CONTROL OF ANTIOXIDANT EFFICIENCY OF CHLOROGENATES IN EMULSIONS: MODULATION OF ANTIOXIDANT INTERFACIAL CONCENTRATIONS

Running title: Antioxidant efficiency of chlorogenates modulated by interfacial concentration

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Abstract

Background. Controlling the interfacial concentrations of antioxidants (AOs) in O/W emulsions can be regarded as an unique approach for increasing the efficiency of AOs in inhibiting the oxidation of lipids. Classical methods to determine the AO distribution in binary systems cannot be employed and their distribution needs to be assessed in the intact emulsion.

Results. We have employed well-established kinetic methods to determine the distribution of a homologous series of AOs derived of chlorogenic acid in olive oil-in-water emulsions and analyse the effects of AO hydrophobicity on their distributions and their efficiencies. Results indicate that the variations in the efficiency of chlorogenates in emulsions are due to differences in their interfacial concentrations. Their interfacial concentrations, (AO_I), were much higher (20-150-fold) than their stoichiometric concentrations. On the other hand, their concentrations in the oil region were 1.5-0.1 fold. Results also show the complex effect of the oil to water ratio employed in the preparation of the emulsions on the (AO_I) values.

Conclusion. Results highlight the key role of the interfacial region and of its composition (interfacial AO molarity, emulsifier concentration, oil to water ratio) in interpreting the efficiency of AOs in inhibiting lipid oxidation in emulsions. Thus, a careful modulation (adjustment?) of these parameters is necessary to ensure ~~an~~ optimum AO efficiency.

Keywords

Antioxidant distribution, antioxidant efficiency, cut-off effect, chlorogenic acid, pseudophase model, emulsions

Introduction

Oxidative stress has been recognized as a key factor in accelerating the aging process and in the development and progression of chronic diseases such as cancers, diabetes, neurodegenerative and cardiovascular diseases.[1] The development of such pathologies generally is a consequence of an increase in radical production or a decrease in antioxidant defenses, leading to the oxidative alteration of important biomolecules such as proteins, lipids, carbohydrates and nucleic acids.[2-5]

Natural polyphenols present in diet are considered to be one of the most important bioactive agents that may help ~~to~~ prevent, or slow down, the progression of ~~the~~ oxidative damage. Their efficiency depends on their chemical nature – mainly the number and position of substituents – but also on their local availability, limiting their applications in functional foods or medicine.[6-8] ~~Multiphasic~~ System such as emulsions, membranes and cells are multiphasic, with regions of different solvent properties. Thus, polyphenols may exhibit differential solubility in each region of the system, partitioning between the different regions. Therefore, their availability is not necessarily the same and may vary widely from one compound to another. The most abundant polyphenols in our diet, or the most reactive against radicals, are not necessarily the most efficient antioxidants (AOs). ~~Therefore~~, Understanding the effects of compartmentalization is crucial to predict their efficiency and in the search of efficient AOs for a given system or application.

The hydrophobicity of the AOs can be modulated by grafting alkyl chains of varying length (1-20 carbon atoms)[9-16] to polyphenolic moieties. This strategy has been exploited for years to analyze the effects of hydrophobicity on ~~their~~ ~~various~~ biological activities, allowing the evaluation of a series of homologous compounds bearing the same reactive moieties but different hydrophobicities.[17,18] By employing a pseudophase kinetic model, we were able to determine the distribution of a series of AOs derived from hydroxytyrosol in intact oil-in-water emulsions and demonstrated that there is a direct relationship between the efficiency of AOs and the percentage of AO in the interfacial region of the emulsion (Figure 1A).[19] This fundamental result implies that the efficiency of AOs can be, in principle, increased by fine-tuning their hydrophobicity to achieve the maximum interfacial concentrations. The effects of hydrophobicity on the

efficiency of a series of homologous antioxidants were examined in ~~a series of~~ recent papers[14,19-22] (Figure 1B) and similar nonlinear variations as those shown in Figure 1A were found. Depending on the parental AO, the optimal alkyl chain length to achieve the highest interfacial concentration has varied from three to eight carbon atoms. Unfortunately, predicting the optimal chain length of an alkyl group grafted to an AO is not possible because no relationship between the hydrophobicity of a molecule and its efficiency has been established so far.

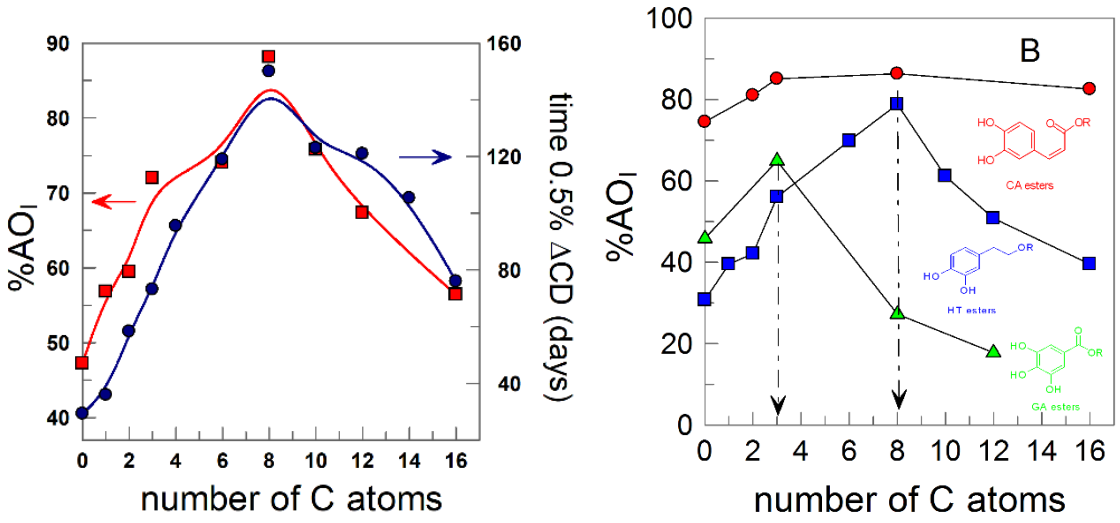
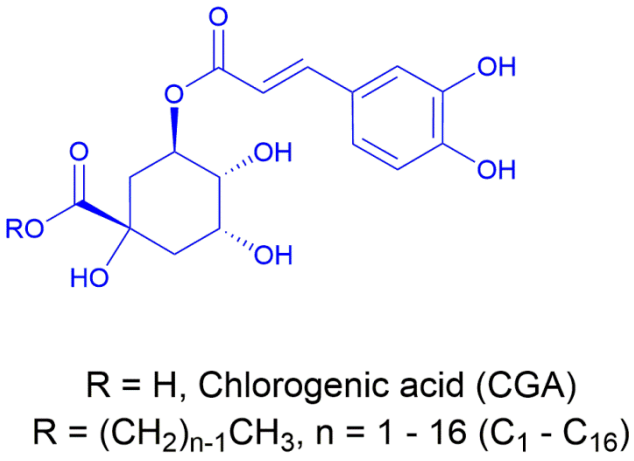


Figure 1. (A) Variation of the time to reach 0.5% conjugated dienes and the values of the percentages of AOs in the interfacial region of 4.6 (O/W) olive oil-in-water emulsions, %AO$_I$, with the number of C atoms at Φ_I = 0.01 for a series of hydroxytyrosol esters. Extracted from reference[19]. **(B)** Variation of %AO$_I$ of 4.6 (O/W) olive oil-in-water emulsions showing the non-linear variation of %AO$_I$ with the hydrophobicity of the AOs(●-caffeates,■-hydroxytyrosol esters,▲-gallates).[14,16,19,22]

Prediction of the most efficient AO in an emulsified system is difficult because the AO efficiency depends, among others, on their distribution between the different regions of the emulsion. Their distribution depends, in turn, on the relative solubility in the regions, which is controlled by both the differences in solvation and on the capabilities of the AOs of intra- and intermolecular hydrogen bonding with the solvent. In emulsions, three regions with different solvent properties, the aqueous, interfacial and

oil regions, can be distinguished and setting the relative importance of each contribution cannot be easily established. Thus, the key questions remain unsolved and structure-reactivity relationships need to be investigated for each series of AOs and oils under various experimental conditions.

Herein, we investigate the antioxidant efficiency and the distribution in model emulsions composed of olive oil, water and the non-ionic surfactant Tween 20 of a series of *n*-alkyl chlorogenates (Scheme 1). Chlorogenic acid (CGA) is a natural antioxidant present in the human diet. Several epidemiological studies have linked CGA consumption to a wide range of health benefits, including neuroprotection, cardioprotection and anti-inflammatory activity.[23,24] However, its high hydrophilicity prevents its application as an antioxidant because of its low bioavailability in protecting lipid tissues. However, modulation of its hydrophilic-lipophilic balance (HLB) by lipophilization may provide an opportunity to improve its antioxidant properties.



Scheme 1. Structures of chlorogenic acid (CGA) and its ester derivatives (C1-C16) employed in this work.

2. Materials and Methods

2.1. Chemicals and Materials

All chemicals were of the highest purity available and used as received. 2,2 Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), chlorogenic acid (CGA), the fatty alcohols employed in the

preparation of CGA esters and the surfactant polyoxyethylene (20) sorbitan monolaurate (Tween 20) were from Acros Organics or Aldrich. Olive oil, stripped of natural tocopherols and phenols, was prepared from commercial virgin olive oil by washing it with a 0.5 M NaOH solution and passing it twice through an aluminium oxide column. Complete removal of tocopherols was confirmed by HPLC according to the *IUPAC method 2.432*. Details can be found elsewhere.[25]

All aqueous solutions were prepared by employing Milli-Q grade water ($\kappa < 0.1$ $mScm^{-1}$). The acidity of aqueous phase was controlled by employing citric acid/citrate buffer (0.04 M, pH 3.65). Solutions of the coupling agent *N*-(1-naphthyl)ethylenediamine (NED, Aldrich) were prepared in a 50:50 (v/v) BuOH:EtOH mixture to give [NED] = 0.02 M.

4-Hexadecylbenzenediazonium tetrafluoroborate, 16-ArN_2BF_4, was prepared under non-aqueous conditions as described in a published method[26] from commercial 4-hexadecylaniline (Aldrich, 97%) and was stored in the dark at low temperature to minimize ~~their~~ decomposition.

2.2. Synthesis of chlorogenic fatty acid esters.

Chlorogenate esters (C2-C4) were synthesized by chemical acylation of the carboxylic group following the procedure described by Reis *et al.*[27] The method was modified for the synthesis of the C6-C16 derivatives (enzymatic acylation) as described in the *Supplementary Material*. Final yields (purified compounds, purity > 98%) were 65-75% for the C1-C4 derivatives and 50-65% for the C6-C16 derivatives. In all cases, ^{1}H and ^{13}C NMR spectra of the synthetized chlorogenates were in accordance with the literature.[28]

2.3. Preparation of oil-in-water emulsion.

Olive oil-in-water emulsions (4:6, O/W) were prepared by mixing stripped olive oil, acidic water (0.04 M citrate buffer, pH 3.65) and Tween 20 as emulsifier (Φ_I = 0.5 - 4%). The mixture was stirred at high speed at room temperature with the aid of a Polytronic PT-1600 homogenizer.

2.4. Methods

2.4.1. DPPH$^{\bullet}$ radical scavenging efficiency of chlorogenates.

The effects of the length of the alkyl chain of chlorogenates on the radical scavenging efficiency were investigated in bulk EtOH solution by exploiting the ability of polyphenols to reduce the DPPH$^{\bullet}$ radical. The relative antiradical activity was assessed by the EC_{50} value, defined as concentration of AO required to lower the initial DPPH$^{\bullet}$ concentration by 50%.[14,22] Details can be found elsewhere[14,22] and in the *Supplementary Material*. Results summarized in Table 1 show that the EC_{50} value is independent of the length of the alkyl tail of the esters (<5% at time 5 min) with an average value for CGA and its esters of 0.244 ± 0.004 mole AO/mole DPPH$^{\bullet}$ at T = 25.0 ± 0.1ºC.

2.4.2. Cyclic voltammetry.

The voltammetric working solutions were prepared, in the electrochemical cell, by diluting 0.1 mL of the stock solution (10 mM in ethanol) in 10 ml of aqueous buffer (potassium dihydrogen phosphate/phosphoric acid 0.1 M, pH 3.65). Final AO concentrations were 0.1 mM. The effects of emulsifier on the first anodic potential of the AOs was checked by carrying out auxiliary experiments both in the presence and absence of Tween 20. See *Supplementary Material* for details. Results displayed in Table 1 show that values for the first anodic potential are independent of the length of the alkyl tail of the esters, in keeping with the results found when employing the DPPH assay and didn't change even in the presence of the emulsifier.

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2.4.3. Antioxidant efficiency in olive oil emulsions.

Antioxidant efficiency in emulsions was determined as in previous works.[14,22] Olive oil-in-water emulsions (4:6, O/W) were prepared as above in the presence and absence of chlorogenates (final AO concentration of 0.24 mM in total volume). Emulsions with no added antioxidant were used as ~~the~~ control. Samples were thermostated at T = 60ºC and vortexed for one minute every 12 hours to minimize creaming. After each vortex, a 50 μl aliquot of the emulsion was diluted to 10 ml with ethanol and the absorbance at 233 nm ~~was~~ measured. The level of oxidation of emulsions was monitored by determination of the conjugated dienes (CDs) according to the *AOCS Official Method Ti 1a 64*.

2.4.4. Determining the partition constant, P_W^O, of chlorogenates in binary olive oil-water mixtures.

The partition constants of chlorogenates in binary olive-water mixtures, P_W^O, were determined in the absence of emulsifier by employing the same shake-flask method as described in previous works.[14,22,29] P_W^O values were estimated by employing equation 1 where the parentheses ~~(-)~~ indicate the concentration of AO in moles per litre of phase volume, V_W and V_O are the volumes of the aqueous and oil phases, respectively.

$$P_W^O = \frac{(\mathrm{AO_O})}{(\mathrm{AO_W})} = \frac{(\%\mathrm{AO_O})}{(\%\mathrm{AO_W})}\frac{V_W}{V_O} \qquad (1)$$

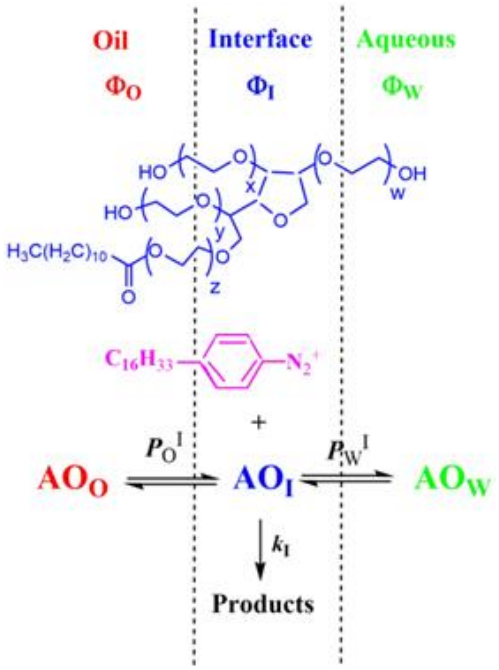
2.4.5. Determining the partition constants and distribution of chlorogenates in intact olive oil-in-water emulsions: application of the pseudophase kinetic model.

Chlorogenates distribute in a different extent between the oil, interfacial, or aqueous regions of emulsions depending upon their HLB, Scheme 2, and their distribution is defined by the partition constants between the oil-interfacial, P_O^I, and aqueous-interfacial, P_W^I, regions (Equations 2 and 3).

$$P_O^I = \frac{(AO_I)}{(AO_O)} \qquad (2) \qquad\qquad P_W^I = \frac{(AO_I)}{(AO_W)} \qquad (3)$$

The distribution of the chlorogenates was determined in the intact emulsions by employing a well-established kinetic method which exploits the rapid reaction between

the chlorogenates and the specifically synthethized chemical probe, 4-hexadecylbenzenediazonium (16-ArN_2^+) ion. The probe is located in the interfacial region of emulsions because 16-ArN_2^+ is itself an ionic surfactant and because it is oil and water insoluble (Scheme 2). Details of the method and the kinetic treatment are given elsewhere[30] and are briefly described in the *Supplementary Material.*



Scheme 2. Representation of an emulsion showing the aqueous, oil and interfacial regions, the location of the reactive group of the hydrophobic ArN_2^+ ions and the distribution of the AO. Subscripts O, I, and W region indicate the oil, interfacial and aqueous regions, respectively, and Φ is the volume fraction of a region (Φ=Vregion/Vtotal)

Statistical Analysis. Kinetic experiments were run in duplicate or triplicate 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 were the average of 6-8 replicates. All the DPPH radical scavenging assays and cyclic voltammetry experiments were run at least in quadruplicate. All oxidation experiments were run in triplicate. 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 $P < 0.05$.

RESULTS AND DISCUSSION

3.1. Oxidative stability of olive oil-in-water emulsions: antioxidant efficiency

Figure 2A shows the typical oxidation kinetic plots found for the chlorogenates as a function of their alkyl chain length. The relative efficiency was determined by measuring the time required to increase the CD content by 0.5%, both in the absence (control experiment) and in the presence of AOs. Measurements were carried out after the propagation step had been initiated (dashed line in Figure 2A), and its variation with the length of the alkyl chain is plotted in Figure 2B.

The efficiency does not correlate with the hydrophobicity of AOs, increasing with increasing length of the alkyl chain to maximum, after which a decrease is observed. This phenomenological, parabola-like, variation of the efficiency is the so-called "cut off" effect already reported by us[16,19-22,31] and others,[9,10,13,32] for several series of homologous AOs bearing the same reactive moiety but ~~of~~ different hydrophobicities. Our results are also qualitatively similar to those reported by Laguerre *et al.* in Brij 35 stabilized sunflower oil-in water emulsions.[10]

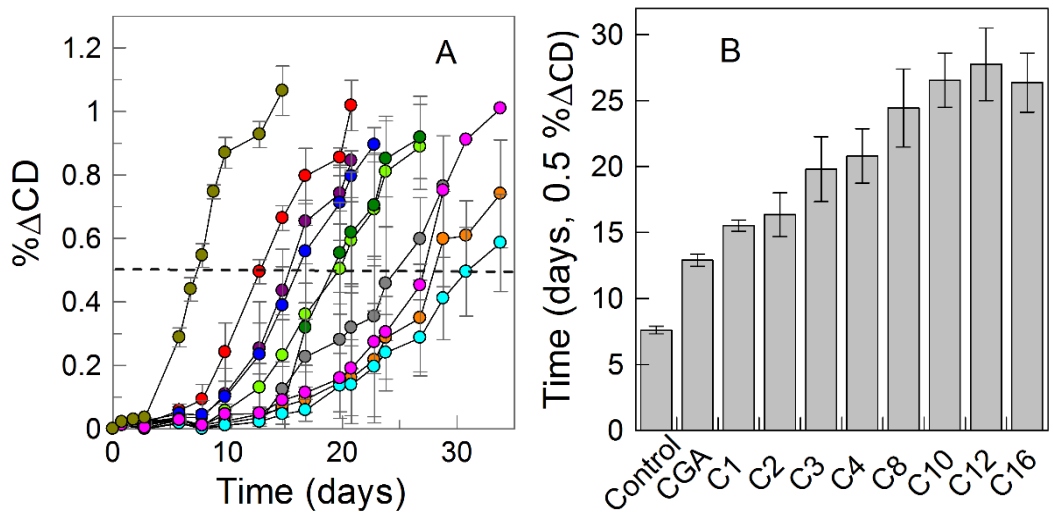


Figure 2. (A) Oxidation kinetic plot in 4:6 (O/W) olive oil-in-water emulsions showing the increase in the conjugated dienes (%ΔCD) content with time at T = 60ºC. ([AO_T] = 0.24 mM in the total volume and 1% Tween 20, ●-control, ●-CGA, ●-C1, ●-C2, ●-C3, ●-C4, ●-C8, ●-C10, ●-C12, ●-C16). **(B)** Time required for an increase in the formation of conjugated dienes of 0.5% as a function of the alkyl chain length of chlorogenates.

Figure 2 shows important differences in the efficiency of chlorogenates in inhibiting the oxidation of the lipids in the olive oil emulsions. We note that the AOs employed here constitute a set of AOs bearing the same reactive group but of different hydrophobicity, and that we previously demonstrated the negligible effect of the alkyl chain length of the chlorogenates on the EC_{50} values and on the anodic peak potential values. However, changes in the hydrophobicity of AOs also change their relative solubilities in the oil, interfacial and water regions and, as a consequence, ~~in~~ their partitioning. Thus, the observed changes in the AO efficiency, Figure 2, can be eventually attributed to changes in their concentrations. To test this hypothesis, we determined the distributions of chlorogenates in the same intact emulsions as those employed in the oxidation experiments.

3.2. Distribution of chlorogenates in binary oil-water mixtures and in olive oil-in-water emulsions: partition constants and interfacial concentrations.

The partition constants of CGA derivatives in binary olive oil–water mixtures, P_W^O, are a thermodynamic measure of the relative hydrophobicity and hydrophilicity of a compound. They provide preliminary qualitative estimations of how AOs distribute in emulsions. Their values, Table 1, were determined at T = 25 ºC as described in the experimental section 2.4.4.

Results show that short chain (C1-C4) CGA derivatives are quite soluble in water, with low P_W^O values in the 10^{-3} – 10^{-2} order of magnitude. As a consequence of the presence of the highly hydrophilic quinic moiety, the value for CGA is much smaller than those found for caffeic (P_W^O =0.02) and for gallic (P_W^O =0.03) acids in olive oil-water mixtures at the same temperature.[14,22] As expected, P_W^O values increase upon increasing the hydrophobicity of AO.

		CGA	C2	C4	C8	C10	C12	C16
EC_{50}	5 min	0.243 ± 0.004	0.243± 0.003	0.248 ± 0.003	0.244 ± 0.003	0.242 ± 0.004	0.249 ± 0.006	0.237 ± 0.006
	15 min	0.235 ± 0.005	0.217 ± 0.005	0.234 ± 0.002	0.234 ± 0.004	0.228 ± 0.005	0.238 ± 0.004	0.223 ± 0.006
	60 min	0.234 ± 0.006	0.172 ± 0.012	0.179 ± 0.005	0.184 ± 0.008	0.176 ± 0.009	0.189 ± 0.005	0.161 ± 0.005
$E_{p,a}$ (mV)	0% Tween 20	0.391	0.410	0.397	0.406	0.393	0.398	0.390
	2% Tween 20	0.398	0.403	0.400	0.409	0.394	0.407	0.400
Binary	P_W^O	$(6.8 \pm 0.4)10^{-3}$	0.086 ± 0.002	0.073 ±0.002	19.6 ± 0.6	26.8 ± 0.9	35.1±1.3	298.5 ± 11.4
Emulsion O/W	P_W^I	40±4	78± 12	141±31	----	---	---	---
	P_O^I	---	---	---	111 ± 17	124 ± 13	159 ± 16	89 ± 6
	$10^2 k_I (M^{-1}s^{-1})$	1.49±0.07	6.15 ± 0.28	7.77 ± 0.30	7.71 ± 0.17	---	5.60± 0.60	5.38 ± 0.07

Table 1. EC_{50} (mole AO/mole $DPPH^{\bullet}$) values obtained at different reaction times with a level of significance $P < 0.05$ and anodic potential ($E_{p,a}$) *vs* Ag/AgCl measured at a glassy carbon electrode for 10^{-4} M solutions of compounds in buffer solution in the absence and presence of 2% Tween 20. P_W^O values in binary olive oil-water systems, in the absence of emulsifier, P_O^I and P_W^I values and the rate constant in the interfacial region, k_I in olive oil emulsions.

Because of the differential hydrophobicity of the CGA derivatives, their distribution between the oil, aqueous and interfacial regions of the emulsions is expected to be different, their partitioning depending on their hydrophilic-lipophilic balance (HLB). The P_O^I, P_W^I and k_I values displayed in Table 1 were obtained from the variations of the observed rate constant, k_{obs}, for the reaction between 16-ArN_2^+ and the chlorogenates with the surfactant volume fraction Φ_I as described elsewhere (section 2.4.5 and references therein).

Consistent with the predictions of equations S1-S3, Figure 3 shows that k_{obs} values decrease asymptotically 3 to 6-fold upon increasing the emulsifier volume fraction from Φ_I =0.005 to Φ_I =0.04 (*Supplementary Material*). The straight lines shown in Figure 3 are plots of $1/k_{obs}$ *vs* Φ_I. The slopes and intercepts for the linear fits of $1/k_{obs}$ *vs* Φ_I were used to calculate the P_W^I, P_O^I and k_I values.

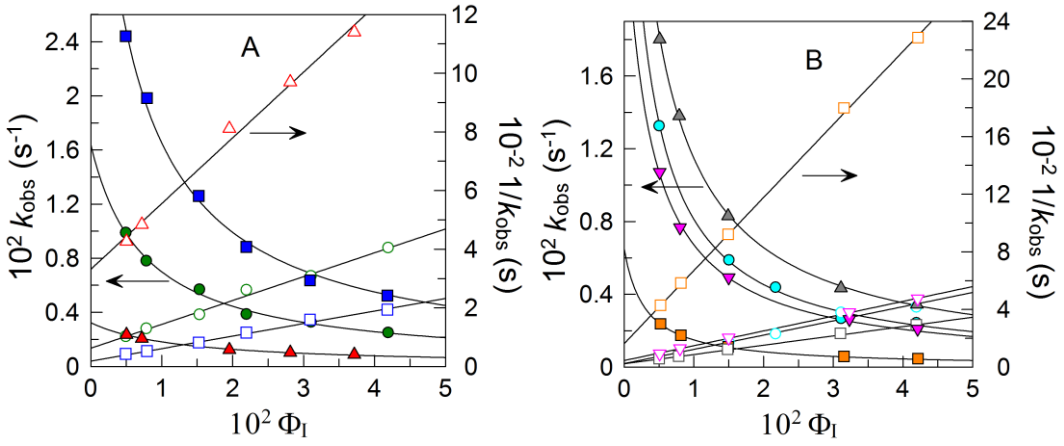


Figure 3. Representative plots of the variation of k_{obs} (curved lines) and $1/k_{obs}$ (straight lines) with Φ_I for the different chlorogenates (▲-CGA, ■-C2, ●-C4, ▲-C8, ■-C10, ●-C12, ▼-C16) in 4:6 olive oil−water emulsions. Solid lines are the theoretical curves obtained by fitting the experimental data to equations S1-S3 (*Supplementary Material)* and to their reciprocals. pH 3.6 (citric acid−citrate buffer, 0.04 M), [AO] = 1.8-2×10^{-3} M, [16-ArN_2^+]~3.0×10^{-4} M, T = 25°C.

Values for k_I are not required to estimate P_W^I and P_O^I values, but they can provide insights into the impact of the medium or mechanistic changes of the reaction of 16ArN_2^+ with chlorogenates. Table 1 shows that k_I values for the CGA derivatives are

independent of the chain length of the chlorogenates, with an average value of k_I ~6.52±1.03%, suggesting that the reactive moiety of the antioxidants is located in the interfacial region of the emulsion.

P_W^I and P_O^I values, Table 1, range from 40 to 160, indicating that all chlorogenates have a natural tendency to incorporate into the interfacial region of the emulsion (the Gibbs free energy for the transfer process from the oil or aqueous to the interfacial region is negative). However, the trend is different and depends on the hydrophobicity of the CGA derivatives. P_W^I values increase with increasing HLB of AO from P_W^I = 41 (CGA) to 141 (C4). In contrast, P_O^I values increase with increasing hydrophobicity up to a maximum at the C12, after which these values decrease.

Since P_W^I and P_O^I are known values, the percentage of the antioxidant in the oil, aqueous and interfacial regions of the olive oil emulsions ($\%AO_O$, $\%AO_W$, $\%AO_I$, respectively) were calculated using equations S4-S6 (*Supplementary Material)*. Figure 4 shows the variation of the percentage of chlorogenates in the aqueous (4A), interfacial (4B) and oil (4C) regions with the surfactant volume fraction Φ_I. For any of the AOs, $\%AO_w$ and $\%AO_O$ decrease upon increasing Φ_I, while $\%AO_I$ increase. For example, $\%CGA_w$ decreases from 80% (Φ_I = 0.005) to $\%CGA_W$ = 30 when Φ_I = 0.45, while $\%CGA_I$ increases from ~ 20% (Φ_I = 0.005) to ~ 70% (Φ_I = 0.45).

The effects of hydrophobicity are more complex. Contrary to what happens in the oil region, in the aqueous region, $\%AO_W$ decreases upon increasing the hydrophobicity. In the interfacial region, the variation in $\%AO_I$ does not correlate with the hydrophobicity of the AOs. $\%AO_I$ Increases upon increasing the hydrophobicity of the AOs up to a maximum at the C12, after which it decreases, following the order %CGA<%C2<%C4 ~%C16<%C8<%C10<%C12, Figure 5. The effects of the alkyl chain length on $\%AO_I$ are more significant at lower than at higher Φ_I, because at high Φ_I most of the AOs are already located at the interfacial region, Figure 5.

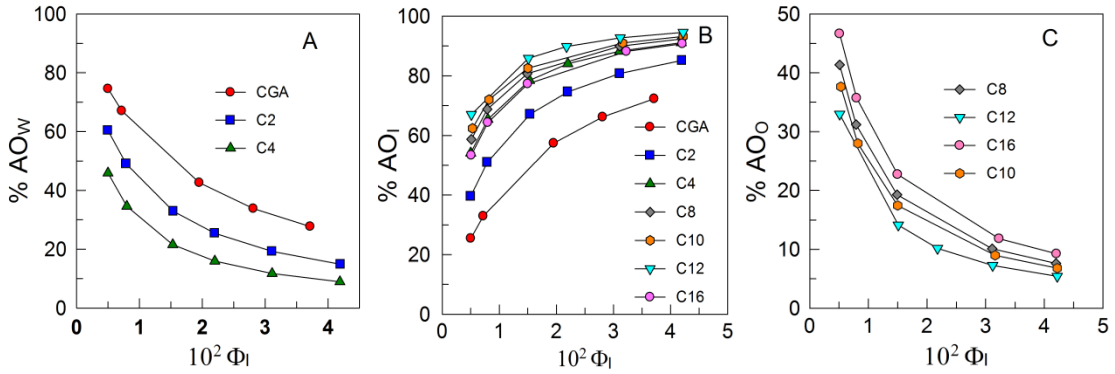


Figure 4. Distribution of chlorogenates between the aqueous **(A)**, interfacial **(B)**, and oil **(C)** regions of 4:6 (O/W) olive oil emulsions.

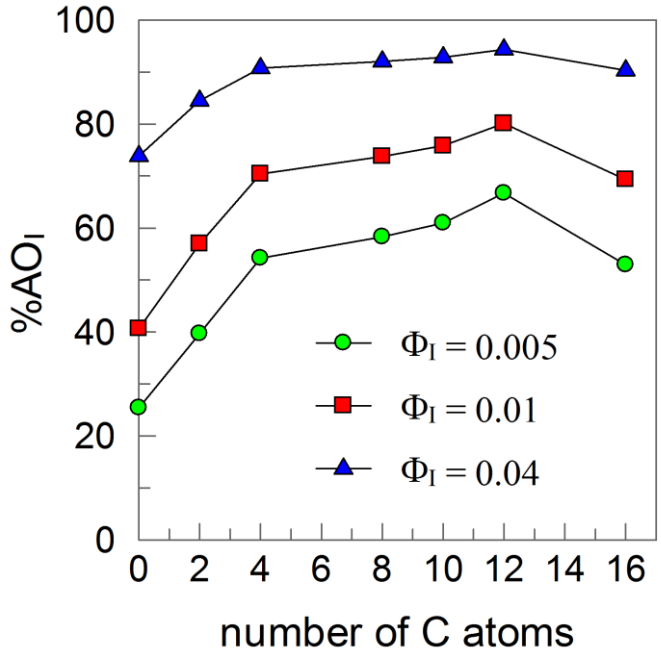


Figure 5. Effects of the hydrophobicity of AO on $\%AO_I$ of olive oil-in-water emulsions at three Φ_I values.

The rate of the reaction between the AOs and the lipid radicals depends, among others, on the concentrations of AOs at the reaction site, which is the interfacial region. The effective concentrations of AOs at the regions of emulsions are not the same as the stoichiometric concentrations. This results from AOs partitioning in different extents between the aqueous, interfacial and oil regions and the volume of the each region is different from the total volume of the emulsion. Thus, we calculated the concentrations

of AOs in the interfacial and oil regions, (AO_I) and (AO_O) respectively, by employing equations 4 and 5 (parenthesis means concentration in moles of AO per liter of the each region).

$$(AO_I) = \frac{(\%AO_I)}{\Phi_I}[AO_T] \quad (4) \qquad (AO_o) = \frac{(\%AO_O)}{\Phi_O}[AO_T] \quad (5)$$

The variations of (AO_O) and (AO_I) with Φ_I are shown in Figure 6A and 6B. For any of the AOs, the interfacial molarities are much higher (20-150 fold) than their stoichiometric AO concentrations ($[AO_T]$~$2.4x10^{-4}$M), while those in the oil region are 0.1-1.5 fold. (AO_I) also decreases asymptotically (6-8 fold) upon increasing Φ_I from Φ_I = 0.005 to Φ_I = 0.04 because interfacial concentrations depend on both $\%AO_I$ and Φ_I, in equation 4, and both parameters work in opposite directions and the increase in the surfactant volume fraction is greater than the increase in $\%AO_I$. For example, an increase in Φ_I from 0.005 to 0.5 would increase the interfacial volume V_I by a factor of 100, but would increase $\%AO_I$ by only 2 - 4 fold (Figure 4B). On the other hand, as Φ_I increases the fraction of AO in the interfacial region approaches a plateau, Figure 4B, and eventually becomes independent of Φ_I because the AO is incorporated to the interfacial region.

To get a better feeling on how hydrophobicity and the oil to water ratio (O/W) employed in the preparation of the emulsion affects the interfacial concentrations, we plotted the variations of (AO_I) with the length of the alkyl chain at three representative O/W ratios, Figure 6C. For any O/W ratio, the variation of (AO_I) with the hydrophobicity of the chlorogenates is parabolic-like, with a maximum at the C12 derivative. Note that the variation of (AO_I) with the number of C atoms in the alkyl chain is more significant at lower than at higher O/W ratios. At relatively high O/W ratios (4:6 and 5:5), the differences in (AO_I) for the C4-C12 derivatives are almost negligible, suggesting that the O/W ratio may change the efficiency order. These results are preliminary but they are currently being analyzed in detail and will be part of future reports. Taken together, the results suggest that changes in either the emulsifier volume fraction and in the O/W ratio may change significantly the interfacial concentrations of the antioxidants, thus affecting their antioxidant efficiency.

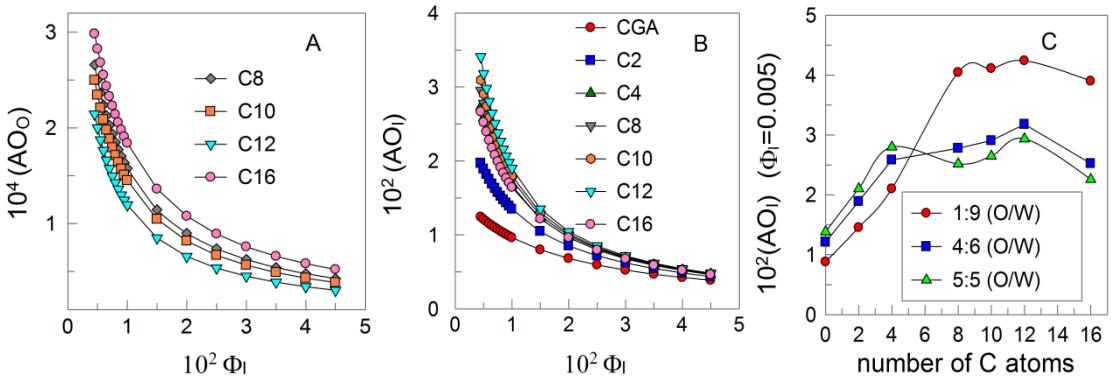


Figure 6. Variation of the concentration of chlorogenates in the oil **(A)** and interfacial **(B)** regions of 4:6 olive oil-in-water emulsions with the emulsifier volume fraction, Φ_I.**(C)** Effect of hydrophobicity of chlorogenates on their interfacial concentrations (AO_I) at different O/W ratio. The concentrations given in parenthesis, ~~( ),~~ refer to the moles of AO per litre of the oil (O) and interfacial (I) regions. Added concentration, [AO_T] = 0.24 mM, refers to the number of moles of AO per litre of the total volume of the emulsion.

3.3. Structure-reactivity relationships: the role of hydrophobicity

Figure 6B shows that, for 4:6 emulsions, the highest interfacial concentration is achieved for the C12 derivative, which is the same antioxidant that shows the higher efficiency in the same emulsions, Figure 2B. This suggests that there may be a similar relationship to that found for other series of homologous antioxidants, between the antioxidant efficiency of chlorogenates and their interfacial concentrations.[14,16,19-22] Thus, we plotted the variations in the interfacial concentrations and the relative antioxidant efficiency (defined as time required to reach an increase in the CD content of 0.5%, figure 2) with the length of the alkyl chain of chlorogenates, Figure 7A. Both variations parallel each other, providing physical evidence that the observed variations in the antioxidant efficiency are a consequence of the differential distribution, i.e., interfacial concentrations of the antioxidants in the emulsion. The variation of either the antioxidant efficiency, or the interfacial concentration, with the length of the alkyl chain of AOs is usually called the "cut off effect". This term has been employed to describe

the variations of biological activities of a series of compounds bearing the same reactive moiety but of different hydrophobicities.

For comparative purposes, Figure 7B displays the variations in the interfacial concentrations of a series of esters derived from caffeic acid (CA) and CGA. The solubility of CA in water is lower than that of CGA as is shown by the P_W^O values obtained in the same binary systems (P_W^O = 0.02 for CA[21] and P_W^O = 0.007 for CGA) and thus CA is more hydrophobic than CGA. Hence, one can expect the interfacial concentrations of chlorogenates to be lower than those of the CA derivatives, as illustrated in Figure 7B. However, due to the larger percentage of CA derivatives in the interfacial region, the relative increase in the interfacial concentrations on going from the acid to the C12 ester derivative is more significant for CGA than for CA (a 3-fold increase for CGA compared to a ~1.3 fold for CA).

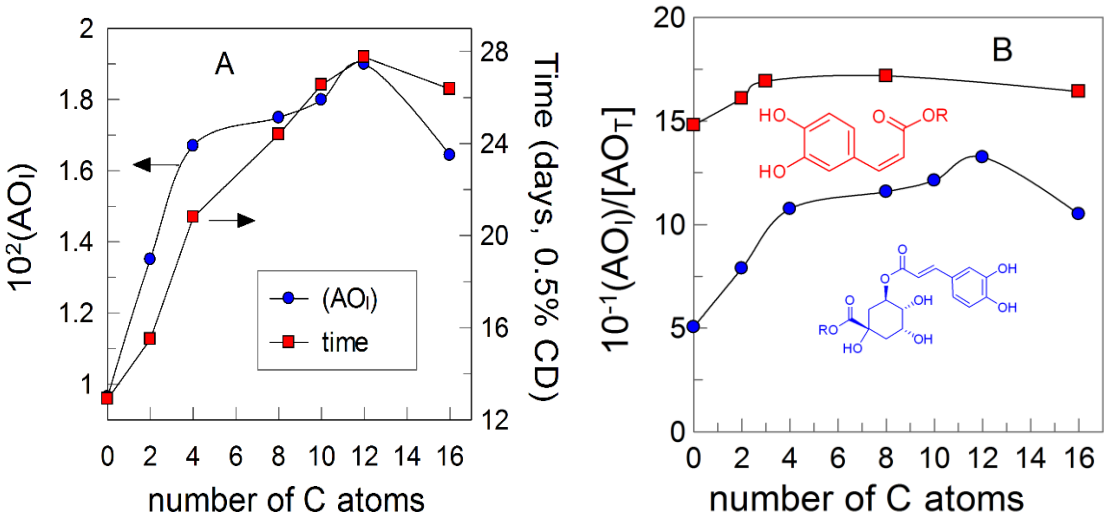


Figure 7. (A) Effect of alkyl chain length on the interfacial concentrations and antioxidant efficiency of chlorogenates in 4:6 (O/W) emulsions containing 1% Tween 20. Values extracted from Figures 2 and 6. **(B)** Effects of the alkyl chain length on the interfacial concentration of chlorogenates (-●-) and caffeates (-■-) relative to stoichiometric AO concentration in 4:6 (O/W) emulsions (Φ_I=0.5%). The concentrations given in parenthesis, ~~(–),~~ refer to the moles of AO per litre of the interfacial region and [AO_T] refers to the number of moles of AO per litre of the total volume of the emulsion.

4. Conclusions

In accordance with our previous work,[16,19-21] the distribution results show that the non-linear effect of the hydrophobicity of chlorogenates in emulsions is due to their differential affinities towards the interfacial region and affect their antioxidant efficiency (verifica se é isto que se queria dizer).

The results highlight the importance of the interfacial region in controlling the antioxidant efficiency. We can emphasis the following aspects:

1) An increase in the HLB of AOs promote their incorporation in the interfacial regions of oil-in-water emulsions, but only up to a point (C12) because a further increase in their HLB may make them ~~to be~~ more soluble in the oil region than in the interfacial one;

2) HLB of antioxidant and emulsifier concentration (Φ_I) are both the main parameters controlling the distribution of all chlorogenates, i.e., a careful choice of both parameters is crucial because they strongly affect the availability of AOs at the reaction site;

3) Changing the O/W ratio from 4:6 (O/W) to 1:9 (O/W) significantly increase the incorporation of very hydrophobic chlorogenates (C8-C16) into the interfacial region of a model emulsion;

4) Due to the higher solubility of CGA in water, changing the HLB of chlorogenates significantly increases their interfacial concentration in a higher extend than that observed for caffeates. These results support the hypothesis that changing HLB of an AO modules significantly the interfacial concentration of very hydrophilic AOs but not so much of AOs of intermediate hydrophobicity;

5) Another important result is that the interfacial concentration of all chlorogenates is higher than the bulk concentration (for example, 115-fold higher than the stoichiometric concentration for C8 at Φ_I =0.005), highlighting that the compartmentalization effects play a key role in defining the antioxidant response against the oxidation of biomolecules.

Finally, we would like to stress that controlling the interfacial antioxidant concentrations of dietary phenolic antioxidants can lead to a more efficient antioxidants in multiphasic systems and can allow the better usage of the existing natural AOs. Future research must be directed towards screening the parameters that control the

optimum length of non-polar chains, the type of lipid radicals employed, the emulsifier nature and O/W ratio ~~among others~~. Verifica se isto que se quer dizer

Conflicts of interest

There are no conflicts to declare.

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