactions of arenediazonium ions with alkyl alcohols and some phenols take place, under acidic conditions, through the rate-determining decomposition of a diazoether adduct of the type Ar−N═N−O−R, formed from the reaction with the neutral, monoanionic, or dianionic form of the alcohol.[49,50] The reaction of HT and HTA with the probe molecule 16-ArN_2^+ was initiated by adding an aliquot (16 μL) of a 0.17 M 16-ArN_2^+ stock solution in acetonitrile to a thermostatted emulsion containing a fixed amount of the antioxidant, so that the final [HT] ≈ [HTA] ~ 3×10^{-3} M. The concentration of antioxidant is at least 10 times higher than that of [16-ArN_2^+] (i.e., pseudo-first-order conditions), so that the variation in its concentration throughout the course of the reaction can be considered negligible.

Kinetic data were obtained by employing our derivatization method (azo dye formation) as described in detail elsewhere.[30] This methodology exploits the rapid reaction of ArN_2^+ ions with a suitable coupling agent, such as NED, yielding a stable azo dye (Figure 3),

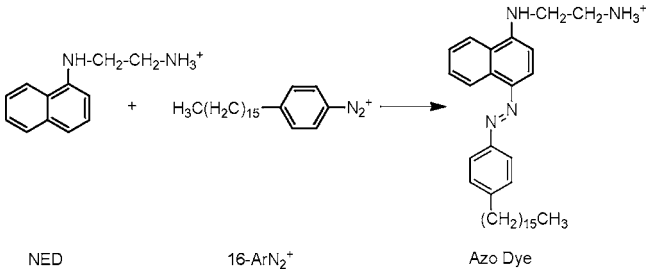

Figure 3. Reaction between the coupling agent NED with 4-hexadecylbenzenediazonium ions yielding a stable azo dye.

whose absorbance can be determined spectrometrically at λ = 572 nm,[19] after dilution with a 50:50 (v/v) BuOH/EtOH mixture that finally yields an optically transparent, homogeneous mixture (Figure 4).

In a typical experiment, a freshly prepared emulsion (10 mL) containing the required amount of antioxidant is placed in a continuously stirred, water-jacketed cell (T = 25 °C) and thermostatted for at least 15 min. Independently, 25 numbered and stoppered test tubes are placed in a thermostatted bath (T = 25 °C). A total of 2.5 mL of a 0.02 M ethanolic solution of NED is added to each test tube and allowed to reach thermal equilibrium for at least 20 min. Once the reaction is initiated, aliquots (200 μL) of the reaction mixture are removed at specific time intervals and added immediately to test tubes to initiate azo dye formation between NED and unreacted 16-ArN_2^+.

Under our experimental conditions, 16-ArN_2^+ reacts with NED much more rapidly than with the antioxidant. Auxiliary experiments showed that the absorbance of the formed azo dye follows Lambert−Beer's law and is a linear function of the concentration of 16-ArN_2^+. Thus, the absorbance of the azo dye at λ = 572 nm is proportional to the concentration of unreacted 16-ArN_2^+, and their variation with time can be used to determine the observed first-order rate constant, k_{obs}. Reactions are monitored for at least $2-3t_{1/2}$. Typical correlation coefficients are >0.995, and duplicate or triplicate experiments gave k_{obs} values within 9%. Values of k_{obs} were obtained by fitting the absorbance versus time data to the integrated first-order rate equation using a nonlinear least-squares method provided by the GraFit 5.0.5 computer program. Details of the method and its limitations can be found elsewhere.[30]

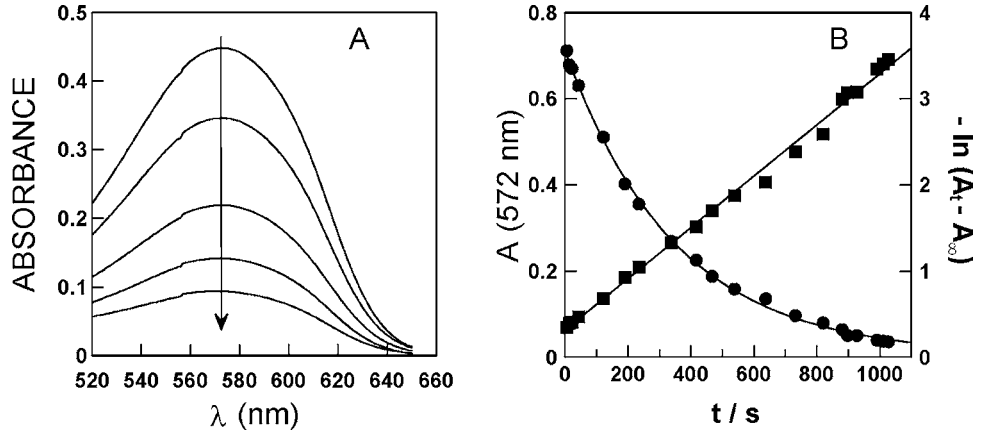

Figure 4. (A) Spectrum of the azo dye (Figure 3) obtained at different reaction times. (B) Variation in absorbance (λ = 572 nm) of the formed azo dye (●) and $\ln[A_t - A_{inf}]$ (■) plots versus time for the reaction of 16-ArN_2^+ with HT in 1:9 (oil-in-water) emulsions at T = 25 °C. Reaction conditions: Φ_I = 0.01 (Tween 80 + Span 80; HLB = 8.05); [16-ArN_2^+] = 2.83 × 10^{-4} M; [HT] = 3.21 × 10^{-3} M; pH 3.57 (0.04 M acetate buffer), and [NED] = 0.02 M.

Connection between the Partition and Observed Rate, k_{obs}, Constants: Determination of Antioxidant Distributions. The conceptual basis of the method is grounded on the pseudo-phase kinetic model for thermodynamically stable microemulsions. Details can be found elsewhere, and only a brief summary will be given here. The basic assumptions are as follows: (i) antioxidant partitioning between the oil, water, and interfacial regions depends upon its relative solubility in each region and not the size and shape of the droplets in the emulsion or the type of emulsion (A/O or O/A), and (ii) the distributions of all components in the emulsion are in dynamic equilibrium; i.e., the rates of transport of the components between the oil, water, and interfacial regions are much faster than that for the chemical reaction.

For a bimolecular reaction in an emulsion, the observed rate, v, is the sum of the rates in each region of the emulsion. Because 16-ArN_2^+ is itself a water- and oil-insoluble ionic surfactant, its concentration in the oil and water regions is negligible, and thus, it is located only in the interfacial region of the emulsions, where it reacts with the antioxidant, as illustrated in Figure 1. Hence, k_{obs} values will depend upon only the rate constant and concentrations of 16-ArN_2^+ and antioxidant in the interfacial region

$$\begin{aligned} v &= k_{obs}[\text{16-ArN}_2^+{}_T] \\ &= k_2[\text{16-ArN}_2^+{}_I][AO_I] \\ &= k_I(\text{16-ArN}_2^+{}_I)(AO_I)\Phi_I \end{aligned} \quad (2)$$

where k_2 and k_I are the observed second-order rate constant and the second-order rate constant in the interfacial region, respectively, square brackets denote the concentration in mol/L of the total emulsion volume, parentheses indicate the concentration in mol/L of the volume of a particular region, subscript T stands for the stoichiometric or total concentration, subscripts O, I, and W indicate the oil, interfacial, and aqueous regions, respectively, and Φ_I is the surfactant volume fraction, defined as $\Phi_I = V_{surfactant}/V_{total}$, which is assumed to be equal to that of the interfacial region.

For those antioxidants whose concentration in the aqueous or oil regions of the emulsions is effectively zero, i.e., for very hydrophilic and very hydrophobic antioxidants, a simplification can be made because only one partition constant is needed to define their distribution. For instance, for very hydrophilic antioxidants (which are oil-insoluble), such as HT (see P_W^O value above) or gallic acid,[45] only the partition constant P_W^I is needed to describe its distribution. Alternatively, for very hydrophobic antioxidants, such as α-tocopherol, only P_O^I is needed.[32] These simplifications are discussed in greater detail elsewhere.[31,32,45]

The final rate expressions for very hydrophilic antioxidants (e.g., HT), eq 3, and for those of moderate hydrophobicity (e.g., HTA), eq 4, were derived previously.[24,25,30,32,45] Equations 3 and 4 describe the dependence of k_{obs} on both the concentration and medium effects, predicting that (a) k_{obs} decreases with an increasing Φ_I because k_I is a constant and [AO_T], Φ_W (volume fraction of water), and Φ_O (volume fraction of oil) are constants in the kinetic experiments, (b) the addition of a surfactant at constant Φ_O/Φ_W increases Φ_I, which increases the value of the denominator and reduces the value of k_{obs}, (c) at very high Φ_I, k_{obs} should approach 0, and (d) the reciprocal forms of eqs 3 and 4, e.g., plots of $1/k_{obs}$ versus Φ_I, should be linear with positive intercepts.

$$k_{obs} = \frac{k_I[AO]_T P_W^I}{\Phi_I P_W^I + \Phi_W} \quad (3)$$

$$k_{obs} = \frac{k_I[AO]_T P_W^I P_O^I}{\Phi_O P_W^I + \Phi_I P_W^I P_O^I + \Phi_W P_O^I} \quad (4)$$

Once the partition constants are estimated, determination of the antioxidant distribution is straightforward. The percentage of the antioxidant in the interfacial region was obtained using eqs 5 and 6 and the calculated values of P_W^I and P_O^I. Similar expressions were employed to obtain the percentage of the antioxidant in the oil and aqueous regions.[24,25,30,32,45]

$$\%\ AO_I = \frac{100\Phi_I P_W^I}{\Phi_I P_W^I + \Phi_W} \quad (5)$$

$$\%\ AO_I = \frac{100\Phi_I P_O^I P_W^I}{\Phi_O P_W^I + \Phi_I P_O^I P_W^I + \Phi_W P_O^I} \quad (6)$$

RESULTS AND DISCUSSION

Figure 5 shows the variation of k_{obs} with the emulsifier volume fraction for both HT and HTA in a 1:9 emulsion. For a given Φ_I, k_{obs}(HT) < k_{obs}(HTA), and for any of the antioxidants, values of k_{obs} decrease asymptotically 3−4-fold on going from Φ_I = 0.005 to 0.045.

The straight lines in Figure 5 are the plots of $1/k_{obs}$ versus Φ_I and were used to calculate the values of P_W^I, P_O^I, and k_I for HT and HTA, and the results are summarized in Table 1. The P_W^I value obtained for HT is similar to that reported for gallic acid in corn oil, $P_W^I \approx 121$,[45] and in olive oil emulsions[51] but lower than that of HTA, as expected from the increase in the lipophilicity upon acylation. The P_O^I value for HTA is higher than that of P_W^I, indicating the tendency of HTA to be located in the interfacial region.

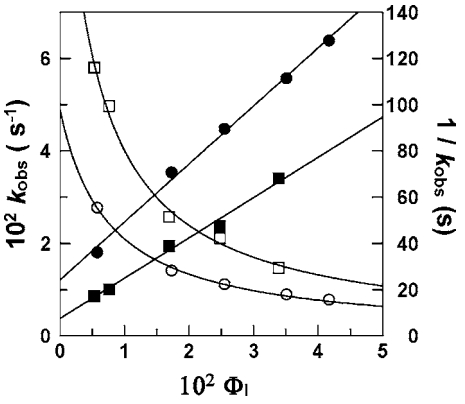

Figure 5. Variation of k_{obs} with Φ_I for the reaction between 16-ArN_2^+ with HT and HTA in a 1:9 stripped olive oil/Tween80−Span80/acidic water (0.04 M acetic acetate buffer, pH 3.57) emulsion. The solid lines were obtained by fitting the experimental data to eqs 3 and 4 or their reciprocal. Experimental conditions: T = 25 °C [16-ArN_2^+] = 3.40 × 10^{-4} M; [HTA] ≈ [HT] ~ 3.3 × 10^{-3} M; and [NED] = 0.02 M.

Table 1. Parameters Obtained by Fitting the Experimental Data in Figure 5 to Equations 3 and 4 Taking into Account the P_W^O Value Obtained in Binary Olive Oil/Water Mixtures in the Absence of an Emulsifier (see the Materials and Methods)

antioxidant	P_W^I	P_O^I	k_I (10^2, $M^{-1}\ s^{-1}$)
HT	120		11.70
HTA	204	331	17.66

The calculated percentages of both compounds displayed in Figure 6 were determined for a 1:9 emulsion from the P_W^I and

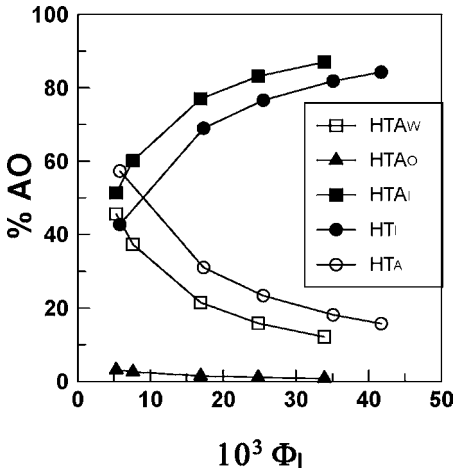

Figure 6. Distribution of HT and HTA between the oil (O), aqueous (W), and interfacial (I) regions of olive oil emulsions.

P_O^I values in Table 1 by employing eqs 5 and 6 and those for the aqueous and oil regions (not shown). Results indicate that a large fraction of the antioxidants, >40%, is located in the interfacial region of the emulsion at the lowest surfactant volume fraction employed, of Φ_I = 0.005. At a fixed Φ_I, % HT < % HTA and the percentage of both antioxidants increases upon increasing Φ_I, so that more than 80% of the antioxidants is located in the interfacial region at Φ_I = 0.05. The percentage of HTA in the oil region is very low, <10%, at any Φ_I.

At the emulsifier concentrations used in this study, the formation of micelles in the water phase cannot be completely ruled out because the interfacial layer saturation threshold may be reached. We do not think the potential presence of micelles in our emulsions affects our partitioning results. Note that our approach is based on the pseudo-phase kinetic model, which was originally developed for micellar and microemulsion solutions, and the basic requirement is the transfer rates of reactants and surfactant between oil, aqueous, and interfacial regions in emulsions to be fast compared to the rate of reaction, in this case, the reaction of 16-ArN_2^+ with the antioxidant. Thus, if micelles do exit (which is possible), the distribution of reactants between all aggregates is in dynamic equilibrium and the measured rate constant is the average value for the reaction in all droplets. Moreover, if micelles are present, they should solubilize a portion of the oil (i.e., swollen micelles) and their interfacial regions should have properties as a reaction medium that are very similar to that of the interfacial region of the large emulsion droplets. That is, the rate constant for the reaction in micelles or in emulsion droplets should be very similar.[24,25,30−32,45]

The oxidative stability of the emulsions was studied at T = 60 °C in the presence and absence of the radical initiator AAPH (Figure 7). For this purpose, two emulsions with Φ_I = 0.005 and 0.01 were employed in each experiment. These values were chosen because, according to the partitioning experiments (Figure 6), the variation in the percentage of the antioxidant in the interfacial region with Φ_I is higher than that at higher Φ_I values. The degree of oxidation over time was monitored by measuring the content of CDs in both experiments (with and without the radical initiator) and the AV in only the experiments without the radical initiator. Emulsions with no added antioxidant were used as controls.

In the absence of radical initiators (panels A and B of Figure 7), only HTA significantly delayed the oxidation of the emulsion at Φ = 0.005. However, at Φ = 0.01, both antioxidants significantly delay the oxidation, showing that the actual delay depends upon the emulsifier volume fraction employed in the preparation of the emulsion, with HTA working as a better antioxidant than HT. When oxidation is accelerated by the addition of the radical initiator AAPH, both antioxidants significantly delay the oxidation of the oil, with HTA working as a better antioxidant than HT. Their efficiency in inhibiting lipid oxidation strongly depends upon the emulsifier volume fraction employed in the preparation of the emulsion.

This order of activity is different from that found in bulk oils. According to literature reports,[33,52] HT has a higher radical scavenging capacity against the DPPH radical than HTA but a similar radical scavenging capacity against the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical. The oxidative results obtained here, therefore, seem to be correlated with the location of the antioxidants in the emulsion, showing the importance of antioxidant distribution for its activity.

Antioxidants have been added for years to control rancidity[9,10,53,54] and are widely used for better food control, and it is currently recognized that several factors affect their effectiveness. It is well-recognized that oxidation of the oil starts in the interfacial region of the emulsion,[5] and the distribution studies indicate that the fraction of HTA in that region is higher than that of HT, and thus, it appears that there is a positive correlation between the fraction of antioxidant in the interfacial region and its ability to minimize lipid peroxidation. Up to our knowledge, this is the first time that such a correlation is

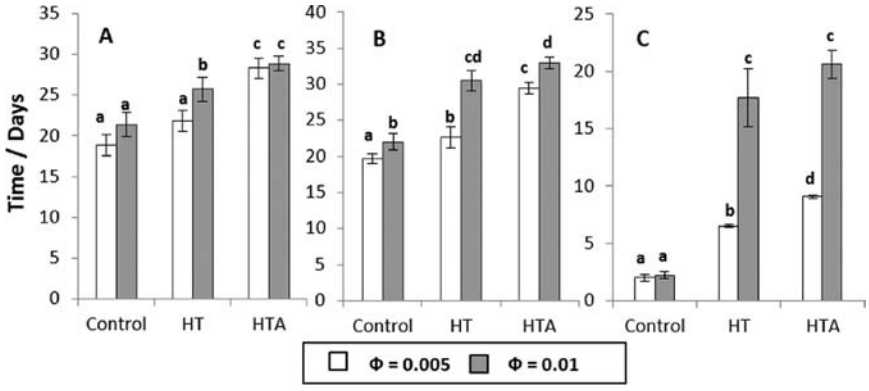

Figure 7. (A and C) Time for samples to reach a ΔCD = 0.6% (values are related to the initial CD content of the oil) and (B) Time for samples to reach (Δ)AV of 20 at 60 °C for the investigated antioxidants at two different emulsifier volume fractions. (A and B) Study without AAPH. (C) Study with AAPH. Mean (error bars represent the standard deviation) of triplicate stored samples. Letters in each graph indicate samples that are significantly different ($p < 0.05$).

established, and application of the methodology to other antioxidants is promising because it may provide new insights to better understand the complex problem of lipid oxidation.

Our results also highlight the importance of determining the percentage of the antioxidants in the interfacial region of the emulsions and the surfactant volume fraction for correlating the chemical/oxidative stability of emulsions. Prior investigations point out that the emulsifier concentration is the main factor controlling the percentage of the antioxidants in the interfacial region and, in to a less extent, the temperature or the oil/water ratio employed to prepare the emulsion.[32,45]

Lipophilic or hydrophilic antioxidants in an edible form are usually employed to stabilize a wide variety of oil-enriched foods, and the choice of the better antioxidant for a given system is usually performed under the light of the so-called polar paradox, which rationalizes the apparently paradoxical behavior of antioxidants in different systems after the introduction of the concept of interfacial oxidation.[55] Briefly, the polar paradox, first coined by Porter,[56] states that polar antioxidants are more efficient in bulk oils, whereas nonpolar antioxidants are more effective in emulsified systems. In the last few years, however, several papers appeared in the literature questioning the validity of the polar paradox, suggesting that factors other than the polarity may be involved.[14,17,44] For instance, it has been proposed that the nonlinear effects found when investigating the chain length effects of a number of antioxidants could be interpreted in terms of the antioxidant location within the emulsion.[17,43,44]

Therefore, it seems crucial to develop methods capable of determining the antioxidant distribution within the emulsified system. Extension of our methodology to determine the distribution of different antioxidants and establish a correlation between their distribution and their antioxidant efficiency is warranted and may be, indeed, of great interest, having far-reaching consequences because the results obtained provide basic information to understand the effects of a number of parameters, such as the temperature, acidity, liphopilicity of the antioxidant, nature of the emulsifier and oil, etc., on the antioxidant distribution, allowing for a better understanding of the factors controlling antioxidant distributions and efficiencies and allowing for a better use of antioxidants and emulsifiers in food processing.

In conclusion, the distribution of two important olive oil antioxidants, HT and HTA, between the interfacial, oil, and aqueous regions in a model olive oil/water emulsion has been determined and, for the first time, correlated with their antioxidant efficiency. Results show that a large percentage, >40%, of both HT and HTA are located in the interfacial region of the emulsion, and the percentage increases upon increasing the surfactant volume fraction. Our results indicate that, in olive oil emulsions, HTA is a better antioxidant than HT and this higher efficiency in inhibiting lipid oxidation may be related with the higher percentage of HTA in the interfacial region than HT. Nevertheless, the antioxidant activity of HT and HTA can be improved in food emulsions by choosing the adequate surfactant volume fraction and probably the type of surfactant.

The chemical kinetic method employed here to assess the distribution of HT and HTA in a model food emulsion is a novel, non-destructive methodology that does not require emulsion breakdown to obtain information on the effects of the emulsifier concentration and other relevant parameters on the distribution of antioxidants in emulsified systems. Up to date, no other methodology capable of evaluating the fraction of antioxidants located in the interfacial region of emulsions is available.

Our approach to determine the distribution of antioxidants between the oil, water, and interfacial regions of an emulsion is focused on determining the partition constants of the antioxidants between the oil and interfacial, P^{I}_{O}, and water and interfacial, P^{I}_{W}, regions of the emulsion rather than determining analyte concentrations in each region as in the past.[15,29,57,58] It is quite general and applicable to a variety of antioxidants and experimental conditions, because common antioxidants are expected to react with our chemical proble 16-ArN_2^+, providing the opportunity of estimating their distributions in the emulsion itself and contributing to a better understanding of the oxidation problem in lipid-based foods.

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Notes

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