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Relationship between Hydrophobicity and Antioxidant Ability of "Phenolipids" in Emulsion: A Parabolic Effect of the Chain Length of Rosmarinate Esters

Mickaël Laguerre,[†,‡] Luis J. López Giraldo,[†] Jérôme Lecomte,[†] Maria-Cruz Figueroa-Espinoza,[†,§] Bruno Baréa,[†] Jochen Weiss,[∥] Eric A. Decker,[‡] and Pierre Villeneuve*[,†]

[†]CIRAD, UMR IATE, Montpellier, F-34398, France, [‡]Department of Food Science, University of Massachusetts, Chenoweth Laboratory, 100 Holdsworth Way, Amherst, Massachusetts 01003, [§]IRC Montpellier SupAgro 1101, av. Agropolis BP 5098 34093, Montpellier Cedex 05, France, and [∥]Department of Food Science and Biotechnology, University of Hohenheim, 70599 Stuttgart, Germany

The polar paradox predicts that hydrophobic antioxidants are more active in emulsions than their hydrophilic homologues, thus assuming a linear dependency between hydrophobicity and antioxidant capacity. In contrast, we formulate in this paper an alternative hypothesis assuming a possible nonlinear dependency. To verify this so-called "nonlinear hypothesis", the antioxidant capacity of a homologous series of rosmarinic acid and its alkyl esters (methyl, butyl, octyl, dodecyl, hexadecyl, octadecyl, and eicosyl) was evaluated using a newly developed conjugated autoxidizable triene (CAT) assay. It appeared that the antioxidant capacity increases as the alkyl chain is lengthened, with a maximum for the octyl chain, after which further chain extension leads to a collapse in antioxidant capacity. This nonlinear effect was discussed in relation to the "cutoff effect" generally observed in studies using cultured cells. This new hypothesis may provide a better understanding of the antioxidant behavior of phenolics in emulsion which is a key to develop new antioxidant strategies to protect lipid substrates from oxidation. Moreover, the lipophilization with medium chain appeared as a promising way to enhance the antioxidant capacity of phenolics since octyl rosmarinate was three times more effective than rosmarinic acid which is already one of the most powerful known phenolic antioxidant. Finally, this work paves the way for systematic investigation of the chain length effect to design new "phenolipids" in a rational fashion.

KEYWORDS: Antioxidant; phenolic compound; phenolipid; rosmarinic acid; lipophilization; lipid oxidation; emulsion; conjugated autoxidizable triene assay; partition; polar paradox; cutoff effect; nonlinear hypothesis

INTRODUCTION

Lipid oxidation in emulsified systems plays an important role for cosmetic and food products, since many of them exist either partly or wholly as emulsion, or have been in an emulsified state at some time during their existence (*1*). However, the reaction mechanisms of lipid oxidation, though extensively investigated since the beginning of the 20th century, are still not completely understood, especially in connection with antioxidants in dispersed systems such as emulsions. Such knowledge is nevertheless highly desirable to develop new antioxidant strategies to protect nutritional lipids from oxidative alterations. To the best of our knowledge, one of the first rationale models to explain antioxidant effect in lipids was suggested by Porter (*2*), and then formulated in the polar paradox hypothesis (*3*). This hypothesis described paradoxes in the effectiveness of antioxidant substances in bulk oil and dispersed lipid systems such as micellar media, emulsions, liposomes, and even whole tissues. Regarding oil-in-water emulsion, the polar paradox proposed that hydrophobic antioxidants are more active than their hydrophilic homologues (*ceteris paribus*), thus implicitly assuming a linear dependency between the antioxidant capacity and the hydrophobicity in such a multiphasic system. Then, Frankel et al. (*4*) strengthened this model introducing the interfacial oxidation concept according to which the higher effectiveness of hydrophobic antioxidants in oil-in-water emulsions would be due to their tendency to concentrate at the interfacial membrane where the oxidation is supposed to occur, while more hydrophilic antioxidants would tend to partition more into the aqueous phase where they would be ineffective. Based upon this polar paradox theory, some authors suggested to design new lipophilized surface active antioxidant molecules, especially phenolics, to improve their ability to counteract lipid oxidation in emulsions and other dispersed lipid systems (*5*). These functionalized molecules resulting from the grafting of

*Corresponding author. E-mail: villeneuve@cirad.fr. Phone: (33) (0)4 67 61 55 18. Fax: (33) (0)4 67 61 55 15.

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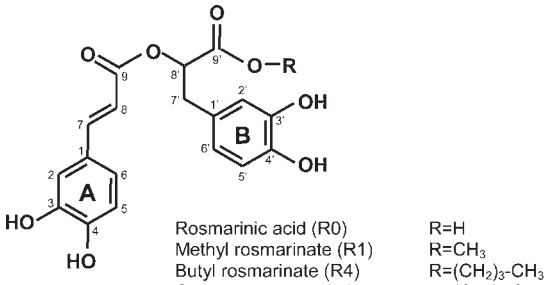

Figure 1. Chemical structure of rosmarinic acid and its alkyl esters.

a lipid on a phenolic moiety (coined as "phenolipids") can be prepared *via* different synthesis strategies such as esterification (*6*−*13*), amidation (*14*) or etherification (*15*, *16*). However, it has been demonstrated in oil-in-water emulsion that lipophilization of phenolics is not always advantageous in terms of antioxidant capacity (*9*, *17*, *18*), thus suggesting that not all antioxidant behave in a manner proposed by the polar paradox theory. These contrasting results highlighted the fact that the behavior of phenolic antioxidants is governed by more complex phenomena than expected, and lead us to postulate that the dependency between the antioxidant capacity and the hydrophobicity does not necessarily follow a linear trend (*19*). Indeed, in a previous study performed in oil-in-water emulsion and using a complete homologous series of chlorogenic acid and its alkyl esters (methyl, butyl, octyl, dodecyl, hexadecyl, octadecyl and eicosyl) we observed that antioxidant capacity increases as the alkyl chain is lengthened, with a threshold for the dodecyl chain, beyond which further chain extension leads to an antioxidant capacity collapse (*19*). This result suggested that the chain length affects antioxidant capacity in a nonlinear manner, at least in our system, providing a new model to understand the behavior of phenolic antioxidants in emulsions and to design promising new phenolipids.

The goal of the present study was to strengthen our understanding of the relationship between antioxidant capacity and hydrophobicity using a new homologous series consisting of rosmarinic acid and its alkyl esters (methyl, butyl, octyl, dodecyl, hexadecyl, octadecyl and eicosyl, **Figure 1**) (*13*). Such a study performed with both hydrophilic and lipophilic antioxidants was possible using a newly developed conjugated autoxidizable triene (CAT) assay (*20*) performed in tung oil-in-water emulsion with slight improvements (*19*, *21*). The partitioning behavior of phenolics into this dispersed system using various emulsifier concentrations was also investigated, along with the ^{1}H NMR data of those compounds.

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. Rosmarinic acid, phosphate buffer solution (PBS) pH 7.2, polyoxyethylene(*23*)lauryl ether (Brij 35, estimated MW = 1198 g/mol) were purchased from Sigma (Saint Quentin, France). All solvents used were 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) from Wako Chemical (Neuss, Germany).

Synthesis of Rosmarinate Esters. The chemoenzymatic esterification of rosmarinic acid to obtain rosmarinic esters was carried out following the procedure described by Lecomte et al. (*13*). Briefly, the chemical esterification of rosmarinic acid (56 μmol) was carried out in sealed brown flasks each containing 5 mL of alcohol (methanol, 123.44 mmol; *n*-butanol, 54.64 mmol; *n*-octanol, 31.905 mmol; *n*-dodecanol, 22.46 mmol; *n*-hexadecanol, 16.95 mmol; *n*-octadecanol, 15.09 mmol and *n*-eicosanol, 13.6 mmol). The reaction mixtures were stirred (orbital shaker, 250 rpm, 55−70 °C) prior to the addition of the catalyst, the strongly acidic sulfonic resin Amberlite IR-120H (5% w/w - total weight of both substrates) previously dried at 110 °C for 48 h. The water generated during the reaction was removed by absorption on molecular sieves (40 mg/mL) added to the medium. Samples (20 μL) were regularly withdrawn from the reaction medium then mixed with 980 μL of methanol, filtered (0.45 μm syringe filter Millex-FH, Millipore Corporation Bedfork, MA), and finally analyzed by reverse phase HPLC with UV detection at 328 nm. After complete (4−21 days) conversion of rosmarinic acid into the corresponding ester, the latter was purified in a two step procedure. First, a liquid−liquid extraction using hexane and acetonitrile was achieved to remove the excess of alcohol. Then, the remaining traces of the alcohol and rosmarinic acid were eliminated by flash chromatography on a CombiFlash Companion system (Teledyne Isco Inc., Lincoln, NE). Separation was achieved on a silica column using an elution gradient of hexane and ether (20% to 100% in 35 min). The yield of purified esters, obtained as pale yellow to yellow amorphous powders, was calculated from calibration curves previously established with pure compounds. Pure esters and rosmarinic acid were then fully characterized by ESI-MS, ^{1}H NMR and ^{13}C NMR as previously described by Lecomte et al. (*13*).

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 sealed brown glass tubes, then flushed with nitrogen, 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 rosmarinic acid and its alkyl esters was measured using the CAT procedure developed by Laguerre et al. (*20*) with slight improvements (*19*, *21*) allowing the analysis of a larger panel of molecules from hydrophilic to lipophilic ones. Briefly, phenolics solutions were prepared as follows: a methanol solution of rosmarinic 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) 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 phenolics contain the same methanol volume (100 μL), which allows any eventual bias among samples to be avoided. All buffered solutions of phenolics were prepared extemporarily and 50 μL/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 for spectral measurement in the UV-domain (absorbance at 273 nm = 0.03). The microplate was then prewarmed and stirred in a thermostatted shaker (PHMT Grant Instruments Ltd., Shepreth, England) for 5 min at 37 °C and 1200 rpm.

Twenty-five milliliters of PBS (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 is 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. This tung oil-in-PBS emulsion (100 μL) was then added to well. To improve repeatability, the microplate was then immediately prewarmed and shaken, in the absence of 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 then added to each well 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 phenolics (from 0.01 to 0.55 μM) in PBS. The progress of reactions was immediately monitored by recording the decrease in absorbance at 273 nm. The possibility to use direct spectrophotometric measurements suggests that our emulsion is likely to be a microemulsion whose droplet diameter is small enough to avoid light scattering. Afterward, 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, Grödig, Austria) equipped with Magellan software. Three independent analyses using different microplates were carried out.

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

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

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 reference for antioxidant capacity measurements. Thus, the antioxidant capacity of a sample relative to Trolox (CAT value) is given as

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

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 internal standard (i.e., analyzed on the same microplate). Finally, the CAT value is expressed as moles of Trolox per mole of tested compound (Trolox equivalents).

Antioxidant Partitioning Measurements. The sunflower oil-in-water emulsions (2 wt %/wt) were prepared using Brij 35 as emulsifier. The final concentrations of emulsifier were 0, 0.017, 0.17, 1, 2, and 10 mM. The procedure was the following: 25 mL of PBS (pH 7.2) containing the previous concentration of Brij 35 were 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 minimize lipid and phenolic oxidation, 100 μM EDTA was added to emulsion sample. All phenolics 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 at room temperature. To measure concentration of phenolics in the continuous phase of the emulsion, 1 mL of emulsion 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 aqueous phase (lower layer) of the emulsion was withdrawn with a syringe. This procedure of PBS addition, 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 rosmarinic acid and its alkyl esters was finally evaluated using HPLC with a detection at 328 nm as previously described by Lecomte et al. (*13*).

RESULTS AND DISCUSSION

Today, the development of new antioxidants such as phenolipids is hampered by the lack of a reliable theory describing and predicting the antioxidant properties of phenolic antioxidants in emulsified systems. The only rational model (known as polar paradox) assumes that hydrophobic antioxidants are more active in oil-in-water emulsion than their hydrophilic homologues, which can be implicitly interpretated as the fact that the relationship follows a linear shape. In contrast, we formulate in this paper an alternative hypothesis assuming that the relationship between antioxidant capacity and hydrophobicity is not necessarily linear, which can lead to a better rationale to design new phenolipids.

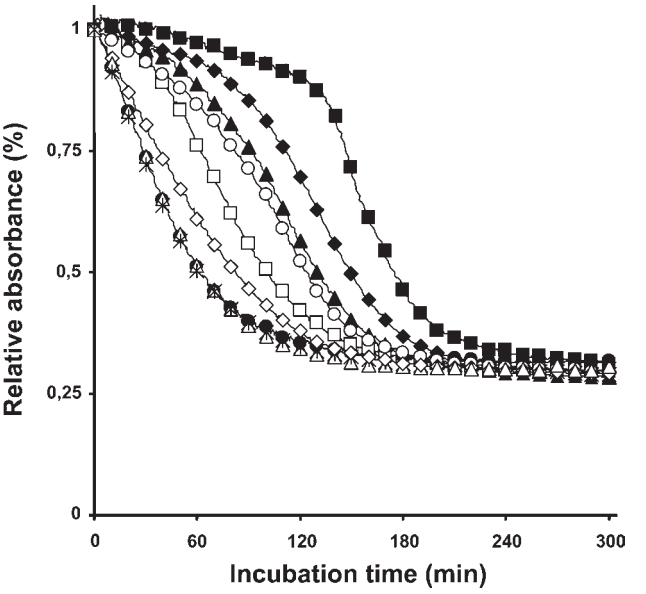

Figure 2. Kinetic of relative absorbance bleaching of stripped tung oil-in-water emulsion in the absence or presence of 0.1 μM of phenolics. The reaction mixture contained 115 μM stripped tung oil, 17 μM Brij 35, and 1 mM AAPH, in PBS pH 7.2 at 37 °C. R0 (□), R1 (▲), R4 (♦), R8 (■), R12 (○), R16 (◇), R18 (△), R20 (*), and phenolic-free control (●).

Antioxidant Capacity Measurement Using the CAT Method. Taking advantage of the possibility to assess both hydrophilic and hydrophobic compounds in the CAT assay, the antioxidant capacity of a homologous series of rosmarinic acid and its alkyl esters (**Figure 1**) was evaluated with the protocol developed by Laguerre et al. (*20*) with slight improvements (*19*, *21*). Except for octadecyl and eicosyl esters, it appeared that all tested phenolics exhibited noticeable antioxidant properties for all tested concentrations (**Figure 2**, data only shown for 0.1 μM). The calculation of their net AUC from eq **3** showed that all phenolics act in a dose-dependent manner (**Figure 3**), which is a prerequisite to calculate their CAT value expressed as moles of Trolox equivalent per mole of test compound through eq **3**. Strong differences were observed between phenolics, with a maximal antioxidant capacity for the octyl ester (15.31 ± 1.33 TE), followed by butyl rosmarinate (10.33 ± 1.39 TE) > methyl rosmarinate (8.37 ± 0.60 TE) > dodecyl rosmarinate (7.67 ± 0.76 TE) > rosmarinic acid (5.21 ± 0.25 TE) ≫ hexadecyl rosmarinate (1.53 ± 0.26 TE) > octadecyl rosmarinate (0.65 ± 0.11 TE) ∼ eicosyl rosmarinate (0.54 ± 0.13 TE) (**Figure 4**). First of all, the lipophilization appeared to induce a drastic modulation of the antioxidant capacity. Rosmarinic acid is known as one of the most powerful phenolic antioxidants since it encompass two catechol (*o*-diphenol) moieties which is an important feature in terms of bond dissociation energy of the phenolic hydroxyl group (O−H BDE) (*22*). In this context, the grafting of an octyl chain leads to a phenolipid three times more effective than rosmarinic acid, demonstrating the interest to modulate the hydrophobicity of phenolics through lipophilization reaction. Moreover, we recently demonstrated that the lipophilization of rosmarinic acid does not necessarily lead to an increase of its free radical scavenging stoichiometry

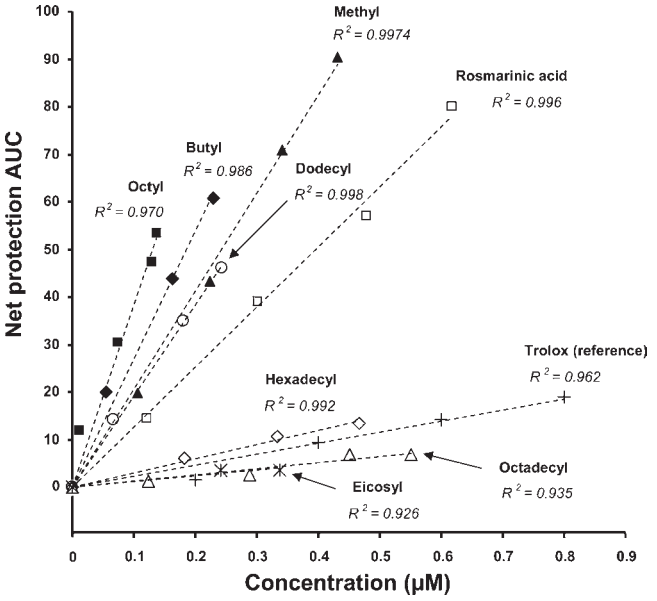

Figure 3. Net protection AUC ($AUC_{sample} - AUC_{blank}$) vs concentration ($\mu$M) for rosmarinic acid, its alkyl esters, and Trolox (reference). Concentration of phenolics has been checked by HPLC and corrected.

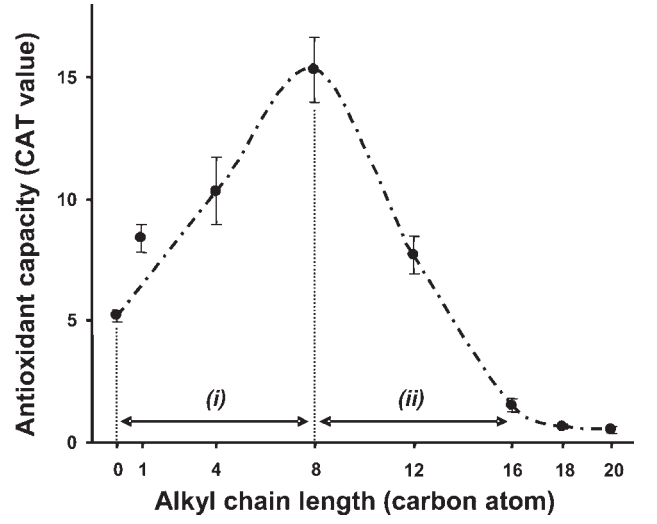

Figure 4. CAT value (moles of Trolox per mole of tested compound) of rosmarinic acid and its alkyl esters vs the alkyl chain length.

against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (*13*). This shows again that the use of two different methodologies can lead to opposite conclusions regarding the interest of lipophilization. One can also notice that noncompetitive free radical scavenging capacity methods (such as DPPH test) do not contain oxidizable substrate (such as lipid), and consequently do not provide a competitive reaction scheme, in which antioxidant and substrate compete for oxidizing species. Thus, unlike the CAT assay, the DPPH methodology does not accurately indicate the ability of a compound to inhibit oxidation.

Of particular interest was the fact that the dependence between the alkyl chain length and the antioxidant capacity followed a parabolic shape with a maximum for the octyl ester (**Figure 4**). This effect can be divided into two virtual domains: a first part (i) with a quasi-linear increase of the antioxidant capacity with the elongation of the alkyl chain, and a second part (ii) for which the antioxidant capacity collapses with increasing hydrophobicity. The fact that such a nonlinear dependence was never reported (apart from our previous article (*19*)) may explain why some lipophilization strategies lead sometimes to an unexpected decrease of the antioxidant effect, especially those using long aliphatic chains. In other words, these results along with those we previously reported with chlorogenate esters (*19*) show that it is not relevant to simply graft any fatty moiety and highlight the prime importance of searching the optimal chain effect to design the fittest phenolipids.

Interestingly, the nonlinear effect observed with the CAT assay is consistent with current literature regarding biological activity, with numerous studies documenting this type of response with homologous series of amphiphilic molecules as antimicrobial (*23*, *24*), anesthetic (*25*, *26*), cytotoxic (*27*), or spermicide (*28*) agents. This particular phenomenon was called "cutoff effect" and can be defined for a homologous series of hydrocarbon chain surface active compounds, as the tendency for various biological activities to increase stepwise (or stay stable) with increasing chain length up to a critical point, beyond which these activities suddenly collapse. It is worth pointing out that the first part of the trend (below the threshold) does not always follow the same trend from a study to another one, while after such a threshold is reached, the trend does always follow a sudden collapse. This definition of the cutoff effect perfectly fits with the parabolic shape depicted in **Figure 4**. In terms of mechanism of action, different hypotheses have been put forward, most of them dealing with a specific interaction between drug(s) and the cell membrane. However, our cutoff effect was observed in a nonliving system (i.e., oil-in-water emulsion) which suggests that this phenomenon may originate from a simple physicochemical process. In our case, among numerous other putative mechanisms of action, we investigated the hypothesis that this effect may come either from (i) an intrinsic modulation of the H-donation ability by the chain length through a π-stacking process, or from (ii) an interaction between phenolics, water and lipids, involving possibly a micellization process as already assumed in a similar case (*19*).

H-Donation Ability, π-Stacking and ^{1}H NMR. In terms of H-donation ability, the grafting of an alkyl chain on rosmarinic acid should not theoretically affect the H-donation ability *via* a mesomeric effect since the carboxylic and the two phenolic groups are not conjugated. However, a self-association may occur between the two aromatic rings via a π-stacking caused by intramolecular overlapping of the p-orbital system which is supposed to lead to an increase of the free radical scavenging ability. It is well-known that a $\pi-\pi$ interaction of two phenolic rings can take place between two phenolic molecules (*29*). Such a self-assembly process can also take place between two aromatic rings present in the same molecule. To the best of our knowledge, this latter type of π-stacking (so-called *intramolecular* π-stacking) is only documented for antocyanins acylated by phenolic acids (*30*). Of particular interest is that rosmarinic acid and its alkyl esters satisfy basic requirements to undergo a folding through *intramolecular* π-stacking. Indeed, the two phenolic rings (A and B) are linked by a suitable spacer, which allows the molecule to fold in such a way that the two rings can interact together. Others requirements involve the protection from hydration, and the planarity of aromatic rings to optimize the contact area between themselves. In an attempt to explain the difference observed for antioxidant capacity between rosmarinic acid and its alkyl esters, it can be hypothesized that, in the case of the rosmarinic acid, the π-stacking configuration (called configuration **1**) may be in competition with another configuration. This latter (called configuration **2**) would involve the establishment of a H-bond from the hydroxyl of the free COOH function and the oxygen of one of the two phenolic hydroxyl groups in the B ring. Let us consider the putative case where the better

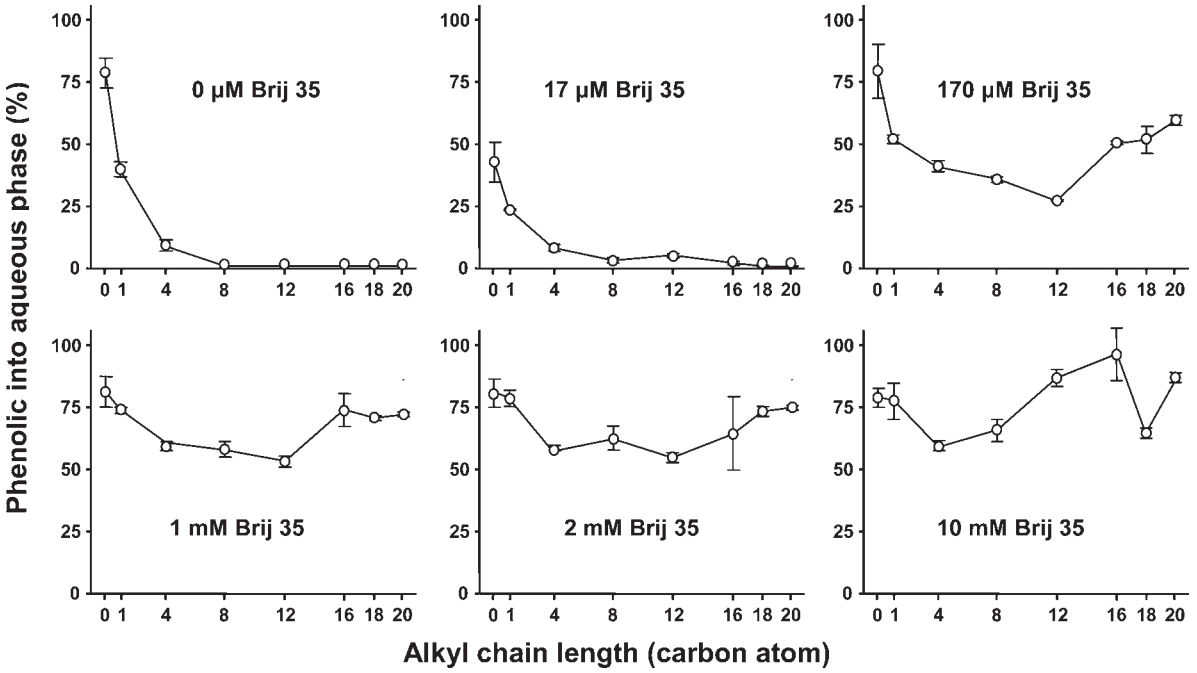

Figure 5. Effect of the alkyl chain length on the partition behavior of phenolics in a mixture of sunflower oil and PBS pH 7.2 without or with 0.017, 0.17, 1, 2, and 10 mM Brij 35 used as emulsifier.

antioxidant capacity for methyl, butyl, octyl, and dodecyl rosmarinates compared to free rosmarinic acid is due to the fact that the configuration **1** is the main configuration for the esters, whereas equilibrium between configuration **1** and **2** takes place for rosmarinic acid. In this case, configuration **1** should confer better antioxidant capacity than configuration **2** (all other things being equal), since the phenolic hydroxyls are more susceptible to establish H-bond with another phenolic hydroxyl group (and consequently to decrease O−H BDE of the molecule by stabilizing the phenoxy radical), than with the carboxylic hydroxyl group. Such a difference in the intramolecular configuration between rosmarinic acid and its alkyl esters should lead to a significant displacement of the chemical shifts of their aromatic protons as indicated by ^{1}H NMR analysis (*31*). However, the investigation of our ^{1}H NMR spectra of rosmarinic acid and its alkyl esters (*13*) did not show such a displacement. Indeed, the chemical shift for rosmarinic acid was 7.08, 6.80−6.78, 7.04−7.02, 6.70−6.69, 6.67−6.65, and 6.56−6.54 δ/ppm for the aromatic protons in position 2, 5, 6, 2′, 5′, and 6′, respectively, whereas their chemical shifts for rosmarinate esters were 7.07, 6.79−6.77, 7.02−7.00, 6.68−6.67, 6.66−6.64, and 6.52−6.50 δ/ppm, respectively. Finally, although the occurrence of a π-stacking between the two aromatic rings seems to be likely for these phenolics, no difference in such a process between rosmarinic acid and its alkyl esters can be deduced from ^{1}H NMR data.

Partitioning Behavior of Phenolics at Low Emulsifier Concentration. Besides intrinsic H-donation ability, the antioxidant capacity of phenolics in heterogeneous systems such as emulsions is supposed to be also governed by their interaction with the environment, especially their partitioning behavior between the different phases (*4*) and their diffusibility toward reactive centers (i.e., oxidizable substrate, free radicals, ...) (*22*). In order to gain insight about a possible mechanism of action, the partitioning of rosmarinic acid and its alkyl esters was studied in a mixture of sunflower oil and PBS (pH 7.2). It appeared in **Figure 5** that, with or without 17 μM Brij 35 (same Brij 35 concentration as in the CAT assay, but different from the Brij 35/phenolic molar ratio used in the CAT assay), the longer the chain length, the lower their level in the water phase is, until octyl chain which totally partitions in the discontinuous phase (oil + interfacial membrane). Then, further increments in the chain length do not significantly change the partitioning behavior. This result was expected and is consistent with literature reports about partitioning behavior of phenolics in oil-in-water emulsion (*18*, *19*). However, a little increase in the concentration of Brij 35 from 17 to 170 μM lead to a drastic change, with the appearance of a weak cutoff effect with a threshold for the dodecyl ester, above which, surprisingly, the longer the chain length, the higher their level in the aqueous phase is. Interestingly, we found the same result with chlorogenate esters in the same system apart from the concentration of Brij 35 which was 17 μM. It has not escaped our notice that the specific cutoff effect in the partitioning behavior with a threshold for the dodecyl chain (in both rosmarinate and chlorogenate cases) may be due the fact that the hydrophobic tail of Brij 35 emulsifier consists of a dodecyl chain. In virtue of their amphiphilic properties, the emulsifier accumulates at the oil−water interface in emulsions and thus is considered to dominate the properties of the interface in terms of solubilization capacity of antioxidants (*17*). Indeed, this structural analogy could enable a good alignment between dodecyl rosmarinate and Brij 35 hydrophobic tails. However, regardless of the mechanism of action, the cutoff effect for a dodecyl chain in the partitioning behavior does not explain the cutoff effect for an octyl chain in the antioxidant properties of rosmarinate esters. This must be connected with the fact that some authors unexpectedly demonstrated that the partitioning behavior of antioxidants is not always a good predictor of the antioxidant effectiveness (*18*).

Besides partitioning, it is worth noting that increased hydrophobic interactions with the environment may lower the diffusion of long chain rosmarinates to the reaction centers, resulting in a reduced antioxidant capacity, as previously suggested by Stockmann et al. (*17*) on a homologous series of gallate esters. In connection, according to Fendler (*32*), the mobility of the hydrocarbon chain is reduced with increasing its length. One can also

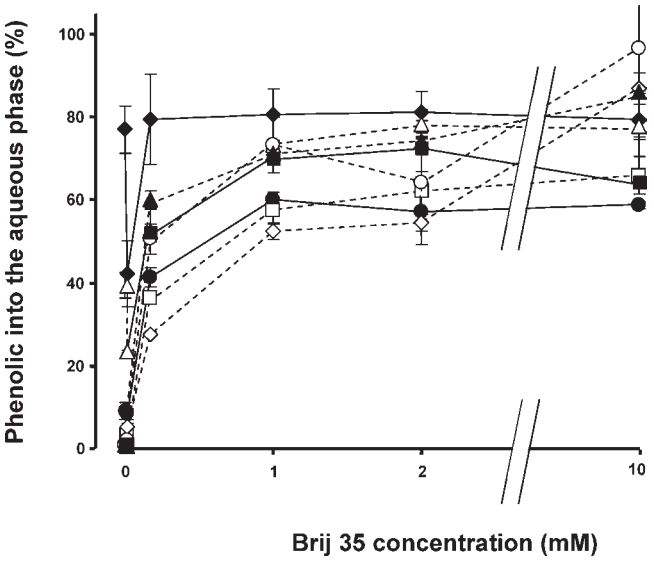

Figure 6. Emulsifier (Brij 35) concentration effect on the partition behavior of phenolics in a mixture of sunflower oil and PBS pH 7.2. R0 (◆), R1 (△), R4 (●), R8 (□), R12 (◇), R16 (○), R18 (■), R20 (▲).

notice that steric hindrance induced by increasing the chain length can render difficult the contact between long chain esters and AAPH-derived free radicals, and may consequently be involved in the cutoff effect observed in this study. In addition, the partitioning behavior of phenolics in the presence of 17 μM (**Figure 5**) may also suggest that increasing the hydrocarbon chain from C8 could drive the antioxidant away from the interface and into the emulsion droplet core where the phenolics would be a poor antioxidant. Interestingly, this putative effect of the alkyl chain length has already been suggested by Medina et al. (*33*) to explain why the octyl ester of hydroxytyrosol exhibits higher antioxidant capacity than the dodecyl one in fish oil-in-water emulsion.

Effect of an Excess of Emulsifier on the Partioning Behavior of Phenolics. Above 17 μM, increasing concentrations of Brij 35 (1, 2, and 10 mM) drastically impact the partition behavior of phenolics and lead to the appearance and then the stepwise disappearance of a partition cutoff trend (**Figure 5**). The depiction of the phenolic concentration in the aqueous phase as a function of the Brij 35 concentration (**Figure 6**) clearly showed that there is a global tendency for esters (but not for free rosmarinic acid which is already located in the aqueous phase) to return in the water phase with increasing emulsifier concentration, especially above 0.017 mM. As already reported by Laguerre et al. (*19*) in a similar case with chlorogenate esters, the simplest hypothesis to explain this trend is that above the critical micelle concentration (CMC, 0.1−0.3 mM in water at room temperature (*34*,*35*)), Brij 35 micelles behave as vehicle for phenolics by means of comicelles to carry them to the water phase. Indeed, the solubilization of phenolics can take place in the palisade layer of the Brij 35 micelles. The formation of micelle with Brij 35 is very likely above CMC since the area of its hydrophilic head (23 polyoxyethylene groups) is much larger than the area of its hydrophobic tail (lauryl chain). Considering the packing parameter (*p*) of Israelachvili (*36*) it is quite evident that p_{Brij35} is less large than 1/3. This implies that, just above the CMC, there would be formation of micelle rather than other lipid polymorphs such as vesicles or bilayer sheets for which a *p* comprised between 1/2 and 1 is theoretically required.

Regarding the carrying of phenolics, the pioneer work of Richards et al. (*37*), and more recently those of Yuji et al. (*9*) and Laguerre et al. (*19*) showed the same partition behavior. For instance, the Brij 700 emulsifier was able to increase the partitioning of propyl gallate into the aqueous phase of an oil-in-water emulsion in a rapid and concentration-dependent manner (*37*). Other related study (*38*) showed that lipid hydroperoxides can be carried from the discontinuous phase to the aqueous one by Brij 76 micelles in a same way as phenolic antioxidants. Taken together, these studies (including the present one) suggest that above CMC, an emulsifier can drastically affect the partition of crucial components in the lipid oxidation pathways by either bringing them together so they can react or isolating them and thus preventing their interaction. Accordingly, the micellization of surfactant may partly shift the oxidation process from a pure interfacial phenomenon to a micellar one. We feel that this may be an important point, since many oil-in-water emulsions contain more surfactant than is needed to completely saturate the emulsion droplet surface (*37*).

Finally, the polar paradox is based on the observations that hydrophobic antioxidants are more active in emulsion than hydrophilic ones, while these latter are more efficient in bulk oil than their hydrophobic homologues. Historically, this hypothesis was put forward to show that it is not relevant to extrapolate uncritically effectiveness data for antioxidants from bulk oil to emulsion (or micelles, membrane and whole tissue). To be clear, the present results along with those previously reported for chlorogenate esters (*19*) do not put into question this part of the polar paradox which was, in the field of lipid oxidation, one of the greatest achievements of recent decades. Our results rather show that the dependence in emulsion between the hydrophobicity and the antioxidant capacity is nonlinear and characterized by a strong cutoff effect for the octyl ester, which cannot be anticipated nor explained on the basis of the polar paradox theory. This finding may be crucial for two reasons. The first one is that the cutoff (or nonlinear) hypothesis (if confirmed with other methods, other oxidation inductors, and other phenolipids) can lead to a much better fundamental understanding of the behavior of phenolic antioxidants in dispersed systems which is a prerequisite to master the current antioxidant strategies. Indeed, at least in the CAT system, our study suggests that the polar paradox theory is not valid for medium and long chain lengths. The second important impact of our nonlinear hypothesis may be the establishment of a rational rule to design and customize new promising antioxidants, such as phenolipids, to optimize the protection of nutritional lipids in multiphasic systems. We recently noted with interest the publication by Medina et al. (*33*) of a supporting evidence for our nonlinear hypothesis, since they found in oil-in-water emulsion that "the presence of a short−medium lipophilic chain (acetate, butyrate or octanoate) improved the antioxidant efficiency of hydroxytyrosol [...], but longer alkyl chain (laurate) maintained or even decreased their antioxidant activity". In addition, we recently observed with chlorogenate esters in human fibroblasts (to be published elsewhere) the same antioxidant cutoff effect with a threshold for the dodecyl chain as already reported in oil-in-water emulsion (*19*), suggesting that this behavior may have important biological consequences. In terms of mechanism of action, neither ^{1}H NMR data nor partitioning studies elucidated the origin of this effect, which remains as unclear for us as for other studies where such a behavior has been observed. Further experiments varying both antioxidant models and methodologies, especially the way to induce oxidation (natural one, transient metal, azo compounds...), must be achieved to confirm or infirm this nonlinear hypothesis, especially the collapse beyond a threshold chain length, and finally to elucidate its mechanism of action.

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