

## A Quantitative Approach to the Free Radical Interaction Between Alpha-tocopherol or Ascorbate and Flavonoids

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**Abstract.** Despite numerous previous studies, the mechanism of the free radical interaction between alpha-tocopherol (VE), or ascorbate and flavonoids, as coantioxidants remains unclear. The synergistic antioxidant effects of VE or L-ascorbyl 2,6-dibutyrate (ASDB, an ascorbate derivative) with the flavonoids (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG) and methyl gallate (MG), were investigated by the induction period method in the polymerization of methyl methacrylate (MMA), initiated by thermal decomposition of 2,2'-azobis(isobutyronitrile) (an alkyl radical, R •), under nearly anaerobic conditions. For VE, a synergistic antioxidant effect was observed with MG, EC, EGC and ECG, whereas this activity was decreased by the addition of EGCG. For ASDB, a synergistic antioxidant effect was observed with EGC and ECG, whereas this activity was decreased by the addition of EGCG or MG. A synergistic antioxidant effect (regeneration of VE) appears to be feasible even though the BDE (phenolic O-H bond dissociation entropy) of the coantioxidants is significantly higher than that of VE. The driving force for the regeneration process may be the removal of the semiquinone radical from the flavonoids MG, EC, EGC and ECG by the VE radical. In the ASDB/flavonoid mixture, flavonoid radicals are scavenged by ASDB. The partial regeneration of flavonoids by ASDB may follow a similar recycling mechanism to that of the well-known VE/ascorbate mixture. The free radical interaction between EGCG and VE or ASDB decreased the antioxidant effect. Such enhancement of prooxidation in EGCG/VE or EGCG/ASDB mixtures oxidized by R • may increase their cytotoxic effects.

Dietary polyphenols, mainly flavonoids, in fruits, vegetables, wines, spices and herbal medicines, possess beneficial antioxidant, anti-inflammatory and anticancer effects.

Recently, the synergistic antioxidant mechanism of alpha-tocopherol (VE) with green tea polyphenols (EC, EGC, ECG, EGCG) and gallic acid was reported, indicating that these polyphenols could reduce the alpha-tocopheroxyl radical to regenerate alpha-tocopherol (1-3). The kinetics of the regeneration of VE by a catechol derivative have been reported (4). Furthermore, the combination of flavonoids and vitamin C was previously reported to produce a synergistic antioxidant effect in an *in vitro* lipoprotein oxidation model (5). Similarly, ascorbate protects (+)-catechin from oxidation under cell-free conditions (6). However, the mechanism of the synergistic antioxidant activity of VE with coantioxidants, such as the flavonoids, has not yet been fully elucidated, since the efficiency of regeneration was found to be dependent not only on the structure of the coantioxidant, but also on its relative quantity and on the microenvironment of the reaction medium (7). For maximum biological relevance, studies on the synergistic antioxidant activity of VE or ascorbate with coantioxidants such as the polyhydroxylated flavonoids should be performed under anaerobic conditions, because biological systems have a low oxygen tension. Although VE possesses potent antioxidant activity *in vitro*, a recent review of clinical studies concluded that there was little evidence that supplementation with VE reduced the risk of cancer (8). Thus, it was of interest to investigate the prooxidant/antioxidant activity of flavonoids with VE or ascorbates at low oxygen tensions.

Ischemia/reperfusion is a frequently encountered phenomenon. Prolonged ischemia followed by reperfusion results in severe oxidative injury to tissues and organs. Carbon-centered free radicals can be involved in damage under hypoxic/anoxic conditions as well as in ischemia/reperfusion injury. We used 2,2'-azobisisobutyronitrile (AIBN) as a source of carbon-centered radicals under nearly

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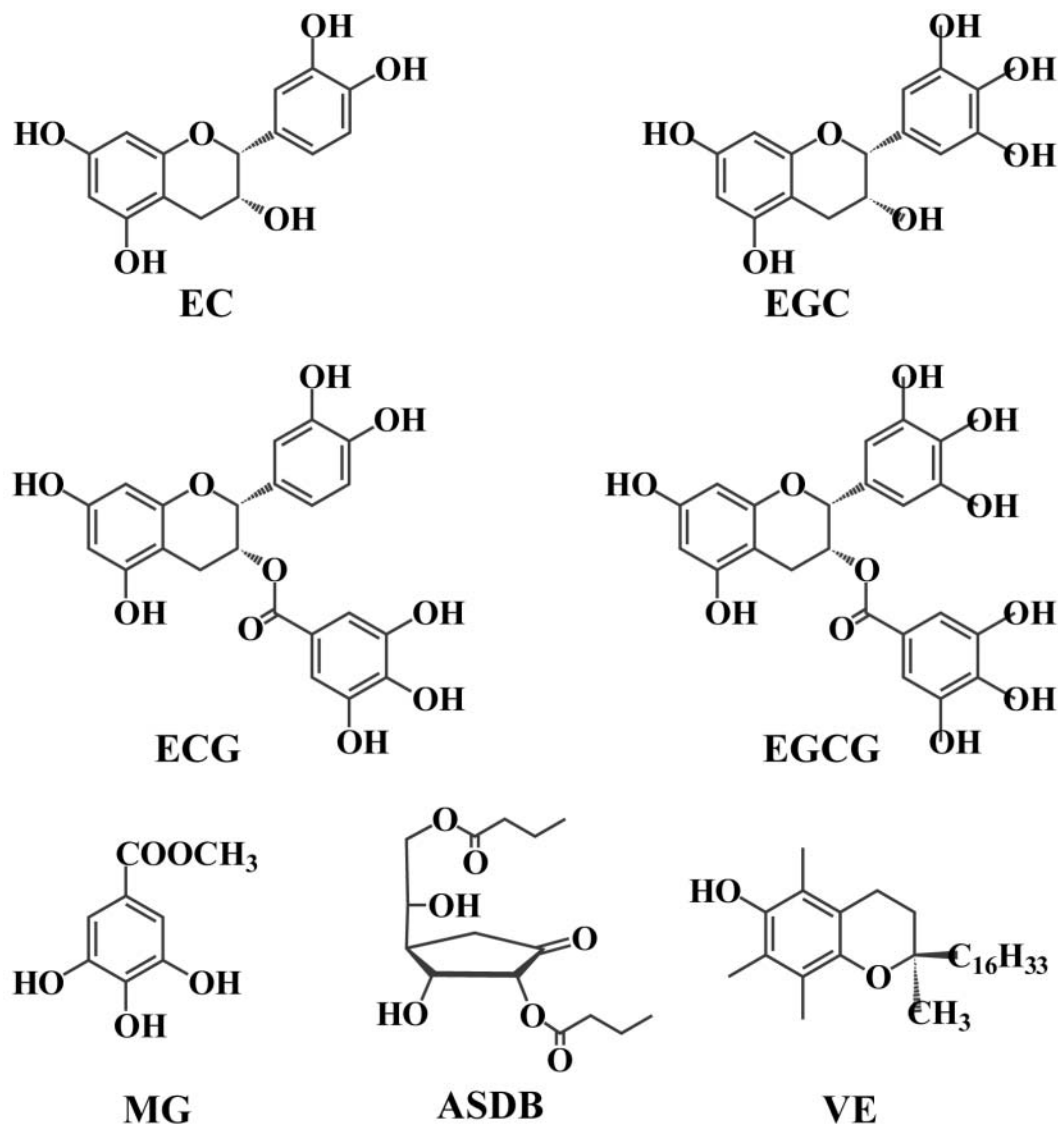


Figure 1. Chemical structures of the investigated antioxidants.

anaerobic conditions (9, 10). There is little information on the interaction between flavonoids and carbon-centered radicals.

With this background, we performed a systematic investigation of the free radical interaction between VE or *L*-ascorbyl 2,6-dibutyrate (ASDB), a vitamin C derivative, and the flavonoid coantioxidants (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG) and methyl gallate (MG) in order to clarify the synergistic behavior of combinations of these antioxidants. The kinetic radical-scavenging activities of mixtures of VE and coantioxidants were investigated by the induction period method in the polymerization of methyl methacrylate (MMA) initiated by the thermal decomposition of AIBN. The reaction was

monitored by the sensitive method of differential scanning calorimetry (DSC), the model being well able to explain the mechanism of the radical-scavenging activities of these antioxidants. ASDB was used as a representative ascorbate derivative instead of vitamin C, which has only limited solubility in MMA.

## Materials and Methods

The following chemicals and reagents were obtained from the indicated companies: EC, ECG, EGC and EGCG (Kurita Kogyo Co., Japan) and MG (Wako Pure Chemical Industries, Ltd., Osaka, Japan), AIBN was obtained from Wako Pure Chemical Industries, Ltd., and was recrystallized from methanol before use. The chemical structures of the investigated antioxidants are shown in Figure 1.

Table I. Bond-dissociation enthalpy (BDE), ionization potential (IP), electron affinity (EA), stoichiometric factors ( $n$ ), the ratio of inhibition rate /propagation rate ( $k_{inh}/k_p$ ) and anti-DPPH radical activity for phenolic antioxidants, VE, MG, EC, EGC, EGCG, and ASDB, a vitamin C derivative.

Chemicals	BDE (kcal/mol)	IP (eV)	EA (eV)	$n$	$k_{inh}/k_p$	DPPH IC <sub>50</sub> (mM)
VE	72.737	8.449	-0.211	1.70	15.1	0.014
MG	81.713	9.264	0.538	1.43	16.2	0.007
EC	80.955	8.913	-0.079	2.39	10.0	0.013
EGC	80.720	8.938	-0.066	2.11	12.1	0.010
ECG	88.034	8.899	0.464	3.85	7.0	0.005
EGCG	80.910	8.938	0.566	3.95	6.5	0.004
ASDB	74.543	9.490	0.260			

BDE, IP and EA were calculated by the PM3 method. The  $n$  and  $k_{inh}/k_p$  value were determined by the induction period method (9.4 mol/l MMA, 1.0 mol% AIBN, 0.01 mol% antioxidant). Anti-DPPH radical activity was calculated as the concentration (mol/l) of antioxidant necessary to decrease the initial DPPH radical concentration by 50%.

**Experimental procedures.** The induction period (IP) and initial rates of polymerization in the presence ( $R_{p_{inh}}$ ) or absence ( $R_{p_{con}}$ ) of an antioxidant were determined by the method previously reported. In brief, the experimental resin consisted of MMA and AIBN in the presence of 0.01 mol% VE, MG, EC, ECG, EGC, EGCG or ASDB and mixtures of VE or ASDB with the coantioxidants MG, EC, ECG, EGC and EGCG at a molar ratio of 1:1. AIBN was added at 1.0 mol%. Approximately 10  $\mu$ l of the experimental resin (MMA: 9.12-9.96 mg) was loaded into an aluminum sample container and sealed by applying pressure. The container was placed in a differential scanning calorimeter (model DSC 3100; MAC Science Co., Tokyo, Japan) kept at 70 °C, and the thermal changes induced by polymerization were recorded for the appropriate periods. The heat due to polymerization of MMA was 13.0 kcal/mole in this experiment. The conversion of all samples, as calculated from the DSC thermograms, was 91.2-95.0%. Polymerization curves were derived from the DSC thermograms using the integrated heat evolved by the polymerization of MMA. Time-conversion curves for ASDB, VE and the five flavonoids for the AIBN system are shown in Figure 2.

Polymerization curves break when an inhibitor is consumed. These breaks are sharp and provide a reliable measure of the IP of the inhibitor. The presence of oxygen retards polymerization, because oxygen reacts with MMA radicals activated by the initiator and subsequently produce a non-radical product. Thus, polymerization of the control was slightly inhibited, even though the reaction was carried out in a sealed DSC pan, because the pan contained a small amount of oxygen, having been sealed in air. Tangents were drawn to the polymerization curves at an early stage in the run. The IP of the test compounds was determined from the length of time between the zero point on the abscissa and the point of intersection of tangents drawn to the early stage of polymerization. The IP was calculated from the difference between the induction period of specimens and that of controls. The initial rates of polymerization in the absence ( $R_{p_{con}}$ ) and presence ( $R_{p_{inh}}$ ) of natural and synthetic antioxidants were calculated from the slope of the first linear portion of the plots of the conversion rate of MMA polymerization (tangent drawn at the early polymerization stage). The rate of initiation ( $R_i$ ) by AIBN was previously reported to be  $5.66 \times 10^{-6} \text{ Ms}^{-1}$  (11).

**Computational details.** Theoretical calculations of heats of formation, bond-dissociation energy (BDE), ionization potential and electron

affinity were carried out by the semi-empirical molecular orbital (MO) method PM3, as implemented in the MOPAC program (CaChe 5.0). In general, the PM3 method cannot give the fine geometry and unpaired electron distribution of a molecule (12). Therefore, after optimization by CONFLEX (Conflex Co., Japan), the geometry was followed by the PM3 method.

**Anti-DPPH radical activity.** The radical-scavenging activities were determined with 2,2'-diphenyl-1-picrylhydrazyl (DPPH) as a free radical. For each antioxidant, various concentrations were tested in ethanol. The decrease in absorbance was determined at 517 nm for 10 min at room temperature. The anti-DPPH radical activity was calculated as the concentration (mol/l) of antioxidant necessary to decrease the initial radical concentration (0.1 mM) by 50% (IC<sub>50</sub>).

## Results and Discussion

**VE, ASDB and flavonoids.** The radical-scavenging activity of flavonoid antioxidants for AIBN was determined from the findings shown in Figure 2, and the results are presented in Table I. The radical-scavenging activity of antioxidants is characterized by two independent parameters (11); one is the stoichiometric factor ( $n$ ) and the other is  $R_{p_{inh}}$ . When the initiating radicals are generated at a constant rate by AIBN, then  $n$ , IP and  $R_{p_{inh}}$  are given by:

$$n = ([IP] R_i) / [IH] \quad 1$$

$$R_{p_{inh}} = \{k_p[MMA]R_i\} / \{n k_{inh}[IH]\} \quad 2$$

where  $R_i$  is the rate of chain initiation and  $[IH]$  is an inhibitor.

Equations 1 and 2 can be combined to give Equation 3. The ratio of rate constants,  $k_{inh}/k_p$ , determines the ratio of the rate of inhibition to the rate of propagation (11):

$$k_{inh}/k_p = [MMA] / ([IP] R_{p_{inh}}) \quad 3$$

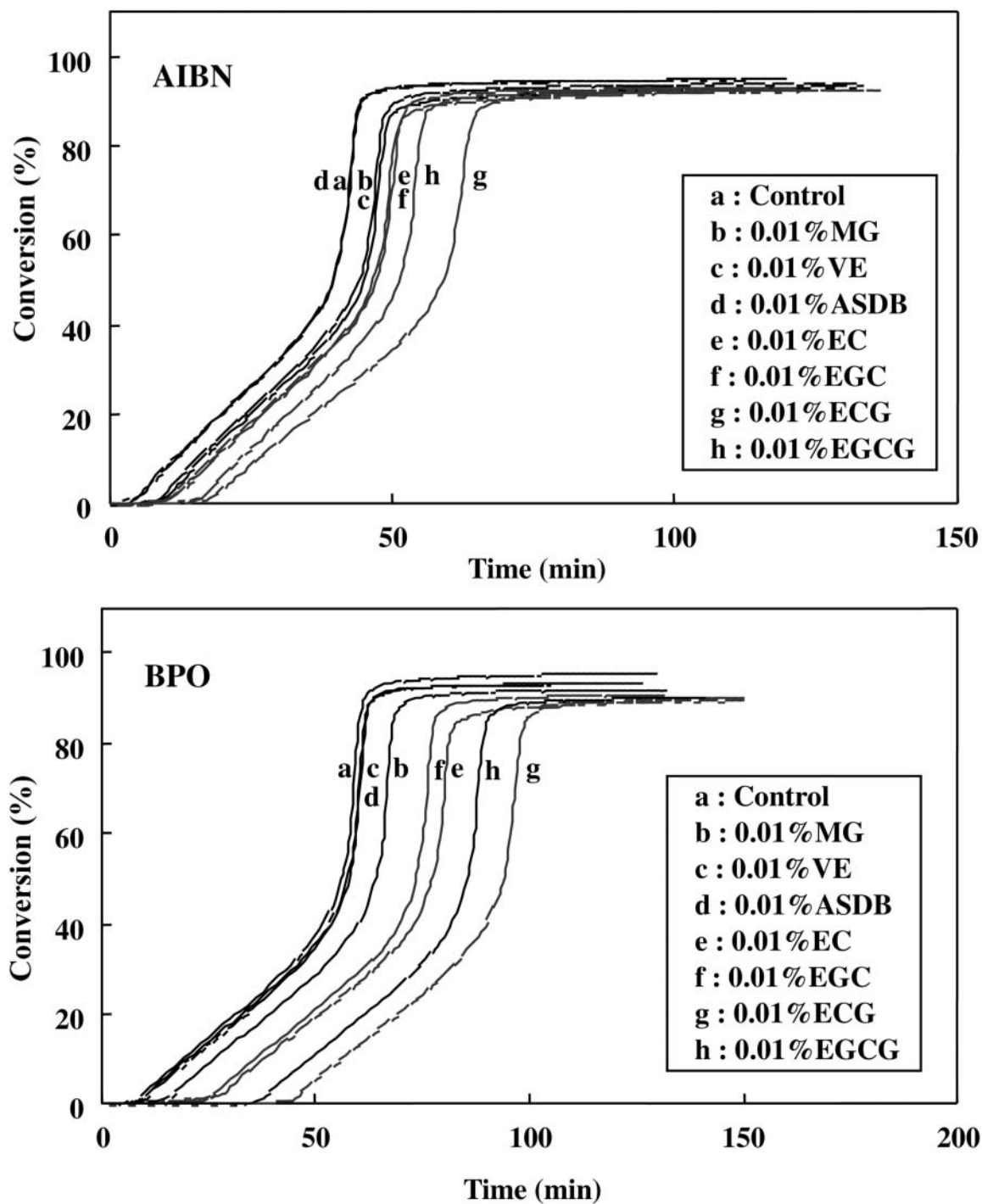


Figure 2. Time-conversion curves for flavonoids (EC, EGC, ECG, EGCG), related compound (MG), VE and ASDB. 9.4 mol/l MMA, 1.0 mol % AIBN, 0.01 mol% antioxidants, 70°C.

The  $n$  and  $k_{inh}/k_p$  values for phenolic antioxidants were calculated from the results in Table I by using Equations 1 and 3, respectively. The calculated  $n$  ( $k_{inh}/k_p$ ) values for the phenolic antioxidants VE, MG, EC, EGC, ECG and EGCG

were 1.70 (15.1), 1.43 (16.2), 2.39 (1.0), 2.11 (12.1), 3.85 (7.0), and 3.95 (6.5), respectively. The  $n$  values declined in the order EGCG>ECG>EGC>VE>MG. DPPH-radical scavenging activity declined in the order EGCG>ECG>

Table II. The free radical interaction between vitamin E (VE,  $\alpha$ -tocopherol) or L-ascorbyl 2,6-dibutyrate (ASDB, a vitamin C derivative) and coantioxidants; catechin (epicatechin, EC; epigallocatechin, EGC; epicatechin gallate, ECG; and epigallocatechin gallate, EGCG) and the related compound (methyl gallate MG) at a molar ratio of 1:1.

System	Inhibitor 0.01 mol%	Observed	Calculated		Rp <sub>inh</sub> / Rp <sub>con</sub>
		IP (min) A	IP* (min) B	B-A	
AIBN	VE	5.00			1.00
	ASDB	0.51			1.00
	MG	4.24			1.10
	EC	7.03			1.08
	EGC	6.22			1.00
	ECG	11.35			0.96
	EGCG	11.57			0.96
	VE+MG	10.17	9.24	-0.93	1.02
	VE+EC	13.18	12.03	-1.15	1.02
	VE+EGC	13.07	11.22	-1.85	0.94
	VE+ECG	16.95	16.57	-0.38	0.94
	VE+EGCG	16.07	16.57	0.50	0.96
	ASDB+MG	3.16	9.24	6.08	1.01
	ASDB+EC	7.05	7.54	0.49	0.94
	ASDB+EGC	7.27	6.73	-0.54	0.90
	ASDB+ECG	13.57	11.86	-1.71	0.93
	ASDB+EGCG	10.58	12.08	1.50	0.90

IP, induction period; Rp<sub>inh</sub> and Rp<sub>con</sub> are the initial rate of polymerization with and without an inhibitor, respectively; AIBN, 1.0 mol%; MMA, 9.4 mol/l; \*simple sum of IP for VE or ASDB and coantioxidant. The IP of controls was 3.28 min. The values are the means of three independent experiments. The computational error was <7%. The procedures are described in the text.

G>EGC>EC>VE (Table I). EGCG showed the highest radical-scavenging activity, as judged by the  $n$  and IC<sub>50</sub> values, as a result of the presence of two antioxidant sites (the two gallate moieties) in this molecule. The k<sub>inh</sub>/k<sub>p</sub> value for VE was two-fold greater than that for the other antioxidants except MG. The k<sub>inh</sub>/k<sub>p</sub> value for VE was identical to that of MG.

*Mixtures of VE with flavonoids.* The observed IPs and calculated IPs for mixtures of VE and the polyphenolic coantioxidants are shown in Table II. Except for EGCG, the observed IPs for mixtures of VE and flavonoids were greater than the corresponding calculated IPs, suggesting a synergistic reaction between VE and these flavonoids in which VE was partially regenerated by the coantioxidant. EGC showed the highest coantioxidant activity. In contrast, the observed IP for EGCG was less than the calculated value, suggesting that the antioxidant activity of EGCG can be suppressed by the addition of VE. To obtain information about the mechanism of these interactions, PM3 calculations were used to investigate energy level differences between the

highest occupied molecular orbital (HOMO) and the lowest occupied molecular orbital (LUMO) of AIBN, MMA, EGCG and VE. Previous studies suggested that a coantioxidant may be effective in regenerating VE when the O-H bond dissociation enthalpy (BDE) of the coantioxidant is lower than, or at least similar to, that of the antioxidant (VE) itself (4). The calculated BDE for EGCG, EGC and EC was approx. 81 kcal/mol, whereas for ECG it was approx. 88 kcal/mol. The BDE for VE was 74.5 kcal/mol. The BDE for the flavonoids was not close to that of VE, suggesting that other factors provide the driving force for the regeneration reaction. Thus, we investigated energy level differences between the HOMO and the LUMO. Possible reaction pathways for EGCG and MMA reacting with cyanoisopropyl radicals (R•) derived from AIBN, and for VE and EGCG reacting with R•, are proposed in Figure 3. The most favorable reaction occurred between R• and VE rather than with EGCG. VE phenoxyl radicals would be scavenged by EGCG in the AIBN system, and polymerization of MMA would occur after VE and EGCG had been completely consumed. The reaction pathways for MG, EC, EGC and ECG were similar to that for EGCG (data not shown). This proposed pathway is supported by previous evidence that the reduction potentials of the flavonoid radicals, E<sub>7</sub>=0.5-0.7V, are higher than that of trolox, E<sub>7</sub>=0.48V, indicating that their reaction with VE is thermodynamically feasible (13).

As shown in Table I, the  $n$  value of 1.7 for VE indicated that it is a good antioxidant against R•, because the  $n$  value of fully oxidized VE should be 2. The  $n$  value of VE in the mixtures with flavonoids ( $n_{VE}$ ) can be calculated from Equation 4:

$$n_{VE} = R_i (IP_{VE+flavonoid} - IP_{flavonoid})/[VE] \quad 4$$

The calculated  $n_{VE}$  values for MG, EC, EGC and ECG were 2.0, 2.1, 2.3, and 1.9, respectively, suggesting that VE in the presence of flavonoids scavenged much more R• than did VE alone (control value of 1.7). In contrast, the calculated  $n_{VE}$  for EGCG was 1.5, much less than that of VE alone, suggesting that VE phenoxyl radicals reduce the antioxidative activity of EGCG. EGCG has the highest electron affinity (0.57eV) among the investigated flavonoids (Table I) and this may cause the strong interaction with VE phenoxyl radicals. A previous study by stopped-flow spectrophotometry of the reaction rates (k(r)) of the 5,7-diisopropyl-tocopheroxyl radical (Toc) with the catechins (EC, ECG, EGC, EGCG) and the related compound (MG) indicated that the k(r) values increased in the order MG<EC<ECG<EGC<EGCG in ethanol and 2-propanol/water solutions (1). This finding suggests a strong interaction between Toc radicals and EGCG.

In the present study, partial regeneration of VE was observed in the presence of MG, EC, EGC and ECG,

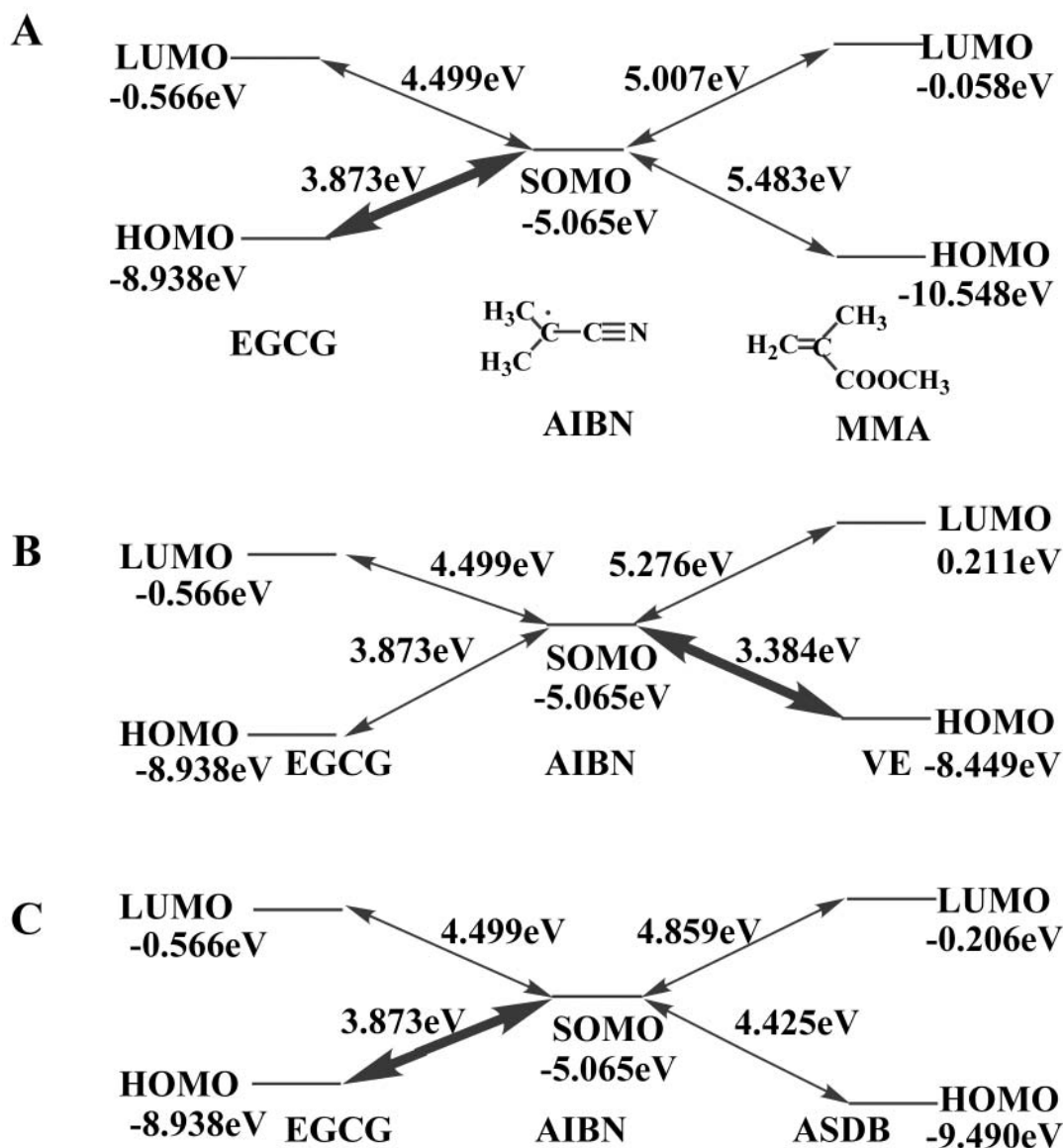


Figure 3. The energy level for SOMO of AIBN and the HOMO and LUMO of MMA or EGCG (A), that of VE or EGCG (B), and of ASDB or EGCG (C).

particularly EGC. The regeneration mechanism of VE by catechol derivatives has previously been reported; the driving force for the regeneration process is the removal of the semiquinone radical from the catechol derivative by the VE radical, which makes the regeneration of VE practically irreversible (4). In terms of BDE values, the BDE for flavonoids (about 81 kcal/mol) was reduced by the formation of semiquinone radicals, probably to about 55 kcal/mol (data not shown). This decrease in the BDE makes regeneration feasible and, thus, the proposed mechanism provides an explanation for the experimental results.

*Mixtures of ASDB and flavonoids.* The results are also shown in Table II. The observed IP values of mixtures of ASDB with MG, EC or EGCG in the AIBN system were less than the corresponding calculated values, whereas the observed IP values of the ASDB/EGC or ASDB/ECG mixtures were greater than the corresponding calculated values. No synergistic antioxidant effect of MG, EC or EGCG with ASDB was observed; rather, ASDB tended to cause prooxidation of these flavonoids. In contrast, the antioxidant effect of EGC and ECG, particularly ECG, were enhanced by ASDB. The PM3 calculation suggested that the reaction between EGCG and R • was

energetically more favorable than that between ASDB and  $R \bullet$  (Figure 3). The PM3 calculation suggests that the other flavonoids are likely to follow a similar reaction pathway (data not shown).

MG showed the greatest prooxidative effect, with the length of its IP declining by 6.1 minutes. This may be related to its high ionization potential (9.26eV), similar to that of ASDB. The reduction potential of radicals from ascorbic acid, (A/A $\bullet$ ) is 300-700 mV. The reduction potential of phenoxyl radicals from EG (ArO $\bullet$ /ArO $^-$ ) is -54 mV and that of radicals from EC is 48 mV (14). The marked difference in reduction potential between ascorbic acid and EG (or EC) indicates that when EG (or EC) and ascorbic acid are present in solution in similar amounts,  $R \bullet$  will react first with the former to give an EG (or EC) radical that immediately reacts with ascorbic acid. This process was also suggested by the PM3 calculation (Figure 3C). MG, with a much lower reduction potential, cooxidized ASDB. The values of reduction potential suggest that the activity of MG against cooxidation is greater than that of EC and, possibly, EGCG.

The following equations explain the cooxidation of ascorbate catalyzed by flavonoid phenoxyl radicals.  $R \bullet$  catalyzes a one-electron oxidation of phenols (PhOH) to form phenoxyl radicals (Eq. 5), which oxidize ascorbate (AsH $_2$ ) to semihydroascorbate radicals (Eq. 6), which then form dehydroascorbate and ascorbate (Eq. 7) (15).



A one-electron oxidation results in ascorbyl radical. Abstraction of a hydrogen atom forms the ascorbate free radical and further oxidation results in the formation of dehydroascorbic acid. Dehydration in aqueous solution forms a dimer.

The  $n$  value of flavonoids in mixtures with ASDB ( $n_{\text{flavonoid}}$ ) can be calculated from Equation 8:

$$n_{\text{flavonoid}} = R_1 (\text{IP}_{\text{ASDB+flavonoid}} - \text{IP}_{\text{flavonoid}}) / [\text{flavonoid}] \quad 8$$

$n_{\text{EGC}}$  and  $n_{\text{EGCG}}$  were 4.43 and 2.29, respectively. These  $n$  values were significantly greater than the corresponding control values of 3.85 and 2.11 (Table I). In contrast,  $n_{\text{EGCG}}$  and  $n_{\text{MG}}$  were 3.41 and 0.90, respectively, significantly smaller than the corresponding control values of 3.95 and 1.43. The antioxidant / prooxidant balance for flavonoids may be modulated by a change in stoichiometric factor ( $n$ ). The high catalytic effectiveness for ascorbate oxidation may be associated with the gallate molecule in MG and EGCG. Both compounds possess high electron

affinity (0.54-0.57eV). EGC also possesses a high electron affinity (0.46eV) arising from the gallate moiety as well as a high BDE value, indicating the existence of a phenol function. The difference between the BDEs of ASDB and EGC was the highest among the compounds investigated, and this may explain the high synergistic antioxidant effect of EGC.

Cancer cells are anaerobic in their metabolism (16, 17) and have very poor mechanisms for absorbing adequate amounts of antioxidants. The addition of EGCG to VE or ASDB oxidized by  $R \bullet$  reduced the antioxidant activity of the vitamin derivatives. This suggests that EGCG may possess potent anticancer activity (18, 19)

Since biological systems tend to have low oxygen tensions, the effectiveness of antioxidants *in vivo* may be considerably different from that in aerobic systems *in vitro*. However, *in vivo* experiments are too complex to be amenable to simple interpretation, and, therefore, we undertook physicochemical studies using the induction period method in the radical polymerization of MMA in the presence of antioxidants as a biomimetic model for radical-scavenging activity *in vivo*. The free radical interaction between flavonoids and VE or ascorbate may contribute to our knowledge of the potential of these compounds in chemoprevention by the induction of cancer cell apoptosis.

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