Behavior of Phenolic Antioxidants in a Partitioned Medium: Structure—Activity Relationship

Marie-Elisabeth Cuvelier*, Vincent Bondet, and Claudette Berset

Département Science de l'Aliment, École Nationale Supérieure des Industries Alimentaires, 91744 Massy-Cedex, France

ABSTRACT: The behavior of phenolic antioxidants is studied in a partitioned medium, composed of linoleic acid dispersed in an aqueous phase. Their efficiency is measured by the diene production during oxidation, induced by Fe (II)/ascorbic acid at 30°C. With a linoleic acid/Fe^{2+} molar ratio of 10 and a Fe^{2+}/ascorbic acid molar ratio of 23, a steady-state propagation rate is reached after 1 h for up to 15 h. The antioxidants cannot avoid the early dienes (30–40% of total dienes), resulting from the inducing reactions; however, they can stop all the dienes produced during propagation reactions by acting on $ROO^{\bullet}$. The inhibition values reveal a great difference between the antioxidants, depending on their structure (number of hydroxyl groups or chelating sites) and on their polarity, confirming the "polar paradox." Thus, α-tocopherol, butylated hydroxytoluene, butylated hydroxyanisole, and isoeugenol appear to be the best antioxidants, but rosmarinic and caffeic acids, generally potent antioxidants, present a weak efficiency. Surprisingly, in such a metal-induced system, the chelator activity seems to play a minor role.

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KEY WORDS: Antioxidant, conjugated dienes, iron-ascorbic acid system, oxidation test, partitioned medium, phenolic compounds, polar paradox, structure–activity relationship.

The antioxidant activity of synthetic and natural compounds has been studied by many methods using model systems. These methods differ in duration, measurement mode, and conditions of temperature, oxygenation, and medium (1–3). The medium can be bulk lipid, emulsified, or aqueous medium. Antioxidants do not present the same behavior under these different conditions. This is well illustrated by Porter's polar paradox (4). Porter *et al.* observed that polar antioxidants are more effective in bulk lipids, whereas nonpolar antioxidants are more active in emulsified media. This paradox can be explained by the interfacial properties of the antioxidants and their partition in the medium (3). As most of food and cosmetic products are constituted of an emulsified matrix, it is useful to test antioxidants in such a medium. To shorten the duration of the test, temperature or initiator systems such as Fe(II) are often used, notably in liposome or lipoprotein oxidation tests (5–7).

The purpose of this work was to measure the efficiency of antioxidants on emulsified linoleic acid peroxidation induced by iron/ascorbic acid, and to analyze how their activity can be explained by their structure. The conditions of the test are described in the accompanying paper (8). The activity of 17 antioxidants was analyzed in relation to their polarity, the presence of radical scavenging phenolic groups, and, for some of them, the presence of chelator sites or substituted groups. The results have been compared to those obtained in an apolar medium with the accelerated test developed in our lab (9).

EXPERIMENTAL PROCEDURES

Materials. Materials required to prepare the emulsified medium are listed in the accompanying paper (8).

The antioxidants butylated hydroxytoluene (BHT) (99%), guaiacol (98%), eugenol (99%), isoeugenol (98%), caffeic acid (97%), butylated hydroxyanisole (BHA) (98%), DL-α-tocopherol (98%), and Trolox (98%) were purchased from Fluka-Aldrich-Sigma (St. Quentin Fallavier, France). Naringenin (90%), eriodictyol (90%), quercetin (90%), DL-catechin (nd), (–)-epicatechin (90%), isoquercitrin (90%), ferulic acid (nd), *p*-coumaric acid (nd), and rosmarinic acid (90%) were purchased from Extrasynthese (Genay, France). The spectrophotometer was a Uvikon 810 (Kontron, Sitzerland).

Oxidation test. A solution containing 14 g/L linoleic acid and the tested antioxidant was prepared in methanol or ethyl acetate, according to the solubility of the antioxidant. Then, 200 μL of this solution was evaporated under nitrogen and emulsified according to the procedure described earlier (8). The standard kinetics of conjugated diene production were obtained from a linoleic acid solution containing no antioxidant.

Inhibition power measurement. The absorbance at 233 nm corresponds to the difference of the conjugated diene amount between the sample cuvette and the reference cuvette. On the curve representing its increase as a function of time (Fig. 1), we took two parameters: the slope of the linear phase of the curve (*S*) and the amplitude corresponding to the intersection of the slope *S* with the *Y* axis (*A*). Antioxidants decreased the

*To whom correspondence should be addressed at ENSIA, Département Science de l'Aliment, 1, avenue des Olympiades, 91744 Massy-Cedex, France. E-mail: cuvelier@ensia.inra.fr

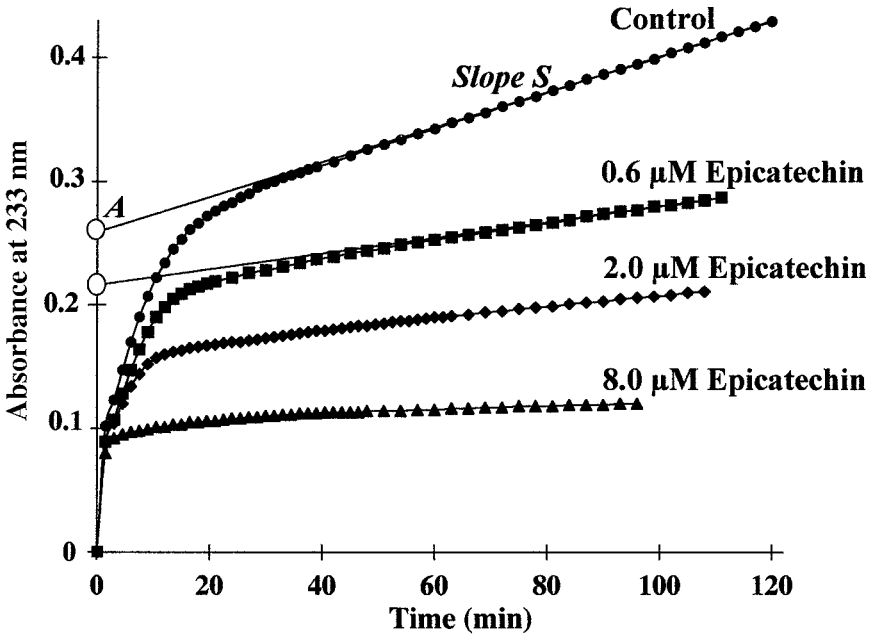

FIG. 1. Kinetics of conjugated diene production during dispersed linoleic acid oxidation induced by iron-ascorbate, in the absence (control) and in the presence of several concentrations of epicatechin. 17 µM linoleic acid, 17 µM $FeCl_2$, 0.7 µM ascorbic acid, pH = 6.45, 30°C. The absorbance level was measured at 3 or 6 min intervals.

value of *A* and/or *S* obtained with the control. For each antioxidant, we searched the quantity required to decrease *A* or *S* by half, the lower it is, the stronger the antioxidant is. For reasons of clarity, the inhibition power was defined as the reciprocal of these quantities and named inhibition power on amplitude (IPa) and inhibition power on slope (IPs).

Polarity measurement. The polarity of the antioxidants was measured by thin-layer chromatography according to Porter *et al.* (4). The eluant was chloroform/methanol/acetic acid (19:1:0.5, vol/vol/vol). A methanolic solution of 300 µM $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) was used to color the plate. Since the tested antioxidants were able to reduce purple-blue $DPPH^{\bullet}$ into discolored DPPH-H, they were detected by white spots on the plate. The apolarity factor (AF) was defined as the retention factor (R_f) of the tested antioxidants.

Statistical analyses. The analysis of variance on 50 standard curves gave a variation of 7% for *A* and 6% for *S*. Each antioxidant concentration was tested twice.

RESULTS AND DISCUSSION

Inhibition parameters. The dienes produced during the iron-induced oxidation of linoleic acid at 30°C were monitored at 233 nm. Figure 1 shows typical kinetics obtained with epicatechin as the antioxidant at several concentrations. To quantify the antioxidant effect, it is usual to consider the starting slope of the kinetic plot. However, this measurement was not sufficiently discriminant and accurate for two reasons: Variations between the different kinetics were too small and there were not enough points during the first few minutes. For the same reasons, we could not use the intersection between the starting slope and the linear phase of the kinetic. Therefore, we decided to use two parameters: *S* and *A* corresponding to the intersection of *S* with the y-axis (Fig. 1). The linear phase of the diene production results only from the propagation Reaction 4, whereas the first phase dienes could be the sum of both initiation Reaction 2 and propagation Reaction 4.

$$ROOH + Fe(II) \longrightarrow RO^{\bullet} + Fe(III) + OH^{-} \quad [1]$$

$$RO^{\bullet} + R'H \longrightarrow ROH + R'^{\bullet} \quad [2]$$

$$R'^{\bullet} + O_2 \longrightarrow R'OO^{\bullet} \quad [3]$$

$$R'OO^{\bullet} + R''H \longrightarrow R'OOH + R'''^{\bullet} \quad [4]$$

The global effect of antioxidants on the diene production can be observed in Figure 1. Antioxidants decrease the total amount of the dienes and *S*. The inhibition depends on the antioxidant concentration. The curves representing the percentage of inhibition on *A* and on *S* show that *A* and *S* are not affected to the same degree by all the antioxidants. Figures 2 and 3 illustrate two cases of antioxidant behavior. In the first case (Fig. 2), the percentages of *A* inhibition and of *S* inhibition increase as a function of antioxidant concentration in a pseudo-exponential manner. Most of the tested antioxidants belong to this case: BHA, Trolox, isoeugenol, eugenol, rosmarinic acid, ferulic acid, *p*-coumaric acid, naringenin, eriodictyol, isoquercitrin, catechin, and epicatechin. In the second case (Fig. 3), the curve presents a sigmoidal form with a concentration threshold. Quercetin and α-tocopherol have this behavior. BHT and caffeic acid present the first behavior (Fig. 2) for *A* and the second behavior (Fig. 3) for *S*; on the

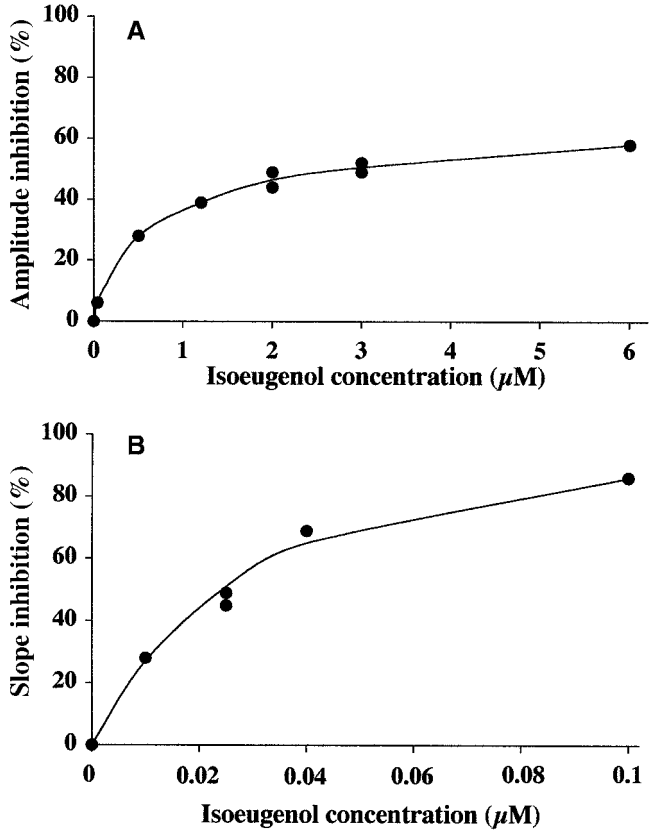

FIG. 2. Percentage of inhibition on amplitude (A) and on slope (B) as a function of isoeugenol concentration.

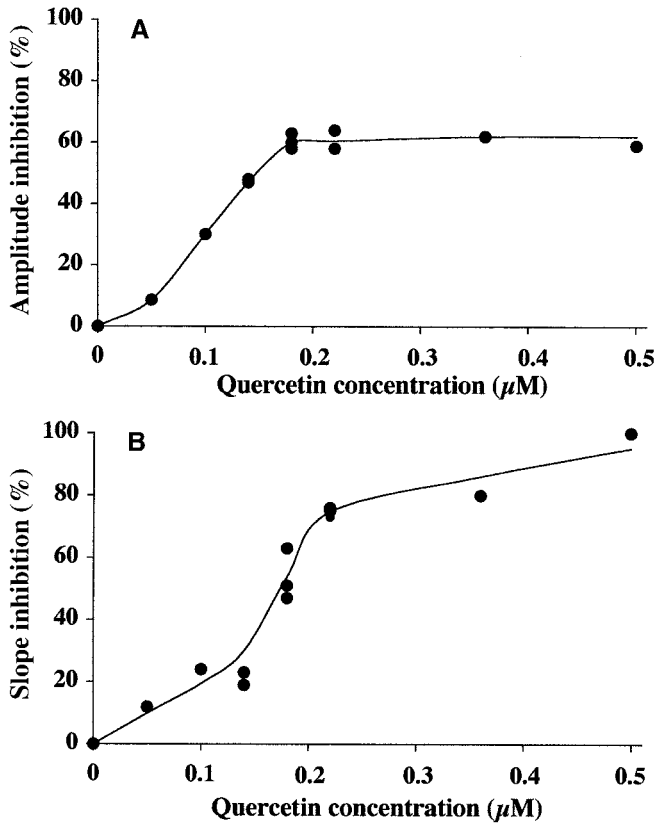

FIG. 3. Percentage of inhibition on amplitude (A) and on slope (B) as a function of quercetin concentration.

contrary, guaiacol presents the second behavior for *A* and the first behavior for *S*.

The threshold can be explained by a segregation phenomenon for α-tocopherol, BHT, and guaiacol, which are the most apolar (Table 1). In fact, they would be located inside the micelles, whereas the first radicals are produced at the interface as proposed by Fukuzawa *et al.* (7). However, this explanation is not valid for caffeic acid and quercetin, which are polar compounds.

TABLE 1
Apolarity Factor (AF), Inhibition Power on Amplitude (IPa), and Inhibition Power on Slope (IPs) for 17 Phenolic Compounds Tested in Iron/Ascorbate-Induced Oxidation

Antioxidants	AF	IPa (mM^{-1})	IPs (mM^{-1})
α-Tocopherol	92	17,000	22,000
Quercetin	19	6,800	5,800
Butylated hydroxytoluene	93	6,500	12,000
Butylated hydroxyanisole	84	3,600	39,000
Isoquercitrin	2	590	15,000
Eriodictyol	31	500	56,000
Isoeugenol	88	360	42,000
Rosmarinic acid	2	230	300
DL-Catechin	5	160	2,600
Eugenol	88	140	12,000
(–)-Epicatechin	5	130	2,000
Trolox	65	120	13,000
Caffeic acid	24	77	12
Naringenin	53	46	93
Guaiacol	90	9.1	170
Ferulic acid	64	5.3	400
p-Coumaric acid	47	1.8	9.6

All the tested antioxidants are able to reach 90–100% inhibition for *S*, and only 60–70% for *A*. This result explains why we calculated the antioxidant power at 50% inhibition level, and it suggests that antioxidants are unable to avoid the formation of 30–40% dienes, probably produced in the earliest phase of the oxidation. Indeed, Fe(II) disappears in a few minutes (8), and the inducing action of Fe(II) is so brief that antioxidants are probably not fast enough to react completely with Fe(II) or $RO^{\bullet}$ (Reaction 1), and therefore not fast enough to avoid the first diene formation (Reaction 2). However, the antioxidants are able to completely block the propagation phase under contions where the substrates (oxygen and linoleic acid) are not limiting (8). Therefore, we assume that the system will probably reveal the efficiency of antioxidants against radicals $ROO^{\bullet}$ rather than against radicals $RO^{\bullet}$.

Inhibition power. The IPa and the IPs are reported in Table 1. The compounds are ranked by decreasing IPa. First, we can note the large range of the results, from 1.8 to 17,000 mM^{-1} for IPa and from 10 to 56,000 mM^{-1} for IPs, which reveals a great difference in efficacy between antioxidants. Second, the values of IPs are globally higher than the values of IPa; their averages are, respectively, 13,000 and 2,100 mM^{-1}. With the exception of quercetin and caffeic acid, IPs is always

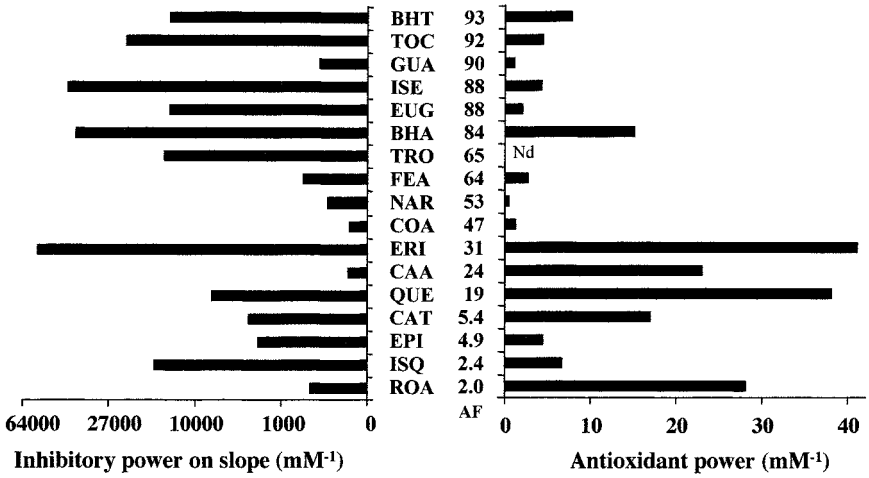

FIG. 4. Comparison between the inhibition power on slope measured in emulsified medium and the antioxidant power measured in bulk lipid medium. Abbreviations: BHT, butylated hydroxytoluene; TOC, α-tocopherol; GUA, guaiacol; ISE, isoeugenol; EUG, eugenol; BHA, butylated hydroxyanisole; TRO, Trolox; FEA, ferulic acid; NAR, naringenin; COA, *p*-coumaric acid; ERI, eriodictyol; CAA, caffeic acid; QUE, quercetin; CAT, DL-catechin; EPI, (–)-epicatechin; ISQ, isoquercitrin; ROA, rosmarinic acid; AF, apolarity factor.

higher than IPa. This result corroborates the fact discussed above about the incapacity of antioxidants to completely avoid first diene production. Third, the ranks according to IPa and to IPs are quite near. Eight compounds are really efficient on *S* and four of them are also very efficient on *A*. The compounds which have a low Ipa have also a negligible effect on *S*.

Nevertheless, we note a troubling fact: α-tocopherol, BHT, and BHA are among the most efficient antioxidants although they have no chelator site and only one radical scavenging phenolic group. Therefore, structure alone cannot predict the antioxidant's behavior. It is necessary to consider the polarity of the molecules since the medium is partitioned.

Polar paradox. We can observe on Table 1 that in a very global manner, with the exception of quercetin and isoquercitrin, the most potent antioxidants are apolar, which confirms the thesis of polar paradox (4). The case of α-tocopherol and Trolox is a good example. Trolox is the polar form of α-tocopherol without a phytyl chain and its apolarity factor is considerably reduced. Its efficiency decreased both on *A* and on *S*. In Figure 4, the compounds are ranked according to their AF. Their IPs is compared with their antioxidant power (AOP), which was measured in an apolar model previously developped in our lab (9–11). This apolar model involves the accelerated oxidation of methyl linoleate in dodecane under strong oxygenation and high temperature (110°C). The global comparison reveals two opposing histograms, which illustrates the polar paradox. According to the hypothesis of Frankel (3), the hydrophilic antioxidants (eriodictyol, caffeic acid, quercetin, catechin, and rosmarinic acid) are more active in a bulk lipid medium by being oriented in the air-oil interface, than the lipophilic antioxidants (BHT, α-tocopherol, isoeugenol, eugenol, and BHA), which remain in solution in the oil phase. In the present micellar system, the lipophilic antioxidants are located in the micelles, whereas the hydrophilic antioxidants remain in the water phase. However, some compounds (eriodictyol and isoquercitrin for IPs, and quercetin for IPa) do not follow this polar paradox rule. Therefore, the polarity may not be the only structural parameter to be taken into account to explain efficiency. Figure 5, by separating monophenols and *ortho*-diphenols, validates the polar paradox in a better manner for each class.

Structure–activity relationship. The number of possible chelator sites on antioxidant molecules may be related to their efficiency on amplitude because Fe(II) acts only during the first phase of the oxidation. In the flavonoid class, eriodictyol and isoquercitrin present two chelating sites, one on ring B and one between rings A and C. As expected, their IPa are higher than that of catechin, epicatechin, and naringenin which have only one site on rings B or C. In the same way, the high IPa of quercetin could be explained by a third possi-

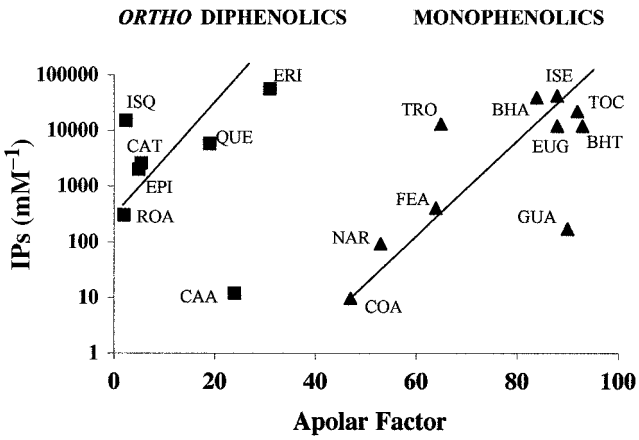

FIG. 5. Inhibition power on slope (IPs) as a function of apolarity factor for the tested phenolic compounds. For abbreviations, see Figure 4.

bility of chelating due to the OH on ring C. Chen and Ahn (12) mentioned that a third Cu^{2+} metal molecule can be chelated between two molecules of quercetin. Phenolic acids have low efficiencies, but we note that rosmarinic acid, with two chelating sites, is more potent than caffeic acid, which has only one site. Caffeic acid is reported as a weak chelator by Chen and Ahn (12). The same efficacy order, quercetin > catechin > caffeic acid > ferulic acid, was also obtained by Chen and Ahn (12) in lipid oxidation induced by Fe(II) using flax-oil emulsion at 37°C and measuring thiobarbituric acid-reactive substances after 30 min of incubation. Nevertheless, in the present study, among the four most efficient antioxidants on amplitude, only quercetin has chelating sites, while α-tocopherol, BHT, and BHA do not have any. This fact proves that this test, even if it is induced by metal, reveals essentially radical scavengers. Foti *et al.* (13) measured linoleic acid oxidation in aqueous micelles of sodium dodecyl sulfate at 50°C induced by a radical initiator [2,2′-azobis(2-amidinopropane) dihydrochloride], and found the range α-tocopherol > quercetin > catechin > naringenin > caffeic acid, which is the same as our finding.

The radical scavenging activity may be closely related to the efficiency on *S*. It is well known that orthodiphenols are more efficient than monophenols (10,14,15). In the present study, as well as in AOP test, naringenin with only one OH on ring B is the weakest of the tested flavonoids. However, it is surprising that eriodictyol and isoquercitrin were more efficient on *S* than quercetin and catechin, in spite of the absence of free OH on ring C. Although phenolic acids are weakly efficient, they proceed on the same logic, rosmarinic acid > caffeic acid > coumaric acid, corresponding respectively to 4 OH, 2 OH, and 1 OH.

Concerning the substitution on an aromatic ring, we note that isoeugenol > eugenol > guaiacol both for IPa and for IPs. All are methoxy-phenols with the same AF. This range is similar to that obtained for AOP and can be explained by the stability of the antioxidant radical, which is higher in the mesomeric group –CH=CH–CH_3 (case of isoeugenol) than in the inductive groups CH_2–CH=CH_2 (case of eugenol) or –H (case of guaiacol).

In conclusion, our results showed that, despite the presence of ferrous ions, the polar paradox is the strongest parameter affecting the efficiency of phenolic antioxidants in the dispersed medium. Thus, α-tocopherol, BHA, BHT, and isoeugenol, which have no chelator site, but are greatly apolar, are the most potent antioxidants against the diene formation. Conversely, rosmarinic acid and caffeic acid, two polar molecules, which are classically ranked among the best antioxidants (10), have poor activity despite one or two chelator sites. The superiority of the free radical scavenging effect with regard to chelating effect leads to a greater efficiency of the phenolics toward propagation than toward initiation.

Two antioxidants need to be studied in more detail. First, the behavior of quercetin is somewhat particular, its inhibition power presents a concentration threshold, and conversely to the others, its efficiency is weaker on *S* than on *A*. Second, eriodictyol does not follow the polar paradox rule as it is powerful both in the emulsified medium and in the apolar medium.

REFERENCES

1. Berset, C., and M.-E. Cuvelier, Methods of Estimating the Degree of Lipid Oxidation and of Measuring Antioxidizing Power, *Sci. Aliments 16*:219–245 (1996).
2. Frankel, E., In Search of Better Methods to Evaluate Natural Antioxidants and Oxidative Stability in Food Lipids, *Trends Food Sci. Technol. 4*:220–225 (1993).
3. Frankel, E., S.W. Huang, J. Kanner, and J.B. German, Interfacial Phenomena in the Evaluation of Antioxidants: Bulk Oils vs. Emulsions, *J. Agric. Food Chem. 42*:1054–1059 (1994).
4. Porter, W., E.D. Black, and A.M. Drolet, Use of Polyamide Oxidative Fluorescence Test on Lipid Emulsions: Contrast in Relative Effectiveness of Antioxidants in Bulk versus Dispersed Systems, *Ibid. 37*:615–624 (1989).
5. Kansci, G., C. Genot, A. Meynier, and G. Gandemer, The Antioxidant Activity of Carnosine and Its Consequences on the Volatile Profiles of Liposomes During Iron/Ascorbate Induced Phospholipid Oxidation, *Food Chem. 60*:165–175 (1997).
6. Aruoma, O., P. Evans, H. Kaur, L. Sutcliffe, and B. Halliwell, An Evaluation of the Antioxidant and Potential Pro-Oxidant Properties of Food Additives and of Trolox C, Vitamin E and Probucol, *Free Radical Res. Commun. 10*:143–167 (1990).
7. Fukuzawa, K., T. Seko, K. Mimami, and J. Terao, Dynamics of Iron-Ascorbate-Induced Lipid Peroxidation in Charged and Uncharged Phospholipid Vesicles, *Lipids 28*:497–503 (1993).
8. Bondet, V., M.-E. Cuvelier, and C. Berset, Behavior of Phenolic Antioxidants in a Partitioned Medium: Focus on Linoleic Acid Peroxidation Induced by Iron/Ascorbic Acid System, *J. Am. Oil Chem. Soc. 77*:813–818 (2000).
9. Cuvelier, M.-E., C. Berset, and H. Richard, Use of a New Test for Determining Comparative Antioxidative Activity of Butylated Hydroxyanisole, Butytated Hydroxytoluene, α- and γ-Tocopherols and Extracts from Rosemary and Sage, *Sci. Aliments 10*:797–806 (1990).
10. Cuvelier, M.-E., H. Richard, and C. Berset, Comparison of the Antioxidative Activity of Some Acid Phenols: Structure–Activity Relationship, *Biosci. Biotechnol. Biochem. 56*:324–325 (1992).
11. Peyrat-Maillard, M.-N., M.-E. Cuvelier, V. Bondet, A. Bocco, and C. Berset, Antioxidant Activity of Phenolic Compounds: Behavior in Different Model Systems and Synergistic Effects, in *Abstracts of the 89th AOCS Annual Meeting and Expo,* Chicago, 1998.
12. Chen, X., and D. Ahn, Antioxidant Activities of Six Natural Phenolics Against Lipid Oxidation Induced by Fe^{2+} or Ultraviolet Light*, J. Am. Oil Chem. Soc. 75*:1716–1721 (1998).
13. Foti, M., M. Piattelli, M. Tiziana Baratta, and G. Ruberto, Flavonoids, Coumarins, and Cinnamic Acids as Antioxidants in a Micellar System. Structure–Activity Relationship, *J. Agric. Food Chem. 44*:497–501 (1996).
14. Pokorny, J., Major Factors Affecting the Autoxidation of Lipids, in *Autoxidation of Unsaturated Lipids*, edited by H. Chan, Academic Press, London, 1987, pp. 141–206.
15. Pratt, D.E., and J.B.F. Hudson, Natural Antioxidants Not Exploited Commercially, in *Food Antioxidants*, edited by J. Hudson, Elsevier Applied Science, London, 1990, pp. 171–192.

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