Effects of pH and surfactant concentration on the local concentrations of antioxidants in binary oil-water mixtures and in oil-in-water emulsions

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Antioxidants (AOs) react with radicals to minimize their harmful effects. The rates of their reactions depend on both the rate constant for the particular reaction and the concentrations of reactants at the reaction site. Thus, knowledge on their concentrations is very useful and desirable to interpret their reactivity (e.g., efficiency) because AOs with high rate constants may have low concentrations at the reaction site (i.e., their bioavailabity is low). Here we analyze the effects of surfactant concentration and acidity on the local concentrations of a model phenolic acid antioxidant in binary oil-water mixtures and, for the sake of comparisons, in corn oil-in-water emulsions. Local concentrations in emulsions can be up to ~160 times higher than the stoichiometric concentrations, thus having a significant effect in AO efficiencies.

Keywords: Antioxidants, emulsions, interfacial concentration

INTRODUCTION

Phenolic and some non-phenolic weak acids (e.g., ascorbic, sorbic, acetic, citric, etc.) are important in the prevention and control of the oxidative stability of lipids and because of their activities as food preservatives and metal chelators[1,2]. Polyphenols function as antioxidants (AOs) because they are excellent hydrogen donors that are accepted by reactive radicals to yield much less reactive radical and non-radical species[3].

Antioxidants minimize the oxidation of bioorganic material through several pathways, which can be simultaneous [3]. Whatever is the exact mechanism of action, an efficient antioxidant can be defined as that molecule whose rate of trapping radicals is equal to, or higher than, the rate of radical production. Chemical kinetics shows us that the rate of any reaction depends on both the rate constant for the particular reaction and the concentration of reactants at the reaction site. The rate of the reaction of AOS with radicals depends on the structure of the AO and on the properties of the reaction site (e.g., its polarity). In general, it is found that AOs with only one –OH group in their aromatic ring are less efficient than those with two –OH (catecholics) or three –OH (gallates) groups [1, 4]. In addition, the chemical structure of AOs strongly affects their bioavailability (concentration) at the reaction site, so that their hydrophilic-lipophilic balance, HLB, controls their location in the water or in less polar regions (e.g., oils, lipid bilayers, interfacial regions of emulsions, etc.) [5, 6]. For instance, we recently demonstrated that the HLB of an AO does not correlate with its fraction in the interfacial region of emulsions [7, 8] and that an increase in the HLB of a series of AOs bearing the same reactive moiety promotes their incorporation into the interfacial regions of oil-in-water emulsions, but only up to a point because a further increase in their HLB may make them to be more soluble in the oil region than in the interfacial one [5, 6, 9, 10].

Many studies focused on the structure-activity relationships of phenols and phenolic acids [1, 11, 12], however, there are only a few experimental contributions on the effects of acidity on their partitioning in food emulsions [13-16]. Partitioning of AOs is of great importance in predicting many of their biological aspects, mainly because of the large pH range experienced by food during its way through the digestive tract, and recent works demonstrated that there is a direct relationship between the AO efficiency and its concentration at the reaction site.[5, 6, 10, 17] The acidity of the medium plays an important role in AOs behavior because their antioxidant activity is largely affected by their degree of dissociation [9, 15]. For instance, the ionized forms of phenolic acids (phenolates) are much more soluble in the water regions than in the interfacial and oil ones.

Here we have investigated the effects of acidity on the partitioning of caffeic acid in binary corn oil-water mixtures and in corn oil-in-water emulsions. We chose caffeic acid (CA) because it is a major representative of hydroxycinnamic and phenolic acids [17-20]. Other phenolic and non-phenolic acids such as Trolox, gallic acid and ascorbic acids also constitute examples of antioxidants that can also be partially ionized in food systems [15] but their behavior in binary oil-water and oil-in-water

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emulsions should be very similar to that of CA because the acidity constants $pK_a(1)$ of their carboxylic groups are all within the same range, 3.9 - 4.5 [21, 22] and thus, they can be present either as neutral or partially ionized molecules at the typical acidities (2-6) of foods [23]. The degree to which

EXPERIMENTAL SECTION

Materials

Caffeic acid (CA), stripped corn oil, (Acros Organics, $d = 0.918$ g/mL) and polyoxyethylene (20) sorbitan monolaurate (Tween 20, Fluka) were of maximum purity available and used as received. The acidity of the aqueous phase was measured potentiometrically and controlled using citric acid buffers (CB, 0.04 M). Other chemicals were from Scharlau or Panreac. Aqueous solutions were prepared by using Milli-Q grade water. Emulsions of 1:9 (v:v) oil to water ratio were prepared by mixing 1 mL of corn oil and 9 mL of acidic (CB buffer) water. CA was solubilized in water (≈ 4.2 mM) and a weighed amount of non-ionic surfactant was added. The volume fraction of surfactant, Φ_I, (defined hereafter as $\Phi_I = V_{surf}/V_{emulsion}$) varied from $\Phi_I = 0.005$ up to $\Phi_I = 0.04$. The mixture was stirred with a high-speed rotor (Polytron PT 1600 E) for 1 min. The emulsion was transferred to a continuously stirred thermostated cell and the stability of the emulsions was checked visually.

Methods

Determining the partition constant P_W^O in binary oil/water mixtures and in oil-in-water emulsions

Hereafter, the term "apparent" partition constant, $P_W^O(app)$, refers to the experimentally determined partition constant of CA between the oil and water phases at any pH (CA may be partially ionized), meanwhile the true partition constant P_W^O refers to the partition constant of the neutral form of CA.

$P_W^O(app)$ values in binary oil-water systems were determined at the different acidities by employing a shake-flask method [25]. CA was dissolved in 9 mL of water and mixed with 1 mL of corn oil, stirred with a high-speed rotor for 1 min and allowed to stay for 30 min to permit phase separation and thermal equilibrium. The phases were then separated by centrifugation and the AO concentrations in the aqueous and oil phases were determined by UV spectrometry with the aid of previously prepared calibration curves. Results were obtained in duplicate or triplicate with deviations less than 5%. P_W^O values were calculated by employing Eqn. 1, where V_W and V_O are the aqueous and oil region volumes, respectively, and the magnitude in

this partitioning may occur depends on their chemical structure (which affects their solubility) but is also pH-dependent, and changes in the acidity of the medium change their distribution between the oil, interfacial and water regions of the emulsions, as well as their antioxidant efficiency[8, 10, 24]. parentheses ( ) means concentrations in moles per liter of the oil (o) and aqueous (w) regions.

$$P_W^O(app) = \frac{(AO_O)}{(AO_W)} = \frac{\%AO_o}{\%AO_w} \times \frac{V_w}{V_O} \qquad (1)$$

The percentages of AOs in the aqueous phase, $\%AO_W$, were determined, at different pH values, by interpolation of the absorbance of aliquots (30 µL) of the aqueous phase of the binary corn oil/acidic water mixtures diluted with buffer solutions of the same pH and the concentration of the AO was determined by using previously prepared calibration curves at each pH.

Determining partition constants in intact oil-in-water emulsions

Determining partition constants in emulsions is a difficult task because methods used in binary oil/water systems cannot be employed because of the physical impossibility of separating the interfacial from the oil and water regions. This means that the partition constants of the AOs need to be assessed in the intact emulsions and for the purpose, a well-established chemical kinetic method was employed. The method exploits the reaction between a hydrophobic arenediazonium ion, 16-ArN$_2^+$, and the antioxidants. The 16 carbon tail and the aromatic ring of the arenediazonium ion makes 16-ArN$_2^+$ to be insoluble in the water region, and also in the oil region because of its cationic –N$_2^+$ headgroup, thus its concentration in the aqueous and oil regions is effectively zero and there is no reaction between the antioxidant and 16-ArN$_2^+$ in these regions. Mathematical relationships (not shown here) between the partition constants and the observed rate constants k_{obs} have been derived on the grounds of the pseudophase kinetic model. Details of the method are described elsewhere in detail [5-7, 17, 26].

RESULTS

Hereafter, magnitudes between parentheses, e.g. (AO), mean concentrations in moles per liter of the volume of a given region, while those between brackets, e.g., [AO] stand for concentrations in moles per liter of the total volume of the system.
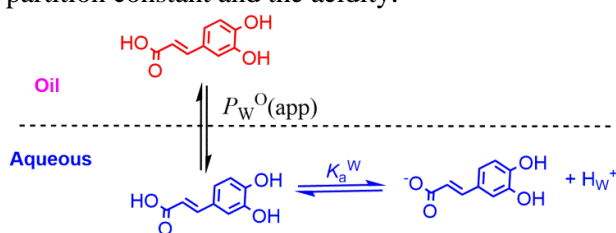
Effects of acidity on the partition constant, P_W^O, and on the distribution of caffeic acid in binary corn oil-water mixtures

The results in Table 1 show that the experimentally determined P_W^O(app) values are pH dependent, decreasing upon decreasing the acidity. This is a consequence of the carboxylic groups on CA: ionized phenolic acids are much more soluble in water than the undissociated acids. Results in Table 1 show that P_W^O(app) values for CA are very low at any pH, $P_W^O < 0.1$, in keeping with the high solubility of the AO in water, $S_{CA} \approx 2.9$ g dm^{-3} at T = 298 K [27]. The P_W^O(app) value of 0.025 at pH ~ 4 is very similar to that found by Huang *et al*. [28] in an unbuffered 1:9 corn oil-water binary mixture P_W^O(app) = 0.028 [29]. Results in Table 1 indicate that more than 99% of the phenolic acid is in the aqueous phase at pH < 2.5, and that this percentage increases upon decreasing the acidity of the medium because of the increase in the fraction of ionized antioxidants. Hence, in emulsified systems, phenolic acids are expected to be mainly distributed between the aqueous and interfacial regions with negligible amounts in the oil phase.

Thermodynamics show us that when a neutral molecule (e.g., the weak acid) partitions between two phases (e.g., oil and water) at equilibrium, the partition constant P_W^O between those phases is defined by Eqn. 2, which assumes that the activities of the molecule in each phase are equal or very close to unity. This approximation usually holds because the concentration of the molecule in each phase is low.

Caffeic acid may both dissociate in the aqueous phase and partition into the oil phase and both, ionized and neutral species are in equilibrium in the oil and water phases. However, ionization constants of acids in oils (O) are usually 5-6 orders of magnitude smaller than in aqueous phases [30], (i.e., $pK_a(O) >>> pK_a(W)$) and, for the sake of simplicity, we can assume that the ionization of the weak acids in the oil phase is negligible, and Scheme 1 applies, from where the apparent (measured) partition coefficient defined by equation 3 can be derived.

Table 1. Values of the partition constant P_W^O for the distribution of CA in binary corn oil-water systems at different acidities, determined at T = 25 °C.

pH	2.50	2.88	3.05	3.35	3.39	3.53	3.67	3.70	3.97	4.01	4.10
P_W^O (app)	0.086	0.079	0.073	---	0.065	0.050	---	0.046	---	0.026	---
P_W^I (app)	603	556	476	351	---	---	278		194	---	102

The dissociation of caffeic acid in water is characterized by the dissociation constant K_a, Eqn. 4. Substitution of Eqn. 4 into Eqn. 2 leads to Eqn. 5, which sets the relationship between the apparent partition constant and the acidity.

Scheme 1. Partitioning of caffeic acid between the oil and water phases of binary mixtures. Ionization is assumed to take place exclusively in water.

$$P_W^O = \frac{[CA_{oil}]}{[CA_{water}]} \quad (2)$$

$$P_W^O(app) = \frac{[CA_{oil}]}{[CA_{water}] + [CA_W^-]} \quad (3)$$

$$K_a = \frac{(CA_W^-)(H^+)}{(CA_W)} \quad (4)$$

$$P_W^O(app) = \frac{P_W^O}{1 + \frac{K_a}{[H^+]}} \quad (5)$$

$$\frac{1}{P_W^O(app)} = \frac{1}{P_W^O} + \frac{K_a}{P_W^O} \frac{1}{[H^+]} \quad (6)$$

Equation 5 predicts a sigmoidal variation of the apparent (measured) partition constant value with acidity. Note that when pH <<< pK_a, P_W^O (app) = P_W^O, that is, there exists an upper limit in the experimentally measured partition constants which corresponds to that of the neutral molecule. The reverse of equation 5 is equation 6, which predicts a linear variation of 1/ P_W^O (app) with 1/[H$^+$], Figure 1A, from where a value of $P_W^O = 0.087 \pm 0.003$ can be determined for the partition constant P_W^O of neutral CA. The local concentration of CA in the water phase can be obtained by bearing in mind the corresponding mass balance for the concentration of the acid in terms of the total volume of the system gives Eqn. 7.

$$[CA_T]V_T = (CA_{oil})V_{oil} + (CA_W)V_W + (CA_W^-)V_W \quad (7)$$

where brackets [ ] mean concentration in moles per liter of the total volume. Defining the volume fraction of the oil phase as $\Phi_O = V_{oil}/(V_o+V_w)$, Eqn. 8 can be derived.

$$\frac{(CA_W)}{[CA_T]} = \frac{1}{\Phi_W + P_W^O \Phi_O + \dfrac{K_a}{[H^+]}} \quad (8)$$
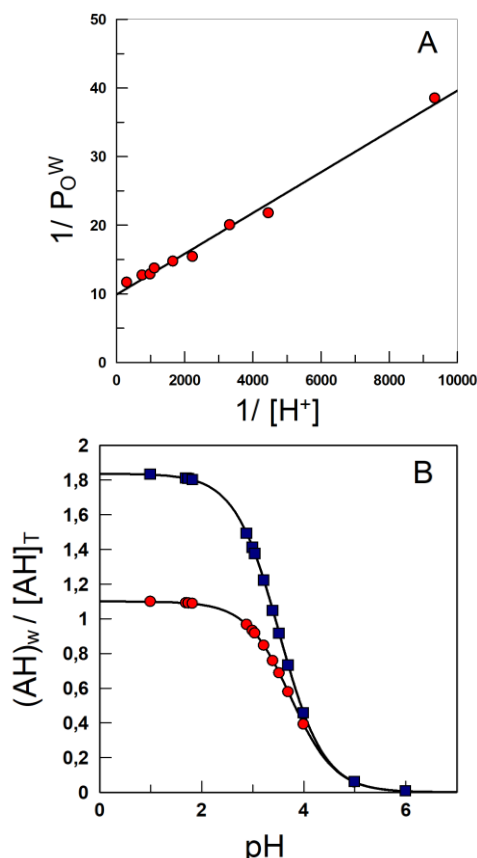

Figure 1. A) Variation of the measured partition constant in oil/water mixtures, P_W^O (app), with pH. B) Variation with pH of the ratio between the local concentration of the AO in the aqueous region and the total or stoichiometric concentration.

Eqn. 8 is a modification of the Henderson-Hasselbach equation and gives the concentration of the neutral weak acid in a two-phase system relative to the total (stoichiometric) concentration as a function of the acid concentration, the volume fractions of each phase, the partition constant of the neutral molecule and the ionization constant. Figure 1B simulates the variation of the concentration of CA in the water phase at two o:w ratios. It becomes apparent that at low pH, the concentration of CA in the water phase of oil-water mixtures with a high content of water (e.g., 1:9 o:w) is very similar to the stoichiometric concentration, while in 1:1 oil:water mixtures, the concentration in the water phase is almost twice that of the stoichiometric

concentration. As expected, the ratio $(CA_w)/[CA_T]$ decreases upon increasing pH as a consequence of the ionization of CA. The results, though somehow expected, illustrate the fact that the local concentrations of AOs may be quite different from the stoichiometric concentrations and constitute an important remark because when AOs partition in multiphasic systems, their interfacial concentration may be by orders of magnitude higher than the stoichiometric concentration (see below).
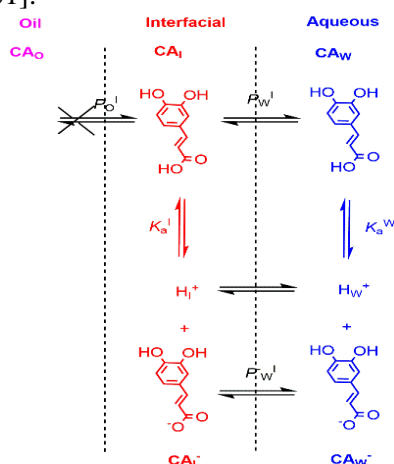
Effects of acidity on the partitioning and on interfacial concentrations of CA in oil-in-water emulsions

Lipids are typically present in foods in the form of oil-in-water emulsions (butter, mayonnaise, dressings, etc.) The interfacial region of the emulsions is highly anisotropic and contains a mixture of oil, water and surfactant, and, therefore, can solubilize both hydrophobic and hydrophilic molecules. Antioxidants added to minimize the oxidation of the lipids distribute between the oil, water and interfacial regions, and their efficiency strongly depends on their partitioning.

In general, the distribution of AOs in emulsions can be described by two partition constants, Scheme 2, that between the oil and interfacial, P_O^I, and that between the water and interfacial, P_W^I, regions, but simplifications can be made when AOs are very hydrophobic or very hydrophilic. For instance, CA is a hydrophilic AO whose solubility in oils is expected to be very low because the measured partition constants between water and corn oil, P_W^O (see above) in corn oil/water mixtures were very low. Thus, one can safely assume that the concentration of CA in the oil region of the emulsions is negligible and, thus, CA will mainly partition between the aqueous (w) and interfacial (I) regions of oil-in-water emulsions and only one partition constant (P_W^I) is needed to describe its distribution, Scheme 2. In general, phenolic AOs containing weakly acidic functional groups, e.g, a carboxylic acid moiety, have very low oil solubility. However, their solubilities in water can be modified by changing the acidity of the system because at the typical acidities (2-6) of emulsified foods [23], phenolic acids may be neutral or partially ionized and a change in emulsion acidity changes their distributions between the interfacial and aqueous regions.

Note that the ionic forms of the AOs are generally oil insoluble, but they are much more water soluble than the neutral forms. The P_W^I values displayed in Table 2 were determined as described elsewhere by

employing a kinetic method in the intact emulsions [6, 7, 26, 31].

Scheme 2. Equilibria for the distribution of the hydrophilic neutral and anionic form of an AO, e.g., Scheme 1, between the oil, interfacial and aqueous regions of the emulsion

Results in Table 1 show that P_W^I values decrease by a factor of ~ 4 upon increasing pH from ~2.5 to ~4.2 but, at any acidity, $P_W^I > 1$, indicating that the Gibbs free energy of transfer from the aqueous to the interfacial region is negative, that is, CA has a natural tendency to incorporate into the interfacial region.

Figure 3A shows their variation with pH. CA may ionize in both the aqueous and interfacial regions and the apparent partition constant P_W^I (app) is defined by Eqn. 9.

The pK_a values of weak acids within interfacial regions of nonionic association colloids and emulsions are somewhat different – but not too much - from their values in aqueous solution because the polarity of the interfacial region is less than that of water[32, 33].

$$P_W^I(app) = \frac{((AO_I) + (AO_I^-))}{((AO_W) + (AO_W^-))} \qquad (9)$$

$$P_W^I(app) = \frac{P_W^I + \dfrac{P_W^{-I}K_a}{[H^+]}}{1 + \dfrac{K_a}{[H^+]}} \qquad (10)$$

Nonionic micellar solutions usually have small effects on the apparent pK_a values of weak acids, unlike cationic and anionic micelles that are known to shift substantially the pK_a values of weak acids compared to water in opposite directions[34, 35]. For example, Jaiswal *et al*. [21] found that the pK_a of ascorbic acid increases by less than 0.3 units in Triton X micelles compared to water. We expect similar behavior between the aqueous and interfacial

regions of emulsions and micelles prepared with nonionic surfactants, and we assume that p$K_{a(w)} \approx$ p$K_{a(I)}$, so that the percentage of phenolic acids in their anionic form will be about the same in the interfacial region of Tween 20 emulsions as in the bulk aqueous phase. Using the equations defining the ionization (eq. 4) and partition (eq. 9) constants and bearing in mind Scheme 2, Eqn. 10 can be derived, where P_W^{-I} stands for the partition constant of the ionized form of CA between the aqueous and interfacial regions.

Eqn. 10 predicts that the variation of P_W^I with pH follows a sigmoidal-shaped curve as can be seen in Figure 2 (solid line). Eqn. 10 predicts that at high acidities (pH << pK_a), the measured P_W^I (app) value is equal numerically to P_W^I, that is, the partition constant of the neutral CA, since at low pH, all CA molecules are neutral and the P_W^I (app) values at such acidities should be independent of pH, as shown in Figure 2. At pH >> pK_a, the measured P_W^I (app) value decreases and approaches that of P_W^{-I} (depending on the magnitude of P_W^{-I} and K_a) because CA is fully deprotonated and the carboxylate has low solubility in the interfacial region of the emulsions as demonstrated by Huang *et al*. [22], who found that at pH = 6, more than 95% of the total added Trolox is located in the aqueous region of corn oil/Tween 20 ($\Phi_I = 0.01$) emulsions and at pH = 7, the percentage of Trolox found in the aqueous region was ~100%. Hence, values of P_W^I (app) are expected to be close to zero at pH > 6, Figure 2.

Bearing in mind the corresponding mass balances, the concentrations of the antioxidant in the interfacial and aqueous regions of the emulsion relative to the stoichiometric concentration can be obtained by means of Eqns. 11 and 12, respectively.

$$\frac{(AO_I)}{[AO_T]} = \frac{P_W^I}{\Phi_W + P_W^I\Phi_I} \qquad (11)$$

$$\frac{(AO_W)}{[AO_T]} = \frac{1}{\Phi_W + \Phi_I P_W^I} \qquad (12)$$

Figure 4 illustrates the variations of the local concentrations of CA in the interfacial and aqueous regions relative to the stoichiometric CA concentration. At the lowest surfactant volume fraction employed, $\Phi_I = 0.005$, the interfacial concentration of CA is as much as ~130 times higher than the stoichiometric concentration (pH = 2). On the contrary, the local concentration in the aqueous region is much lower than the stoichiometric concentration.
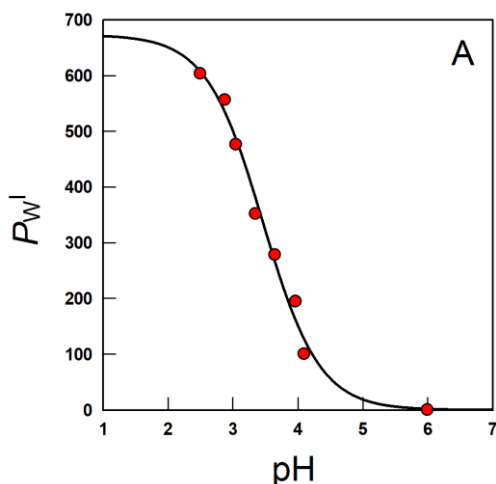
Figure 3A. Effects of acidity on the partition constant P_W^I of caffeic acid in emulsions.

At a constant acidity, the interfacial and aqueous concentrations decrease to almost zero upon increasing the surfactant volume fraction from $\Phi_I = 0.005$ to $\Phi_I = 0.045$, highlighting the enormous impact of the surfactant concentration on the local concentrations of antioxidants. Note that the largest variations in the local concentrations are achieved for Φ_I values ranging 0.005 to $\Phi_I \approx 0.015$. Further increases in Φ_I do not change substantially the local concentrations. Alternatively, at constant Φ_I, the interfacial concentration decreases upon decreasing the acidity by a factor of ~2 on going from pH = 2 to pH $\approx$ 4 because of the ionization of CA. Note that the local aqueous concentrations increase upon increasing pH, but still is a fraction of the stoichiometric concentration. Eventually, at high pH (>6), the local aqueous concentration will approach the stoichiometric concentration.

CONCLUSIONS

Antioxidant efficiency depends on several factors including the rates of the reaction between the AO and the radicals (which depends on the physical properties of the AO) and its concentration at the reaction site. It is currently accepted that the oxidation of the oil starts in the interfacial region of the emulsion [17, 36] and we recently showed [10, 37] that there exists a direct correlation between the mole fraction of antioxidants in the interfacial region of emulsions and their antioxidant efficiency. Interfacial concentrations are much higher than stoichiometric concentrations and this is part of the reason why only small amounts of AOs are needed to minimize the oxidation of lipids.

The results obtained here are important to understand the relative efficiency of AOs in emulsified systems. They show, among others: 1) the partition constants in binary oil-water systems cannot be employed to predict the relative distribution of AOs in multiphasic systems. 2) In binary oil-water mixtures, local AO concentrations
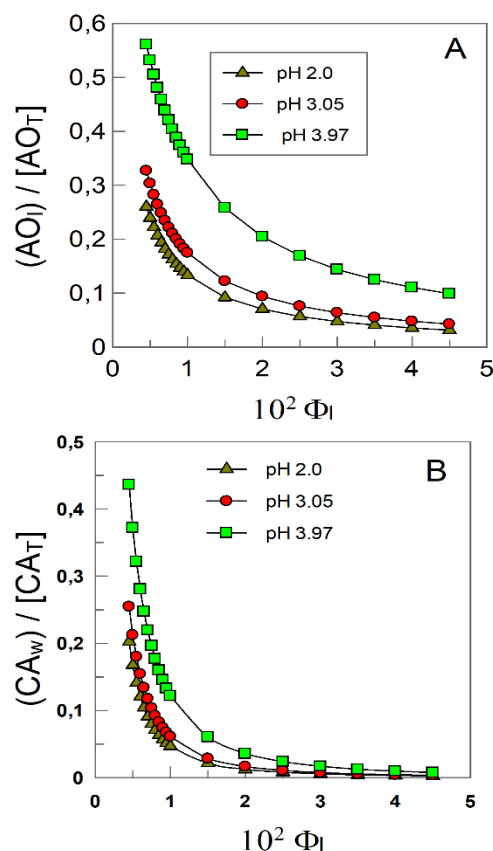

Figure 4. Variation of the interfacial (A) and aqueous (B) concentrations of CA in corn oil emulsions with the surfactant volume fraction at selected acidities. The values at pH= 2 were determined theoretically by employing equations 11 and 12 and the P_w^I value determined by fitting the experimental data in Figure 2 to equation 10 (P_w^I = 650).

increase modestly with respect to the stoichiometric concentrations. 3) In emulsions, the interfacial region has a significant role because it contains concentrated AOs so that interfacial AO concentrations are by orders of magnitude higher than the stoichiometric concentrations. 4) The acidity plays a significant role in the behavior of phenolic acids, which upon ionization, decrease significantly their interfacial concentration and therefore a decrease in their efficiency upon increasing pH is expected.

Indeed, 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, which in turn also affects their partitioning between the different regions of the emulsions [2, 38]. 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. [39, 40] Thus, strategies exploiting changes in their relative solubility leading to changes in their local concentrations (e.g., the interfacial concentration in emulsions) are needed to improve their efficiency while maintaining their antioxidant properties. Therefore, improvement of strategies to modulate interfacial concentrations deserve further investigations.

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ВЛИЯНИЕ НА РН И КОНЦЕНТРАЦИЯТА НА ПОВЪРХНОСТНО АКТИВНО ВЕЩЕСТВО ВЪРХУ ЛОКАЛНИТЕ КОНЦЕНТРАЦИИ НА АНТИОКСИДАНТИ В БИНЕРНИ СМЕСИ ОЛИО-ВОДА И ЕМУЛСИИ НА ОЛИО ВЪВ ВОДА

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(Резюме)

Антиоксидантите (АО) реагират с радикалите за минимизиране на вредното им влияние. Скоростта на реакциите зависи от скоростната константа на съответната реакция и от концентрациите на реактантите на мястото на реакцията. Познаването на техните концентрации на мястото на реакцията е много полезно за интерпретиране на реактивоспособността им (например, ефективността), тъй като АО с високи скоростни константи може да са с ниски концентрации на мястото на реакцията (т.е., тяхната бионаличност е ниска). В настоящата работа е изследвано влиянието на киселинността и концентрацията на повърхностно активно вещество върху локалните концентрации на моделен антиоксидант фенолна киселина в бинерни смеси олио-вода и за сравнение в емулсии на царевично олио във вода. Локалните концентрации в емулсиите може да са до ~160 пъти по-високи от стехиометричните концентрации, което има значителен ефект върху ефективността на АО.