JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Use of Reference Compounds in Antioxidant Activity Assessment

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The choice of reference compounds is examined as a "critical control point" of antioxidant activity assessment. Gallic, caffeic, sinapic, uric, and ascorbic acids, isoeugenol, and Trolox were tested using different redox (FRAP, Folin−Ciocalteu) and radical scavenging (DPPH$^{\bullet}$, ABTS$^{\bullet+}$, CBA, ORAC) assays. The ability to chelate transition metals was assessed to support some of the findings. Analytes were also tested in liposomes. On the basis of the findings, we do not recommend uric acid (due to solubility constrains) and ascorbic acid (due to fast degradation kinetics) as references. The behavior of the rest of the compounds could not always be attributed to typical structural characteristics. Selection of suitable reference compounds for in vitro antioxidant activity assays is not an easy task to achieve. The choice of reference compounds has to remain at the convenience of the researchers, with regard to the aim of the study.

KEYWORDS: Antioxidants; antioxidant activity assessment; reference compound; radical scavenging assays; redox assays; liposome oxidation; gallic acid; caffeic acid; sinapic acid; uric acid; ascorbic acid; isoeugenol; Trolox

INTRODUCTION

Evaluation of antioxidant activity of matrixes such as plasma, beverages, vegetables, and fruits as well as of pure compounds (i.e., phenols, peptides) has become a rather disputable issue during the past decade. Methods and data are questioned for providing meaningful information to interested parties. Data are expressed either in absolute values or indirectly with regard to a control (e.g., antioxidant index) or a reference (e.g., Trolox equivalents) (*1*, *2*). On the basis of analytical chemistry principles, apart from the antioxidant activity, a suitable reference should fulfill some other requirements. It should be stable, inexpensive, and structurally affiliated to the tested compounds to ensure reliability of results (*3*, *4*). As a consequence, over the years, a series of antioxidants have been used as references depending on the aim of each study (*1*, *2*, *5*). Some of them were abandoned (e.g., BHT) with the advance of consumer preferences in natural products. Lately, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of vitamin E, gained an unequivocal position as a reference in antioxidant activity assays, mainly because of its effectiveness in both lipophilic and hydrophilic systems (*6*, *7*). However, it is still under question as to whether its chemical structure substantiates this use, in particular, when the results of in vitro assays serve to extrapolate a potential in vivo activity (*5*).

Our work aimed at examining the choice of reference compounds as a "critical control point" of the antioxidant activity assessment process and to add to the ongoing discussion on the issue. Gallic and caffeic acids were chosen as two representative natural phenolics (bearing a pyrogallol and a catechol moiety, respectively) often used as references in antioxidant activity studies (*8*, *9*). Sinapic acid and isoeugenol were also included as prominent antioxidants (*10*, *11*) and possible references in activity studies of methoxy-substituted phenolics. Uric (2,6,8-trioxypurine) and ascorbic (2-oxo-L-threo-hexono-1,4-lactone-2,3-enediol) acids were tested because of their known in vivo antioxidant potency (*12*). Trolox was included for the reasons previously stated. The assays chosen were redox (FRAP, F−C) as well as radical scavenging activity (DPPH$^{\bullet}$, ABTS$^{\bullet+}$, ORAC, and CBA) ones. The behavior of all the compounds was also tested in liposomes, a model system that mimics cell membranes and shares common characteristics with the above assays. Other experiments were carried out where it was considered necessary.

MATERIALS AND METHODS

Materials. Ascorbic acid (99.7%) and caffeic acid (98%) were purchased from Riedel de Haën (Seelze, Germany), and isoeugenol (98%) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (97%) were from Aldrich Chemical Co. (Steinheim, Germany). Gallic acid (99.5%) and sinapic acid (98%) were obtained from Sigma Chemical Co. (St. Louis, MO), and uric acid (98%) was from BDH Chemicals, Ltd. (Poole, England). 2,2′-Azobis (2-amino propane) dihydrochloride (AAPH) was purchased from Fluka Chemie (Buchs,

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10.1021/jf070473q CCC: $37.00
Published on Web 06/19/2007

Switzerland). L-a-Phosphatidylcholine (lecithin, ~40%) from soybean, 2,4,6-tripyridyl-*s*-triazine (TPTZ), ABTS, DPPH•, $FeCl_3 \cdot 6H_2O$, or Cu $(CH_3COO)_2 \cdot H_2O$ were from Sigma Chemical. Folin−Ciocalteu and fluorescein sodium salt were from Panreac Quimica, S.A. (Barcelona, Spain). Saffron red stigmas were donated by Saffron Cooperative of Kozani (Greece).

For the following assays, mother solutions of 10 mM in methanol (ascorbic acid, Trolox, phenolic acids, and isoeugenol) or water in the presence of alkali (uric acid) were used. Suitable aliquots were dissolved properly to prepare the working solutions for each assay.

FRAP Assay. FRAP reagent (1680 μL), freshly prepared and prewarmed at 37 °C, was mixed with 120 μL of test sample or methanol (blank) (*13*). The working solutions of the tested compounds were prepared in methanol in the range of 100−750 μM (final dilution of the test sample in the reaction mixture was 1/14). The FRAP reagent contained 2.5 mL of a 10 mM TPTZ solution in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 mL of 0.3 M acetate buffer, pH 3.6. The reaction was monitored at the absorption maximum (593 nm) for up to 4 min with a Shimadzu UV-1601 spectrophotometer (Kyoto, Japan), while the temperature was maintained at 37 °C with the aid of an outer water-circulating bath. For each compound and each concentration, measurements were made in triplicate with suitable blank solutions each time. Graphs of antioxidant concentration vs ΔA_{593} at 4 min ($\Delta A_{593} = A_{AH} - A_{cont}$) were then constructed. FRAP value was considered the slope of the linear curve ($\times 10^3$) derived from the constructed graphs.

Folin−Ciocalteu Assay. A series of methanolic solutions of 0.5−2.0 mM were prepared for each compound under investigation. Deionized water (5 mL), antioxidant solution (0.5 mL), and Folin−Ciocalteu reagent (0.5 mL) were transferred to a 10-mL volumetric flask, and after 3 min exactly, 1 mL of a saturated Na_2CO_3 solution was added and the volume was brought to 10 mL with deionized water. Absorbance at 725 nm was recorded 1 h after the addition of the Na_2CO_3 solution (*14*). For each compound and each concentration, measurements were carried out in triplicate using suitable blank solutions each time. Graphs of antioxidant concentration vs absorbance were then constructed. The F−C value was considered the slope of the linear curve derived from the constructed graphs.

ABTS•+ Assay. The ABTS•+ solution was prepared by reaction of 5 mL of a 7 mM aqueous ABTS solution and 88 μL of a 140 mM (2.45 mM final concentration) potassium persulfate ($K_2S_2O_8$). After storage in the dark for 16 h, the radical cation solution was further diluted in phosphate buffer solution pH = 7.4 (PBS) until the initial absorbance value of 0.7 ± 0.05 at 734 nm was reached (*2*). Solutions of each tested compound were prepared in PBS (pH = 7.4) so that their final concentration after the addition of 20 μL to the radical solution (2 mL) was 0−15 μM. The decrease in absorbance was recorded at 0 and after 6 min. For each compound and each concentration, measurements were made in triplicate with suitable blank solutions each time. Graphs of antioxidant concentration vs % inhibition were then constructed. The ABTS value was considered the slope of the linear curve derived from the constructed graphs.

DPPH• Assay. An aliquot (2.9 mL) of a 0.1 mM ethanolic DPPH• solution was transferred in a glass cuvette (10 mm) and then mixed with 0.1 mL of an antioxidant solution at different ratios [AH]/[DPPH•] depending on the activity of the tested compound (*14*). The decrease of the DPPH• concentration was monitored at 516 nm until the steady state was reached. The working solution of the stable radical DPPH• was prepared with magnetic stirring 1 day before the analysis, to remain overnight at 4 °C. For each compound and each concentration, measurements were made in triplicate. Graphs of percent DPPH• remaining concentration (%) versus the ratios [AH]/[DPPH•] were then constructed. The above graphs were used for the calculation of EC_{50}, that is, the amount of antioxidant necessary to decrease the initial [DPPH•] by 50%. Moreover, the reaction times needed to reach the steady state for EC_{50} (T_{EC50}), and the antiradical efficiency, $AE = 1/EC_{50} \times T_{EC50}$, were also calculated. All measurements were performed in triplicate.

CBA Kinetic Study. Estimation of crocin concentration to ca. 10 μM was based on extinction coefficient reported in the literature $\epsilon_{433}^{MeOH} = 133\,000\ M^{-1}\ cm^{-1}$. A certain volume of crocin working solution was diluted with methanol to 5 mL (total volume) so that the A_{433} value was ~1.3. The same volume of crocin working solution was then transferred into a 5-mL volumetric flask, along with 0.1 mL of AHs from a 0.5 mM solution in methanol so that [AH]/[C] = 1 was achieved as proposed by Ordoudi and Tsimidou (*15*). Stock AAPH solution (0.25 M) was prepared daily in 0.01 M PBS and stored at 4 °C during the different sets of experiments. The reaction started with the addition of AAPH (250 μL) (t = 0 min). After dilution to 5 mL (total volume) with PBS and being stirred for ca. 30 s, the test solution was transferred into a 3-mL quartz cell, and absorbance monitoring (440 nm) started at exactly 1 min after the addition of initiator. Recordings were taken every 6 s for a period of 10 min. For each compound, measurements were made in triplicate with suitable blank solutions each time. Percent inhibition of crocin bleaching value (% Inh) was calculated as % Inh = $[(\Delta A_0 - \Delta A)/\Delta A_0] \times 100$, where ΔA_0 and ΔA are the difference in absorbance during the bleaching in the absence and the presence of the AH, respectively.

ORAC Assay. In a 5-mL volumetric flask, 4 mL of an 8.6 nM fluorescein solution (preincubated at 37 °C for 15 min) prepared daily from a 0.11 mM stock solution was transferred. Then 250 μL of the antioxidant solution (final concentration 1 μM for all compounds) or phosphate buffer for the control reading was added. The reaction started with the addition of 120 μL of a 125 mM AAPH solution. All the referred solutions were diluted with a 75 mM phosphate buffer (PB), pH = 7.0. Then the volume was brought to 5 mL with buffer solution (pH = 7.0), and the reaction mixture was vortexed for 0.5 min. The fluorescence was recorded every 0.5 min (excitation 490 nm, emission 515 nm) until zero fluorescence occurred on a Shimadzu RF 1501 spectrofluorometer (Kyoto, Japan). During the whole experimental procedure, the temperature was maintained at 37 °C and the reaction mixture was stirred. For each compound and each concentration, measurements were made in triplicate. The net area under the curve (AUC) was obtained by subtracting the area under the curve (AUC) of blank (AUC_{blank}) from that of the tested compound (AUC_{test}). Calculations were carried out by means of the RF 1501-PC software.

Statistical Analysis. Statistical comparisons of the mean values for each experiment were performed by one-way analysis of variance, followed by the multiple Duncan test ($p < 0.05$ confidence level).

Phosphatidylcholine Liposome Oxidation. Lecithin was suspended in doubly distilled water at a concentration of 8 mg/mL by stirring with a glass rod and sonicating for ~5 min. Liposome formation was obtained through additional sonication with a rod (UP 200S, Dr. Hielscher, GmbH, Berlin, Germany) (2.5 min for 10-mL aliquots of the liposome sample). A quantity of 0.5 mL of methanolic solutions of the tested compounds (60 μM final concentration) was added to Erlenmeyer flasks (100 mL). Liposome aliquots were weighed into the flasks and diluted with doubly distilled water to a final lecithin concentration of 0.8% w/w. The samples were then set for 2 min in a bath-type sonicator and oxidized by addition of cupric acetate (3 μM) on a shaker at 37 °C in the dark. The course of oxidation was monitored through measurement of conjugated diene formation at 234 nm (*14*). All measurements were performed in triplicate.

Calculation of Partition Coefficient (Log *P*). Calculation of the log *P* values, simulating partitioning of tested compounds in an *n*-octanol/water (1:1, v/v) system, was based on Broto's fragmentation method and was accomplished using the CS ChemDraw Ultra 5.0 software (*16*).

Study of Ascorbic Acid Degradation Kinetics. The stability of ascorbic acid (100 μM final concentration in each assay and also at 40 μM only for CBA and ORAC assays) was examined by monitoring the decrease in absorbance at λ_{max} of the corresponding solutions (246 nm at pH 3.6, 269.3 nm in the presence of Na_2CO_3, and 266 nm in all other solutions). For details, refer to the experimental part of each method.

Chelating Effect on Ferric and Cupric Ions. The ability to chelate ferric or cupric ions of the tested compounds was measured as follows. A quantity of 3 mL of a 0.13 mM antioxidant solution was transferred to a quartz cuvette (10 mm), and the spectrum was recorded in the region 200−800 nm. Then 20 μL of a 0.02 M $FeCl_3 \cdot 6H_2O$ or

Table 1. Redox, Radical Scavenging, and Chelating Ability of Compounds Tested as Potential References

AH	redox assays		radical scavenging assays					other tests
	FRAP	F–C	DPPH•		ABTS•+	CBA	ORAC	Fe^{3+} chelation
	slope[a,g] × 10^3	slope[a,g]	EC_{50}[b,g]	AE[c,g]	slope[a,g]	%Inh[d,g]	ΔAUC[e,g]	
gallic acid	2.83 ± 0.15^{A}	0.94 ± 0.02^{A}	0.11 ± 0.01^{A}	2.15 ± 0.22^{A}	6.61 ± 0.18^{A}	61.0 ± 3.0^{A}	3209 ± 388^{A}	+
caffeic acid	1.43 ± 0.06^{B}	0.84 ± 0.06^{B}	0.20 ± 0.01^{B}	2.80 ± 0.15^{A}	2.26 ± 0.06^{B}	72.0 ± 0.9^{B}	8079 ± 632^{B}	+
sinapic acid	0.90 ± 0.00^{C}	0.57 ± 0.04^{C}	0.32 ± 0.03^{C}	0.17 ± 0.01^{B}	4.70 ± 0.13^{C}	76.5 ± 1.3^{B}	5035 ± 335^{C}	–?
isoeugenol	1.07 ± 0.06^{D}	0.53 ± 0.04^{C}	0.74 ± 0.02^{D}	1.65 ± 0.04^{A}	3.16 ± 0.07^{D}	26.4 ± 2.7^{C}	6394 ± 280^{D}	+?
ascorbic acid	1.37 ± 0.06^{B}	0.83 ± 0.01^{B}	0.20 ± 0.01^{B}	19.6 ± 1.50^{C}	2.00 ± 0.05^{E}	55.9 ± 4.4^{D}	931 ± 129^{E}	–
uric acid	1.30 ± 0.00^{B}	0.32 ± 0.01^{D}	N[f]	N[f]	2.00 ± 0.07^{E}	39.4 ± 0.5^{E}	2214 ± 179^{F}	+?
Trolox	1.43 ± 0.06^{B}	0.25 ± 0.01^{E}	0.21 ± 0.01^{B}	2.04 ± 0.10^{A}	2.06 ± 0.05^{E}	39.8 ± 4.3^{E}	3057 ± 260^{A}	+?

[a] Activity is expressed as the slope value of a linear curve describing the dependence of activity as a function of [AH]. [b] Efficient [AH] for scavenging 50% of [DPPH•]. [c] AE values [$AE = 1/(EC_{50} \times TEC_{50})$]. [d] % Inhibition at final [AH] = 10 μM. [e] $\Delta AUC = AUC_{AH} - AUC_{control}$ at final [AH] = 1.0 μM. [f] N = not determined. [g] Each value is the mean of triplicate determinations ± SD; values within the same column with different uppercase letters are significantly different at $p < 0.05$

$Cu(CH_3COO)_2 \cdot H_2O$ solution was added (C_{Fe3+}/C_{AH} or $C_{Cu2+}/C_{AH} = 1$), and the spectrum of the compound was recorded again within 1 min of addition and mixing. Testing was carried out at room temperature and for Fe^{3+} at pH = 3.6 and 5.6 and for Cu^{2+} only at 5.6.

RESULTS AND DISCUSSION

The results obtained for all the tested compounds using both redox and radical scavenging activity assays are presented in **Table 1**.

On the basis of the FRAP values, it seems that all the compounds may reduce Fe^{3+} at acidic pH (3.6). Among selected phenolics, gallic acid, bearing a pyrogallol moiety, was by far the most potent one. Caffeic acid, having a catechol moiety, followed in activity (gallic acid vs caffeic acid = 1.9) in line with other investigators (*17*, *18*). Sinapic acid and isoeugenol, having one available hydroxyl group, were found to be less active (gallic acid vs sinapic acid or isoeugenol = 3.1 and 2.64, respectively). Ascorbic acid, a compound well known for its reducing properties, presented a FRAP value lower than that of gallic acid. This ene-diol was found to exert an activity similar to that of caffeic acid and Trolox. The latter finding was in accord with published data (*7*, *13*), though such efficiency cannot be justified by structural characteristics. The heterocyclic uric acid was found to be equally potent to ascorbic acid and Trolox (*7*, *13*). Uric acid has been reported to oxidize via one-electron oxidation and subsequent hydrogen atom abstraction from a NH group (*19*); however, its in vivo activity is mainly attributed to metal-chelating properties and scavenging of free radicals (*12*).

FRAP assay values express electron-donating ability of compounds so that the relative activity of molecules should be in accordance to the oxidation potential. Still, an "unusually" large number of electrons involved in redox reactions of some polyphenols have been attributed to subsequent chemical reactions (dimerization, polymerization) (*20*). Thus, Hotta and co-workers reported that oxidation of gallic acid involves 4.6 electrons, whereas two electrons are involved in ascorbic, sinapic, and caffeic acids oxidation. In this view, under FRAP assay conditions such chemical reactions may explain the superiority of gallic acid toward ascorbic acid.

This was not the case when the Folin–Ciocalteu assay was used (pH = 11). The obtained results were much closer to classical structure–activity relationship (SAR) principles. Therefore, in the case of phenolic compounds the order of activity was in agreement with the available hydroxyl groups in the aromatic ring. Ascorbic and caffeic acids were equally potent. Gallic acid was slightly better than these two compounds in accordance to comments made by Singleton et al. (*21*), who reported two reacting groups for gallic acid. Uric acid was twice less potent than ascorbic acid, and Trolox was the least active of all. The low reactivity of Trolox toward F–C reagent has been reported by Stratil and co-workers (*18*) who, for this reason, did not recommend it as reference in the evaluation of total phenol content in a series of plant extracts. The low activity of Trolox when F–C assay is used is not unexpected. The particular acid has a single hydroxyl group and lacks other type of substituents, that is, methoxy ones that could further enhance the reducing activity (*21*). Indeed, in the present study, sinapic acid and isoeugenol, being monophenols but having methoxy substituents, presented a twofold higher reducing capacity than that of Trolox.

The evidence thus far shows that the tested compounds may participate in redox reactions with transition metals (Fe, Mo, W) and reduce them to a more pro-oxidative valence status. However, when the structural characteristics of an antioxidant and conditions are suitable, binding of transition metals cannot be precluded (chelating effect). In this way, the pro-oxidant activity of metal ions may be moderated. To screen the chelating ability of the compounds, the UV–vis spectra of aqueous solutions (at pH 3.6 and 5.6) of all of them were recorded in the absence or presence of Fe^{3+} ions ($[Fe^{3+}]/[AH] = 1$). The data shown in **Figure 1** illustrate interactions at pH 5.6.

As expected, gallic and caffeic acids tend to form such complexes. Intense changes in the spectra with characteristic shifts in the visible region were observed for both acids. Spectral observation could not support such an effect in the case of sinapic acid. The finding was in line with literature data suggesting that the latter participates in electron-transfer reaction with Fe^{3+} ions, without forming a complex (*22*). On the other hand, though isoeugenol bears a methoxy group at the 3-position and the single hydroxyl group that is not expected to be ionized at such pH values, it seems that it may form complexes with ferric ions. Such a claim is supported by the fact that isoeugenol is used as a chelating agent in sol technology by controlling the sol polymerization process via stabilization of transition metal intermediates (i.e., Ti or Zr) inhibiting, thus, undesirable precipitation (*23*). Ascorbic acid is rather prone to oxidative degradation in the presence of ferric ions (see also the Supporting Information); chelation does not seem to prevail in this case. Indeed, the decrease in the absorbance was great when the ions were introduced to the solution. On the basis of spectral information, both uric acid and Trolox presented a tendency to form complexes with ferric ions. In the case of uric acid, complex formation was expected as it is reported to tightly bound to transition metal ions in vivo (*12*), thus inhibiting lipid

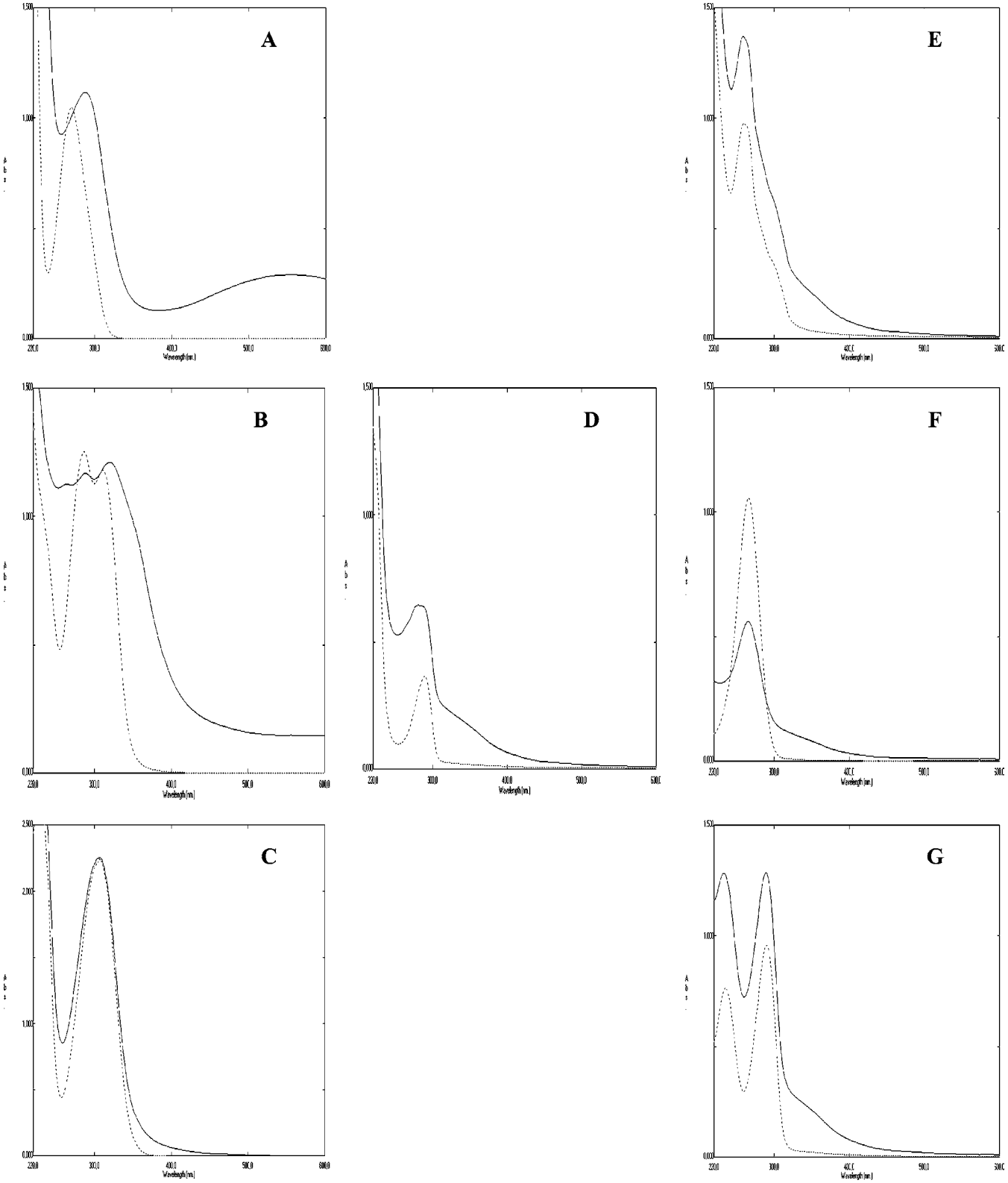

Figure 1. Screening of interactions of (**A**) gallic acid, (**B**) caffeic acid, (**C**) sinapic acid, (**D**) Trolox, (**E**) isoeugenol, (**F**) ascorbic acid, and (**G**) uric acid with ferric ions (pH = 5.6, $[Fe^{3+}]/[AH] = 1$). – – – depicts the original spectrum, and — depicts spectrum after 1 min of addition and mixing of ferric ions.

oxidation. Concerning Trolox, there is no strong evidence to support a chelating ability. A suggestion for complex formation between Trolox and ferric ions has been proposed as an explanation of its pro-oxidant activity in induced oxidation of erythrocyte membrane lipids (*24*). A similar suggestion for iron binding has been made in the past for *a*-tocopherol (*25*), the natural chroman counterpart of Trolox.

On the basis of the above discussion, it seems that a suggestion for a suitable reference compound for the FRAP assay is rather ambiguous. The choice should be strictly based

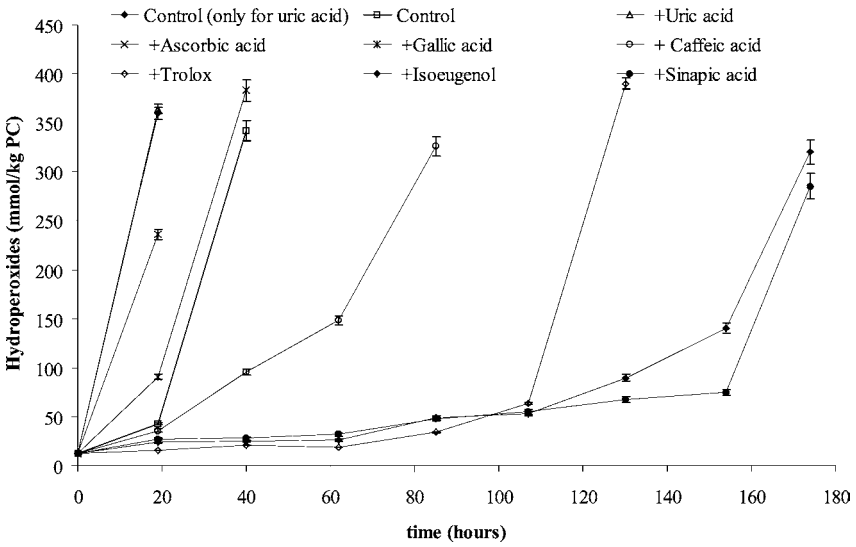

Figure 2. Copper-induced liposome oxidation in the absence or presence of reference compounds (60 μM final concentration) at 37 °C. Each value is the mean of triplicate determinations ± standard deviation.

on the aim of the study. On the other hand, an antioxidant activity trend as estimated by the F−C assay can assist SAR studies, and in this case any of the tested compounds can be used as reference.

The trend of the radical scavenging ability of the compounds varied depending on the assay used. When DPPH• was selected, uric acid was the only compound that could not be tested, as it is not soluble in methanol. A careful literature search showed that uric acid is tested toward DPPH• (*7*, *26*) as an aqueous solution, although the radical is dissolved in alcohol. In our study, it was evidenced that uric acid could not be dissolved in an aqueous environment (pure water of 10 MΩ or PB/PBS, at concentrations of 10 and 5 mM) unless a few drops of alkali were added as described in reference handbooks (*27*). Such a practice is not clearly stated in published articles (*2*, *7*, *13*). Our trials, previous experience on the dependence of DPPH• results on the environment (organic solvent or mixture with water) (*28*, *29*), and the information that "under ordinary conditions the radical does not oxidize purines" (*30*) did not support further attempts to study uric acid. The scarce data reporting an either low (*7*) or high activity (*26*) toward DPPH• for uric acid, apart from being contradictory to each other, are expected to be affected by the percentage of water in the reaction media. For example, the presence of ∼20% (v/v) water (*26*) is expected to enhance activity of compounds toward the DPPH• (*28*). On the basis of the EC_{50} values, the order of activity for the rest of the compounds was gallic acid > caffeic acid ≈ ascorbic acid ≈ Trolox > sinapic acid > isoeugenol. The results are considered satisfactory. The activity of phenols is related to the number of available hydroxyl groups and of electron-donating substituents in the aromatic ring. Ascorbic acid (stable in methanol in the form of monoanion based on absorbance at $\lambda_{max} = 266$ nm) and Trolox were equally potent to caffeic acid. The activity of the former is justifiable since it is an ene-diol and may form a quinone by donating two hydrogen atoms to the free radical. On the other hand, Trolox has only one available hydoxyl group to react with the free radical, so that its high activity should be the result of a more complex mechanism of action. As a matter of fact, a recent investigation of the mechanism of reaction between Trolox and DPPH• indicated that, after a hydrogen atom abstraction, Trolox radical is either dimerized or disproportionated to a quinoid structure. The measured stoichiometry was, thus, 2 (*31*), equal to that of caffeic acid.

When the reaction kinetics was considered (AE values), the order was much different and not in line with classical SAR criteria. A close inspection of the values shows that gallic acid, caffeic acid, isoeugenol, and Trolox were not very different in activity. Great discrepancies were observed for sinapic and ascorbic acids. Because of the slow reaction with DPPH• ($T_{EC50} = 18$ min), sinapic acid appears to present a slow tendency to donate hydrogen atoms. Such an observation may be related to the protection of the phenolic hydroxyl group by the two adjacent methoxy groups. In this way, donation of hydrogen atom to the radical is delayed or even hindered. On the contrary, ascorbic acid was by far the most reacting compound. This is due to its instant reaction with the radical that may be enhanced by the electron-donating effect of the ionized hydroxyl group.

When the DPPH• assay is used, the selection of result expression is important for the choice of a reference compound. When EC_{50} values are about to be used, it seems that selection is relatively easy. In the case where Trolox is chosen, the activity of tested compounds will be somewhat underestimated. When a kinetic approach is carried out (i.e., in terms of AE values), more than one compound should be a candidate as references depending on kinetic behavior (fast, intermediate, and slow reacting) of tested compounds. In this view, the use of ascorbic acid should be excluded, as all other compounds will appear as almost nonreactive.

In the case of the $ABTS^{\bullet+}$ assay, gallic acid was by far the most active compound. Caffeic, Trolox, uric, and ascorbic acids (ascorbic was relatively stable at pH 7.4) were of equal potency, in line with existing data (*2*, *7*, *18*). A low activity of caffeic acid (when compared to that of monophenols) has been also observed by some investigators (*2*, *32*). Because the method has been scrutinized for inherent limitations (*32*), we did not try to rationalize further the choice of a particular compound as a reference.

Since peroxyl radicals are involved in oxidative stress, CBA and ORAC were also employed. These two assays have been used to assess the antioxidant status of plasma in the recent past (*33*, *34*). Despite the similarities between the conditions

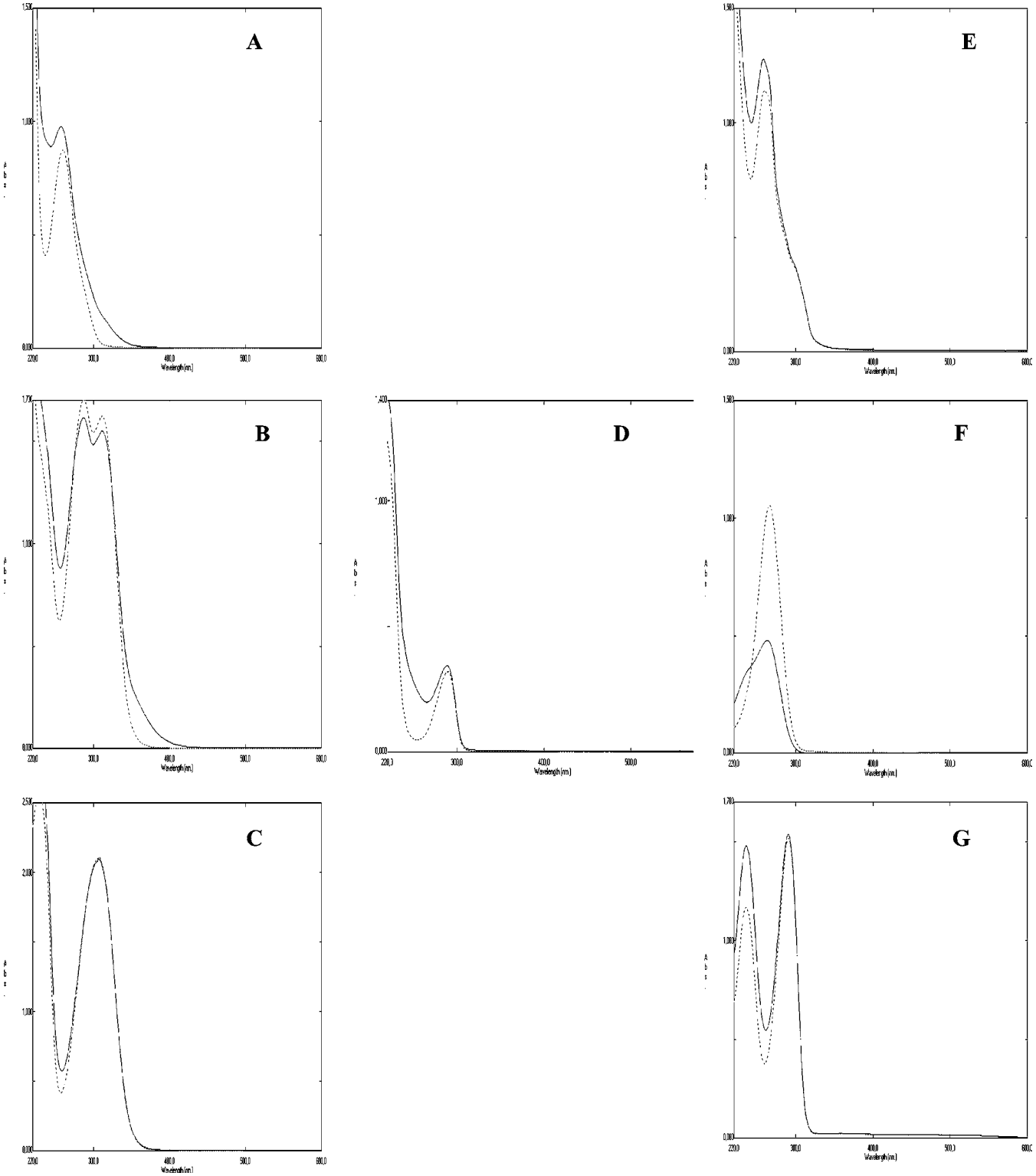

Figure 3. Screening of interactions of (**A**) gallic acid, (**B**) caffeic acid, (**C**) sinapic acid, (**D**) Trolox, (**E**) isoeugenol, (**F**) ascorbic acid, and (**G**) uric acid with cupric ions (pH = 5.6, $[Cu^{2+}]/[AH] = 1$). – – – depicts the original spectrum, and — depicts spectrum after 1 min of addition and mixing of cupric ions.

used in the two assays (same azo initiator, similar temperature and pH), the obtained results varied.

On the grounds of CBA data, sinapic and caffeic acids appear to be the most efficient among the compounds tested. Sinapic acid was found to be slightly better, as reported once before in the literature (*10*). Gallic acid was found to be less efficient, despite the presence of a pyrogallol moiety. Ascorbic acid was even less active (relatively stable under the conditions of the assay and within the 10 min of test duration) but twice more efficient than uric acid or Trolox, in line with data of other investigators (*33*). Isoeugenol was the least active of all. Ordoudi and Tsimidou, studying 31 acid phenolic compounds, suggested that contribution of anions or even dianions may be crucial for the results obtained with CBA (*35*). The same authors also illustrated that prioritization among compounds belonging to different classes of phenols is rather questionable, but within

each class of compounds the information obtained is to a great extent in line with physical organic chemistry principles. It seems that selection of a proper reference with the particular assay should be carried out on the basis of structural characteristics of tested compounds when SARs are about to be drawn.

In the case of the ORAC assay, results were unexpected only in the case of gallic and ascorbic acids. Both of them, especially ascorbic acid, were found to have low activity. The latter has been also observed by other investigators (*36*, *37*), who, however, did not make any comments on the performance of the two compounds. The low activity of gallic acid cannot be explained in terms of stability. Under the pH conditions of the assay, formation of dianions for gallic acid ($pK_{a2} = 8.45$) is possible to a certain extent. Ionization may result in ionic interactions with the azo initiator. The latter (AAPH), which is in excess, during thermal degradation may form positively charged peroxyl radicals (*38*). In this way, anions or dianions of gallic acid may be blocked from reacting with peroxyl radicals, thus being unavailable to protect fluorescein from oxidation. Obviously, further substantiation of this hypothesis is needed. In the case of ascorbic acid, the low activity may be attributed to the degradation of the compound under the experimental conditions. During constant heating at 37 °C and at pH = 7.0 using a PB, the degradation of ascorbic acid at 40 and 100 μM final concentration was rapid (Supporting Information). If this is the case at such concentrations, then the effect at only 1 μM final concentration used in the test should be critical. Once again, instability of ascorbic acid was proved to be a limiting factor for its use as a reference.

All of the compounds were also tested in liposomes, a model system that shares common characteristics with the above in vitro assays. In such a model, the activity of an antioxidant is mainly governed by lipophilicity, which determines partitioning of compounds in the lipid phase (*4*, *6*). The order of increasing lipophilicity on the basis of calculated partition coefficient values (log *P*) was ascorbic acid (−1.54), uric acid (−0.36), gallic acid (0.06), caffeic acid (1.15), sinapic acid (1.83), isoeugenol (2.49), and Trolox (3.19). On the basis of the results obtained (**Figure 2**), the order of activity was sinapic acid ≈ isoeugenol > Trolox > caffeic acid >> gallic acid > ascorbic acid > uric acid at the level used (60 μM final concentration).

What was observed is that not all of the compounds acted as antioxidants. Some of them (namely, gallic, ascorbic, and uric acids) were pro-oxidants. The rest of the compounds protected liposomes efficiently from oxidation. The compounds presenting a pro-oxidative behavior were the most polar ones. We also examined whether other factors may also contribute to pro-oxidant behavior of the compounds. Because liposome oxidation is induced by copper ions (Cu^{2+}), the tested compounds could interact with the ions through electron transfer or even to chelate them. Screening for possible cupric ion chelation is presented in **Figure 3** (pH = 5.6).

On the basis of spectral observations, it seems that only gallic and caffeic acids show a tendency to form a complex with Cu^{2+} under the experimental conditions of the assay. This trend was less strong when compared to that observed with Fe^{3+} (**Figure 1**). The rest of the compounds seem not to interact with Cu^{2+} and unable to form a complex under the same conditions. Moreover, ascorbic acid degradation was fast in the presence of Cu^{2+} ions.

Ascorbic acid, the most polar of all compounds, was the least active probably because it is located in the aqueous phase where it can reduce Cu^{2+} to the more pro-oxidative Cu^{+}. In this way, an enhancement in oxidation process is observed. Similarly, uric and gallic acids, also found in the aqueous phase, participate in analogous redox reactions. Judging from conjugate diene formation data and spectral observations for a possible chelating effect, it seems that redox reactions should prevail, and a pro-oxidative effect is observed. Because antioxidant concentration is also critical for an anti-/pro-oxidant behavior of compounds, the activity of ascorbic and gallic acids was tested at different levels of addition (30, 60, 120, 240, and 480 μM).

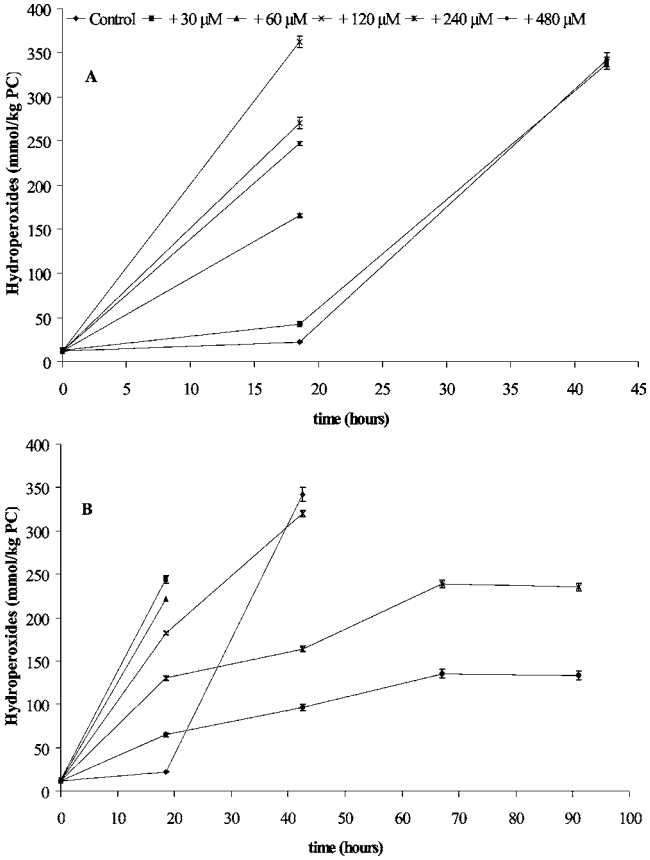

Figure 4. Copper-induced liposome oxidation in the absence or presence of (**A**) ascorbic acid and (**B**) gallic acid at different levels of addition at 37 °C. Each value is the mean of triplicate determinations ± standard deviation.

As given in **Figure 4**, ascorbic acid acts only as pro-oxidant possibly because it is oxidized rapidly in the presence of Cu^{2+} ions (Supporting Information) and the redox reaction with the ions that takes place in parallel. On the contrary, gallic acid at low levels of addition acts as pro-oxidant but at higher ones turns to behave as antioxidant.

Caffeic acid that is less polar than the previously mentioned compounds may chelate Cu^{2+} and also reduce it. The overall result, at the level used, was protective toward liposome oxidation. Sinapic acid was found to be very active. It was more effective than caffeic acid and of similar activity with the highly lipophilic isoeugenol. Trolox was less efficient than sinapic acid and isoeugenol. The estimated activity at a fixed level of addition seems to be the sum of its pro-oxidative (metal reduction) and antioxidative (free radical scavenging) activity (*39*). The latter is significantly affected by lipophilicity. On the basis of FRAP values, we found that sinapic was the least reducing compound when compared to caffeic acid, Trolox, and isoeugenol. This reduced activity may partially explain its high activity in liposomes. Lipophilicity was revealed as a "critical control point" for a reference selection in liposomes and consequently in other lipid substrates.

Table 2. Redox and Radical Scavenging Ability of Selected Reference Compounds Expressed in Terms of Trolox, Ascorbic, and Gallic Acids Equivalents

	redox assays		radical scavenging assays				
AH	FRAP	F–C	DPPH		ABTS	CBA	ORAC
gallic acid	1.98[a]	3.76	0.52[d]	1.05[e]	3.21	1.53	1.05
	(2.07)[b]	(1.13)	(0.55)	(0.11)	(3.30)	(1.09)	(3.28)
	[1][c]	[1]	[1]	[1]	[1]	[1]	[1]
caffeic acid	1	3.36	0.95	1.37	1.10	1.81	2.64
	(1.04)	(1.01)	(1)	(0.14)	(1.13)	(1.29)	(8.68)
	[0.51]	[0.88]	[1.82]	[1.30]	[0.34]	[1.18]	[2.52]
sinapic acid	0.63	2.28	1.52	0.08	2.28	1.92	1.65
	(0.66)	(0.69)	(1.60)	(0.009)	(2.35)	(1.37)	(5.41)
	[0.32]	[0.61]	[2.91]	[0.08]	[0.71]	[1.25]	[1.57]
isoeugenol	0.75	2.12	3.52	0.81	1.53	0.66	2.09
	(0.78)	(0.64)	(3.70)	(0.08)	(1.58)	(0.47)	(6.87)
	[0.38]	[0.56]	[6.73]	[0.77]	[0.48]	[0.43]	[1.99]
ascorbic acid	0.96	3.32	0.95	9.61	0.97	1.40	0.30
	(1)	(1)	(1)	(1)	(1)	(1)	(1)
	[0.48]	[0.88]	[1.82]	[9.11]	[0.30]	[0.92]	[0.29]
uric acid	0.91	1.28	N[f]	N	0.97	0.99	0.72
	(0.95)	(0.39)	N	N	(1)	(0.70)	(2.38)
	[0.46]	[0.34]	N	N	[0.30]	[0.65]	[0.69]
Trolox	1	1	1	1	1	1	1
	(1.04)	(0.30)	(1.05)	(0.10)	(1.03)	(0.71)	(3.28)
	[0.51]	[0.27]	[1.91]	[0.95]	[0.31]	[0.65]	[0.95]

[a] Activity expressed as Trolox equivalents. [b] Activity expressed as ascorbic acid equivalents. [c] Activity expressed as gallic acid equivalents. [d] EC_{50} values. [e] AE values. [f] N = not determined.

All of our findings and also those of other investigators prove that there is no universal type of reference for antioxidant activity assessment. The merits and drawbacks of the tested compounds were in close relation with the characteristics of the methods used. A realistic proposal should be the choice of one reference as a result of a compromise/agreement among researchers working in the field. Toward this direction, Trolox is an interesting candidate. Furthermore, if a relative expression of results is adopted (i.e., equivalents), then the published results may be compared easier. This is exemplified in **Table 2**, which contains transformation of results of **Table 1** in terms of Trolox, ascorbic, and gallic acid equivalents. Trolox equivalents have been introduced in the ABTS (TEAC) assay, and since then this expression using the same or other reference compound has been reported for other methods (*5*, *8*). Researchers are aware of such an indirect expression of results and have postulated that such a proposal can be accepted as part of standardization of antioxidant activity assessment protocols.

The selection of suitable reference compounds for in vitro antioxidant activity assays is not an easy task to achieve, and it obviously remains a "critical control point" in the process of antioxidant activity assessment. The choice of reference compounds has to remain at the convenience of the researchers with regard to the aim of the study.

ABBREVIATIONS

AAPH, 2,2′-azobis (2-amino propane) dihydrochloride; $ABTS^{\bullet+}$, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical cation; CBA, crocin bleaching assay; $DPPH^{\bullet}$, 1,1-diphenyl-2-picrylhydrazyl; F−C, Folin−Ciocalteu; FRAP, ferric reducing antioxidant power; ORAC, oxygen radical absorbance capacity; TPTZ, 2,4,6-tripyridyl-*s*-triazine.

Supporting Information Available: Figure 1: Stability of ascorbic acid (100 μM final concentration) under the experimental conditions employed in all assays used. **Figure 2**: Stability of ascorbic acid (40 μM final concentration) under the experimental conditions employed in Crocin and ORAC assays, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review February 17, 2007. Revised manuscript received May 10, 2007. Accepted May 11, 2007. N.N. thanks the Research Committee of Aristotle University of Thessaloniki for financial support (postdoctoral fellowship).

JF070473Q