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ARTICLE

Optimizing the efficiency of antioxidants in emulsions by lipophilization: tuning interfacial concentrations

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Optimization of the efficiency of antioxidants, AOs, in lipid-based emulsions *via* chemical modifications of their reactive moieties is not always possible because of the inherent experimental difficulties and because of the regulatory status of AOs. Esterification of hydrophilic AOs may be a practical, convenient, alternative approach. Here we employed a series of caffeic acid derivatives bearing the same reactive moiety but of different hydrophobicity (alkyl chain lengths of 1 to 16 carbon atoms) to investigate the effects of hydrophobicity on the oxidative stability of stripped corn oil-in-water emulsions. AO efficiency was determined by monitoring the production of primary oxidation products (conjugated dienes) with time and a non-linear, parabolic-like, variation of their efficiency with the number of C atoms in their alkyl chain, with a maximum at the C8 derivative, was found. To rationalize the results, we also determined the distribution of the AOs between the oil, interfacial and aqueous regions of the same emulsions by employing a recently developed kinetic method that provides the partition constants of the AO between the oil-interfacial, P_O^I, and water-interfacial, P_W^I, regions of the intact emulsions. Values of both P_O^I and P_W^I range 180-2000, suggesting that the transfer of the AOs to the interfacial region is spontaneous. Results indicate that the variations of both the percentage of AO in the interfacial region, $\%AO_I$, of the emulsions and the AO efficiency with the number of C atoms in the AO alkyl chain parallel each other with a maxima at the C_8 derivative. Results illustrate an effective and convenient way to control lipid oxidation by modulation of the hydrophobicity (HLB) of the AOs. An increase in the alkyl chain length of the AOs promote their incorporation into the interfacial region of emulsions but only up to a critical chain length, after which a further increase makes their efficiency to decrease as a consequence of the decrease in their $\%AO_I$.

Introduction

Consumer demands for healthy foods lead to the preparation of functional foods containing bioactive compounds such as omega-3, 6 and 9 polyunsaturated fatty acids (PUFAs), which are added as ingredients with well-recognized positive health benefits.[1-4] However, food manufacturers must face numerous challenges during the production, transportation and storage of fortified foods because PUFAs are extremely susceptible to oxidative degradation. This undesirable (radical) reaction leads to the development of off-flavors, loss of nutrients and other bioactive molecules and, eventually, to the formation of potentially toxic compounds, making the foods to be either unsuitable for consumption or rejected by consumers.[5, 6] It is not surprising therefore, the continuous efforts to developing novel methods to control lipid oxidation aiming at retarding the effects of free radicals damage in various food products as well as in the human body. [7-11]

Improvement of the oxidative stability of the lipids can be achieved by several methods including the encapsulation of the lipids to release them under specific conditions, the control of the concentrations of oxygen or radical initiators in the vicinity of the lipids and the addition of antioxidants (AOs). The latter is probably the most effective, convenient and economical method.[12, 13] The efficiency of AOs in inhibiting lipid oxidation depends on several factors including the nature of the AO and their concentration at the reaction site.[7, 11, 14, 15] The molecular structure of phenolic AOs (position and number of hydroxyl or other substituents on their aromatic ring) has a considerable effect on their antioxidant properties.[3, 5] For instance, the efficiency of phenolic AOs increases with the number of hydroxyl groups in their structure, so that the efficiency of phenolic AOs with only one hydroxyl group (e.g., 3- or 4-hydroxybenzoic acids) is much lower than that of AOs bearing two hydroxyl groups, e.g., caffeic (3,4-dihydroxycinnamic) or protocatechuic (3,4-dihydroxybenzoic) acids, and much lower than that of phenolic AOs bearing three hydroxyl groups, e.g, gallic (3,4,5- trihydroxybenzoic) acid. The position of the hydroxyl groups on the aromatic ring is also relevant because strongly affects the stability of the resulting radicals. Phenolic AOs with –OH groups in the *ortho* or *para* positions are much more efficient those with the –OH groups in *meta*. Insertion of methylene (3,4-dihydroxyphenylacetic acid) or ethylene groups (caffeic acid) between the phenyl ring and the carboxylic group also has significant effects on their antioxidant activity as a consequences of

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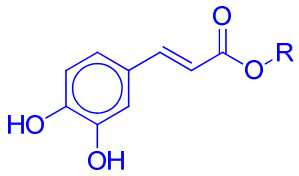
the different resonance stabilization and conformation of the molecules.[16, 17] For example, the esters of caffeic acid and hydroxytyrosol have different reactivity because the restrictions in the rotation of the phenyl group due to the double (caffeic) or single (hydroxytyrosol) bond connecting the phenyl and alkyl groups.

However, chemical modifications of the reactive moieties to modulate their efficiency are not always possible because of the inherent experimental difficulties and because of the regulatory status of AO_s cannot be ignored when selecting antioxidants for use in particular foods. [18, 19] An alternative approach to modulate the AO efficiency is to use of potent, natural, antioxidants and modify their hydrophobicity so that their reactive moiety is maintained but their HLB is chemically modified by insertion of, for instance, alkyl chains of different length.[20] This strategy exploits the changes in their relative solubility in the distinct regions of the emulsions as a consequence of the changes in their hydrophilic-lipophilic balance (HLB) of the AOs, leading to changes in their concentrations at the reaction site (the interfacial region of emulsions).[21, 22] Thus, AO efficiency can be improved by increasing their concentrations at the reaction site while maintaining their antioxidant properties. This approach represents the aim of the present study in which we have investigated the antioxidant efficiency and the distribution of a series of *n*-alkyl caffeates in a model food emulsion composed of corn oil, water and the nonionic surfactant Tween 20.

We chose caffeic acid, CA, because CA attracts more interest than synthetic antioxidants as a consequence of its broad spectrum of biological activities, including antioxidative, antimicrobial and antitumor activities.[23-25] The study was performed in an attempting to define an optimum chain length of the molecules to achieve the maximum antioxidant efficiency by increasing the percentage the AO in the interfacial region of the emulsions. The results reported are the first - to our knowledge – obtained by employing stripped corn oil emulsions and shed some light on the complex structure-activity relationships governing the efficiency of AOs in multiphasic systems. Previous experiments on the effects of the HLB of the emulsifier and of AOs were carried out in different oils (octane, soybean, olive),[21, 26-28] but extrapolation to other oils, different oil-to-water ratios or environmental conditions (pH, T, etc.) cannot be done because the fatty composition of oils is different and the distribution of AOs between the different phases or regions depends on both the differences in solvation of the solutes by solvent molecules or emulsifiers and on the capabilities of solutes of intra- and intermolecular hydrogen bonding with solvent.



R = H, caffeic acid (CA)
Cn, R = $(CH_2)_{n-1}CH_3$, n = 1-16

Scheme 1. Structure of the caffeic acid derivatives employed in this work

The relative importance of each contribution cannot be easily established, and thus the structure-efficiency relationships between need to be determined for each oil and set of homologous AOs under the same experimental conditions. Results are relevant to the food industry because of the economic importance of corn oil–based products and can provide useful information for the design/availability of new AOs radical scavenging properties.[29]

Experimental Section

Materials

Caffeic acid (Across Organics), the emulsifier Tween 20 (polyoxyethylene (20) sorbitan monolaurate, Fluka), stripped corn oil (Across Organics, d = 0.918 g cm^{-3}) were of the highest purity available and used as received. Aqueous buffered (citric acid/citrate 0.04 M, pH = 3.6) solutions were employed in the preparation of emulsions. The chemical probe, 4-n-hexadecylbenzenediazonium tetrafluoroborate, 16-ArN_2BF_4, was prepared in high yield and purity from commercial 4-n-hexadecylaniline (Aldrich 97%) by diazotization following a published method.[14] 2,2 Diphenyl-1-picrylhydrazyl (DPPH●) and reagents employed in the preparation of CA esters (bezaldehyde, Meldrum´s acid, β-alanine and pyridine) were purchased from Acros Organics or Sigma-Aldrich and used as received. Milli-Q grade water (conductivity < 0.1 mS cm^{-1}) was used in all experiments. Thin layer chromatography (TLC) analyses were performed on aluminum silica gel sheets 60 F254 plates (Merck, Darmstadt, Germany) and spots were detected by using a UV lamp at 254 nm after treatment with iodine.

Scheme 2 summarizes the synthetic route employed for the preparation of the CA esters according to published procedure.[21, 30] The C_1–C_{16} derivatives were synthesized from manomalonates through Verley-Doebner modification of Knoevenagel condensation with the 3,4-dihydroxybenzaldehyde. In a typical experiment, Meldrum's acid (0.4 M) was added to a mixture of the corresponding alcohol (0.4 M) and 5 mL toluene in a dry round bottom flask and stirred for 4 hours.

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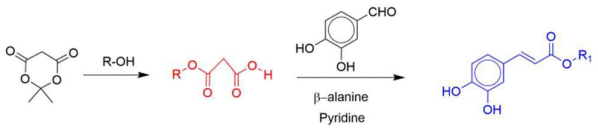

Scheme 2. Synthetic route employed to prepare CA esters of different alkyl chain lengths.

The reaction mixture was allowed to reach room temperature and 10 mL of toluene was added. The mixture was extracted with aqueous 0.6 M Na_2CO_3 (3 x 30 mL). The combined aqueous solution was neutralized with HCl 1 M, was extracted with diethyl ether (3 x 30 mL) and dried over Na_2SO_4. The solvent was evaporated and the obtained product (1g) was added to a mixture of 3,4-dihydroxybenzaldehyde (1g), β-alanina (37mg) and 2.5 mL pyridine in a dry round bottom flask and stirred 24 hours at T = 60ºC. The product was purified by flash column chromatography over silica gel using ethyl acetate/petroleum ether as the eluent (1:1, v:v). Final yields were > 90% for all derivatives. ^{1}H and ^{13}C NMR spectra for CA and its alkyl esters agree with literature results.[30]

Methods

Preparation of Emulsions. Emulsions of different oil to water ratios (4:6 and 1:9 v:v, V_T = 10 mL) were prepared from stripped corn oil, acidic water (0.04 M citrate buffer, pH 3.65) and Tween 20 as emulsifier (0.5 - 4% w/w). Before homogenization, hydrophilic AOs (C_1 - C_3) were added to the aqueous buffer and hydrophobic AOs (≥ C_4) were added to the oil phase. The mixtures were stirred at high speed at room temperature with the aid of a Polytronic PT-1600 homogenizer for 1 min and the resulting emulsions were visually stable for at least 12 hours, a much longer time than that required to complete the chemical reaction between 16-ArN_2^+ and the AOs, which is typically < 30 min for 3-4 half-lives of reaction (see below).

Determination of the antioxidant efficiency in emulsions: oxidative stability. The oxidative stability of food lipids is usually estimated by determining the extent of oxidation under a set of standardized conditions.[5] In previous works, we estimated the efficiency of the AOs by measuring the degree of oxidation from the production of conjugated dienes (CD) with time and here we used the same method because it is a sensitive and reproducible (and identical with the AOCS official standard method[31] Ti-1a-64) for following the early stages of lipid oxidation and for the sake of comparisons with previous results with other AOs and oils.[21] In addition, we have shown in previous work[22, 32] that monitoring primary and secondary products under similar conditions to those used here lead to the same conclusions. No visual phase separation was observed in the emulsions and the small increase constant up to sudden increase in the formation of conjugated dienes suggests that phase separation, if any, does not have a significant effect on the kinetics of the reaction.

4:6 (v:v) oil-in-water emulsions (V_T = 10 mL) were prepared as above in the presence and absence of AOs (final AO concentration of 0.6 mM) and transferred to screw–capped sample vials (25 mL volume). Each sample was vortexed for 1 min and thermostated at 37 ºC by employing an orbital Incubator Heidolph 1000 orbital stirrer equipped with a Heidolph thermostat 1010 to control temperature (±1 ºC). Aliquots (50 µl) of each emulsion were diluted to 10 ml with ethanol, producing a homogeneous solution, and the absorbance at 233 nm was measured.

Determining the partition constant of P_W^O in binary oil-water mixtures in the absence of emulsifier. The partition constants, P_W^O, of CA and its esters in binary oil-water mixtures, equation 1, were determined in binary stripped corn oil–water mixtures in the absence of emulsifier by employing a shake-flask method.[28, 33] For each AO, a number of binary mixtures were prepared by stirring at high speed for one minute a mixture of 4 mL of stripped corn oil and 6 mL of aqueous buffer (0.04 M citrate buffer. pH = 3.65). Caffeic acid and the most polar derivatives, C_1 – C_3, were dissolved in the aqueous solution and the more hydrophobic derivatives, C_4 - C_{16}, were dissolved in the corn oil.

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

The final AO concentration in the total 10 mL emulsion volume was 3.5 mM in all samples. The mixtures were gently shaken for at least 1 minute and allowed to equilibrate at T = (25 ± 1) ºC for at least 24 hours. The two phases were separated by centrifugation, and the concentrations of the AO in the aqueous and in the oil phases were determined spectrometrically (UV-Vis) at λ = 320 nm by interpolation using previously prepared calibration curves. Each P_W^O value is an average of duplicate or triplicate measurements. The results are summarized in **Table 1**. Complete experimental details are published elsewhere[28, 34]

AO	P_W^O	P_W^I	P_O^I	$10^2\ k_I$ ($M^{-1}s^{-1}$)
CA	0.04 ± 0.01	268 ± 10	---	3.52 ± 0.3
C_1	3.3 ± 0.3	747 ± 44	226 ± 25	5.95 ± 0.3
C_2	11 ± 0.4	2000 ± 130	188 ± 13	6.00 ± 0.2
C_3	38 ± 3	---	177 ± 25	5.60 ± 0.1
C_8	651 ± 55	---	513 ± 27	5.30 ± 0.3
C_{16}	1200 ± 150	---	385 ± 1	5.31 ± 0.2

Table 1. Values of the partition constant in binary oil-water systems in the absence of emulsifier, P_W^O, the partition constants between the oil-interfacial, P_O^I, and water-interfacial, P_W^I, regions and the rate constant in the interfacial region, k_I, for CA and alkyl caffeates.

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Determining the partition constants by employing the pseudophase kinetic model: Determination of k_{obs} values for the reaction between 16-ArN_2^+ and the AOs in intact emulsions. The reaction between 16-ArN_2^+ and the CA and its esters was followed spectrometrically by employing the dye derivatization method (azo dye formation) described in detail elsewhere.[28, 32, 34 14] The methodology exploits the rapid reaction of 16-ArN_2^+ ions with a suitable coupling agent such as *N*-(1-naphthyl)ethylenediamine dihydrochloride, NED, yielding an stable azo dye. Its absorbance was measured spectrometrically at λ = 572 nm after dilution with a 50:50 (v:v) BuOH:EtOH mixture that was an optically transparent, homogeneous solution.

In a typical experiment, a freshly prepared emulsion (10 mL) containing the required volume of AO stock solution is placed in a continuously stirred, water-jacketed cell (T = 25 ºC) and thermostated for at least 15 min. Independently, 25 numbered and stoppered test tubes were placed in a thermostated bath (T = 25 ºC) and 2.5 mL of a 0.02 M EtOH–BuOH (50:50 v:v) solution of NED were added to each test tube and allowed to reach thermal equilibrium for at least 20 min. After the reaction is initiated, aliquots (200 µL) of the reaction mixture were removed at specific time intervals and added immediately to test tubes to quench the reaction by initiating azo dye formation between NED and unreacted 16-ArN_2^+. The absorbance of the azo dye at λ = 572 nm is proportional to the concentration of unreacted 16-ArN_2^+ and the decrease in absorbance with time was used to determine the observed first order rate constant, k_{obs}. Values of k_{obs} were obtained by fitting the absorbance *vs* time data to the integrated first order rate equation by using a non-linear least squares method provided by the GraFit 5.0.5 computer program.

Figure 1 shows examples of absorbance versus time plots for azo dye formation and the fitting curves to the integrated and linearized first-order equations for the reactions of 16-ArN_2^+ with the C_1 and C_{16} derivatives in 4:6 corn oil-in-water emulsions. The excellent fits of the (A, t) data pairs to the first-order kinetic equation demonstrate that any changes in droplet size are not kinetically significant.[14, 22, 32] The results also show that diffusion of reactants is not rate-limiting because if it was, we would not get good first order kinetics.[35]

Kinetic analyses of data: application of the pseudophase kinetic model to determine the interfacial rate constants and the partition constants of the AOs between the oil-interfacial and aqueous-interfacial regions of the emulsions. Addition of a surfactant to produce a kinetically stable emulsion creates a new interfacial region between the oil and water regions, and the distribution of AOs is now described by two partition constants, **Scheme 3,** that between the aqueous and interfacial regions, P_W^I, and that between the oil and interfacial regions, P_O^I, which are defined by equations 2 and 3, respectively. Note that the value of the ratio P_W^I / P_O^I is equal to that of the partition constant between the oil and water in the absence of surfactant, P_W^O, as shown in equation 4.

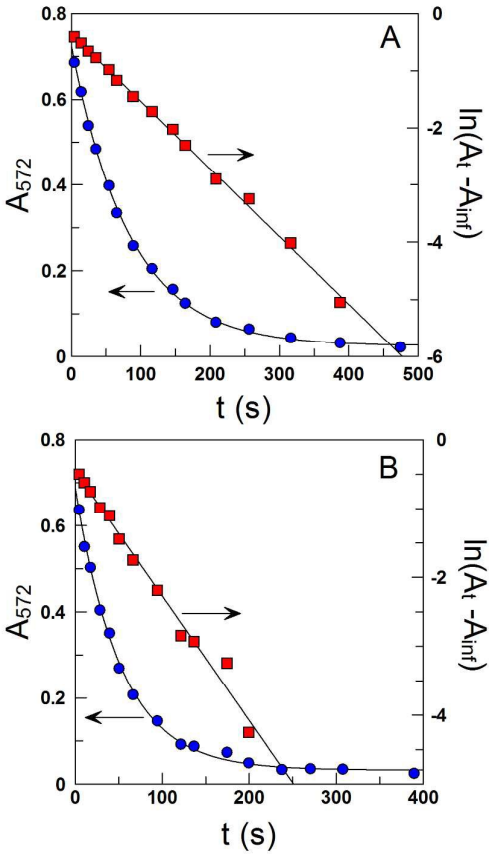

Figure 1. Typical kinetic plots obtained for the reaction of 16-ArN_2^+ with the C_1 (A) and C_{16} (B) CA derivatives in 4:6 corn oil-in-water emulsions composed of acidic water (citric/citrate buffer, 0.04 M, pH = 3.65) and Tween 20 (Φ_I = 0.0284), [16-ArN_2^+] ≈ 2.9 x 10^{-4} M, [AO] = 3 x 10^{-3} M, T = 25 ºC.

$$P_W^I = \frac{(AO_I)}{(AO_W)} \quad (2)$$

$$P_O^I = \frac{(AO_I)}{(AO_O)} \quad (3)$$

$$\frac{P_W^I}{P_O^I} = \frac{(AO_I)/(AO_W)}{(AO_I)/(AO_O)} = \frac{(AO_O)}{(AO_W)} = P_W^O \quad (4)$$

In pseudophase models emulsions are divided into three reaction regions, the oil, interfacial and aqueous regions, **Scheme 3**. The volume fraction of each region is determined by the volumes of added oil, V_O, surfactant, V_I, and aqueous solution, V_W, and is defined as the ratio between the volume of the region and the total volume, e.g., $\Phi_I = V_I / (V_W + V_I + V_O)$.

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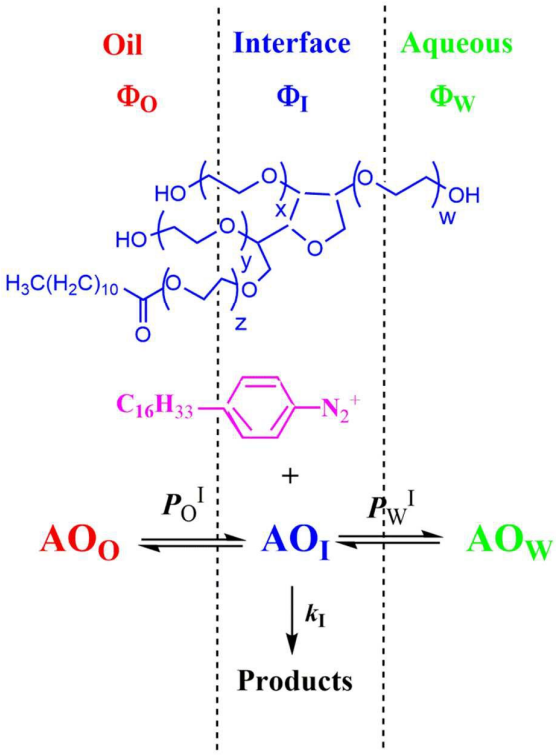

Scheme 3. Conceptual division of an emulsion droplet showing the oil (O), interfacial (I) and aqueous (W) regions. The distribution of an AO between the regions is defined by the partition constants P_O^I and P_W^I, Φ stands for the volume fraction of a region and k_I is the rate constant for reaction between 16-ArN_2^+ and the AO in the interfacial region.

No visual breakdown of the emulsions was observed for at least 6 hours, a time much higher than that required to complete the reaction between 16-ArN_2^+ and AOs ($t_{1/2}$ = 2-30 min). After bulk mixing is complete, the distributions of reactants are determined by their relative solubilities in the oil, interfacial and aqueous regions. 16-ArN_2^+ is located only in the interfacial region and oriented with its tail in the hydrophobic core and its headgroup in the interfacial region in contact with water,[11, 14] where it reacts with the AO, **Scheme 3.** The chemical reaction does not significantly perturb the distribution of the reactants because both reactants and emulsion components are in dynamic equilibrium, and because the surfactant and AO concentrations are in large excess with respect to that of 16-ArN_2^+. Thus, overall rate of the bimolecular reaction, v, in the interfacial region of an emulsion, **Scheme 3**, depends only on the measured (or observed) rate constant, k_{obs}, and on the concentrations of 16-ArN_2^+ and antioxidant, AO, in the interfacial region, equation 5, where square brackets, [ ], denote the concentration in molarity of total emulsion volume, parenthesis, e.g., (AO_I), stand for concentrations in moles per liter of interfacial volume; k_I ($M^{-1}s^{-1}$) is the second order interfacial rate constant and Φ_I the volume fraction of the interfacial region.

$$\mathrm{v} = k_{\mathrm{obs}}[16\text{-}\mathrm{ArN}_{2\mathrm{T}}^{+}] = k_2[16-\mathrm{ArN}_{2\mathrm{T}}^{+}][\mathrm{AO}_\mathrm{T}] = \\ = k_\mathrm{I}(16-\mathrm{ArN}_{2\mathrm{I}}^{+})(\mathrm{AO}_\mathrm{I})\Phi_\mathrm{I} \quad (5)$$

Equations 2 and 3 are combined with the relevant mass balance equation to give the relationship between k_{obs} and partition constant values, equation 6,[11] where subscripts W, O, and I stand for water, oil and interfacial regions. The mathematical treatment can be simplified for very hydrophilic and very hydrophobic AOs. For AOs that are oil insoluble, i.e., that distribute between the aqueous and interfacial region only the partition constant P_W^I is needed to define its distribution and the derivation of equation 6 simplifies to give equation 7.[11, 14] For water insoluble AOs, i.e., that distribute between the oil and interfacial regions, only the partition constant P_O^I is needed and equation 6 simplifies to equation 8.

$$k_{obs} = \frac{[\mathrm{AO}_\mathrm{T}]k_\mathrm{I}P_\mathrm{W}^\mathrm{I}P_\mathrm{O}^\mathrm{I}}{\Phi_\mathrm{O}P_\mathrm{W}^\mathrm{I}+\Phi_\mathrm{I}P_\mathrm{W}^\mathrm{I}P_\mathrm{O}^\mathrm{I}+\Phi_\mathrm{W}P_\mathrm{O}^\mathrm{I}} \quad (6)$$

$$k_{\mathrm{obs}} = \frac{k_\mathrm{I}[\mathrm{AO}]_\mathrm{T}P_\mathrm{W}^\mathrm{I}}{\Phi_\mathrm{I}P_\mathrm{W}^\mathrm{I}+\Phi_\mathrm{W}} \quad (7)$$

$$k_{\mathrm{obs}} = \frac{k_\mathrm{I}[\mathrm{AO}]_\mathrm{T}P_\mathrm{O}^\mathrm{I}}{\Phi_\mathrm{I}P_\mathrm{O}^\mathrm{I}+\Phi_\mathrm{O}} \quad (8)$$

Equations 6-8 predict that k_{obs} values should decrease asymptotically with increasing Φ_I. Equation 9 has the same form as the reciprocal of equation 6, where the parameters *a* and *b* are given by equations 10 and 11, respectively. Equations 12 and 13 are the reciprocals of equations 7 and 8, respectively, and they predict that plots of $1/k_{obs}$ *vs* Φ_I should be linear with positive intercepts. Equations 9, 12 and 13 were employed to calculate the partition constants P_O^I and P_W^I and the rate constant k_I in the interfacial region as described elsewhere.[11, 14]

$$\frac{1}{k_{obs}} = \frac{1}{a} + \frac{b}{a}\Phi_I \quad (9)$$

$$a = \frac{[\mathrm{AO}_\mathrm{T}]k_\mathrm{I}P_\mathrm{W}^\mathrm{I}P_\mathrm{O}^\mathrm{I}\left(1+\Phi_\mathrm{W}/\Phi_\mathrm{O}\right)}{P_\mathrm{W}^\mathrm{I}+\Phi_\mathrm{W}/\Phi_\mathrm{O}\,P_\mathrm{O}^\mathrm{I}} \quad (10)$$

$$b = \frac{P_W^I P_O^I\left(1+\Phi_W/\Phi_O\right)}{P_W^I+\Phi_W/\Phi_O\,P_O^I} - 1 \quad (11)$$

$$\frac{1}{k_{obs}} = \frac{\Phi_\mathrm{W}}{k_\mathrm{I}[\mathrm{AO}_\mathrm{T}]P_\mathrm{W}^\mathrm{I}} + \frac{1}{k_\mathrm{I}[\mathrm{AO}_\mathrm{T}]}\Phi_\mathrm{I} \quad (12)$$

$$\frac{1}{k_{\mathrm{obs}}} = \frac{\Phi_\mathrm{O}}{k_\mathrm{I}[\mathrm{AO}]_\mathrm{T}\mathrm{P}_\mathrm{O}^\mathrm{I}} + \frac{1}{k_\mathrm{I}[\mathrm{AO}]_\mathrm{T}}\Phi_\mathrm{I} \quad (13)$$

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Once the partition constants are known, the distribution of the antioxidant can be determined as the percentage of the antioxidant in each region. Equations 14-16 were employed to calculate the fraction of AO in the interfacial region for AOs that distributes between the three regions and for those that distribute between the aqueous-interfacial and oil-interfacial regions, respectively. Similar equations used to calculate the percentage of AO in the oil and aqueous regions were derived previously and are published elsewhere.[11, 14]

$$\%AO_I = \frac{100\Phi_I P_W^I P_O^I}{\Phi_O P_W^I + \Phi_I P_W^I P_O^I + \Phi_W P_O^I} \quad (14)$$

$$\%AO_I = \frac{100\Phi_I P_W^I}{\Phi_I P_W^I + \Phi_W} \quad (15)$$

$$\%AO_I = \frac{100\Phi_I P_O^I}{\Phi_I P_O^I + \Phi_O} \quad (16)$$

Statistical Analysis. Duplicate or triplicate kinetic experiments were run for 2-3 $t_{1/2}$. The k_{obs} values were within ± 7 – 9 % with typical correlation coefficients of > 0.995. Reported partition constants in binary oil-water systems are the average of 6 - 8 replicates. The Dixon´s Q-test was employed in deciding whether to accept or reject the datum before calculating the average of the set of replicates. All oxidation experiments were

run in triplicate and SPSS 21.0 software was used for statistical analysis by one-way analysis of variance (ANOVA, with Tukey's HSD multiple comparison) with the level of significance set at $P < 0.05$. Data are presented as mean values ± standard deviation.

Results

Effects of AO chain length on the reactivity with radicals: DPPH• radical scavenging activity and EC_{50} values for CA esters. The procedure for determining EC_{50} values as a function of AO chain length has been published in detail.[21, 32] The rate of disappearance of DPPH• from reaction with each AO was monitored at 515 nm by using a Powerwave XS Microplate Reader (Bio-Tek Instruments, Inc) thermostated at T = 25.0 ± 0.1 °C. The wells of a 96-well microplate contained a methanolic solution of AO (3-20 μM) and 80 μM DPPH•. The absorbance of each well was recorded at one min intervals for a 60 min period. The absorbance of each solution was subtracted from the blank (80 μM DPPH• solution without AO).

The value of EC_{50} for each AO was obtained in a two-step process. First, the DPPH• absorbance as a function of time (up to 60 minutes) was plotted for six mole AO/mole DPPH• ratios between 3-20 μL of AO, **Figure 2A**. The percentage of the remaining DPPH• at a certain time of reaction was determined as %DPPH• = 100[DPPH• (time = t)/ DPPH• (time = t_o)]. Second, the %DPPH• was plotted against the mole AO/mol DPPH• ratio at selected times of reaction (5, 15, 30 and 60 min), **Figure 2B**. On these plots, the value of EC_{50} is equal to the value the intersection of the line at %DPPH• = 50%. Representative plots are shown below. Each sets of experiments was performed in quadruplicate. The EC_{50} values given in **Table 2** are the average of these repetitions. Note that the variations in EC_{50} times are small and virtually independent of AO chain length.

Effects of the alkyl chain length on the oxidative stability of corn oil-in-water emulsions. The relative efficiency of CA and its esters in 4:6 (v:v) corn oil-in-water emulsions was determined by monitoring the formation of the primary oxidation products (conjugated dienes) with time until a suitable end point is reached. We run the experiments at low temperature (T = 37 ºC) to minimize hydroperoxide side reactions (thermal decomposition) that may difficult the interpretation of the results. **Figure 3A** illustrates a typical kinetic plot obtained by monitoring the formation of conjugated dienes, CDs, in corn oil-in-water emulsions showing the initiation (very slow increase in the formation of CDs), propagation (much faster increase where the scavenging antioxidant has been consumed and does not inhibit the reaction) and termination steps of the radical reaction. The oxidation level defined as the time required to increase the content of conjugated dienes in 0.6% , a time higher than that necessary to reach the propagation step both in the presence and absence (control experiments) of AOs. All runs were in triplicate. The uniformity of the induction period for different AOs indicates minimal effects of phase separation on the kinetics of oxidation because, otherwise, the values should have shown more scatter.

Figure 3B shows that the variation of AO efficiencies at 0.6% diene formation goes through a maximum at C_8 followed by a decrease. The variation illustrated in **Figure 3B** is qualitatively similar to the maxima reported by ourselves[11, 33] in emulsions prepared with other oils and by Laguerre and others when employing different AOs.[36-39] This behavior is known as the "cut-off effect", indicating a non-linear dependence of the various chemical or biological activities of homologous series of reactants with the length of their alkyl, reaching a maximum at a critical chain length after which their activity decreases.

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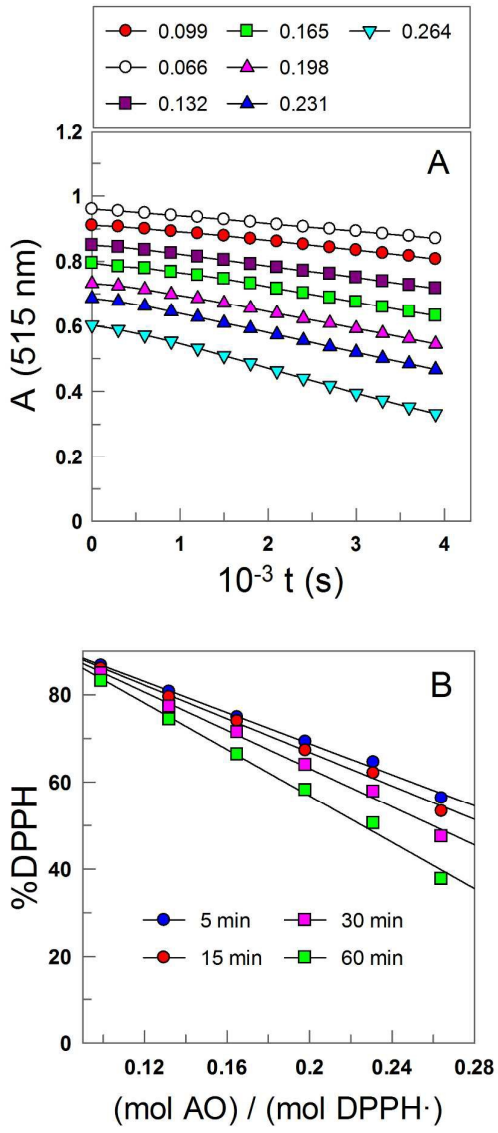

Figure 2. A) Illustrative time course for the change in DPPH$^{\bullet}$ absorbance, (initial DPPH$^{\bullet}$] = 100 μM) with different concentrations of propyl caffeate (C_3) expressed as mole ratios of C_3 to DPPH$^{\bullet}$. [C3] = 0.047 – 0.282 M ([C3] ~ 5% to 25% of [mole AO/mole DPPH$^{\bullet}$). **B)** Linear variation of the percentage of initial DPPH radical with the mole ratio of AO to DPPH$^{\bullet}$ at selected times of reaction. The EC_{50} values obtained at each time interval are listed in **Table 2**.

Table 2. EC_{50} (mole AO / mole DPPH$^{\bullet}$) values for CA derivatives obtained at different reaction times with a level of significance P < 0.05. [a]Standard deviation (n = 4).

AO	$EC_{50} \pm \sigma^a$			
t (min)	5	15	30	60
CA	0.330 ± 0.005	0.329 ± 0.001	0.334 ± 0.002	0.344 ± 0.005
C_1	0.308 ± 0.012	0.290 ± 0.008	0.262 ± 0.005	0.222 ± 0.010
C_2	0.323 ± 0.005	0.277 ± 0.005	0.263 ± 0.003	0.233 ± 0.006
C_3	0.322 ± 0.002	0.291 ± 0.006	0.260 ± 0.017	0.225 ± 0.001
C_4	0.310 ± 0.006	0.293 ± 0.009	0.259 ± 0.005	0.216 ± 0.006
C_6	0.315 ± 0.005	0.262 ± 0.008	0.243 ± 0.006	0.198 ± 0.007
C_8	0.293 ± 0.012	0.269 ± 0.013	0.239 ± 0.005	0.199 ± 0.005
C_{10}	0.333 ± 0.003	0.285 ± 0.003	0.254 ± 0.009	0.212 ± 0.008
C_{12}	0.315 ± 0.004	0.294 ± 0.007	0.266 ± 0.006	0.211 ± 0.002
C_{14}	0.317 ± 0.008	0.298 ± 0.001	0.257 ± 0.003	0.199 ± 0.005
C_{16}	0.314 ± 0.003	0.282 ± 0.007	0.244 ± 0.001	0.196 ± 0.006

The large variations in antioxidant efficiency in emulsions cannot be attributed to changes in the length of the alkyl chain of the AOs (HLB) because we previously demonstrated the independence of the rate constant of the reaction of AOs with the free radical DPPH (no statistically significant differences in the EC_{50} values were detected , see **Table 2**). The DPPH$^{\bullet}$ test measures the H radical/electron-transferring ability of an antioxidant versus the stable free radical DPPH and it gives a rough index of the scavenging activity of the AO_S because it is carried out in homogeneous solution, and the AOs bear the same reactive phenolic moiety both in homogeneous solution and in emulsions.

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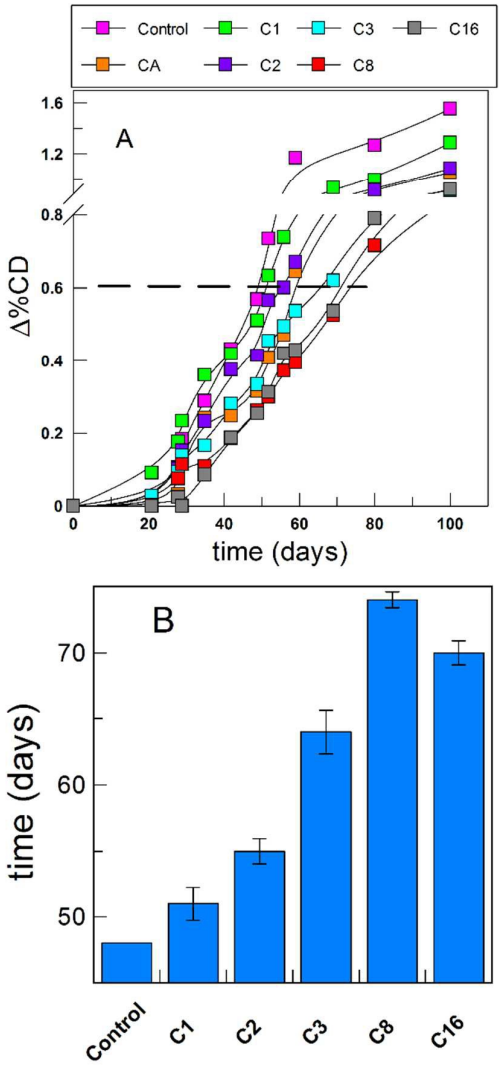

Figure 3. A) Illustrative kinetic plot for the oxidation of the corn lipids at T = 37ºC (1% emulsifier volume fraction), showing the initiation, propagation and termination steps. B) Variation of the time necessary increase the percentage of conjugated dienes 0.6% as a function of the alkyl chain length of the caffeic acid derivatives.

Thus, we hypothesized as in previous works that the observed differential rate of the reaction with the lipidic radicals, **Figure 3B**, can be caused by a differential partitioning of the AO_S between the different regions of the emulsion caused by the variation in their hydrophobicity and analyzed the effects of the alkyl chain length on their distribution within the emulsion.

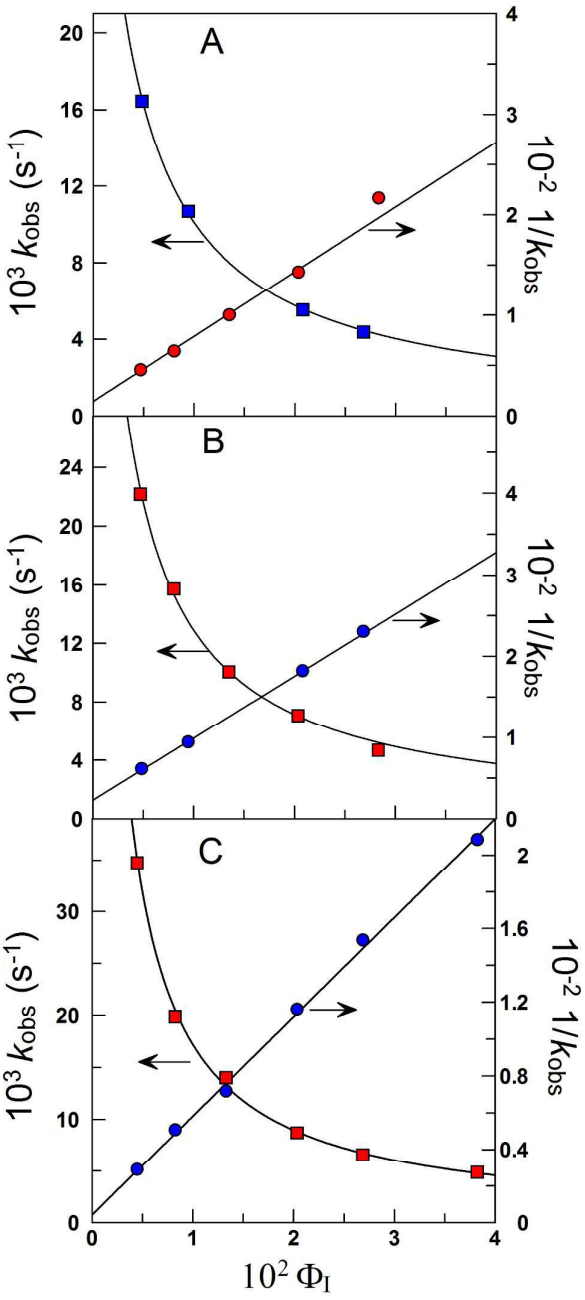

Figure 4. Illustrative variations of k_{obs} with Φ_I for the reaction of 16-ArN_2^+ with CA in 1:9 (A) and 4:6 (B) corn oil emulsions and with octyl caffeate(C) in 4:6 emulsions (citric citrate (0.04 M) buffer , pH 3.6). [CA] = $4.4x10^{-3}$ M, [C_8] = $3.5x10^{-3}$ M, [16-ArN_2^+] = $2,8x10^{-4}$ M, T = 25 ºC.

Effects of alkyl chain length on the distribution of CA and its alkyl esters in corn oil emulsions

The distribution of AOs in emulsions is defined by the partition constants between the oil-interfacial, P_O^I, and that between the

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aqueous-interfacial, P_W^I, regions, equations 2 and 3, respectively. For hydrophilic antioxidants such as caffeic acid, which is oil

insoluble, only the partition constant P_W^I is needed to describe their distributions, meanwhile for very hydrophobic AOs such as the C12 or C16 alkyl derivatives only P_O^I is needed. These simplifications are discussed in greater detail elsewhere.[34, 40, 41]

The partition constants P_O^I and P_W^I, as well as the interfacial rate constant k_I were determined in the intact emulsions by employing the method described before. **Figure 4** is illustrative and shows the variations of k_{obs} for the reaction of 16-ArN_2^+ with CA and C_8 in 1:9 (CA) and 4:6 (CA, C_8) stripped corn oil/Tween 20/water emulsions.

The excellent fit of the (k_{obs} , Φ_I and $1/k_{obs}$, Φ) data sets, **Figure 4**, demonstrate that the assumptions of the pseudophase model are met. Values of k_{obs} decrease asymptotically and $1/k_{obs}$ increase linearly with increasing Φ_I at constant AO concentration and the 6-9 fold decreases in k_{obs} with increasing Φ_I are similar to those found for other AOs.[14, 28, 34] Values of k_{obs} decrease asymptotically on going from Φ_I = 0.005 to Φ_I = 0.05, consistent with the predictions of equations 7 and 8. The straight lines in **Figures 4A** and **B** are plots of $1/k_{obs}$ *vs* Φ_I, equations 12 - 13, from where the partition constants and the k_I values displayed in **Table 1** were determined.

Changes in the oil to water (o:w) ratio from 1:9 to 4:6 (CA) leads to differences in the partition constants P_W^I and P_O^I less than 20%. This low dependence of the ratio o:w on the partition constants was previously observed for hydrophilic and hydrophobic AOs, and for this reason we only employed 4:6 (o:w) emulsions to investigate the distribution of AOs of moderate and high hydrophobicity.

k_I is the rate constant of the reaction of 16-ArN_2^+ with AOs, which takes place in the interfacial region of the emulsion, Scheme 3. k_I values are not needed to assess the distribution of AOs, however, their values are important because changes in k_I values may detect changes in the reaction mechanism. For example, the variation of k_I with temperature provides the activation parameters for the reaction between 16-ArN_2^+ and AOs.[42] Changes in k_I with acidity may also allow to determining whether the reactive species is the anionic, dianionic or neutral form of the AO.[41, 43] In addition, comparison of k_I values for a number of AOs could be used as a basis for assessing a scale of AO activity that is independent of the AO distribution in the emulsion because the same chemical probe (16-ArN_2^+) is used in all distribution experiments.[14]

The positive P_W^I and P_O^I values in Table 1 **(**ranging 177-2000) indicate that the transfer process of the AOs from the oil or aqueous to the interfacial region is spontaneous (Gibss free energy negative) highlighting the natural tendency of these AOs to be mainly located in the interfacial region.

P_W^I values increase with increasing the alkyl chain length (i.e., the HLB of the AO) in keeping with the hydrophobnic effect. Comparisons of P_W^I values for different AOs highlight the effect of changing HLB. The partition constant for caffeic acid, P_W^I = 268, is significantly higher than those obtained for gallic acid (P_W^I = 125)[34], consistent with caffeic acid having the lowest solublity in water.[44] The higher P_W^I values for the C1 (P_W^I = 747) and for C2 (P_W^I = 2000) caffeates indicate that their solubility in water relative to the interfacial region is reduced upon increasing their HLB. Moreover, the P_W^I values for C_1 and C_2 are much higher than the P_O^I values, **Table 1**, consistent with the expected higher solubility of C_1 and C_2 in corn oil compared to water.

The variation of P_O^I seems to be more complex because the P_O^I value for the the C_8 derivative is much higher than those for the C_1-C_3 and C_{16} derivatives. However, this complexity is more apparent that real as shown when calculating the distribution of the AOs.

Comparisons of P_W^I values for different AOs highlight the effect of changing HLB. The partition constant for caffeic acid, P_W^I = 268, is significantly higher than those obtained for gallic acid (P_W^I = 125)[34], consistent with caffeic acid having the lowest solublity in water.[44] The higher P_W^I values for the C1 (P_W^I = 747) and for C2 (P_W^I = 2000) caffeates indicate that their solubility in water relative to the interfacial region is deeply reduced upon increasing their HLB. Moreover, the P_W^I values for C_1 and C_2 are much higher than the P_O^I values, **Table 1**, consistent with the expected higher solubility of C_1 and C_2 in corn oil compared to water.

The percentage of the antioxidant in the interfacial region of the corn oil emulsions was calculated using equations 14-16 and the calculated P_W^I and P_W^O values (**Table 1**). Similar expressions were used to determine the fraction of AO in the oil and aqueous regions. **Figure 5** shows the variation of %AO with Φ_I for the different alkyl caffeates. For any given AO, the fraction of AO in the interfacial region increases upon increasing Φ_I so that at Φ_I = 0.005 more than 60% of the AOs are located in the interfacial region and the percentage increases up to 95% at Φ_I = 0.04. It is worth noting that, at any Φ_I, $\%AO_I$ does not correlate with the hydrophobicity of the AO, following the order $\%C_1 < \%C_2 < \%CA \approx \%C_3 < \%C_4 < \%C_6 < \%C_{16} < \%C_8$. The most hydrophilic AOs (CA and C_1) are more soluble in the aqueous region than in the oil, despite their much lower solubility in water than that of other hydrophilic AOs such as gallic or ascorbic acids.[44, 45]

However, for each antioxidant type, as the alkyl chain length increases, its solubility in water decreases and increases in the oil region, e.g., following P_W^O values (**Table 1**), which means the HLBs of different antioxidants depend on the interactions of their polar groups with water and oil as well as on the length of their alkyl chains.

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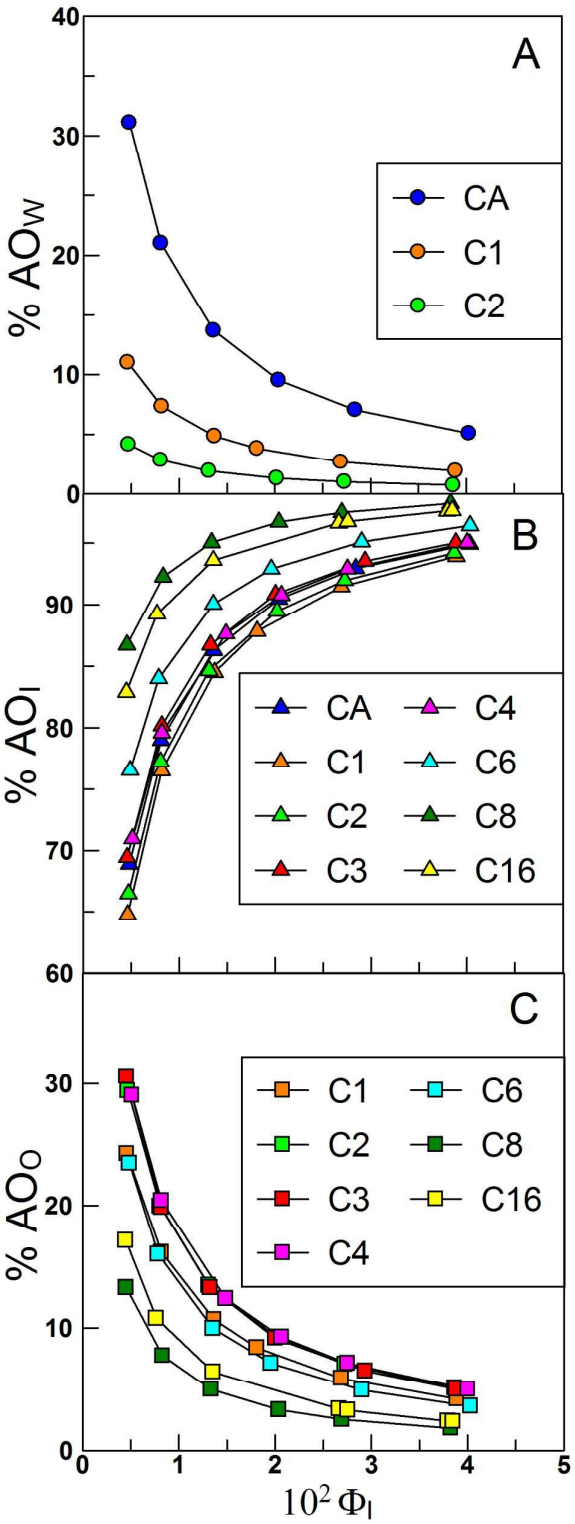

Figure 5. Distributions of caffeic acid derivatives between the aqueous (A), interfacial (B) and oil (C) regions of corn oil-in-water emulsions.

Relationships between the AOs efficiency and the fraction of AOs in the interfacial region of the emulsions. We note that the set of AOs we employed here constitute of a group of AOS with the same reactive moiety (catechol) but of different hydrophobicity. Because AO efficiency depends, other things being equal, on the concentration of AOs at the reaction site which, in emulsions, is believed to the interfacial region, we analyzed if there is any relationship between $\%AO_I$ and their efficiency by plotting their variations with the alkyl chain length. The non-linear, parabolic-like, variation in the efficiency of the AOs, with a maximum at C_8, **Figure 6**, parallels that of $\%AO_I$, confirming that there is a direct relationship between AO efficiency and $\%AO_I$. Such a parabolic variation is known as the "cut-off "effect, and has been observed for several series of AOs bearing the same reactive moieties but different aliphatic chain lengths in oil-in-water emulsions.[21, 22]

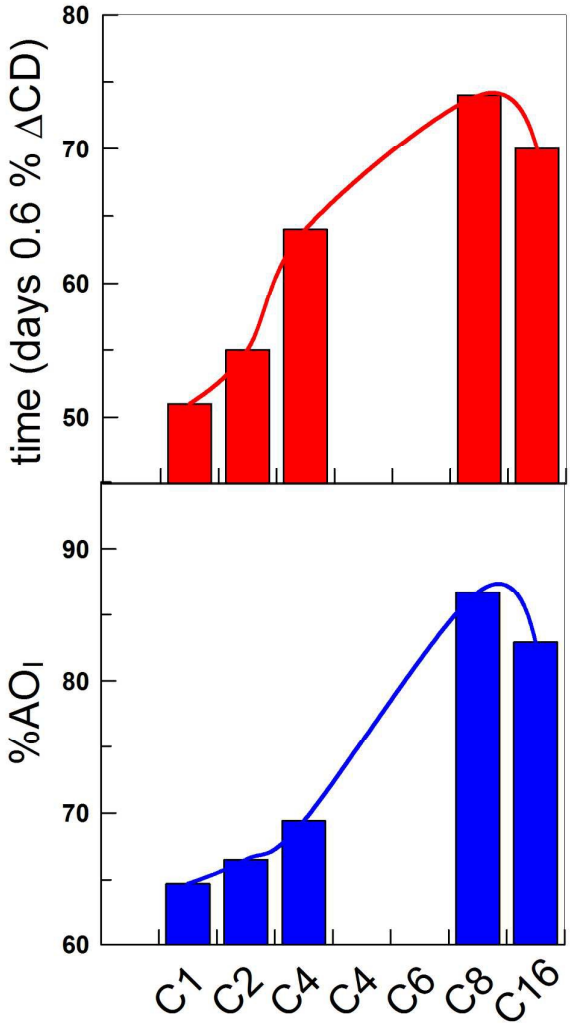

Figure 6. Variations of the effects of alkyl chain length on the percentage of AO in the interfacial region of 4:6 corn oil-in-water emulsions (Φ_I = 0.01) and the AO efficiency (measured as the time necessary to reach an increase of 0.6% in the production of conjugated dienes).

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Conclusions

We have determined the effects of the alkyl chain length of a homologous series of caffeic acid derivatives on their efficiency in inhibiting the oxidation of the lipids of corn oil-in-water emulsions. We also determined the partition constants of the AOs between the oil-interfacial, P_O^I, and water-interfacial, P_W^I, regions. For any AO, both P_O^I and P_W^I are >> 1, indicating all AOs have a natural tendency to be located in the interfacial region. P_W^I values increase upon increasing the alkyl chain length in keeping with the increase in their hydrophobicity. The variation of P_O^I is, however, more complex though apparent as shown when determining the distributions of the AOs.

The contrasting behavior of AOs in bulk oil *vs* water-in-oil emulsions has been discussed for a long time and usually referred as "the polar paradox": in bulk oil, hydrophilic AOs are more effective than their hydrophobic analogs; however they are less effective in emulsified systems. The phenomenon was interpreted on the basis of the differential affinities of the AOs towards the air-oil interface in bulk oils and the water-oil interface in emulsified systems. More recently, the results obtained in experiments analyzing the role of hydrophobicity on AO efficiency were puzzling because the efficiencies of AOs increased with increasing the hydrophobicity (alkyl chain length) up to a critical length after which the efficiency decreased. This phenomenon, observed for the first time more than 100 years ago, is known as the "cut-off" effect and was also observed in the various biological activities of series of homologous compounds. Several hypotheses were put forward in attempting to rationalize the "cut-off" effect, including the possibility that changes in the AO efficiency may be induced by different orientation - positioning of the radical scavenging nucleus (the phenoxy moiety). While this hypothesis cannot be completely discarded, the parallel variations of the AO efficiency and $\%AO_I$ with the number of C atoms in the alkyl chain, **Figure 6**, suggest that the changes in the efficiency of AOs are a consequence of the differential partitioning of the AOs between the oil, interfacial and aqueous regions of the emulsions.

In summary, our results show that modulation of the interfacial concentrations of AOs *via* modification of their HLB is a convenient, practical method to improve the efficiency of AOs in emulsions. However, results indicate that efficiency of the AOs does not increase linearly with the AO alkyl chain length but is parabolic-like, with a maximum at the C8, suggesting that there is a critical HLB value after which their efficiency decreases as a consequence of the increasing solubility of AOs in the oil phase. To date, the number of series of homologous AO_S and oils investigated is rather limited and therefore no reliable structure-efficiency relationships can be established so far. Indeed, the presence of various regions in emulsions may induce changes in their location and concentrations at the reaction site, thus having an important impact on quest for the best AO or set of AOs to minimize lipid oxidation efficiently, and deserve further investigations.

Acknowledgements

We would like to thank Prof. Larry Romsted for encouragement and extremely fruitful iscussions. Support from the following institutions is acknowledged: Xunta de Galicia (10TAL314003PR), Programa Operacional Factores de Competitividade – COMPETE) and Fundação para a Ciência e a Tecnologia (FCT, grant UID/QUI/50006/2013 – POCI-01-0145-FEDER-007265), Universidades de Vigo and Porto.

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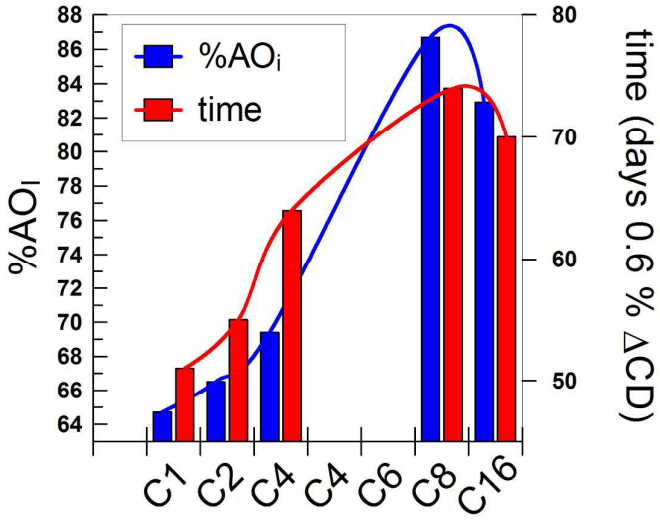

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