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Chain Length Affects Antioxidant Properties of Chlorogenate Esters in Emulsion: The Cutoff Theory Behind the Polar Paradox

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Twenty years ago, Porter et al. (*J. Agric. Food Chem.* **1989**, *37*, 615−624) put forward the polar paradox stating among others that apolar antioxidants are more active in emulsified media than their polar homologues. However, some recent results showing that not all antioxidants behave in the manner proposed by this hypothesis led us to investigate the relationship between antioxidant property and hydrophobicity. With a complete homologous series of chlorogenic acid esters (methyl, butyl, octyl, dodecyl, hexadecyl, octadecyl, and eicosyl), we observed in emulsified medium that antioxidant capacity increases as the alkyl chain is lengthened, with a threshold for the dodecyl chain, after which further chain extension leads to a drastic decrease in antioxidant capacity. The antioxidant capacity evaluation in emulsion was possible using a newly developed conjugated autoxidizable triene (CAT) assay, which allows the assessment of both hydrophilic and lipophilic antioxidants. The nonlinear behavior was mainly explained in terms of antioxidant location since it was found from partition analysis that the dodecyl ester presented the lowest concentration in the aqueous phase and also that the quantity of emulsifier drastically changes the partition of antioxidant. In addition, this nonlinear influence was connected to the so-called cutoff effect largely observed in studies using cultured cells. Taken together, these different results allow one to make the proposal of a new scenario of the behavior of phenolic compounds in emulsified systems with special emphasis on the micellization process. Finally, in the CAT system, the polar paradox appeared to be the particular case of a far more global nonlinear effect that was observed here.

KEYWORDS: Antioxidant capacity; phenolic compound; chlorogenic acid; lipophilization; lipase; lipid; emulsion; conjugated autoxidizable triene assay; partition; polar paradox; cutoff effect

INTRODUCTION

Unsaturated lipid substrates are prime targets of oxidation in foods, cosmetics, and biological environments. In vitro lipid oxidation is a major concern for agrifood and cosmetics industries since they utilize unsaturated fatty acids to an increasing extent, such as those derived from the highly oxidation-sensitive n-3 (ω-3) family. Besides altering the taste (rancidification) and nutritional quality (loss of vitamins and essential fatty acids) of foodstuffs, the ultimate oxidation of lipids into highly reactive and toxic compounds (malondialdehyde and 4-hydroxynonenal) is a real danger for consumers (*1*). In this context, researchers have turned their interest toward the use of antioxidant molecules, especially phenolic compounds, to counteract lipid oxidation in various lipidic systems from simple ones such as vegetable oils to more complex systems such as heterogeneous lipid based systems including micellar dispersion, emulsion, liposome, and lipoprotein. Concerning heterogeneous systems, the presence of water results in the partitioning of antioxidants according to their hydrophobicity between the aqueous phase and the apolar one, and this should be considered when explaining antioxidant activity (*2*). This particularity leads Porter (*3*) and Porter et al. (*4*) to put forward the polar paradox hypothesis (not to be confused with the antioxidant paradox reported by Halliwell (*5*)) stating among others that apolar antioxidants are more effective in systems of high surface-to-volume ratio such as emulsions (but also micelles, membranes, and whole tissues) than their polar homologues. This paradoxical behavior was explained later by the concept of the interfacial oxidation (*6*) that leads to the polar paradox shift from an empirical observation to a putative theory. Accordingly, in emulsions, lipophilic antioxidants would concentrate in the oil−water interfaces (which are assumed to be the site

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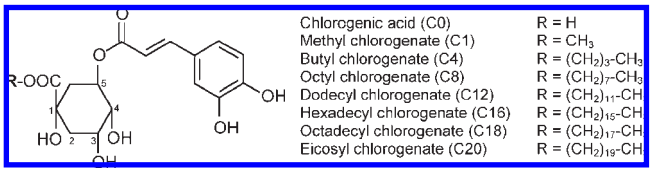

Figure 1. Chemical structure of chlorogenic acid and its alkyl esters.

where oxidation occurs) and inhibit lipid oxidation more efficiently than hydrophilic antioxidants that partition into the water phase. Although this paradoxical behavior was confirmed in various studies performed in heterogeneous systems (*7*−*12*), not all antioxidants behave in the manner proposed by this hypothesis (*13*, *14*), suggesting that antioxidant activity in such systems are possibly governed by more complex phenomena. These inconsistencies led us to hypothesize that the relationship between the antioxidant capacity and the hydrophobicity of a molecule in emulsion is not as linear as expected.

The first challenge to test this hypothesis was to use a relevant way to modulate hydrophobicity. Indeed, in the case of phenolics, the antioxidant capacity is partly governed by the O−H bond dissociation enthalpy (BDE), i.e., good phenolic antioxidants have low O−H BDEs, all other things being equal (*15*). However, since O−H BDEs are strongly dependent on the nature of ring substituents (*16*, *17*), the modulation of the hydrophobicity through addition or removal of subtituents seems to be inappropriate to test this hypothesis. In such a case, the modification of the antioxidant capacity can much more likely result from a BDE modulation than a simple change of the hydrophobicity. Consequently, the studies using this kind of model compounds are not able to give insight into the relevancy of the polar paradox hypothesis. To exclusively study the impact of the hydrophobicity of a molecule on its antioxidant capacity, it is thus required that one uses a homologous series of antioxidants. In this way, we recently showed that chlorogenic acid (5-caffeoylquinic acid) exhibiting antioxidant (*18*) and antiradical (*19*) properties can be lipophilized by fatty alcohols in a two step lipase-catalyzed transesterification strategy (*20*). This reaction leads to a homologous series of alkyl chlorogenate esters (from methyl to eicosyl, **Figure 1**) which constitutes an ideal phenolic model to investigate the relationship between hydrophobicity and antioxidant capacity.

The second challenge is to use an appropriate analytical tool to assess the antioxidant properties of both hydrophilic and lipophilic compounds in a relevant and reliable manner (*1*, *21*). With regard to this matter, we recently developed a method called conjugated autoxidizable triene (CAT) assay (*18*). This in vitro test is based on spectral properties of tung oil (*Aleurites fordii*) exhibiting a strong UV absorption at 273 nm. In emulsion in water and under the constant flow of peroxyradicals produced by thermal decomposition of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37 °C, the degradation of tung oil is accompanied by a bleaching at 273 nm. Addition of antioxidant molecules, plant extracts, or fruit juices rich in antioxidants results in a delay of oxidation, thus allowing the measurement of their antioxidant capacity in comparison with a reference standard (Trolox).

Finally, besides the importance of the hydrophobicity of the molecule, the influence of the type (cationic, anionic, and nonionic) and the quantity of the emulsifier present in the emulsion play a crucial role in the evaluation of the antioxidant capacity of phenolics. Indeed, Mei et al. (*22*), Stöckmann et al. (*23*), Richards et al. (*24*), and more recently Yuji et al. (*14*) demonstrated that these parameters can drastically modify the distribution of species within media and consequently interfere with the antioxidant properties evaluation. It is well known that above its critical micelle concentration, the emulsifier saturates the interfacial membrane and can accumulate in the water phase by a micellization process. The formation of micelles can, however, bias the antioxidant capacity evaluation in such a system since the antioxidant can be trapped in these self-assembled structures.

In this article, new insights on the behavior of phenolic compounds in heterogeneous lipid-based systems are given, and the influence of the emulsifier quantity on the partition of a homologous series of chlorogenic acid and its alkyl esters was studied. Taken together, these different results allow one to propose a new scenario of the behavior of phenolic compounds in emulsified systems.

MATERIALS AND METHODS

Chemical. Tung oil (averaged MW = 872 g/mol) was purchased from Aldrich (ref. 440337). Sunflower oil was purchased in a local supermarket. Chlorogenic acid, phosphate buffer solution (PBS) at pH 7.2, and polyoxyethylene(23)laurylether (Brij 35, estimated MW = 1198 g/mol) were purchased from Sigma (Saint Quentin, France). All solvents used were of HPLC or analytical grade and were also purchased from Sigma. Trolox was from Acros Organic (Geel, Belgium), and finally, 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) was from Wako Chemical (Neuss, Germany).

Synthesis of Chlorogenate Esters. The chemo-enzymatic esterification of chlorogenic acid to obtain chlorogenate esters was carried out following the procedure described by López-Giraldo et al. (*20*). Briefly, in a 500 mL glass vessel, 10 mmol of 5-CQA was dissolved in 240 mL of methanol. Amberlite IR-120 H (10 g), previously dried at 110 °C for 48 h, was added to the reaction mixture, which was then stirred in an orbital shaker (250 rpm) for 9 h at 55 °C. After cooling to room temperature, the reaction medium was filtered on a 1.6 μm glass microfiber filter (Whatman International Ltd., Maidston, England), and methanol was removed under vacuum. Chloroform (150 mL) was then added, and the solution was dried over sodium sulfate, filtered on a 1.6 μm glass microfiber filter, and evaporated under vacuum at 50 °C. The resulting methyl chlorogenate (5 mmol) was then added to 375 mL of desired fatty alcohol, and the mixtures were then placed in sealed flasks and stirred on an orbital shaker (250 rpm, 55 °C) until complete dissolution of methyl chlorogenate. *Candida antarctica* B lipase 5 wt %/wt (calculated from the total weight of both substrates) was then added to start the transesterification step. The suspension was then stirred at 55 °C for 96 h under nitrogen flow in order to continuously eliminate the formed methanol and favor the displacement of the reaction equilibrium toward synthesis. The final lipophilized esters were then purified in a two step procedure. First, a liquid−liquid extraction using hexane and a solution of acetonitrile/water (3:1, v/v) was realized to remove the fatty alcohol in excess. In a second step, the alcohol traces were eliminated using silica gel column chromatography (length 25 cm. i.d. 1.6 cm), using toluene/ethyl acetate (90:10, v/v) as eluant. All recovered esters were then characterized by mass spectrometry as described by López-Giraldo et al. (*20*).

Preparation of Tocopherol-Free Tung Oil Samples. The polar compounds of tung oil (including β-tocopherol) were removed by passing 25 mL of a 200 mg/mL tung oil solution in hexane, followed by 25 mL of pure hexane through an alumina column prepared as follows: 25 g of alumina in hexane was introduced into a glass column, and the excess hexane was eliminated until it rose to the alumina surface. After complete removal of tocopherols, hexane was evaporated under vacuum at 35 °C using a rotatory evaporator equipped with a vacuum pump (Laborport, KNF Neuberger GmbH, Freiburg, Germany). It is worth noting that all experiments must be carried out under shelter from light, as much as possible. Finally, the stripped tung oil was aliquoted into brown glass tubes, then inerted under nitrogen stream, and stored at −18 °C until use. The use of disposable stripped tung oil aliquots (i.e., 15 aliquots for 15 microplates) avoids the necessity for successive withdrawals from one aliquot and minimizes any eventual oxidation.

Conjugated Autoxidizable Triene (CAT) Assay Protocol. Antioxidant capacity of chlorogenic acid and its alkyl esters was measured

using the CAT procedure described by Laguerre et al. (*18*) with slight improvements previously brought by Laguerre et al. (*25*). Briefly, antioxidant solutions were prepared as follows: a methanol solution of chlorogenic acid, its alkyl esters, or Trolox (reference) was prepared at the desired concentration. Then, various volumes of this solution (25, 50, 75, and 100 μL) were added to 24.9 mL of phosphate buffer solution (PBS) at pH 7.2 and then completed to 25.0 mL with pure methanol (75, 50, 25, and 0 μL, respectively). In this way, all buffered solutions of antioxidants contain the same methanol volume (100 μL), which allows one to avoid any eventual bias among samples. This new CAT procedure presents the advantages, compared to those the former protocol (*18*), in enabling the analysis of a larger panel of molecules from hydrophilic to lipophilic ones. Moreover, phenolic compounds in methanol solutions are much more stable than in PBS at neutral pH, thus allowing storage at −20 °C during several days, which is not possible with PBS at pH 7.2 for which the stability does not exceed few hours (unpublished results). In this way, all buffered solutions of antioxidant were prepared extemporarily. Fifty microliters/well of these solutions were transferred using a multichannel micropipet into a UV-Star 96-well microplate (Greiner, Frickenhausen, Germany), a well adapted plastic-based microplate to spectral measurement in the UV-domain (absorbance at 273 nm = 0.03). The plastic-based microplate was then prewarmed and stirred in a thermostatted shaker (PHMT Grant Instruments Ltd., Shepreth, England) at 37 °C for 5 min at 1200 rpm.

Twenty-five milliliters of PBS solution, pH 7.2, containing 34 μM Brij 35 (neutral emulsifier, estimated MW = 1198 g/mol) was added to 5 mg of stripped tung oil in a brown glass flask. Afterward, it was crucial to premix this mixture by stirring it for 10 s using a Vortex apparatus, before its homogenization in an Ultra Turrax homogenizer (Janke & Kunkel, Staufen, Germany) at approximately 2400 rpm for 90 s. Each well was then completed with 100 μL of this tung oil-in-PBS emulsion. To improve repeatability, the microplate was then immediately prewarmed and shaken, under shelter from light, in a thermostatted shaker (PHMT Grant Instruments Ltd.) at 37 °C for 1 min at 1200 rpm.

Fifty microliters of a freshly prepared AAPH solution in PBS (4 mM) was added with a multichannel micropipet. Finally, each well contained 200 μL of the final mixture consisting of 115 μM stripped tung oil, 17 μM Brij 35, 1 mM AAPH, and various concentrations of antioxidants (from 0 to 0.8 μM) in PBS. The progress of reactions was immediately monitored by recording the decrease in absorbance at 273 nm. Measurements were performed each minute for 5 h at 37 ± 0.1 °C, with 5 s stirring before each measure, using a Saffire 2 microplate reader (Tecan, Gröedig, Austria) equipped with Magellan software. Three independent analyses using different microplates were carried out.

Expression of the Results of the Conjugated Autoxidizable Triene Assay. To normalize data, the raw absorbance signal was transformed in relative absorbance according to the following equation:

$$\text{Relative absorbance} = A_t/A_0 \quad (1)$$

where A_t and A_0 are absorbances read at times t and 0 min, respectively. It is worth mentioning that if the measurement is not rapid enough after AAPH addition, the A_0 for the blank (without antioxidant) may be lower than that of the sample containing antioxidant. In this case, to normalize A_0, the experimental A_0 for the blank can be artificially replaced with the A_0 of samples in eq 1.

The area under the curve (AUC) corresponding to relative absorbance decay was then calculated as follows:

$$\text{AUC} = 1 + A_1/A_0 + A_2/A_0 + ... + A_{299}/A_0 + A_{300}/A_0 \quad (2)$$

The net protection area provided by an antioxidant sample was then calculated using the difference between the AUC in the presence of an antioxidant sample (AUC_{Sample}) and the AUC of the blank (AUC_{Blank}), the latter consisting of the same mixture without antioxidant.

Trolox was used as a calibrator for antioxidant capacity measurements. Thus, the antioxidant capacity of a sample relative to Trolox (CAT value) is given as follows:

$$\text{CAT value} = [(AUC_{Sample} - AUC_{Blank})/(AUC_{Trolox} - AUC_{Blank})] \times [\text{moles of Trolox/moles of sample}] \quad (3)$$

where (AUC_{Sample} − AUC_{Blank}) and (AUC_{Trolox} − AUC_{Blank}) are the net protection areas in the presence of a sample and Trolox, respectively. It must be mentioned that Trolox was used as the internal calibrator (i.e., analyzed on the same microplate). Finally, the CAT value is expressed as moles of Trolox per mole of tested compound (Trolox equivalents).

Partition Measurements. The sunflower oil-in-water emulsions (2 wt %/wt) were prepared using Brij 35 as the emulsifier. The final concentrations of emulsifier were 0.017, 2, 5, and 10 mM. The procedure was the following: 25 mL of a phosphate buffer solution at pH 7.2 containing the previous concentration of Brij 35 was added to 0.5 g of sunflower oil in a brown glass flask. Afterward, the solution was vortexed for 10 s before its homogenization using an Ultra Turrax homogenizer (Janke & Kunkel, Staufen, Germany) at approximately 2400 rpm for 90 s. To prevent lipid oxidation, 100 μM EDTA was added to the emulsion sample. All antioxidants were dissolved in methanol and added to the emulsion at a final concentration of 25−100 μM. Afterward, the previous solutions were kept for 24 h in the dark. To measure antioxidant concentration in the continuous phase of the emulsion, 1 mL of emulsions and 0.5 mL of PBS were mixed in a centrifuge tube and centrifuged at 10,000*g* at 4 °C for 40 min. After centrifugation, 1 mL of the continuous phase (lower layer) of the emulsion was withdrawn with a syringe. This procedure of centrifugation and collection of the continuous phase of the emulsion was repeated three times. After the final centrifugation, the continuous phase was filtered through a 0.20 μm syringe filter to remove any residual emulsion droplets. The concentration of chlorogenic acid and its alkyl esters was finally evaluated using HPLC with a detection at 327 nm as previously described by Lopez Giraldo et al. (*20*).

RESULTS AND DISCUSSION

Numerous in vitro studies performed in heterogeneous systems have shown that the antioxidant capacity of phenolics is governed not only by intrinsic properties such as O−H BDE or steric hindrance but also by physicochemical phenomena such as partitioning in the different phases (*6*, *7*, *26*), diffusion and intermicellar diffusion (*27*), micellization, etc., which are in turn mainly governed by hydrophobicity. However, the influence of hydrophobicity on phenolic antioxidants is not yet well understood, especially in complex systems such as lipid-based ones. With regard to this matter, Porter et al. (*4*) put forward twenty years ago the polar paradox stating among others that in emulsified media, apolar antioxidants are more active than their polar homologues. However, it has been demonstrated that not all antioxidants behave in the manner proposed by this hypothesis, which led us to postulate that the relationship between hydrophobicity and antioxidant capacity does not necessarily follow a linear shape.

Antioxidant Properties of Chlorogenic Acid and Its Esters.

To test this hypothesis, the antioxidant capacity of a homologous series of alkyl esters of chlorogenic acid (noted as C1, C4, C8, C12, C16, C18, and C20) and chlorogenic acid (noted as C0) (**Figure 1**) was evaluated using the conjugated autoxidizable triene (CAT) assay (*18*) in its improved version using methanolic predissolution of antioxidants (*25*). **Figure 2** shows the kinetics of relative absorbance bleaching for C0, C1, C4, C8, C12, and C16 at 0.2 (**Figure 2a**), 0.4 (**Figure 2b**), 0.6 (**Figure 2c**), and 0.8 (**Figure 2d**) μM. First of all, it can be seen that all antioxidants inhibit the AAPH-mediated oxidation of tung oil following a biexponential reciprocal trend, which can be divided in three regions: (i) a pseudolag phase for which chlorogenic acid and its esters compete with tung oil to reduce AAPH-derived free radicals, (ii) a logarithmic-like phase beginning when all initial antioxidants are transformed in oxidation product(s), most of them still endowing residual antioxidant capacity, and finally (iii) a steady state beginning when tung oil is totally oxidized. From **Figure 2**, it seems that there is no significant difference in the lag-phase duration between phenolics. In contrast, differences can be noticed in the logarithmic-like phase, with a maximal duration for both dodecyl and hexadecyl esters (**Figure 2**, phase (ii)).

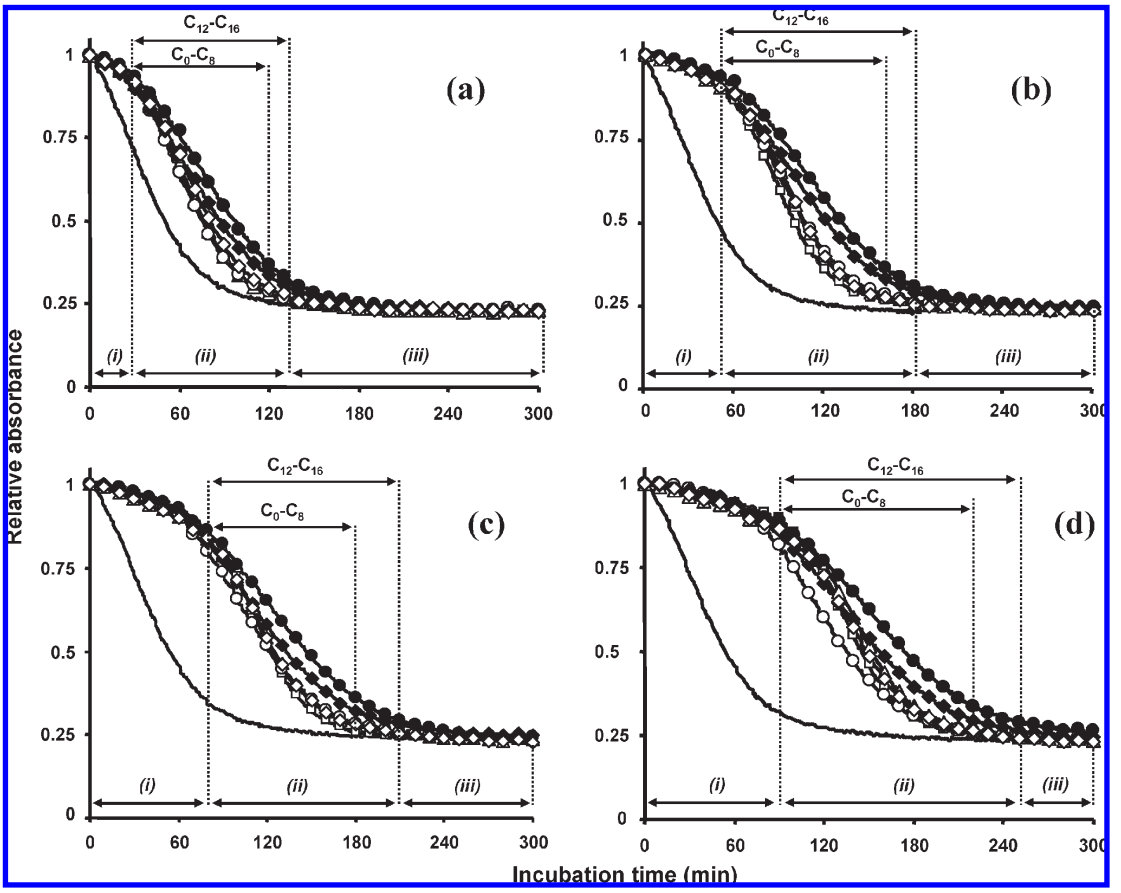

Figure 2. Kinetics of relative absorbance bleaching in the absence (—) or presence of 0.2 (**a**), 0.4 (**b**), 0.6 (**c**), and 0.8 μM (**d**) phenolics. The reaction mixture contained 115 μM stripped tung oil, 17 μM Brij 35, and 1 mM AAPH, in PBS at pH 7.2 at 37 °C. C0 (◇), C1 (△), C4 (□), C8 (○), C12 (●), and C16 (♦).

Through calculation of the AUC (see the Materials and Methods section), good linear relationships ($R^2 \geq 0.94$) were established between net area protection ($AUC_{Sample} - AUC_{Blank}$) and antioxidant concentration for all compounds tested (data not shown), which is a prerequisite to calculate their CAT values. When eq 3 was applied, the dodecyl ester was shown to exhibit the highest CAT value (2.97 ± 0.13 Trolox equivalent, TE), followed by C16 (2.46 ± 0.23 TE) > C0 (2.05 ± 0.03 TE) > C8 (1.95 ± 0.07 TE) ≃ C1 (1.94 ± 0.02 TE) > C4 (1.76 ± 0.08 TE) ≫ C18 (0.88 ± 0.04 TE) > C20 (0.32 ± 0.02 TE). The depiction of the CAT value as a function of the alkyl chain length showed a strong nonlinear influence of this latter parameter on antioxidant capacity (**Figure 3**). This nonlinear effect can be divided into three virtual domains: (i) below 12 carbons for which no important effect on the CAT value was observed, (ii) 12 carbons for which an optimal CAT value was measured, and finally (iii) above 12 carbons for which a CAT value collapse occurred. First of all, regardless of the mechanism of action, this result is interesting because to the best of our knowledge, it is the first time such a nonlinear effect has been demonstrated in an emulsion. Moreover, this result may suggest that the polar paradox theory is not valid for long chain esters, and therefore, we have to look beyond to gain a new understanding.

Besides the antioxidant properties, such a nonlinear influence of the chain length has been largely observed in a diverse range of biological activities, such as anesthetic (*28*, *29*), antimicrobial (*30*, *31*), and cytotoxic (*32*) properties. More interestingly, with the same homologous series analyzed in cultured human fibroblasts, we previously observed a similar nonlinear effect of the alkyl chain, with a maximal antioxidant capacity for the dodecyl chlorogenate (to be published elsewhere). In these studies performed in cellular systems, the relationship is quasi-parabolic, which means that the efficiency of the interaction of such compounds with biological membranes grows with an increase in their hydrophobic parts up to a certain length and then begins to diminish. Ferguson (*33*) was one of the first to document this type of effect in 1939 when compiling a combination of studies related to a homologous series of compounds. Sometimes named the parabolic case, this effect is now known under the name the cut-off effect, a term adopted by Ferguson (*33*). Balgavy and Devinsky (*34*) reported that this effect is a general phenomenon observed in various biological and toxic activities with practically every amphiphilic homologous series tested so far. On the contrary, to the best of our knowledge, a cutoff effect has never been observed in in vitro antioxidant measurements involving an emulsified system. This is possibly due to the fact that the homologous series currently used do not cover a large hydrophobic range, while in pharmacological studies, the range and the number of compounds in the same series are generally much larger. This study, thus, constitutes a first attempt to connect the behavior of phenolics in emulsions to the so-called cutoff effect in order to fill out the gap existing between these chemical and biological fields.

Partition Behavior As a Possible Mechanism of Action. Taking into account that the location of the phenolics toward the oil droplet is of prime importance in their antioxidant capacities, the partition behavior of chlorogenic acid and its alkyl esters was investigated as a possible mechanism of action for the cutoff effect. Regarding this matter, Walters et al. (*35*) reported that it is difficult to rationalize the cutoff effect because most of the properties supposed to be involved in this behavior (partition coefficients, CMC, ...) do not show maxima or minima at these

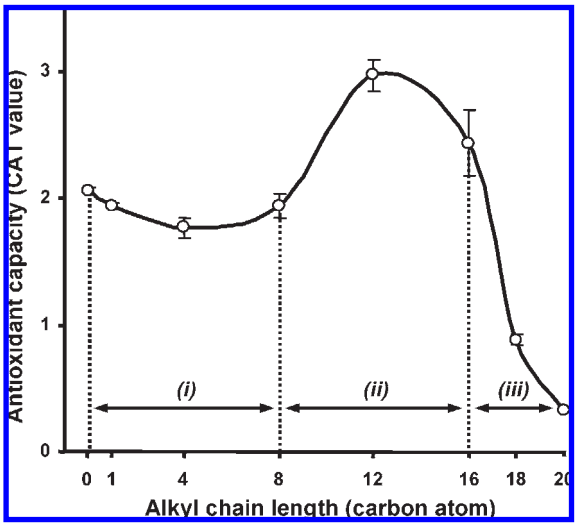

Figure 3. CAT value (mol of Trolox per mol of tested compound) of chlorogenic acid and its esters vs the alkyl chain length.

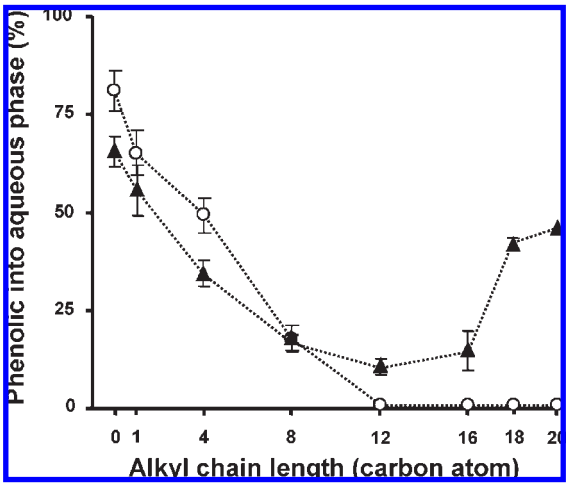

Figure 4. Alkyl chain length effect on the partition behavior of phenolics in a mixture of sunflower oil and PBS at pH 7.2 without (○) or with (▲) 17 μM Brij 35 used as emulsifier.

chain lengths. In other words, the cutoff theory lacks physicochemical descriptors to explain its nonlinear shape. In order to find a rational basis to understand our results, the experimental partitioning of phenolics in a mixture of oil and PBS (without emulsifier) was investigated (**Figure 4**, curve ○). As can be anticipated from the remark of Walter et al. (*35*), no maximum or minimum appeared from these curves, which renders impossible an explanation of the nonlinear shape of our CAT results. However, since the cutoff trend has been observed in the CAT assay involving an emulsifier membrane, the same experiment was repeated using the same molar concentration of emulsifier already used in the CAT assay (i.e., 17 μM) (**Figure 4**, curve ▲). It can be seen that the addition of a very small quantity of emulsifier drastically changes the partition behavior of chlorogenic acid and its alkyl esters between the continuous phase (water) and the discontinuous one (oil + membrane). Indeed, results showed that the concentration of phenolics into the aqueous phase decreases as the alkyl chain length increases, until a threshold was reached for the dodecyl chain. But above 12 carbon atoms, surprisingly, further increments in alkyl chain length led to an increase of the antioxidant level into the aqueous phase. Although the curves depicted in **Figures 3** and **4** (curve ▲) are not perfectly symmetric, the partitioning behavior of chlorogenic acid and its esters in **Figure 4** (curve ▲) showed the same threshold for a dodecyl chain length. This is interesting because this partitioning behavior allows the proposal of a new putative scenario which can possibly explain the cutoff influence of the alkyl chain length on the antioxidant capacity in the CAT assay. Actually, it can be hypothesized that until 12 carbon atoms the corresponding esters are not close enough to the interface where oxidation is supposed to occur (**Figure 5a**). Consequently, the weaker antioxidant capacity of the corresponding esters may be seen as a consequence of their bad localization toward oxidizable substrate. In contrast, at 12 carbons, one can hypothesize that the dodecyl chain provides a closer location to the oil droplet than other alkyl chains along with an adequate orientation of the phenolic head toward the aqueous phase where the AAPH-derived free radicals are concentrated (**Figure 5b**). In complement to our partitioning analysis, this putative scenario is also supported by the recent study of Sasaki et al. (*36*) demonstrating that dodecyl chlorogenate decreases interfacial tension (in water/hexadecane) much more than octyl and butyl esters. Finally, above 12 carbons the antioxidant capacity collapse could be due to the fact that chlorogenate esters mainly exist as aggregates, possibly as micelles (**Figure 5c**). Indeed, it is well-known that as amphiphilic molecules increase in chain length, their tendency toward aggregate formation is greater, noted by the lower critical micelle concentration (CMC) at higher chain lengths (*14*). In connection, Vorum et al. (*37*) have reported that the solubility of the dodecanoic acid in phosphate buffer solution at pH 7.4 (approximately the same conditions as those in the CAT assay) is greater than 500 μM, whereas the hexadecanoic acid showed a tendency to aggregation even at concentrations below 1 μM (approximately the same concentration as that in the CAT assay). Even though it is difficult to extrapolate from free fatty acids to chlorogenate esters, at least this result demonstrated the strong influence of an increment of four carbon atoms on the micellization properties. Accordingly, we assumed that this tendency to form aggregates for the longer chains (C16, C18, and C20) becomes greater than the tendency to move toward the interfacial membrane and that thus the concentration of antioxidant at the site of oxidation decreased. Consequently, the micellization process could confer two major drawbacks to the esters concerned to counteract lipid oxidation: a removal of the antioxidant for the site of lipid oxidation and/or a weak mobility toward AAPH-derived free radicals.

Of course this scenario (summarized in **Figure 5**) has to be strengthened. Indeed, we neglected the fact that the differences in partitioning behavior among C8, C12, and C16 (**Figure 4**) are far less important than their differences in the CAT values (**Figure 3**). Moreover, it is difficult to explain why there is no improvement of the antioxidant capacity from C0 to C8, while a constant decrease into the aqueous phase was demonstrated (**Figure 4**, curve ▲). This difficulty shows that a study of the exact location of phenolics into the discontinuous phase (i.e., interfacial layer or oil droplet) is crucial to understand these results more deeply. However, this point highlights that other parameters may also contribute to the antioxidant action of chlorogenic acid and its alkyl esters in this system. It is indeed possible that the mobility of the phenolic decreases as its alkyl chain is lengthened, consecutively decreasing its ability to move toward the oxidation site. One can also observe (**Figure 2**) that the pseudolag phase (phase *i*) of C12 is almost identical to that of C8, while the difference in the exponential reciprocal phase (phase *ii*) of C8 and C12 is more important. Assuming that the pseudolag phase is rather linked to the antioxidant action of nonoxidized forms, whereas the exponential reciprocal phase is linked to the reaction of oxidized

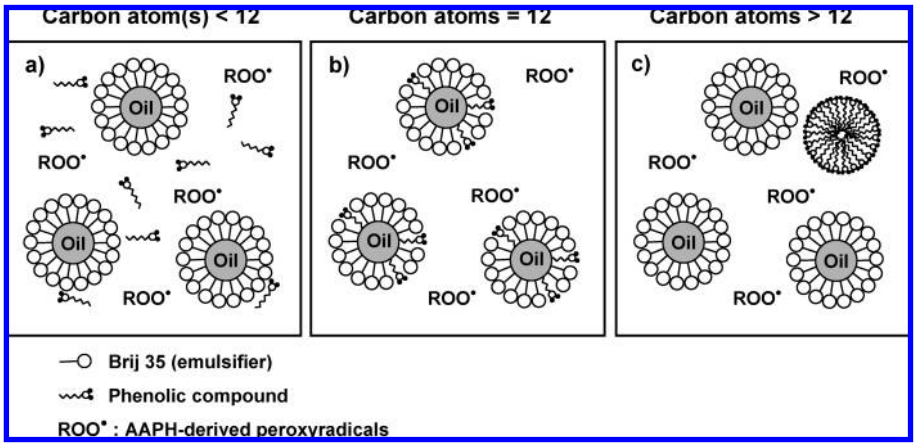

Figure 5. Putative scheme of the distribution of antioxidant in an emulsified system.

form(s) of the antioxidant (still endowing antioxidant activity), it is logical to consider that the higher CAT value of C12 is rather due to a better location of the C12-oxidized form(s) than a better location of the C12 nonoxidized form However, this hypothesis cannot be verified by our experiments (**Figure 4**), which only measure the partition behavior of the nonoxidized forms of antioxidants. For this purpose, work is in progress to measure the partition under oxidative conditions in order to give insight on the partition behavior of the oxidation product(s) of phenolics.

However, it has not escaped our notice that at neutral pH, the phenolic groupings of chlorogenic acid and its alkyl esters must be partly ionized. This means that the reaction mechanism with AAPH-derived peroxyradicals may involve an electron transfer (ET) process from the phenolate anion to the peroxyradical. Interestingly, we have previously found with this homologous series that the ET process is the most probable pathway in the reduction of the 2,2-diphenyl-1-picrylhydrazyl radical in methanol (*19*). In addition, such an ET mechanism must be favored since AAPH-derived radicals bear a positive charge (*38*).

Finally, it appeared that until 12 carbons, our results are in partial agreement with the polar paradox (*4*). However, above 12 carbons, there is a strong difference. In our case, when the antioxidant hydrophobicity increases, the polar paradox theory does not take into account the progressive predominance of the antioxidant's accumulation in the water phase possibly by a micellization process, compared to its anchorage in the oil-in-water interphase. In other words, our results suggest that in the CAT system the polar paradox is simply a *particular case* of a much more global *cutoff effect*. Further experiments have to be done on different emulsified systems (varying oxidant, emulsifier, pH, ...) to validate or invalidate this nonlinear behavior in other oil-in-water emulsion systems.

Effect of the Emulsifier Quantity on Partition Behavior. Taking into account that the emulsifier concentration used in the CAT assay (17 μM) is much lower than those usually employed in similar procedures, we investigated the influence of the concentration of emulsifier on the partition behavior of chlorogenic acid and its alkyl esters. In this way, increasing concentrations of Brij 35 (2, 5, and 10 mM) were added to sunflower oil-in-buffered water (pH 7.2) emulsion at 37 °C (**Figure 6**). As can be seen, the partition behavior of chlorogenic acid and its esters was strongly different from those previously observed (**Figure 4**). Instead of a quasi-parabolic trend, the present data do not show any significant influence of the alkyl chain length on the partition behavior. All of the phenolics are mainly present in the water phase of the emulsion (~60−80%). Since it is difficult to observe the influence of the emulsifier concentration on the basis of **Figure 6**, the

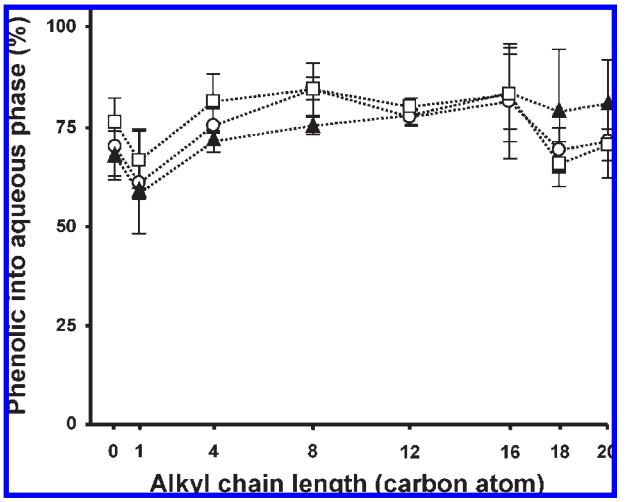

Figure 6. Alkyl chain length effect on the partition behavior of phenolics in sunflower oil-in-water emulsion using an excess of Brij 35 as emulsifier (2 mM, ▲; 5 mM, ○; 10 mM, □).

antioxidant concentration in the water phase was plotted as a function of Brij 35 concentration for each molecule (**Figure 7**). It appeared that the concentration in the water phase for all antioxidants increases, especially for octyl, dodecyl, and hexadecyl esters when the concentration of Brij 35 was shifted from 17 μM to 2000 μM and also that further addition of Brij 35 does not lead to a noticeable change in the partition behavior.

Once again, this observation allows one to propose a putative scenario describing the multiple interactions occurring between the chemical species involved in this emulsified system. It is generally assumed that until the CMC, an emulsifier such as Brij 35 is mainly located around the oil droplet and that at the CMC, saturation occurs at the interfacial monolayer. When the interface is fully covered with emulsifier, the additional molecules of surfactant would no longer adsorb at interfaces and would instead form a wide range of thermodynamically stable self-assembled structures such as micelles, vesicles, or bilayer sheets according to the geometry of the surfactant molecule. Consequently, it can be speculated that such an aggregation process for Brij 35 (occurring between concentrations of 17 and 2000 μM) can lead to a drastic change of the partition behavior of phenolics (*24*). In terms of the mechanism of action, it is likely that Brij 35 micelles act as vehicles for chlorogenic acid and its esters, especially dodecyl ester, to carry them into the aqueous phase. In the future, a better knowledge of the proportion of the

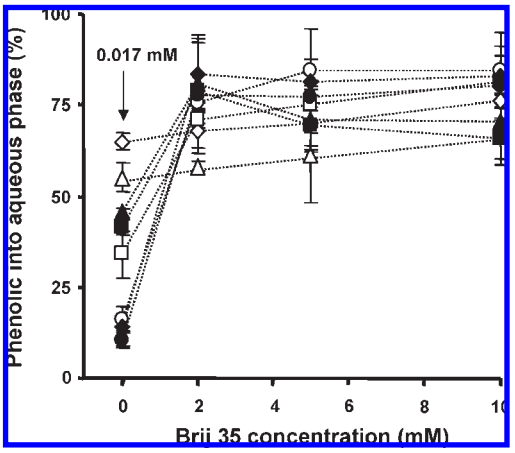

Figure 7. Emulsifier concentration effect on the partition behavior of phenolics in sunflower oil-in-water emulsion. C0 (◇), C1 (Δ), C4 (□), C8 (○), C12 (●), C16 (♦), C18 (■), and C20 (▲).

phenolics into the interfacial membrane and the oily phase will be required to specify the exact mechanism. Finally, it has been demonstrated that the increase of the emulsifier concentration above its CMC can drastically affect the partition behavior of antioxidant and can hide the cutoff effect. This could explain why most of the studies in this field using large emulsifier concentrations did not report a nonlinear effect of the hydrophobicity on the antioxidant properties in emulsions, whereas such an effect is almost always observed in pharmacological studies which do not use any emulsifiers.

In conclusion, although the polar paradox states among others that apolar antioxidants are more active in emulsions than their polar homologues, we observed with a homologous series of chlorogenic acid esters that chain extension from dodecyl ester leads to a drastic decrease in antioxidant capacity. This nonlinear behavior was mainly explained in terms of antioxidant location since it was found from partition analysis that the dodecyl ester presented the lowest concentration in the aqueous phase and also that the quantity of emulsifier drastically changed the partition of antioxidant. In addition, this nonlinear influence was connected to the so-called cutoff effect largely observed in studies using cultured cells. These different results allow one to propose a new scenario for the behavior of phenolic compounds in emulsified systems with special emphasis on the micellization process.

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