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Kinetic and Stoichiometry of the Reaction of Chlorogenic Acid and Its Alkyl Esters against the DPPH Radical

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The lipophilization of polar antioxidants such as phenolics is an efficient way to enhance their solubility in apolar media. Thus, in emulsified systems, lipophilized antioxidants are supposed to locate at the lipid/aqueous phase interface and to lead to a better protection of unsaturated lipids. Herein, the antiradical activity of chlorogenic acid (5-CQA) and its corresponding esters with seven fatty alcohols (from methanol to eicosanol) have been achieved using the well-known 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Hydrophobation was shown to significantly improve the antiradical activity of 5-CQA esters which reached a maximum for butyl- and octyl-chlorogenate. In addition, for both 5-CQA and its esters, it was demonstrated that the global mechanism of DPPH$^{\bullet}$ stabilization proceeded likely by electron transfer (ET), while it appeared that the pathways of DPPH$^{\bullet}$ stabilization were different between 5-CQA and its esters, as confirmed by the LC-MS characterization of reaction products. Finally, strong differences were found between the tested molecules allowing the proposal of different DPPH$^{\bullet}$ stabilization pathways by electron transfer for 5-CQA and its esters.

KEYWORDS: Antioxidant capacity; DPPH radical; chlorogenic acid; lipophilization; electron transfer; hydrogen atom transfer

1. INTRODUCTION

The relative low solubility of phenolic compounds in apolar media may be seen as a drawback when considering their use as antioxidants for stabilizing unsaturated lipids in formulated complex emulsified systems. Recently, some modifications of these phenolics by lipophilization reactions have been suggested in order to enhance their hydrophobicity (*1, 2*) and improve their resulting antioxidant properties by a better location at the lipid/aqueous phase interface where the oxidation occurs (*3*). Therefore, various recent studies have been carried out describing the grafting of an aliphatic chain to a phenolic compound in reactions that are generally performed by lipase-catalyzed processes (*2*). For example, Buisman et al. (*4*), Stamatis et al. (*5*), and Priya et al. (*6*) studied the esterification of phenolic acids (hydroxybenzoic and hydroxycinnamic acid derivatives) with fatty alcohols (C_4, C_6, C_{12}, C_{14}, C_{16}) to obtain antioxidants with emulsifying properties which could be used in hydrophobic media. A similar strategy was also applied for the direct grafting of phenolic acids on triglyceridic structures. For example, dihydrocaffeic acid was the first used to transesterify trilinolein or trilinolenin (*7*), leading to different ratio of obtained mono- and diacylglycerol phenolics depending on the chosen reaction parameters. More complex structures such as flavonoids have been also described as potential candidates for lipophilization reactions. Indeed, Kontogianni et al. (*8*) performed some lipase-catalyzed acylation of flavonoid glycosides, taking advantage of the lipase catalytic action which generally allows a highly regioselective esterification. In this study, the enzymatic acylation of rutin and naringin was carried out with medium chain fatty acids (C_8, C_{10}, C_{12}) using a lipase from *Candida antarctica* B. One can also cite the work of Ardhaoui et al. (*9*) who studied the acylation of the flavonoid structure by lipase-catalyzed esterification with fatty acids. Among the numerous tested flavonoids, satisfactory reaction rates were obtained for esculin, rutin, and hesperidin whereas lipophilization was not possible for quercetin. Various publications then deal with the impact of such lipophilization on the resulting antioxidant activity of the modified molecules. Silva et al. (*10*) measured the antiradical

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activity by the DPPH method of methyl, ethyl, and propyl esters of caffeic and dihydrocaffeic acids and observed that alkyl caffeates had a higher 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical scavenging ability than the native molecule, in contrast to the dihydrocaffeic acid esters which presented a lower scavenging activity than that of dihydrocaffeic acid. Similarly, Nenadis et al. (*11*) demonstrated that ethyl ferulate was more effective than ferulic acid in both bulk oil and oil-in-water emulsions. Others studied the antioxidant properties of gallic acid and its esters (C_1, C_3, C_{12}, C_{18}) using the DPPH method and a liposome-based multiphase system (*12*). Overall, the gallic acid esterification increased the antioxidant activity of the resulting esters in both assays. More precisely, it was observed that propyl gallate was the most powerful DPPH• radical scavenger, while in AAPH-induced peroxidation of egg yolk PC liposomes lauryl gallate was the strongest antioxidant.

However, some other studies underline the fact that lipophilization is not always advantageous in terms of antioxidant efficiency. Indeed, Kikuzaki et al. (*12*) showed that esterification of ferulic acid by alcohols (from C_1 to C_{12}) leads to a decrease in the antiradical capacity in all cases, as measured by the DPPH method. Additionally, Yuji et al. (*13*) observed that, although butyl or dodecyl esters of *p*-hydroxyphenylacetic acid (HPA) concentrated at lipid/water interfaces more than free HPA, free HPA was more effective at inhibiting the oxidation of Brij 35-stabilized Menhaden oil-in-water emulsions. The authors suggested that this result could be due to the higher concentration of the free HPA in the lipid phase compared to its esters in the presence of surfactant micelles.

Recently, we showed that 5-caffeoylquinic acid (5-CQA), belonging to a family of mono- and diesters of *trans*-hydroxycinnamic acids (caffeic, ferulic, and *p*-coumaric) with quinic acid and exhibiting antioxidant properties (*14*), could be lipophilized by fatty alcohols in a two step lipase-catalyzed transesterification strategy (*15*). This reaction leads to homologous series of 5-CQA and its alkyl chlorogenate esters (C_1, C_4, C_8, C_{12}, C_{16}, C_{18}, and C_{20}) for which the impact of such hydrophobation must be evaluated in terms of antioxidant activity. However, as mentioned by Laguerre et al. (*16*), this kind of evaluation can be difficult, namely because (i) the antioxidant activity can be conveyed via many different pathways and also (ii) because there is a large number of available assays performed in dissimilar conditions that leads to the measurement of different properties. Taking into account this complexity, the first arising question is what are the more appropriate methods to use in order to evaluate antioxidant capacity? The aim of this study was not to assess antioxidant capacity with regard to biological relevance but more particularly to study the kinetic and stoichiometry of the reaction of chlorogenic acid and its esters against free radicals. Although the DPPH assay has been criticized for its artificiality and for the fact that it does not involve an oxidizable substrate (*17*), it constitutes however a widespread and easy-to-use protocol to study antiradical reactivity. In this purpose, neglecting natural environment interactions, we investigated by kinetic and stationary studies the influence of the grafted alkyl chain on the antiradical mechanisms of chlorogenic acid (5-CQA) and its esters.

2. MATERIALS AND METHODS

2.1. Chemicals. Commercially immobilized lipase from *C. antarctica* lipase B (Novozym 435, >2 U/mg (U corresponds to the amount of enzyme which liberates 1 μmol butyric acid per minute at pH 8.0 and 40 °C (tributyrin, Fluka no. 91010, as substrate)) was purchased from Novozymes A/S (Bagsvaerd, Denmark). Ion exchange resin (Amberlite IR120 H), molecular sieve (3 Å), 5-CQA (95%), 2,2-diphenyl-1-picrylhydrazyl (DPPH•), 1-butanol (99.8%), 1-octanol (99%), 1-dodecanol (99.5%), 1-hexadecanol (99%), methanol, acetonitrile, and toluene HPLC grade and hexane, ethyl acetate, and formic acid analytical grade were purchased from Sigma-Aldrich (Saint Quentin, France). Silica (granulometry 0.06−0.2 mm, pore diameter 0.6 nm) was from Acros organics (Geel, Belgium).

2.2. Synthesis of Chlorogenate Esters. The chemo-enzymatic esterification of 5-CQA to obtain chlorogenate esters was carried out following the procedure described by López-Giraldo et al. (*15*). Briefly, in a 500 mL glass vessel, 10 mmol 5-CQA was diluted in 240 mL of methanol. Amberlite IR120 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 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 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 suspensions were then heated at 55 °C for 96 h under a nitrogen flow in order to eliminate continuously the formed methanol and favor the displacement of the reaction equilibrium toward the synthesis. 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 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. (*15*).

2.3. Measurement of Antiradical Activity of 5-CQA and Its Esters by the DPPH Method. The procedure for the determination of stationary parameters of antiradical activity was performed by spectrometry as described by Brand-Williams et al. (*18*), wherein the bleaching rate of a stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) is monitored at 515 nm in the presence of a phenolic sample as reducing agent. The solutions were prepared as follows: 1.95 mL of methanolic DPPH• solution (60 μM) was placed into a 1 cm quartz cuvette and completed with 50 μL of methanolic solution of phenolic compound (Φ-OH) (0.154−1.15 mM). These latter concentrations correspond to molar ratios of Φ-OH/DPPH• ranging from 0.05 to 0.5. The decay of absorbance at 515 nm due to the reduction of DPPH• radical after Φ-OH addition was monitored each minute until it reached a steady state (6 h) at 20 °C, using a spectrometer Lambda 25 UV (Perkin-Elmer, Courtaboeuf, France) equipped with a stirring and temperature control system (Peltier Temperature Programmer 6+6, Perkin-Elmer). The homogeneity of the reaction medium was guaranteed by magnetic stirring at 1000 rpm. A correction of the intrinsic DPPH• bleaching was done in the absence of Φ-OH in order to avoid a possible overestimation of the Φ-OH capacity (eq 1).

$$\mathrm{Abs}^{c} = \mathrm{Abs}_t + \mathrm{Abs}^{b}_{t0} - \mathrm{Abs}^{b}_{t} \quad (1)$$

where Abs^{c} is the corrected absorbance of the sample, Abs_t is the absorbance of the sample at time t, and Abs^{b}_{t0} and Abs^{b}_{t} are the absorbance of the blank (1.95 mL of DPPH• and 50 μL of methanol) at 0 min and t min, respectively. Afterward, the percentage of remaining DPPH• was calculated using eq 2.

$$\%\ \mathrm{DPPH} = (\mathrm{Abs}^{b}_{t0}/\mathrm{Abs}^{b}_{t}) \times 100 \quad (2)$$

where Abs^{c}_{t0} is the corrected absorbance of sample at 0 min and Abs^{c}_{t} is the corrected absorbance at each time interval. For each Φ-OH concentration tested, the % DPPH• remaining was plotted as a function of time (**Figure 1**), and the % DPPH• remaining at the steady state (6 h) was determined. The percentage of residual DPPH• at the steady state was then established as a function of the molar ratio of Φ-OH to

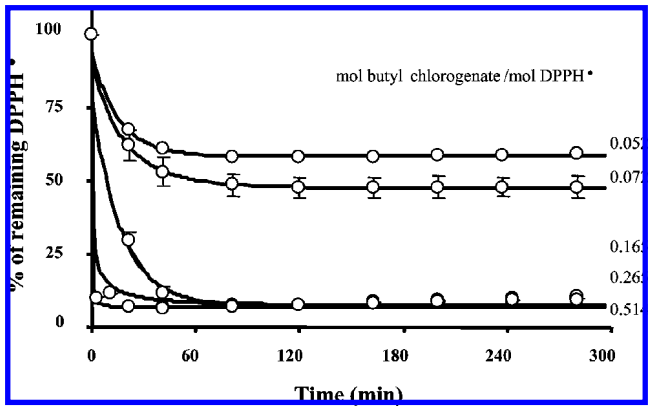

Figure 1. Example of classical behavior of percent remaining DPPH• for various butyl chlorogenate/DPPH ratios.

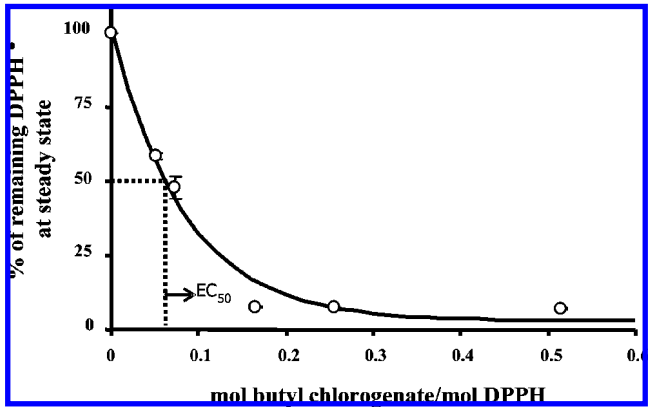

Figure 2. Values of percent remaining DPPH• calculated at steady state as a function of the molar ratio of butyl chlorogenate/DPPH.

DPPH• (**Figure 2**). The antiradical capacity was thus defined as the amount of antioxidant necessary to decrease the initial DPPH• concentration by 50% and expressed as EC_{50}. For clarity, antiradical activity was expressed in Trolox equivalent (TE).

Concerning the study of kinetic parameters, slight modifications of the experimental protocol were done. Briefly, the main changes were as follows: (i) a final concentration of tested compounds of 1.76−17.6 μM and (ii) an acquisition frequency of 0.1 s for 1−2 min. It is worth noting that although the addition of Φ-OH was done manually, careful attention was taken to minimize the information loss during the first seconds of reaction. In this way, the addition of Φ-OH solution was achieved under stirring conditions and after temperature equilibration of the reaction medium and recording started.

2.4. LC-MS Analysis. The products obtained from a reaction medium containing 2.16 mM DPPH• and 1.8 mM Φ-OH were analyzed after 2 min of reaction by liquid chromatography coupled to a mass spectrometer (LC-MS). The LC equipment (Thermo Fisher, San Jose, CA) was made of a Surveyor MS pump, an autosampler with a 25 μL loop, and a photodiode array (PDA) detector (recording at 327, 280, and 254 nm and scanning from 200 to 600 nm). The separation was done on an ACE C18 column (3 μm, 50 × 2.1 mm, AIT, Houilles, France) in a gradient system. Solvent A was water/formic acid (99.9/0.1 v/v), and solvent B was acetonitrile/water/formic acid (80/19.9/0.1 v/v/v). The gradient profile consisted of a linear increase of solvent B from 3% to 35% in 50 min, 35% to 50% in 5 min, 50% to 80% in 5 min, 80% to 100% in 5 min, followed by 10 min isocratic conditions, and return to 3% B in 85 min. The solvents were delivered at a total flow rate of 300 μL/min. The LC equipment was interfaced with a mass spectrometer fitted with an ESI source (Thermo Fisher, San Jose, CA) and operating in zoom scan mode. MS operating conditions (negative mode) were optimized using 5-CQA with an ionization voltage of 4.5 kV, a capillary temperature of 270 °C, a sheath gas (nitrogen) flow rate of 80 arbitrary units, and an auxiliary gas (helium) flow rate of 20 arbitrary units.

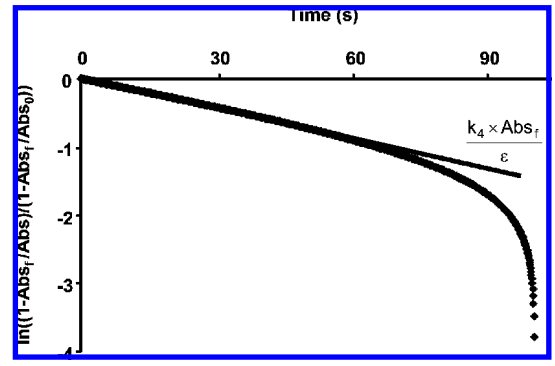

Figure 3. Example of determination of rates constants (k_3) of 5-CQA and its esters.

3. RESULTS AND DISCUSSION

It is well-known that one of the main characteristic responsible for the antioxidant activity of a phenolic compound is its ability to scavenge free radicals. In order to evaluate this capacity, one of the most widespread and easy-to-use methods consists of measuring the ability of a molecule to reduce the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) (*19*). Nevertheless, as pointed out by Goupy et al. (*20*), this popular test generally pays no attention to the kinetics of H atom transfer, which however could be as much important as the total stoichiometry or EC_{50} typically evaluated. In the present study, the behavior of 5-CQA and its fatty esters as antiradical molecules were evaluated in the DPPH assay considering both kinetic and static parameters. The kinetic study was performed in order to obtain a more reliable and specific insight about both pathways and mechanisms of hydrogen atom transfer (HAT) versus electron transfer (ET) involved during early stages (2 min) of stabilization of DPPH•. Moreover, in order to confirm the reaction pathway obtained from kinetic study, the identification of the stabilization products occurring in the early stages of the reaction between DPPH• and Φ-OH were carried out by LC-MS. Finally, the classical determination of scavenging activity at steady state (6 h) was performed with the purpose to determine whether the pathways involved during the early stages of reaction impact the final stoichiometry of the tested molecules.

3.1. Kinetic Parameters for the HAT Mechanism. In order to obtain information about pathways involved in the DPPH• stabilization by 5-CQA and its esters, the reaction kinetic parameters were determined. It is worth mentioning that only the early stages of the reaction (2 min) were detailed in terms of kinetic analysis, where it is commonly accepted that the stabilization of DPPH radicals results from the transfer of the most labile H atoms of the Φ-OHs (eq 3).

$$\text{Φ-OH} + \text{DPPH}^{\bullet} \xrightarrow{k_3} \text{Φ-O}^{\bullet} + \text{DPPH-H} \qquad (3)$$

The rate constant of reaction (k_3) was determined in methanol at 20 °C monitoring the DPPH• absorbance decay at 515 nm (see Materials and Methods). Under these conditions, various authors (*20, 21*) propose that the rate of DPPH• bleaching can be defined as follows:

$$-\frac{d[\text{DPPH}^{\bullet}]}{dt} = k_3[\Phi - \text{OH}][\text{DPPH}^{\bullet}] \qquad (4)$$

In order to obtain the rate constant (k_3), eq 4 was integrated considering that the concentration of DPPH• can be expressed as [DPPH•] = Abs/ε, where Abs is the absorbance and ε (experimentally determined to be 10 680 M^{-1} cm^{-1}) is the molar

extinction coefficient of DPPH•. Another consideration was that the concentration of Φ-OH can be expressed as [Φ-OH] = [DPPH•]/n^{es}, where n^{es} is the number of reduced DPPH• during the early stage of the reaction per molecule of antioxidant. This n^{es} parameter can be then determined as follows:

$$n^{es} = \frac{Abs_0 - Abs_f}{[\Phi\text{-OH}]_0 \varepsilon} \quad (5)$$

The subscripts 0 and *f* correspond to the initial and final (2 min) conditions, respectively. Finally, values of k_3 can be calculated from eq 6 which is valid for second order kinetics in the early stages of reactions.

$$\ln\left(\frac{1 - Abs_f/Abs}{1 - Abs_f/Abs_0}\right) = -k_3 \frac{Abs_f}{\varepsilon} t \quad (6)$$

Thus, when $\ln[(1 - Abs_f/Abs)/(1 - Abs_f/Abs_0)]$ is plotted as a function of time, a straight line with zero intercept is obtained over most of the 2 min (**Figure 3**). The slope of the curve readily gives access to k_3. The values of k_3, stoichiometric coefficients ($\sigma = 1/n^{es}$), and n^{es} for 5-CQA and its esters are summarized in **Table 1**. It appeared that, during the early stages of the reaction, the DPPH• stabilization mechanism is different for 5-CQA and its esters. Indeed, the number of reduced DPPH• during the early stages of reaction suggests that 5-CQA ($n^{es} = 1.81 \pm 0.11$) can transfer two labile H atoms to DPPH•, while its esters can transfer only one H atom to DPPH• ($n^{es} \sim 1.4$, except for octadecyl chlorogenate). However from our data, it seems more likely that there was not a single pathway but a combined one resulting in fractional numbers for the parameter n^{es}. Indeed, an n^{es} value near 1.4 for methyl, butyl, octyl, dodecyl, hexadecyl, and eicosyl chlorogenates could mean that the donation of one H atom was favored and an n^{es} value near 1.8 for chlorogenic acid that rather two H atoms were donated. However, probably there was not a situation of pure 1 or 2 donating pathways. Concerning octadecyl chlorogenate, its n^{es} value of 1.85 ± 0.34 seems to indicate that it can transfer two H atoms in a similar trend as 5-CQA. Nevertheless, it is worth mentioning that, despite eight independent repetitions, the relative standard deviation (RSD) of its n^{es} determination (18.4%) is ~3 fold higher than the global RSD for other investigated compounds. Due to this variability, it cannot be determined the number of H atoms donated by octadecyl chlorogenate at the early stage of the reaction.

Besides the number of H atoms transferred to DPPH•, the type of mechanism (hydrogen atom transfer or electron transfer) involved in this stabilization remains unresolved. In this way, the k_3 rate constant, which gives more global information regarding the species present in the reaction, is a key parameter that could clarify this question. First, it is shown in **Table 1** that the k_3 values of all chlorogenate esters were higher than those of 5-CQA, which is likely due to the fact that it is faster to transfer one H atom (in the case of esters) than two atoms (in the case of 5-CQA). A complementary explanation could come from the hypothesis made by Foti et al. (*21*), according to whom the differences of antiradical activities between a phenolic acid (hydroxycinnamic acid derivatives) and its esters could be due to the quantities of phenolate and carboxylate anions after dissociation in alcoholic media. Assuming that the reaction mechanism between DPPH• and Φ-OH involves a fast electron-transfer process from the phenolate anion to DPPH•, the dissociation of the free carboxylic group in the case of 5-CQA could reduce the quantity of phenolate anions and cause a reduction of its k_3 value for the early stage of the reaction. Consequently, it could be hypothesized that the esterification of the carboxylic function of 5-CQA greatly favors the electron-transfer process compared to the nonesterified 5-CQA.

Second, of particular interest was the strong influence of the initial concentration of Φ-OH on k_3, as we found that increasing all antioxidant concentrations results in a drastic decrease of k_3 (**Figure 4** and **Table 1**). This observation demonstrates that the hypothesis of an elemental mechanism is unlikely. Indeed, a comparison of stoichiometric coefficients (σ) (**Table 1**) and partial reaction orders at the early stage of reaction (m^{es}, **Table**

Table 1. Kinetic (for HAT and ET Mechanisms) and Stationary Parameters of DPPH Stabilization by 5-CQA and Its Esters

compound	kinetic parameters, HAT mechanism			kinetic parameters, ET mechanism			stationary parameters
	n^{es} (mol DPPH•/mol Φ-OH)	σ (mol Φ-OH/mol DPPH•)	k_4 (L mol^{-1} s^{-1})	A	m^{es}	A'	n^{ss} (mol DPPH•/mol Φ-OH)
5-CQA	1.81 ± 0.11	0.55 ± 0.03	7198 ± 323^a 523 ± 84^b	$2.8 \times 10^{-3} \pm 2.1 \times 10^{-3}$	1.25 ± 0.2	$2.68 \times 10^{-5} \pm 8.5 \times 10^{-6}$	4.88 ± 0.48
methyl chlorogenate	1.36 ± 0.09	0.73 ± 0.05	$20\,000 \pm 3447^a$ 8870 ± 838^b	89.3 ± 1.4	0.35 ± 0.05	26.5 ± 3.7	6.25 ± 0.20
butyl chlorogenate[c]	1.40 ± 0.12	0.71 ± 0.06	$12\,470 \pm 1947^a$ 4322 ± 136^b	22 ± 11.0	0.39 ± 0.09	18.9 ± 0.88	8.21 ± 0.40
octyl chlorogenate	1.46 ± 0.03	0.68 ± 0.01	$18\,463 \pm 1542^a$ 9090 ± 294^b	261 ± 17.3	0.31 ± 0.07	38.2 ± 4.6	8.52 ± 0.08
dodecyl chlorogenate	1.49 ± 0.05	0.67 ± 0.02	$41\,202 \pm 2614^a$ 7451 ± 162^b	1.76 ± 0.2	0.5 ± 0.08	31.3 ± 8.1	6.64 ± 0.13
hexadecyl chlorogenate	1.32 ± 0.08	0.76 ± 0.05	$40\,024 \pm 3311^a$ 8987 ± 409^b	9.1 ± 4.9	0.47 ± 0.05	30.3 ± 5.6	6.33 ± 0.16
octadecyl chlorogenate	1.85 ± 0.34	0.54 ± 0.10	$10\,169 \pm 1692^a$ 3991 ± 842^b	117 ± 155	0.32 ± 0.07	16.7 ± 1.2	6.70 ± 0.21
eicosyl chlorogenate	1.42 ± 0.16	0.70 ± 0.08	$27\,774 \pm 2841^a$ 8013 ± 562^b	23 ± 8.2	0.34 ± 0.05	29.2 ± 3.3	6.17 ± 0.08

[a] Calculated at a final concentration of 17.6 μM antioxidant. [b] Calculated at a final concentration of 1.76 μM antioxidant. [c] Calculated at 12 °C.

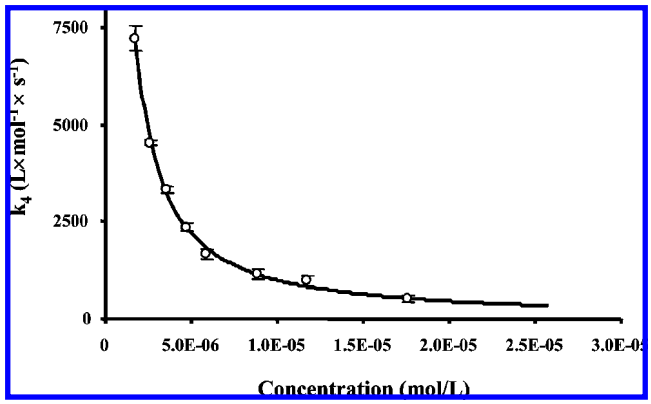

Figure 4. Rate constant k_3 calculated in methanol at 20 °C versus initial concentration of 5-CQA. The solid line represents the (best-fit) equation $A[\Phi\text{-OH}]_0^{m^{es}}$.

1; see section 3.2 for its calculation) confirms that the reaction pathway is not elemental, and consequently, we can theorize that reduction of the DPPH• radical involves intermediary steps that could include, among others, (i) the formation of charged species, (ii) the electron-transfer mechanism, or (iii) the short life products reaction. A similar behavior was found by Foti et al. (*21*), who concluded that an electron-transfer mechanism was the most important pathway in DPPH• stabilization by the hydroxycinnamic acid and its esters. Additionally, Litwinienko and Ingold (*22*−*24*) observed an abnormal increase of rate constants of DPPH• stabilization in alcoholic media which was attributed to partial ionization of the phenols and a very fast electron transfer from phenolate anion to DPPH•. These studies, together with our results, suggest that, in alcoholic media, the ET mechanism predominates over the HAT mechanism for chlorogenic acid and its esters.

3.2. Kinetic Parameters for the ET Mechanism. The hypothesis of an ET mechanism implies that the stabilization of DPPH• is not done in one step and therefore eq 3 has to be decomposed for each step involved in this type of mechanism. Indeed, as described by numerous studies (*21*−*24*), the ET mechanism involves (i) the dissociation of the phenolic hydroxyl group (eq 7), (ii) the electron transfer from the phenolate anion to DPPH• (eq 8), and finally (iii) the stabilization of the $DPPH^-$ anion by a proton present in the medium (eq 9).

$$\Phi\text{-OH} \overset{K_{OH}}{\rightleftharpoons} \Phi\text{-O}^- + H^+ \tag{7}$$

$$\Phi\text{-O}^- + DPPH^\bullet \overset{k_8}{\rightarrow} DPPH^- + \Phi\text{-O}^\bullet \tag{8}$$

$$DPPH^- + H^+ \overset{k_9}{\rightarrow} DPPH\text{-H} \tag{9}$$

Accordingly, the rate of DPPH• reduction by the tested Φ-OH can be expressed as follows:

$$-\frac{d[DPPH^\bullet]}{dt} = k_8[DPPH^\bullet][\Phi\text{-O}^-] \tag{10}$$

where the phenolate anion concentration $[\Phi\text{-O}^-]$ can be determined from its dissociation constant (K_{OH}, eq 7) as follows:

$$[\Phi\text{-O}^-] = \frac{K_{OH}[\Phi\text{-OH}]}{[H^+]^\alpha} \tag{11}$$

Here, the α factor represents the possibilities for dissociation of the two hydroxyl phenolic groups present in the 5-CQA and its esters. In the case of 5-CQA, the quantity of H^+ resulting from the dissociation of phenol in phenolate was replaced by the quantity of H^+ derived from the dissociation of the carboxylic acid in the carboxylate anion, which was determined from the dissociation constant of the carboxylic group ($K_{COOH} = [H^+]^2/[\Phi\text{-OH}]_0$), as proposed by Foti et al. (*21*). In the ester case, this quantity can be theoretically determined from the residual antioxidant quantity at a given time. Consequently, $[H^+] = \theta\chi[\Phi\text{-OH}]_0$, where θ represents the stoichiometric coefficient ratio and χ represents the change from Φ-OH to its dissociated form H^+. Accordingly, the DPPH• stabilization rate can be expressed by eq 12 for 5-CQA and eq 13 for its alkyl esters.

$$-\frac{d[DPPH^\bullet]}{dt} = \frac{k_8 K_{OH}[\Phi\text{-OH}]_0^{-\alpha/2}}{K_{COOH}^{\alpha/2}}[\Phi\text{-OH}][DPPH^\bullet] \tag{12}$$

$$-\frac{d[DPPH^\bullet]}{dt} = k_8 K_{OH}[\theta\chi[\Phi\text{-OH}]_0]^{-\alpha}[\Phi\text{-OH}][DPPH^\bullet \tag{13}$$

k_3 (eq 4) can then be expressed by the term $((k_8K_{OH}[\Phi\text{-OH}]_0^{-\alpha/2})/K_{COOH}^{\alpha/2})$ in eq 12 for 5-CQA or by the term $(k_8K_{OH}(\theta\chi[\Phi\text{-OH}]_0)^{-\alpha})$ in eq 13 for esters. These expressions clearly show that k_3 is directly proportional to the phenolate anion formed in the early stage of the reaction (which is governed by K_{OH}) and diminishes with increases of initial concentration of 5-CQA and its esters. Additionally, as expected, in the case of 5-CQA, k_3 decreased as the amount of carboxylate anion increased (K_{COOH}). In others words, k_3 can be expressed as a function of initial concentration of 5-CQA and its esters as follows:

$$k_3 = A[\Phi\text{-OH}]_0^{-m^{es}} \tag{14}$$

where all constants (k_8, K_{OH}, K_{COOH}, θ, and χ) were grouped in a single A constant which takes into account the capacity of dissociation of chlorogenic acid and its ester, while m^{es} is the partial reaction order for the antioxidant compound during the early stage of reaction.

Under previous circumstances, it is possible to calculate the partial reaction order of phenolic compounds (m^{es}) and the constant (A) by fitting k_3 versus the initial Φ-OH concentration for each compound (**Figure 4**). The m^{es} values (**Table 1**) were essentially 1.5 and 0.5 for 5-CQA and its esters, respectively. However, small differences in this exponent have a strong effect on the A values. Therefore, we have also reported the A' values obtained by setting $m^{es} = 1.5$ for 5-CQA and $m^{es} = 0.5$ for its esters and doing a linear regression of k_3 versus $[\Phi\text{−OH}]_0^{m^{es}}$. First, as expected, the experimental orders of reaction for 5-CQA and its esters confirm that the reactional pathway shown in eq 4 is not elemental, which is probably due to the fact that the molecular species actually involved in the DPPH• stabilization are not present in the neutral form and consequently that the hypothesis of an ET mechanism seems to be very realistic. Additionally, the strong difference between the orders of reaction for 5-CQA and its esters suggests that the stabilization of DPPH• is made by different reactional pathways. This observation is then in agreement with what was observed regarding the different values obtained in the previous section for the number of reduced DPPH• during the early stages of reaction per molecule of antioxidant.

3.3. LC-MS Analysis of the Products Obtained from DPPH• Stabilization. In the previous sections, we have shown that the stabilization of DPPH• radicals by 5-CQA and its esters could be attributed to two different reactional pathways. This hypothesis was based on the kinetic results suggesting that 5-CQA is rather able to reduce two molecules of DPPH• while its esters are rather able to reduce only one DPPH• molecule

Table 2. Retention Time and Full Negative Ion Data for the DPPH• Stabilization Reaction with 5-CQA and Its Esters[a]

compound	retention time (min)	MS negative ion
5-CQA	10.2	351.7 (100)−703.5 (83)
	13.2	353.7 (38)−707.7 (100)
	65.4	394.5 (100)
butyl chlorogenate	41.7	409.8 (15)−819.6 (100)
	55.6	817.9 (94)−1635.6 (100)
	56.6	817.8 (100)−1635.7 (82)
	58.7	817.8 (48)−1635.7 (100)
	65.4	394.5 (100)
dodecyl chlorogenate	65.4	394.5 (100)
	66.0	1043.5 (100)−521.6 (20)
	75.5	1041.8 (100)

[a] Parentheses values represent the relative intensity of ions.

during the early stages of reaction. In order to confirm this hypothesis, the characterization of reaction products was achieved by LC-MS. The reactions were performed with 1.3 equiv of DPPH• (see Materials and Methods), because preliminary experiments revealed that a larger amount of radicals resulted in complex mixtures and polymeric products that hindered the identification of primary products by LC-MS and consequently the information on the initial reactional pathways. All LC-MS analyses were achieved after 2 min of reaction, which corresponds to the duration of the early stage considered in the kinetic study. Under these conditions, the LC-MS results obtained for 5-CQA (MW = 354.7 g/mol), butyl (MW = 410.5 g/mol), and dodecyl chlorogenate (MW = 522.6 g/mol) confirmed the existence of two different pathways (**Table 2**).

Indeed, for 5-CQA, after 2 min of reaction, a molecular ion at 351.7 uma was detected, which corresponds to the molecular ion of the 5-CQA *o*-quinonic form. These MS data confirmed the preceding kinetic study according to which the stabilization of DPPH• rather proceeded by two successive fast electron transfers from a phenolate anion to DPPH• followed by a stabilization of the resulting 5-CQA radical in its quinonic form (pathway a, **Figure 5**).

Concerning butyl ester, a molecular ion at 817.9 uma was detected, which corresponds to the dimeric form resulting from stabilization (pathway b, **Figure 5**) of two semiquinonic radicals of butyl chlorogenate. The same observation was also made for dodecyl chlorogenate for which a molecular ion at 1043.5 uma was detected. These values show that, after one fast electron transfer, the semiquinonic radicals derived from chlorogenate esters are more prone to rapidly form a dimer (**Figure 5**) than to donate a second electron. Consequently, these MS data match with the hypothesis based on our kinetic study according to which, during the early stages of reaction, 5-CQA is rather able to donate two H atoms (via the ET mechanism), while its esters are rather able to donate a single H atom (via the same ET mechanism).

These radical dimerization pathways can involve diverse types of linkages between semiquinonic radicals. Considering that the unpaired electron can be delocalized from a cycle to carboxylic function (or ester function, **Figure 5**), and also that all combinations are possible, 21 different forms of dimers can be theoretically found. Among them, the most reported (*20*) being (i) a C−C linkage between aromatic rings leading to the formation of a biphenyl dimer (or C−C dimer, b1 in **Figure 5**) and (ii) a C−O linkage between aromatics rings leading to the formation of a diaryl ether (or C−O dimer, b2 in **Figure 5**).

Additionally, the fact that after 2 min the semiquinonic radicals of both butyl and dodecyl chlorogenate esters were not detected in MS analysis (**Table 2**) suggests that all semiquinonic forms are transformed into their corresponding dimers. Interestingly, this observation could explain the fractional number of reduced DPPH• observed at the early stage of the reaction for esters ($n^{es} \sim 1.4$) in the kinetic study (see section 3.1). Indeed, if the steric hindrance of both DPPH• and dimer do not hinder their contact, it is likely that most of the possible dimers which still possess hydroxyl groups could stabilize DPPH•. In the specific case of C−C (b1, **Figure 5**) and C−O (b2, **Figure 5**) dimers which still possess two hydroxyl groups available for stabilizing DPPH•, two subsequent H atom donations (via an ET mechanism) can occur to form an *o*-quinonic dimer radical (b3 and b4, **Figure 5**). This H-donation could thus explain that the n^{es} value for the two first minutes of the stabilization of DPPH• by chlorogenate esters is not equal to 1 but is between 1.36 and 1.49 (except for octadecyl chlorogenate). In other words, during early stages of the reaction, the n^{es} value takes into account H atom transfers from both initial esters and dimer.

Finally, both kinetics and LC-MS data allow the proposal of a general scenario, during which the semiquinonic radical derived from the monoelectronic reduction of one DPPH radical could quickly donate a second electron to a second DPPH radical to form an *o*-quinone (pathway a, **Figure 5**), which is nonreactive in terms of antiradical capacity. Although this is not documented, this reaction may take place in competition with a semiquinonic radical dimerization by C−C linkage (e.g., biphenyl dimer), C−O linkage (e.g., diaryl ether dimer), or other type(s) of linkage(s). Thus, a competition could occur between DPPH• and semiquinonic radical to react with another semiquinonic radical (pathway b, **Figure 5**). In this way, the fate of semiquinonic radicals could be governed by their contact between themselves. From LC-MS data, it is clear that 5-CQA does not form a dimer, while both butyl and dodecyl chlorogenates can. These results suggest that the presence of a carboxylic acid function does not favor the contact of two semiquinonic radicals to form a dimer. The reason for this behavior remains unclear to us, especially when considering the higher apparent steric hindrance of esters than 5-CQA.

Otherwise, the fact that LC-MS data for butyl chlorogenate showed three dimer forms while only one dimer was detected as an oxidation product of dodecyl chlorogenate (**Table 2**) strongly suggests a steric effect. Indeed, it is possible that mesomeric forms of the semiquinone radical resulting from the first H donation of butyl chlorogenate are in better contact between themselves than those of docecyl chlorogenate, which was more hindered.

3.4. Stationary Parameters of Antiradical Activity of 5-CQA and Its Esters. Because the scavenging action of 5-CQA and its esters toward DPPH• does not exclusively concern the two first minutes, a determination of their global stoichiometry was performed after 6 h of reaction, when all reactive Φ-OH are consumed. The total number of reduced DPPH• (n^{ss}, expressed as mol DPPH•/mol Φ-OH) at steady state was calculated as $1/(2 \times EC_{50})$ and summarized in **Table 1**. This stoichiometric parameter provides a second opportunity to compare tested molecules. It appeared that the n^{ss} value was ∼4−5 for 5-CQA and ∼6−7 for chlorogenate esters, except for butyl and octyl chlorogenates for which n^{ss} values of ∼8−9 were found. However, neither of the two distinct reaction pathways previously described (pathways a and b, **Figure 5**) can lead at the steady state to an n^{ss} value above 2. In addition, all n^{ss} values were greater than the number of available phenolic hydroxyl groups (known as being responsible for antiradical activity) present in each tested molecule. This observation

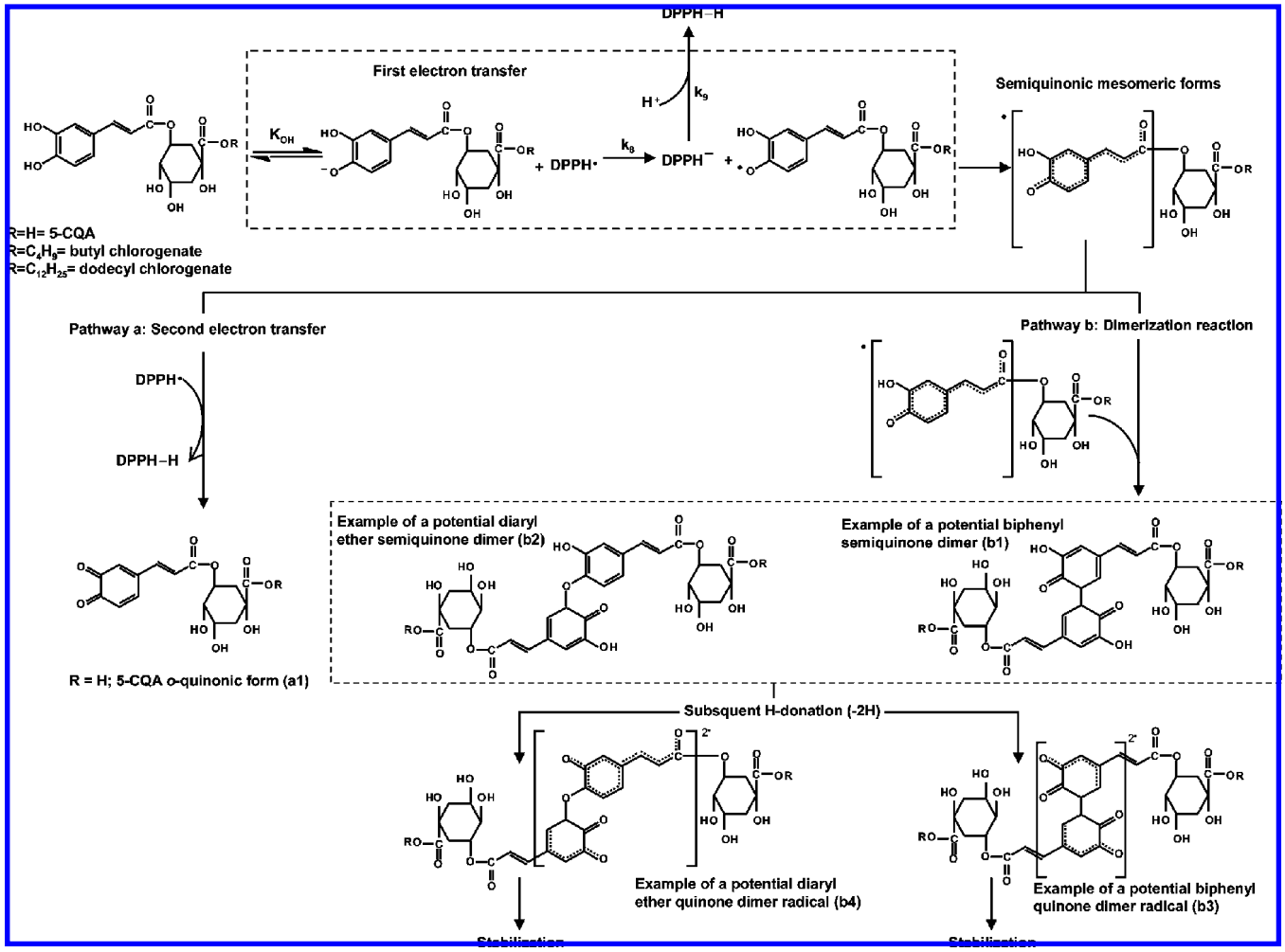

Figure 5. Probable reaction pathways involved during the early stages of the reaction between DPPH• and 5-CQA (a) or its esters (b).

suggests that further reaction pathways occurred in both cases of 5-CQA and its esters. Interestingly, many authors (*18, 20, 25−27*) observed that the number of reduced DPPH• exceeded the number of available phenolic hydroxyls of Φ-OH. Among possible explanations, the most reported one is a nucleophilic attack of the oxidation product derived from Φ-OH. Thus, we can speculate that the solvent (methanol) which is present in great excess into the system can act as a strong nucleophilic agent and consequently can regenerate the nonreactive forms (monomeric and dimeric *o*-quinone, **Figure 5**) to their corresponding reduced ones (which possess available hydroxyl phenolic groups), which is supposed to increase their stoichiometry n^{ss}. This hypothesis is in accordance with Saito and Kawabata (*27*), who observed that the number of reduced DPPH• at 30 min for protocatechuic methyl ester is 2.2 in acetonitrile and 5 in methanol, suggesting that the presence of methanol drastically influences the DPPH• scavenging activity. Moreover, these authors identified the methanol adduct of protocatechuic methyl esters by means of NMR.

Considering the difference in terms of antiradical behavior between 5-CQA and its esters, it is worth mentioning that the n^{ss} value of esters was higher than that of 5-CQA. Furthermore, from a kinetic standpoint, the bleaching of DPPH• by 5-CQA reached steady state at ∼5−6 h, while in the case of esters it was reached at ∼2 h. Interestingly, this strong kinetic difference between acid and ester has already been observed for carboxylic acids, such as protocatechuic (*26*) and caffeic (*10, 28*) ones but also for sulfonic (3,4-dihydroxybenzoic acid (*26*)) and phosphonic (3,4-dihydroxyphenylphosphonic acid (*26*)) acids. Saito and Kawabata (*26*) postulated that the dissociation of the carboxylic function leads to a decrease of the mesomeric electron-withdrawing effect of the COOH group compared to its ester. Consequently, the ring would be richer in electrons for the acid than for its esters, decreasing the electrophilicity of the carbons. In this way, the ability of ring carbons to undergo a nucleophilic addition by methanol decreases as the ring carbon electrophilicity decreases. From this point of view, the quinonic form of phenolic acid is expected to be far less regenerated in its *o*-diphenolic form by methanol addition than esters.

Surprisingly, in our case, a better methanolic addition induced by such a mesomeric effect is impossible because, in the case of 5-CQA and its esters, there is no conjugation between the ring and the carboxylic function. This could imply that the higher DPPH• scavenging activity generally observed for ester compared to acid (*10, 26−28*) is not due to a mesomeric electron withdrawing effect but to another mechanism which is still unknown.

Finally, it is clear that the reactivity of oxidation product(s) derived from phenolics is by far the most important contributor in the final stoichiometry, accounting for 63% for 5-CQA, while higher values were observed for esters, with a maximum of 83% for butyl and octyl. This great important contribution of the oxidation product could also explain the conflicting results reported in the literature for the same compounds analyzed with the DPPH method, as duration assays are generally not harmonized from one study to another. Finally, further experiments using other assays involving more representative oxidizable substrates and media regarding food and biological media

have to be performed in order to elaborate a rational basis to study antiradical and antioxidant capacity especially toward the contribution of oxidation products derived from an antioxidant.

In conclusion, the study of the kinetic parameters showed that the electron-transfer mechanism is the most probable pathway in the DPPH• stabilization in the case of 5-CQA and its esters. With regard to this matter, the partial orders of reaction and the number of DPPH• reduced at the early stages of the reaction suggest that the involved pathways are different for 5-CQA and its esters. It seems that, during the first two min, 5-CQA can transfer two labile H atoms to DPPH•, while its esters can transfer only one H atom to DPPH•, which has been confirmed by LC-MS characterization of the reaction products. At steady state, in contrast, 5-CQA can transfer approximately five labile H atoms to DPPH•, while its esters can transfer approximately six to nine H atoms to DPPH•. This study illustrates the great complexity of the free radical scavenging ability of phenolic compounds even in the simplest case of the DPPH assay. Finally, 5-CQA, which exerts the highest stoichiometry against DPPH• at the early stages, exhibits nevertheless the lowest stoichiometry at the steady state. So, it was evidenced that the reactivity of oxidation products derived from the antioxidant is the most important contributor in the final stoichiometry.

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