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Antioxidant Properties of Chlorogenic Acid and Its Alkyl Esters in Stripped Corn Oil in Combination with Phospholipids and/or Water

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ABSTRACT: In bulk oil, it is generally thought that hydrophilic antioxidants are more active than lipophilic antioxidants. To test this hypothesis, the antioxidant activity of phenolics with increasing hydrophobicity was evaluated in stripped corn oil using both conjugated diene and hexanal measurements. Chlorogenic acid and its butyl, dodecyl, and hexadecyl esters were used as model phenolic antioxidants with various hydrophobicities. Results showed that hydrophobicity did not correlate well with antioxidant capacity. The combination of chlorogenic acid derivatives with dioleoylphosphatidylcholine (DOPC) and/or water was also studied to determine if the physical structure in the oil affected antioxidant activity. DOPC alone made hexadecyl chlorogenate a less effective antioxidant, but it did not change the antioxidant capacity of chlorogenic acid. In contrast, the combination of DOPC and water (~400 ppm) renders chlorogenic acid a less active antioxidant, whereas it does not change the activity of hexadecyl chlorogenate. These results show, in bulk oil, that *intrinsic* parameters such as the hydrophobicity of lipophilized phenolics do not exert a strong influence on antioxidant capacity, but they can be highly influential if potentialized by *extrinsic* factors such as physical structures in the oil.

KEYWORDS: antioxidant, chlorogenic acid, corn oil, lipid oxidation, conjugated diene, hexanal, polar paradox

INTRODUCTION

Among food products, lipid-based foodstuffs are particularly prone to oxidation induced and mediated by free radicals, photon impact, and/or transition metals. In this context, phenolic compounds have attracted broad interest as food antioxidants due to their potential to prevent or delay oxidation in those systems. Most phenolics (monomeric forms) are rather hydrophilic, and yet they are paradoxically regarded as strong antioxidants in lipid systems such as bulk oils. As early as 1976, it was observed in bulk oil that antioxidant effectiveness in a series of compounds is inversely correlated with hydrophobicity.[1] During the 1980s, Porter[2] and Porter et al.[3,4] postulated in a series of papers the general rule that in food systems of low surface-to-volume ratio (e.g., bulk vegetable oils) polar antioxidants are more effective than their nonpolar homologues. Until recently, the only available working hypothesis to explain this polar paradox assumed that the increased effectiveness of hydrophilic antioxidants in bulk oils is due to their ability to migrate and concentrate at the air−oil interface where oxidation is prevalent, whereas lipophilic antioxidants are solubilized throughout the bulk oil, where they would be less effective.[5]

It can be argued, however, that air is less polar than oil because the dielectric constant of air is 1.0 compared to approximately 3 for food oils.[6] Accordingly, there would not be a major driving force for polar antioxidants to migrate to the air−oil interface, and thus they would not be more likely to concentrate at the air−oil interface than hydrophobic antioxidants. Chaiyasit et al.[6] recently postulated another mechanism of action, which is partly based on the pioneering work of Koga and Terao[7] using the concept of association colloids. Although often thought of as a simple homogeneous medium, oil is a complex multiphasic system; it contains small amounts of water and also various minor components such as mono- and diacylglycerols, phospholipids, sterols, and free fatty acids. These minor components are surface active and thus tend to entrap traces of water and to create an oil−water interface within the so-called "bulk" oil. Such structures are known as association colloids. According to Chaiyasit et al.,[6] the polar paradox can be explained by the fact that hydrophilic antioxidants are more active in bulk oil than their nonpolar homologues because they have a better affinity for the interface of association colloids. Increased effectiveness of the hydrophilic antioxidants would occur if the association colloids were a major site of oxidation due to their ability to also concentrate transient metals and surface active lipid hydroperoxides. Therefore, oxidation is postulated to be likely to take place at the interfaces of these association colloids, rather than at the air−oil interface.[6]

Besides this legitimate questioning of the lipid−air interfacial oxidation concept, recent results show that in bulk oil not all antioxidants behave in a manner dictated by the polar paradox,[8,9] which sheds some doubts on the "universality" of this hypothesis and raises some crucial questions. What if the observation that hydrophilic antioxidants are more active than lipophilic antioxidants in oil (i.e., the polar paradox hypothesis) is not as general as expected? What if the reverse trend (i.e., lipophilic antioxidant are more active than hydrophilic antioxidants in bulk oil) is more widespread? And, finally, how do environmental/structural

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factors such as the presence and nature of association colloids modulate the ability of phenolics to affect lipid oxidation?

To address these questions, the antioxidant activity of phenolics with increasing hydrophobicity was investigated in stripped corn oil by measuring both conjugated diene and hexanal formation. Chlorogenic acid and its butyl, dodecyl, and hexadecyl esters were used as model phenolic antioxidants of various hydrophobicities because much is already known about their antioxidant capacity in emulsified lipids,[10−12] methanolic medium,[13] and reactive oxygen species-overexpressing fibroblasts.[14] To extend matters further, the combination of these antioxidants with phospholipids (dioleoylphosphatidylcholine) and/or water was studied to shed light on the role of association colloids on antioxidant activity. Understanding how the chain length and the presence of phospholipids and water affect phenolic antioxidants in stripped bulk oil could provide fundamental knowledge that could be used to develop more efficient antioxidant strategies for food preservation and protection of nutritionally important bioactive lipids.

MATERIALS AND METHODS

Materials. Corn oil was purchased from a local retail store. Chlorogenic acid, silicic acid (100−200 mesh, 75−150 μm, acid washed), *Candida antarctica* B lipase, silica gel G60, and activated charcoal (100−400 mesh) were purchased from Sigma Chemical Co. (St. Louis, MO). Amberlite IR 120 H was purchased from Rohm and Haas (Philadelphia, PA). All of the solvents used in this study were of analytical grade or purer. Glassware was soaked in 3 mM HCl overnight to remove metal traces followed by rinsing with double-distilled water before use.

Synthesis of Chlorogenate Esters. The chemoenzymatic esterification of chlorogenic acid to obtain chlorogenate esters was carried out following the procedure described by López-Giraldo et al.[15] Briefly, 10 mmol of chlorogenic acid 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., Maidstone, U.K.), and the 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 the desired fatty alcohol, and the mixture was placed in sealed flasks and stirred on an orbital shaker (250 rpm, 55 °C) until complete dissolution of methyl chlorogenate. *C. antarctica* lipase B (5 wt %/wt; calculated from the total weight of both substrates) was then added to start the transesterification reaction. The suspensions were heated at 55 °C for 96 h under a nitrogen flow to continuously eliminate any methanol formed and favor the displacement of the reaction equilibrium toward the synthesis of fatty acid esters. The final lipophilized esters were then purified in a two-step procedure. First, a liquid−liquid extraction using 250 mL of hexane and 1000 mL of a solution of acetonitrile/water (3:1, v/v) was used to remove excess fatty alcohol. In a second step, alcohol traces were eliminated using silica gel column chromatography (length = 25 cm, i.d. = 1.6 cm) with toluene/ethyl acetate (90:10, v/v) as eluent. All recovered esters were then characterized by mass spectrometry as described by Lopez-Giraldo et al.[15]

Preparation of Minor Component Stripped Oils. To remove minor components such as phospholipids, water, and endogenous antioxidants (tocopherols), corn oil was stripped according to the method of Boon et al.[16] and was used in all experiments. In short, silicic acid (100 g) was washed three times with a total of 3 L of double-distilled water followed by filtering with Whatman filter paper in a Buchner funnel and drying at 110 °C for 20 h. The washed silicic acid (22.5 g) and activated charcoal (5.5 g) were suspended in 100 and 70 mL of hexane, respectively. A chromatographic column (3.0 cm i.d. × 35 cm height) was then packed sequentially with 22.5 g of silicic acid, followed by 5.5 g of activated charcoal, and then another 22.5 g of silicic acid. Thirty grams of oil was dissolved in 30 mL of hexane and passed through the column by elution with 270 mL of hexane. To minimize lipid oxidation during stripping, the collected triacylglycerols were held in an ice bath, which was covered with aluminum foil. Hexane was then evaporated using a vacuum rotary evaporator at 37 °C, and traces of the remaining solvent were removed by flushing with nitrogen, until a constant weight was achieved. Finally, the stripped corn oil was aliquoted into brown glass tubes, then flushed with nitrogen, and stored at −80 °C until use.

Critical Micelle Concentration (CMC) Evaluation Using 7,7,8,8-Tetracyanoquinodimethane (TCNQ) Probe. The CMC of dioleoylphosphatidylcholine (DOPC) in stripped oil was assessed according to the procedure previously described by Kanamoto et al.[17] Briefly, stripped corn oil containing 1 mg of TCNQ/g oil was prepared and gently blended for 12 h in a 16 mL glass test tube at room temperature. This oil was aliquoted into different test tubes, and various volumes of DOPC were added as a methanolic solution into each tube. The different aliquots were flushed with nitrogen for 30 min to evaporate methanol. Then, all samples were allowed to equilibrate at 55 °C for 1 h under agitation with a magnetic stirrer. Absorbance values at 480 nm were recorded using a spectrophotometer (Shimadzu 2014, Tokyo, Japan), and DOPC-free stripped corn oil was used as a blank. The CMC was determined as the inflection point in the curve plotting absorbance as a function of DOPC concentration (semilog plot). All experiments were conducted in triplicate samples and were expressed as the mean ± standard deviation.

Conjugated Diene Measurement. Prior to any measurements, known volumes of methanolic solutions of phenolics were added into stripped corn oil to get the desired concentration. Methanol was then evaporated by flushing with nitrogen.

Conjugated dienes, which are primary polyunsaturated fatty acid oxidation products, were spectrophotometrically measured every day to monitor lipid oxidation. Weighted oil samples were dissolved in isooctane at the appropriate dilution factor, and conjugated diene absorbance was measured at 234 nm in a Shimadzu spectrophotometer (UV-2101PC, Shimadzu, Kyoto, Japan). Results were expressed as millimoles of conjugated dienes per kilogram of stripped corn oil using an absorptivity of 26000 for linoleate hydroperoxides.[18] All experiments were conducted in triplicate samples and were expressed as the mean ± standard deviation.

Hexanal Measurement. Hexanal, which is a major secondary oxidation product of corn oil, was measured as previously described by Boon et al.[16] using a GC-17A Shimadzu gas chromatograph (GC) equipped with an AOC-500 autosampler (Shimadzu, Kyoto, Japan). One gram of stripped bulk oil (in the absence or presence of different antioxidants and phospholipid concentrations) was dispensed in 10 mL brown glass vials and capped with aluminum caps with PTFE/silicone septa. Samples were shaken and heated at 55 °C for 13 min in an autosampler heating block before measurement. A 50/30 μm DVB/Carboxen/PDMS solid-phase microextraction (SPME) fiber needle from Supelco (Bellefonte, PA) was injected into the vial for 1 min to adsorb volatiles present in the headspace and then was transferred to the GC injector port (250 °C) for 3 min to desorb volatiles from the SPME fiber. The injector port was operated in split mode, and then the split ratio was set at 1:5. Volatiles were separated on a Supelco 30 m × 0.32 mm Equity DB1 column with a 1 μm film thickness at 65 °C for 10 min. The carrier gas was helium at 15.0 mL/min. A flame ionization detector was used at a temperature of 250 °C. Hexanal concentrations were

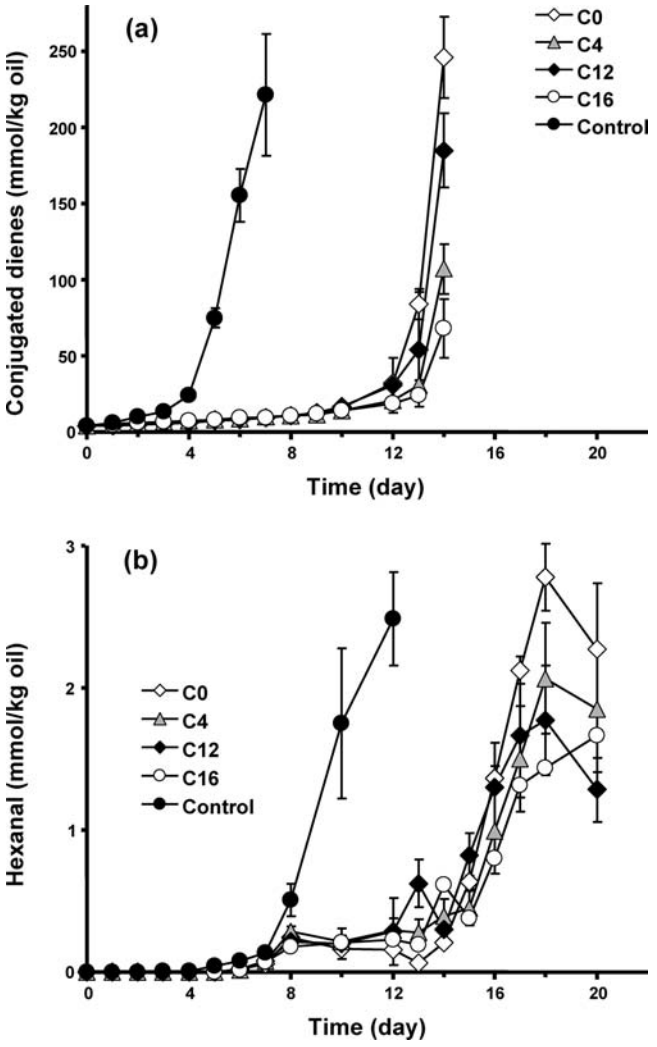

Figure 1. Time course oxidation of stripped corn oil at 55 °C in the dark: evolution of conjugated diene (a) and hexanal (b) contents in the absence (control) or presence of 200 μmol/kg of oil of phenolic compounds. C0, chlorogenic acid; C4, butyl chlorogenate; C12, dodecyl chlorogenate; C16, hexadecyl chlorogenate.

determined from peak areas using a standard curve prepared from authentic hexanal and expressed as nanomoles of hexanal per kilogram of oil. All experiments were conducted in triplicate samples and were expressed as the mean ± standard deviation.

Measurement of Water Content. The determination of the water content in stripped oil was done using a classical Karl Fischer apparatus.[19] Additional double-distilled water was added to obtain the desired moisture content.

Statistical Analysis. Statistical analysis of lipid oxidation kinetics was performed using a one-way analysis of variance. A significance level of $p < 0.05$ between groups was accepted as being statistically different. In all cases, comparisons of the means of the individual groups were performed using Duncan's multiple-range tests. All calculations were performed using SPSS17 (http://www.spss.com; SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

By virtue of their redox properties, phenolic compounds have received increasing attention over the past few decades regarding their potential to protect food lipids from being oxidized. Even though great progress has been achieved, we are still unable to predict how the *intrinsic* chemical structure of phenolics affects their antioxidant behavior in different food systems. One striking example is the complex influence of hydrophobicity on antioxidant effectiveness. To complicate matters further, these antioxidant properties are also highly dependent upon their *extrinsic* physicochemical environment, especially the presence and nature of lipid−water interfaces. To address this issue within the framework of bulk oil oxidation, this study investigated the effect of the alkyl chain lengthening on antioxidant capacity of chlorogenic acid derivatives, first, in a homogeneous system without any association colloids and then in a multiphasic system containing phospholipid reverse micelles.

Antioxidant Activity of Phenolics without Association Colloids. To prevent biases and pitfalls that could occur with a comparison of antioxidants with different functional groups, the following experiments were conducted with chlorogenic acid and a series of its alkyl esters (butyl, dodecyl, and hexadecyl chlorogenates) such that all antioxidants had the same active phenolic group yet had differences in polarity. In all experiments lipid oxidation was evaluated by measuring both conjugated dienes (primary oxidation products) and hexanal (secondary oxidation product). Chlorogenic acid and its butyl, dodecyl, and hexadecyl esters (200 μM) exhibited strong antioxidant activity by delaying the onset of conjugated diene and headspace hexanal formation in corn oil stripped of its minor components (Figure 1). Few if any differences in antioxidant activity were found between chlorogenic acid and its esters with a lag phase of 11 days for chlorogenic acid and its dodecyl ester and 12 days for the butyl and hexadecyl esters on the basis of conjugated diene formation (Figure 1a). When lipid oxidation was measured by hexanal, all of the chlorogenic acid esters had similar activity with a lag phase of approximately 14−15 days (Figure 1b). These results are somewhat unexpected if the polar paradox was accurate[3] because the most polar antioxidants were not consistently the more effective when compared to the more nonpolar antioxidants with long hydrocarbon chains.

To explain the quasi absence of a polarity (e.g., chain length) effect, let us consider the working hypothesis according to which the antioxidant capacity is influenced by hydrophobicity not through a simple change of the reactivity of the antioxidant but rather via a change in the partition/distribution of antioxidant species into the medium. Karl Fischer measurements have shown that the water concentration in stripped corn oil was 19 ppm, which is the lowest level of water we have ever been able to produce in bulk oil. Previous research has shown that in the absence of phospholipid or other surface active minor components (removed by the stripping process, see Material and Methods) and with these low amounts of water, little or no association colloid can be formed.[20] Thus, the system can be considered as close to a homogeneous bulk oil as possible. If the oil−air interface was the driving force for the polar paradox in bulk oils, one would have expected to see a difference in the antioxidant activity of the chlorogenic acid esters. However, these results suggest that hydrophobicity of the antioxidants was not an important factor in activity because there was very little water−lipid interface for phenolics of different chain lengths to partition into and, consequently, no means to discriminate them according to their hydrophobicity. However, it should be kept in mind that although often thought as homogeneous media, commercial vegetable oils are multiphasic systems because they contain small amounts of water (~200−400 ppm) and various minor

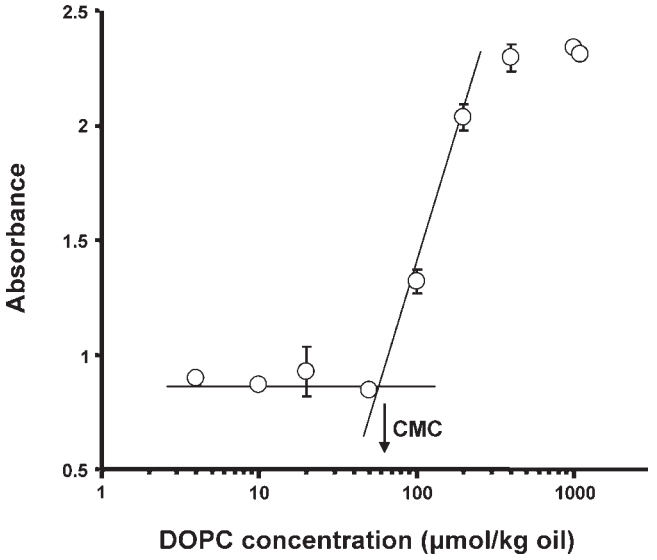

Figure 2. Determination of the dioleoylphosphatidylcholine critical micelle concentration using 7,7,8,8-tetracyanoquinodimethane probe.

components, such as mono- and diacylglycerols, phospholipids, sterols, and free fatty acids.[6] To investigate how the transition from a homogeneous (artificial) to a more heterogeneous (natural) system affects the antioxidant effectiveness of phenolic compounds, further experiments involving reverse micelles of phospholipids were carried out.

CMC Measurement of DOPC in Corn Bulk Oil. In nonaqueous media such as oil, surface-active molecules form association colloids in a concentration-dependent manner. Reverse micelles are structurally inverse analogues to normal micelles, containing a nanoscale aqueous core stabilized by a monolayer of surfactant molecules.[21] Reverse micelles are dynamic aggregates that are dispersed randomly following Brownian motion that are capable of exchanging their water content.[22,23] Reverse micelles are typically formed by surfactants with low hydrophilic−lipophilic balance (HLB) values such as free fatty acids (HLB ≈ 1.0), diacylglycerols (HLB ≈ 1.8), and monoacylglycerols (HLB ≈ 3.4−3.8),[24] but also with phospholipids, having intermediate HLB values (HLB ≈ 8.0).[25]

To study the antioxidant behavior of phenolic compounds in heterogeneous systems containing association colloids such as reverse micelles, we first investigated the ability of phospholipids to form structures in stripped corn oil. DOPC was used as a phospholipid model because it is widespread and, above all, because its oleyl residues contain only one unsaturation per fatty acyl chain, which allows it to be liquid and easily handled and yet is not as prone to lipid oxidation as the linoleic acid in the stripped corn oil. Although the experimental techniques to understand self-assembly in aqueous systems are very well-established, application of these techniques to nonaqueous systems such as bulk oil are not so well developed.[25] In this context the TCNQ method has been used to measure the CMC of DOPC, that is, the concentration at which DOPC begins to aggregate into association colloids and, thus, the concentration at which an oil starts to behave as a multiphasic system. This technique has been found to be consistent with interfacial tension measurement, especially for phospholipids in bulk oil.[20,26] Figure 2 shows that at low concentration in bulk oil, DOPC does not change the absorbance of TCNQ, which lies in a

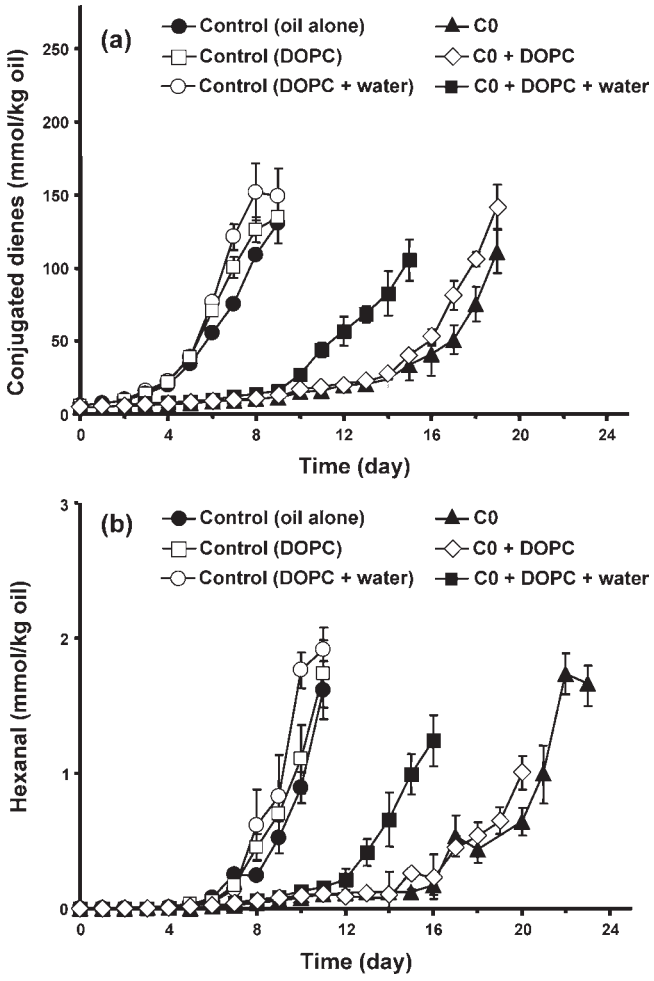

Figure 3. Time course oxidation of stripped corn oil at 55 °C in the dark: evolution of conjugated diene (a) and hexanal (b) contents with or without 200 μmol/kg oil of chlorogenic acid (C0), 200 μmol/kg oil of dioleoylphosphatidylcholine (DOPC), and 400 ppm exogenous water.

plateau, until a drastic increase is reached at 65 μM DOPC as determined by tangent-based calculation. This break corresponds to the CMC of DOPC in bulk oil and is assumed to be due to a charge transfer between TCNQ and phospholipids when they self-assemble in physical structures. Chen and coworkers[20] recently reported that the structures formed by DOPC in stripped corn oil under similar conditions are reverse micelles as determined by small-angle X-ray diffraction.

Antioxidant Activity of Phenolics in the Presence of Reverse Micelles. To ensure the presence of association colloids in oil, DOPC (200 μM) was added to freshly stripped corn oil at a concentration well above its CMC in combination with 400 ppm of exogenous water to mimic water concentrations in natural oils. At this point, it is worth specifying that the added water will be termed "*exogenous water*" in order not to confuse it with traces of "*endogenous water*" (<40 ppm in all further experiments) already present in stripped corn oil.

First, addition of DOPC and 400 ppm exogenous water exacerbates the trend already seen in Figure 1. In the presence of the DOPC reverse micelles, the lag phase for conjugated diene formation was in the order free chlorogenic acid < butyl ester < dodecyl ester ~ hexadecyl ester, whereas for hexanal formation, free chlorogenic acid was the least effective, with all of the esters

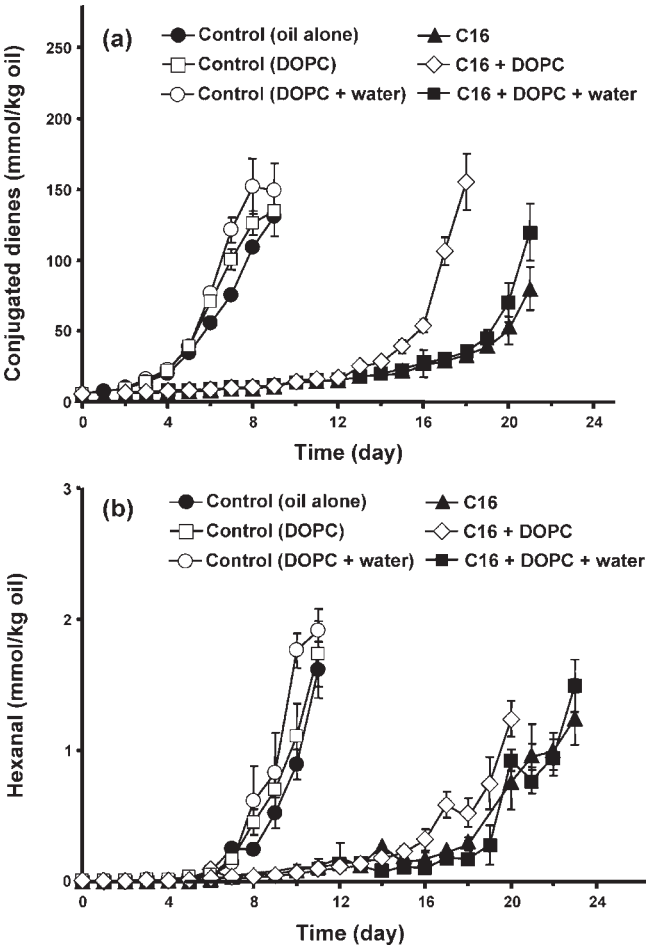

Figure 4. Time course oxidation of stripped corn oil at 55 °C in the dark: evolution of conjugated diene (a) and hexanal (b) contents with or without 200 μmol/kg oil of hexadecyl chlorogenate (C16), 200 μmol/kg oil of dioleoylphosphatidylcholine (DOPC), and 400 ppm exogenous water.

having similar activity (data not shown). The inability of the more polar chlorogenic acid derivatives to more effectively inhibit lipid oxidation confirms our previous observation that the polar paradox is not applicable to the antioxidant behavior of chlorogenic acid and its alkyl esters in stripped corn oil. However, because both DOPC and exogenous water were added to corn oil, it is worth investigating whether the chain length effect is induced by the addition of DOPC alone or by a combination of DOPC and exogenous water.

Because the following experiment contained numerous variables, only the most polar (free chlorogenic acid) and least polar (hexadecyl chlorogenate) antioxidants were tested. Data from Figure 3 clearly show that addition of 200 μM DOPC alone or in combination with 400 ppm exogenous water does not exhibit any antioxidant or prooxidant effect because the conjugated diene and hexanal lag phases for the controls (oil + DOPC and oil + DOPC + water) were similar.

The combination of DOPC (without exogenous water) and hexadecyl chlorogenate led to an unexpected result, because DOPC decreased the antioxidant activity of the hexadecyl chlorogenate (antagonistic effect) (Figure 4). Although a combination of DOPC and phenolic compounds leads to an antagonistic effect with hexadecyl ester, it does not noticeably change the antioxidant capacity of chlorogenic acid (Figure 3).

In contrast to the sole addition of DOPC, the addition of both 200 μM phospholipids and 400 ppm exogenous water induces a reverse phenomenon, characterized by an antagonistic effect with chlorogenic acid (Figure 3), but does not change the antioxidant effectiveness of hexadecyl chlorogenate (Figure 4). One possible cause of this observation could be the ability of phenolics to reduce iron (and/or other transient metal cations), thus increasing the prooxidant activity of the metals. Addition of water to stripped oil with DOPC will produce reverse micelles with a water core.[20] This water core could promote the partitioning of free chlorogenic acid into the reverse micelle, where it could interact with water-soluble metals or metals bound to the anionic phospholipids to increase the prooxidative activity of the metals. Hexadecyl chlorogenate could be too nonpolar to partition into the water phase and thus might not increase the prooxidative activity of metals. These results again show that the hydrophobicity of antioxidants is not always a good predictor of their ability to inhibit lipid oxidation in bulk oils. The difficulty of predicting the antioxidant effectiveness of phenolics in bulk oils could be due to their ability to interact with association colloids such as the reverse micelles formed by DOPC and water in this study.

In conclusion, it appears that *intrinsic* parameters such as the hydrophobicity of chlorogenic acid do not exert a strong influence by themselves on antioxidant capacity, but can become highly influential if potentialized by *extrinsic* factors such as the presence and nature of association colloids. This enables us to state again that it clearly does not make sense to just consider the magnitudes of the antioxidant capacities as being values universally applicable for all conditions.[27,28]

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