Antioxidant Activity of Phenolic Compounds in 2,2′-Azobis (2-amidinopropane) Dihydrochloride (AAPH)-Induced Oxidation: Synergistic and Antagonistic Effects

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ABSTRACT: Interactions between phenolic antioxidants in binary systems were determined by adding two antioxidants simultaneously in equimolar proportions to an aqueous dispersion of linoleic acid that was then subjected to 2,2′-azobis (2-amidinopropane) dihydrochloride-induced oxidation and by evaluating the protective effect of the antioxidant mixture. The antioxidant power of the mixture was then compared with the expected antioxidant activity calculated by the sum of efficiencies of each compound separately, relative to their proportions in the mixture. If it was higher, a synergy was pointed out whereas a lower value was representative of an antagonism. Thus, synergistic effects were observed between rosmarinic acid and quercetin, or rosmarinic acid and caffeic acid, whereas antagonistic effects were obtained with the following mixtures: α-tocopherol/caffeic acid; α-tocopherol/rosmarinic acid; (+)-catechin/caffeic acid; and caffeic acid/quercetin. These mixture effects are partly explained by regeneration mechanisms between antioxidants, depending on the chemical structure of molecules and on the possible formation of stable intermolecular complexes.

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KEY WORDS: AAPH, antagonism, antioxidant, flavonoids, phenolic acids, synergism, α-tocopherol.

Plant phenolics are well known to protect food against lipid oxidation, which leads to the production of undesirable off-flavors (1). The antioxidant activity of these compounds has been studied largely in model systems. Although the results obtained by such accelerated oxidative tests are not easily extrapolated to the usual storage conditions of foodstuffs, such methods allow fast screening of pure antioxidants and plant extracts, and are suitable to determine the structure–activity relationships of molecules (2).

One of the current tests deals with the oxidizability of linoleic acid in an aqueous dispersion. The formation of conjugated diene hydroperoxides produced by a hydrophilic azo radical initiator, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) is followed at 234 nm (3). Indeed, azo compounds such as AAPH are able to generate free radicals through spontaneous decomposition at 37°C (reaction 1). The produced radicals $R^\bullet$ react immediately with oxygen (reaction 2) and cause the oxidation of lipids (reactions 3–5).

$$R{-}N{=}N{-}R \longrightarrow (1-e)R{-}R + 2e\,R^\bullet + N_2 \quad [1]$$

$$R^\bullet + O_2 \longrightarrow ROO^\bullet \quad [2]$$

$$ROO^\bullet + LH \longrightarrow ROOH + L^\bullet \quad [3]$$

$$L^\bullet + O_2 \longrightarrow LOO^\bullet \quad [4]$$

$$LOO^\bullet + LH \longrightarrow LOOH + L^\bullet \quad [5]$$

where R–N = N–R is the radical initiator, LH linoleic acid, $L^\bullet$ a linoleic radical, $LOO^\bullet$ a linoleic peroxy radical, and *e* the efficiency of free radical production.

Primary antioxidants (AOH) acting as hydrogen donors stabilize the free radicals and delay the oxidation of lipids (reactions 6, 7).

$$L^\bullet + AOH \longrightarrow LH + AO^\bullet \quad [6]$$

$$LOO^\bullet + AOH \longrightarrow LOOH + AO^\bullet \quad [7]$$

Although the antioxidant activity of some phenolics has already been described by the AAPH test (3), no systematic work on the structure–activity relationship by this method has been reported until now. Moreover, only a few studies have considered the possible interactions between phenolics, whereas a potent regeneration of an antioxidant by another one can increase or decrease the activity of a mixture of antioxidants. The present investigation was undertaken to confirm structure–activity relationships already pointed out by other measurement methods and to study the synergistic and antagonistic effects occurring between pairs of phenolic antioxidants in a mixture.

EXPERIMENTAL PROCEDURES

Reagents. Linoleic acid (purity >98%), Tween 20, AAPH, sodium borate, boric acid, and sodium hydroxide were obtained from Sigma-Aldrich (St Quentin Fallavier, France).

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Sodium dihydrogen phosphate dihydrate and sodium hydrogen phosphate dihydrate were purchased from Fisher Scientific (Elancourt, France). Water was purified with an Elix 3 system (Millipore, Milford, MA).

Caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, rosmarinic acid, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, quercetin, and rutin were obtained from Extrasynthèse (Genay, France). BHT, propyl gallate (PG), and α-tocopherol were from Sigma-Aldrich.

Antioxidant activity determination. The antioxidant activity of pure phenolic compounds and mixtures of phenolics was determined by measuring spectrophotometrically at 234 nm the formation of conjugated diene hydroperoxides produced by AAPH according to a method previously described by Liégeois *et al.* (3), with minor modifications.

(i) Procedure. Substrate solution (30 µL; 16 mM linoleic acid) and 10 µL of freshly prepared methanolic solution of antioxidant were added to 2.81 mL of 0.05 M phosphate buffer pH 7.4 previously thermostated at 37°C. Then 150 µL of AAPH solution (40 mM) was added, and the progress of oxidation was monitored by recording the absorbance increase at 234 nm vs. a blank cuvette containing the same mixture except without substrate solution. At least four different concentrations of antioxidants, prepared from three different solutions, were tested. The final concentrations in cuvettes varied from 0 to 1 µM for quercetin; 0 to 4 µM for rosmarinic acid; 0 to 5 µM for (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, rutin, caffeic acid, and chlorogenic acid; 0 to 10 µM for ferulic acid, BHT, PG, and α-tocopherol; and 0 to 30 µM for *p*-coumaric acid.

(ii) Expression of the results. The antioxidant power (AOP) is defined as the slope of the curve representing the inhibition time of oxidation (T_{inh}) vs. the concentration of antioxidant (Fig. 1) and is expressed in min/µM. The higher this value, the stronger the antioxidant.

Determination of the mixture effect (ME). The ME is defined by comparing the real T_{inh} of a mixture of compounds (noted "experimental T_{inh}") with the expected T_{inh}, calculated by the sum of efficiencies of each compound separately, relative to their proportions in the mixture (noted "calculated T_{inh}"). It was expressed as follows:

$$\text{ME} = \text{experimental } T_{inh} / \text{calculated } T_{inh} \quad [8]$$

A value >1 defines a synergistic effect between the implicated antioxidants, whereas a value <1 corresponds to an antagonism. A value =1 means neither a synergistic nor antagonistic effect. Data are the mean of at least three replications.

Equimolar concentrations of both antioxidants were tested. The value was fixed as the efficient concentration of the more active antioxidant that is necessary to obtain a T_{inh} of 50 min.

RESULTS AND DISCUSSION

Assessment of the antioxidant activity of phenolic compounds in the AAPH test. The antioxidant activity of five phenolic acids, five flavonoids, and α-tocopherol was determined and compared with the activity of two synthetic antioxidants, BHT and PG (Table 1).

Although *p*-coumaric acid had no activity in this reaction medium, the other natural phenolic compounds showed good antioxidant properties. Except for ferulic acid, they were all more efficient than the synthetic molecules.

The absence of activity of *p*-coumaric acid is probably due to the limit of detection of the oxidation products by spectrophotometry. In fact, this hydroxycinnamic acid is always described as a poor antioxidant (4–6).

As already shown, the efficiency increases with the number of OH groups or the presence of a methoxy group (3–8). It is well known that a catechol group enhances the radical-scavenging activity of the molecule owing to internal resonance stabilization or *o*-quinone formation, as in the case of caffeic, rosmarinic, and chlorogenic acids. On the other hand, *ortho* substitution with the electron donor methoxy group increases the stability of the produced radical (e.g., ferulic acid).

The esterification of caffeic acid by quinic acid leading to chlorogenic acid does not influence the AOP, whereas glycosylation of quercetin in rutin clearly decreases its efficiency. There is also a stereochemical effect of phenolic compounds and particularly of the stereoposition of the OH group in C_3, with (−)-epicatechin more active than (+)-catechin, as already mentioned by Saint-Cricq de Gaulejac *et al.* (9) and Maillard *et al.* (10).

ME between phenolic compounds. To evaluate the interactions occurring between molecules, some of the previously tested phenolic compounds were added at the same time and in equimolar proportions to the aqueous dispersion of linoleic acid.

TABLE 1
Antioxidant Power (AOP) of Phenolic Compounds Determined by the 2,2′-Azobis (2-Amidinopropane) Dihydrochloride (AAPH) Test

	AOP	
Phenolic compound	min/µM	R^{2a}
Phenolic acids		
p-Coumaric acid	0	
Ferulic acid	16	0.9528
Caffeic acid	40	0.9862
Rosmarinic acid	73	0.9976
Chlorogenic acid	40	0.9968
Flavonoids		
(+)-Catechin	33	0.9831
(−)-Epicatechin	41	0.9991
(−)-Epigallocatechin	44	0.9976
Quercetin	55	0.9873
Rutin	41	0.9939
Others		
BHT	17	0.9914
Propyl gallate (PG)	19	0.9998
α-Tocopherol	17	0.9687

[a]Correlation coefficient of the slope of the curve representing the inhibition time vs. the concentration of antioxidant.

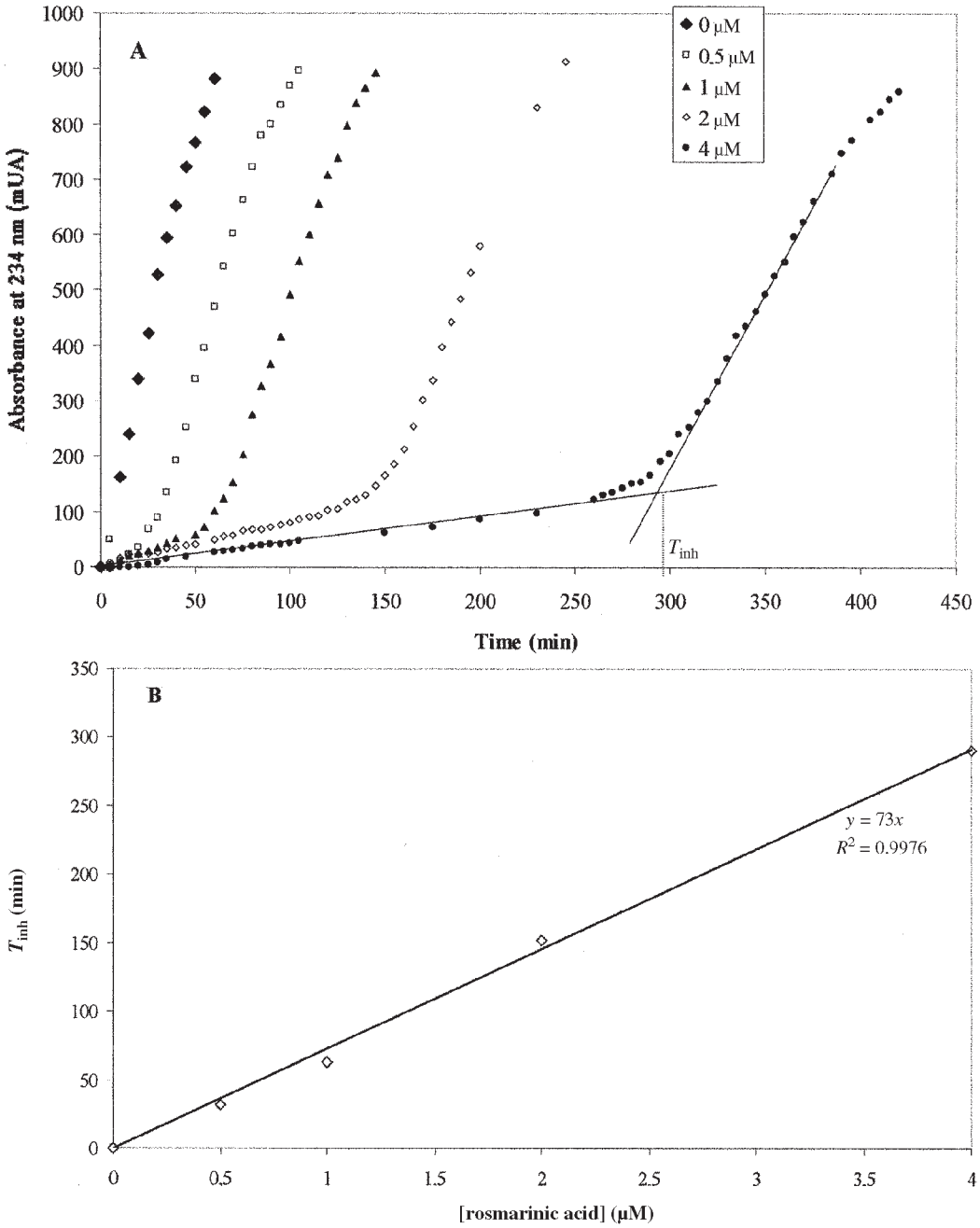

FIG. 1. Effect of rosmarinic acid on 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced linoleic acid oxidation. (A) Kinetics of hydroperoxide production; (B) inhibition time of oxidation (T_{inh}) vs. antioxidant concentration. mUA, milli absorbance unit.

Many phenomena are probably involved in these interactions. We have considered the case in which ME are explained by the regeneration of one antioxidant (e.g., A_1OH) by another (e.g., A_2OH). Three different results can be obtained: (i) a synergistic effect if the less efficient antioxidant regenerates the more efficient one, (ii) an antagonistic effect if the more efficient molecule regenerates the less efficient one, or (iii) no ME if both antioxidants have the same efficiency. Thus, in such binary systems, the following model can be established: A_2OH can react either by giving hydrogen to a lipidic radical (alkyl $L^\bullet$ or peroxy $LOO^\bullet$) (reactions 6, 7) or by regenerating A_1OH (reaction 9), as shown in the following reaction:

$$A_2OH + A_1O^\bullet \longrightarrow A_2O^\bullet + A_1OH \qquad [9]$$

In our study, because both antioxidants have been introduced in the reaction medium in equimolar proportions, we have considered that a 1:1 stoichiometry occurred between them, 1 mol of an antioxidant regenerating 1 mol of another one. Thus, we have determined which part of antioxidant A_2OH regenerated A_1OH (named "fraction X"), and which

TABLE 2
Mixture Effect[a] Between Two Phenolic Compounds in Equimolar Proportions on the Protection of Linoleic Acid from Oxidation[b]

	Caffeic acid				
Ferulic acid	0.99 ± 0.02	Ferulic acid			
Rosmarinic acid	1.07 ± 0.04	1.01 ± 0.00	Rosmarinic acid		
(+)-Catechin	0.82 ± 0.07	0.98 ± 0.06	0.98 ± 0.03	(+)-Catechin	
Quercetin	0.94 ± 0.03	1.01 ± 0.03	1.13 ± 0.03	0.95 ± 0.17	Quercetin
α-Tocopherol	0.81 ± 0.00	1.10 ± 0.01	0.93 ± 0.05	0.99 ± 0.07	1.00 ± 0.05

[a]A value >1 corresponds to a synergistic effect, and a value <1 corresponds to an antagonistic effect.
[b]SD are calculated from at least three values.

part of A_2OH had an antiradical action on lipids (named "fraction [1 − X]"). If X = 0, the totality of A_2OH reacts with lipids and no ME is observed. If X = 1, A_2OH reacts only as a regenerator of A_1OH and only A_1OH protects lipids from oxidation. The T_{inh} that is obtained experimentally is thus expressed as follows :

$$\text{experimental } T_{inh} = T_{inh_1} + X \cdot T_{inh_1} + (1-X) \cdot T_{inh_2} \qquad [10]$$

where T_{inh_1} corresponds to the protection of lipids by A_1OH; T_{inh_2} is the corresponding inhibition time of A_2OH introduced in the reaction medium at the beginning of the experiment; $X \cdot T_{inh_1}$ is the inhibition time corresponding to the protection of lipids by a fraction of A_1OH, which has previously been regenerated by A_2OH; and $(1 - X) \cdot T_{inh_2}$ is the inhibition time corresponding to the protection of lipids by A_2OH.

From Equation 10, we can deduce the X part of antioxidant A_2OH that reacts as a regenerator of A_1OH.

Behavior of the α-tocopherol in mixture. Significant ME were found between α-tocopherol and cinnamic acids (Table 2). They were negative with caffeic acid (ME = 0.81) and rosmarinic acid (ME = 0.93), showing that α-tocopherol with low AOP would be regenerated by the more powerful phenolic acid. Indeed, because caffeic and rosmarinic acids are more efficient than α-tocopherol, this antagonistic effect reveals that a part of them is used to regenerate α-tocopherol. It was surprising to obtain a higher antagonism with caffeic acid than with rosmarinic acid, which has four phenolic groups instead of two. Only 11% of α-tocopherol would be regenerated by rosmarinic acid against 47% for caffeic acid (Table 3). Therefore, rosmarinic acid probably acts as a free radical scavenger of lipidic radicals rather than of phenolic radicals. The reaction rate of each antioxidant with lipidic radicals and phenolic radicals should also be considered to better understand this behavior.

The synergistic effect observed between α-tocopherol and ferulic acid cannot be explained by a regeneration phenomenon, for both molecules have a similar activity.

The regeneration of α-tocopherol by plant phenolics, such as hydroxycinnamic acids from malt rootlets (11) or rosmarinic acid from Moldavian dragonhead (12) often has been pointed out. However, we did not confirm in this study the ME between α-tocopherol and flavonoids as already described (13–15).

Behavior of flavonoids in mixture. ME were shown between flavonoids and some phenolic acids: A synergistic effect was observed between quercetin and rosmarinic acid (ME = 1.13), whereas an antagonism was pointed out between quercetin or (+)-catechin and caffeic acid (ME = 0.94 and 0.82, respectively). No ME was observed between flavonoids and ferulic acid. A slight antagonism between quercetin and (+)-catechin was also shown but was not statistically significant.

The quercetin/rosmarinic acid synergy could be explained by the regeneration of the more efficient molecule, rosmarinic acid (AOP = 73 min/μM), by the less active one, quercetin (AOP = 55 min/μM). Such a regeneration of OH groups of phenolic acids by quercetin could also explain the slight antagonism observed between quercetin and caffeic acid, the latter having a smaller AOP (=40 min/μM). Stable intermolecular complexes between flavonols and cinnamic acids could be formed (Fig. 2), as has already been suggested with anthocyanin and caffeic acid or rutin in the co-pigmentation mechanism (16). These interactions could be due to π-π stacking between the aromatic ring of phenolic acid and the B-ring of flavonol, but hydrogen-bonding effects would also help to

TABLE 3
Importance of Regeneration Mechanisms in the Antioxidant Activity of Phenolic Antioxidants

Antioxidant combinations A_1OH^a/A_2OH^b	Mixture effect	X[c] (%)
α-Tocopherol/rosmarinic acid	Antagonism	11
α-Tocopherol/caffeic acid	Antagonism	47
Rosmarinic acid/caffeic acid	Synergy	24
Rosmarinic acid/quercetin	Synergy	92
Caffeic acid/quercetin	Antagonism	38
(+)-Catechin/caffeic acid	Antagonism	188

[a]A_1OH is the antioxidant that acts only as a hydrogen donor to lipids.
[b]A_2OH is the antioxidant that acts as a hydrogen donor to lipids and as a regenerator of A_1OH.
[c]X is the part of A_2OH that regenerates A_1OH.

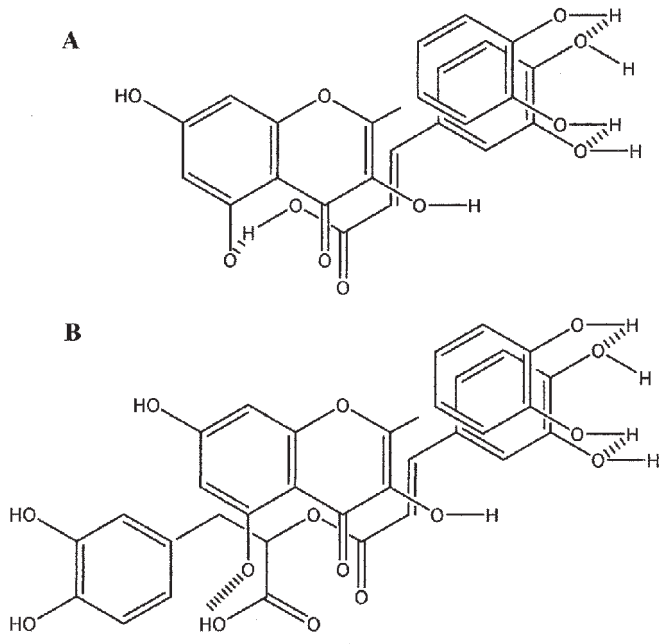

FIG. 2. Possible interactions occurring in quercetin/caffeic acid complex (A) and quercetin/rosmarinic acid complex (B).

stabilize the complex (17). A higher stability of the complex quercetin/rosmarinic acid, due to better structural analogy and additional bondings between the two molecules, could partially explain why 92% of quercetin regenerates rosmarinic acid whereas only 38% does so with caffeic acid (Table 3).

In the case of a mixture of (+)-catechin/caffeic acid, the great antagonistic effect (ME = 0.82) is apparently in contradiction to the absence of an interaction of (+)-catechin with the rosmarinic acid. Moreover, the regeneration model that is proposed on the basis of a 1:1 stoichiometry is not validated here because the X value is greater than 100% (Table 3). The stoichiometry of complexes should be investigated further, considering, for example, that a stoichiometry of 4:1 was obtained between caffeic acid and the stable radical 2,2-diphenyl-1-picryl hydrazyl (DPPH$^{\bullet}$) (6). Moreover, other phenomena are probably involved in these interactions.

Behavior of phenolic acids in mixture. As previously described, caffeic and rosmarinic acids could regenerate α-tocopherol and could be regenerated by quercetin (Table 2). No ME was observed between ferulic acid and other phenolic acids. The slight synergy observed between caffeic acid and rosmarinic acid seems to indicate that caffeic acid would have more affinity for lipidic radicals than for phenolic radicals, as only 24% is used for the regeneration mechanism (Table 3).

We found that synergistic and antagonistic effects occurring between pairs of antioxidants during the oxidation of linoleic acid in an aqueous dispersed system can be partly explained by regeneration mechanisms, depending on the chemical structure of molecules and on the possible formation of stable intermolecular complexes. However, many other phenomena are probably also involved, among them the polarity of molecules, the reaction rates of antioxidants with lipids, and the influence of the microenvironment, particularly the effective concentration of the antioxidant at the site of oxidation (5,18,19).

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