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Use of a Free Radical Method to Evaluate Antioxidant Activity

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The antiradical activities of various antioxidants were determined using the free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH•). In its radical form, DPPH• has an absorption band at 515 nm which disappears upon reduction by an antiradical compound. Twenty compounds were reacted with the DPPH• and shown to follow one of three possible reaction kinetic types. Ascorbic acid, isoascorbic acid and isoeugenol reacted quickly with the DPPH• reaching a steady state immediately. Rosmarinic acid and δ-tocopherol reacted a little slower and reached a steady state within 30 min. The remaining compounds reacted more progressively with the DPPH• reaching a steady state from 1 to 6 h. Caffeic acid, gentisic acid and gallic acid showed the highest antiradical activities with a stoichiometry of 4 to 6 reduced DPPH• molecules per molecule of antioxidant. Vanillin, phenol, γ-resorcylic acid and vanillic acid were found to be poor antiradical compounds. The stoichiometry for the other 13 phenolic compounds varied from one to three reduced DPPH• molecules per molecule of antioxidant. Possible mechanisms are proposed to explain the experimental results.

Introduction

The oxidation of lipids in foods is responsible for the formation of off-flavours and undesirable chemical compounds which may be detrimental to health. Antioxidants are used by the food industry to delay the oxidation process. Many different methods (1) have been used to measure the resistance of a lipid to oxidation when in the presence of potential antioxidants. These tests are generally performed in either a lipid or emulsion medium. Autoxidation is a slow, radical process which proceeds via a chain reaction including induction, propagation and termination steps. During the induction period, alkyl radicals are formed which undergo reaction with oxygen molecules to form hydroperoxides and peroxide radicals during the propagation phase. Termination proceeds via association of two radicals to form a stable adduct.

The majority of tests are performed by shortening the induction period of the chain reaction, either by using high temperature or an increased oxygen supply. From these tests, the antioxidative activities of a number of pure compounds and plant extracts have been determined by measuring the oxygen consumption or production of hydroperoxides or other degradation products.

In our laboratory, an accelerated test has been developed (2) which follows the disappearance of methyl linoleate using gas chromatography.

Recently, a method using a different approach has been cited in the literature (3–6). To evaluate the antioxidative activity of specific compounds or extracts, the latter are allowed to react with a stable radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH•) in a methanol solution. The reduction of DPPH• as indicated below is followed by monitoring the decrease in its absorbance at a characteristic wavelength during the reaction. In its radical form, DPPH• absorbs at 515 nm, but upon reduction by an antioxidant (AH) or a radical species (R•), the absorption disappears.

$$\text{DPPH}^\bullet + \text{AH} \rightarrow \text{DPPH-H} + \text{A}^\bullet$$
$$\text{DPPH}^\bullet + \text{R}^\bullet \rightarrow \text{DPPH-R}$$

In this paper we have attempted to explain the results obtained using the DPPH• method for a number of phenolic compounds as well as ascorbic and isoascorbic acids. The results are compared to those obtained using the methyl linoleate test (2).

Materials and Methods

Reagents

The methanol used was of spectrophotometric grade (990 g/L) from Analyticals Carlo Erba (Milano, Italy). 2,2-Diphenyl-1-picrylhydrazyl (DPPH• 950 g/kg), BHT (990 g/kg), BHA (980 g/kg), isoascorbic acid (980 g/kg), γ-resorcylic acid (980 g/kg) and isoeugenol (980 g/kg) were from Aldrich (St Quentin Fallavier, France). δ-tocopherol (900 g/kg) was from Sigma (St Quentin Fallavier, France). Ascorbic acid (997 g/kg) eugenol, gallic acid (990 g/kg), phenol (990 g/kg) and guaiacol were from Prolabo (Paris, France). Protocatechuic acid, gentisic acid, rosmarinic acid, ferulic acid, vanillic acid, zingerone and caffeic acid were from Extrasynthèse (Genay, France). Vanillin (990 g/kg) was a product of Fluka.

Apparatus

All spectrophotometric data were acquired using a Uvikon 810 Kontron spectrophotometer. Disposable cuvettes (1 cm

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× 1 cm × 4.5 cm) from Muller Ratiolab (Dreieich, Germany) were used for visible absorbance measurements.

Antiradical activities of potent antioxidants by DPPH

The antioxidant activities were determined using DPPH as a free radical. For each antioxidant, different concentrations were tested (expressed as the number of moles of antioxidant/mole DPPH•). Antioxidant solution in methanol (0.1 mL) was added to 3.9 mL of a 6 × 10^{-5} mol/L methanol DPPH• solution. The decrease in absorbance was determined at 515 nm at 0 min, 1 min and every 15 min until the reaction reached a plateau. The exact initial DPPH• concentration (C_{DPPH}) in the reaction medium was calculated from a calibration curve with the equation,

$Abs_{515\,nm} = 12{,}509 \times (C_{DPPH}) - 2.58 \times 10^{-3}$, as determined by linear regression.

For each antioxidant concentration tested, the reaction kinetics were plotted (**Fig. 1**). From these graphs, the percentage of DPPH• remaining at the steady state was determined and the values transferred onto another graph showing the percentage of residual DPPH• at the steady state as a function of the molar ratio of antioxidant to DPPH• (**Fig. 2**). Antiradical activity was defined as the amount of antioxidant necessary to decrease the initial DPPH• concentration by 50% (Efficient Concentration = EC_{50} ((mol/L) AO/(mol/L) DPPH•). For reasons of clarity, we will speak in terms of $1/EC_{50}$ or the antiradical power (ARP): the larger the ARP, the more efficient the antioxidant. Nineteen pure compounds have been tested. Their formulas are given in **Fig. 3**.

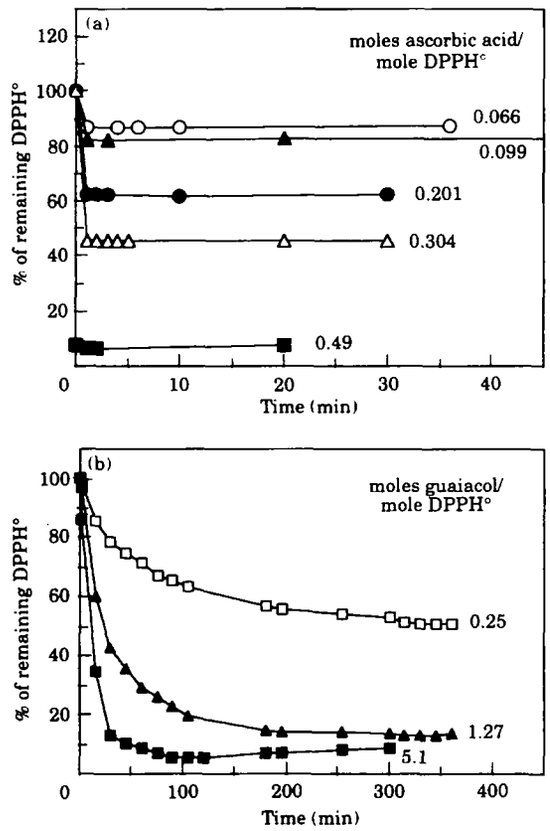


Fig. 1 Examples of the two observed types of reaction kinetics. (**a**) Kinetic behaviour of ascorbic acid; (**b**) kinetic behaviour of guaiacol

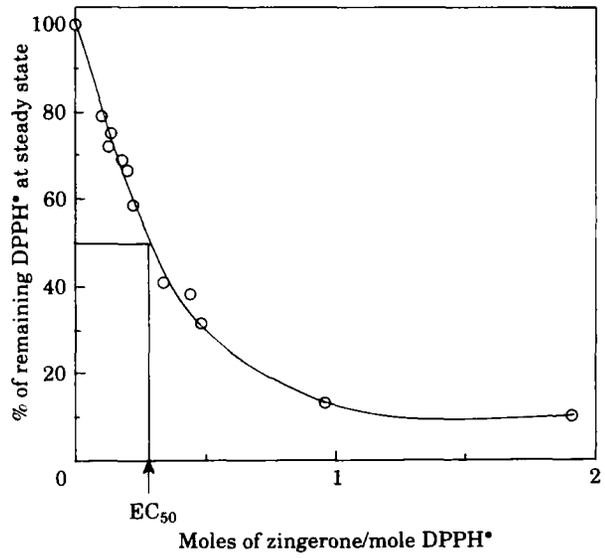
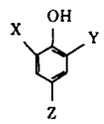


Fig. 2 The disappearance of DPPH• as a function of the number of moles of zingerone/mole DPPH•



Compounds	X	Y	Z
Phenol	H	H	H
BHA	H	$C(CH_3)_3$	OCH_3
BHT	$C(CH_3)_3$	$C(CH_3)_3$	CH_3
p-Coumaric acid	H	H	CH = CH - COOH
Vanillin	H	OCH_3	CHO
Vanillic acid	H	OCH_3	COOH
Isoeugenol	H	OCH_3	CH = CH - CH_3
Ferulic acid	H	OCH_3	CH = CH - COOH
Eugenol	H	OCH_3	CH_2 - CH = CH_2
Zingerone	H	OCH_3	CH_2 - CH_2COCH_3
Guaiacol	H	OCH_3	H
Protocatechuic acid	H	OH	COOH
Caffeic acid	H	OH	CH = CH - COOH
Gallic acid	OH	OH	COOH
Gentisic acid	H	COOH	OH

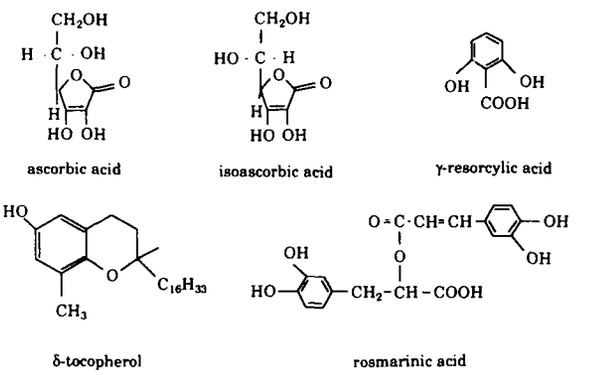


Fig. 3 Chemical structures of the tested compounds

Results and Discussion

Antiradical measurements

The evolution of the different reaction kinetics depends on the nature of the antioxidant being tested. Three types of behaviour were observed. In **Fig. 1(a)**, an example of rapid kinetic behaviour is shown. Only three of the 20 compounds tested, including ascorbic acid, isoascorbic acid and isoeugenol reacted rapidly with the DPPH•, reaching a steady state in less than 1 min. The second type of behaviour was intermediate and concerned only rosmarinic acid and δ-tocopherol. For these reactions, the steady state was reached

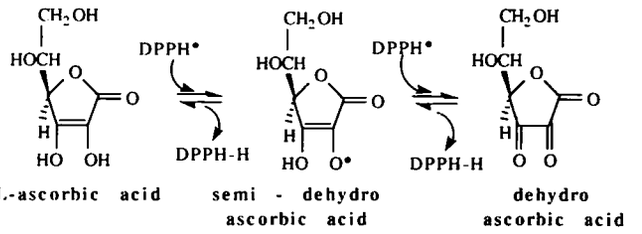
after approximately 5 and 30 min for rosmarinic acid and δ-tocopherol, respectively. The 15 remaining compounds reacted more slowly with the DPPH•. An example of their behaviour (i.e. guaiacol) is shown in **Fig. 1(b)**. These slower kinetics were all hyperbolic curves taking anywhere from 1 to 6 h to reach a steady state.

As indicated in the methods section, the antiradical activity was evaluated from the plot of the percentage DPPH remaining when the kinetics reached a steady state as a function of the molar ratios of antioxidant to DPPH (**Fig. 2**). In contrast to other researchers (4,6,7) who determined the EC_{50} after 30 min of reaction time, the antiradical activities were analysed at the steady state. For those compounds which react rapidly with the DPPH• radical no difference was observed in the ARPs. However, in the case of slower kinetic behaviour, an ARP determined at 30 min would be erroneous because the reaction would still be progressing (i.e. for BHT $ARP_{30\,min}$ = 1.06 and $ARP_{240\,min}$ = 5.30; for protocatechuic acid $ARP_{30\,min}$ = 4.0 and $ARP_{120\,min}$ = 7.14). It was therefore decided to analyse the data at the steady state.

Another way to analyse the antiradical activity could be to determine the amount of antioxidant necessary to decrease the initial DPPH• concentration by 100% (EC_{100}). In this case, the classification of the antiradical efficiency would be different and certain compounds tested (i.e. coumaric acid and vanillin) never react with more than 75% of the initial DPPH•, even after 7 h of reaction time and at very high concentrations. Therefore, the classification of the antiradical efficiencies has been established from an EC_{50} determined as shown in **Fig. 2**. In **Table 1**, all compounds are classified in increasing order of ARP according to their kinetic behaviour.

Reactions stoichiometry

The stoichiometry was obtained by multiplying the EC_{50} of each antioxidant by two which gives the theoretical efficient concentration of each antioxidant needed to reduce 100% of the DPPH•. In **Table 1**, these values are presented for all compounds together with their inverse values (the number of DPPH• moles reduced by one mole of antioxidant). According to these data, the compounds that have rapid or intermediate kinetics, have stoichiometries that correspond approximately to the number of hydrogens available for donation on hydroxyl groups except δ-tocopherol. One isoeugenol molecule reduces one DPPH• molecule. Ascorbic acid and isoascorbic acid each reduce nearly two DPPH• molecules as is shown in the following reaction (8):



The ARP value found by Lamaison *et al.* (4,9) for ascorbic acid was 4 slightly higher than our value of 3.7. This corresponds to two reduced DPPH• molecules per molecule of antioxidant. A stoichiometric value of 0.3 was determined for rosmarinic acid whereas Lamaison *et al.* (4,9) found 0.25. Rosmarinic acid has four hydroxyl groups which could reduce four DPPH• molecules. δ-tocopherol has a stoichiometry of 0.5, reducing two DPPH• molecules. However, it only has one available hydroxyl group. A dimerization may occur between two tocopherol radicals. The new compound formed would then be able to reduce a second DPPH• molecule (10).

For the remaining 'slower' kinetic reactions, the stoichiometry was more difficult to interpret. Only ferulic acid, with one hydroxyl group, reduces one DPPH• molecule. Phenol, coumaric acid, vanillin, vanillic acid and γ-resorcylic acid react very poorly with the DPPH•.

Table 1. Classification of antiradical efficiencies and stoichiometry, according to kinetic behaviour

	Compound	ARP	Stoichiometric Value	Number of reduced DPPH•
Rapid kinetic behaviour	Isoeugenol	1.94	1.03	0.97
	Ascorbic acid	3.70	0.54	1.85
	Isoascorbic acid	3.70	0.54	1.85
Intermediate kinetic behaviour	δ-tocopherol	4	0.50	2
	Rosmarinic acid	6.90	0.30	3.33
Slow kinetic behaviour	Phenol	0.002	270	<1
	Coumaric acid	0.02	98	<1
	Vanillin	0.05	44	<1
	Vanillic acid	0.17	11.8	<1
	γ-resorcylic acid	0.36	5.6	<1
	Ferulic acid	2.33	0.86	1.16
	Eugenol	3.7	0.54	1.85
	Zingerone	3.7	0.54	1.85
	Guaiacol	4	0.5	2
	BHA	4.17	0.38	2.63
	BHT	4.20	0.38	2.63
	Protocatechuic acid	7.14	0.28	3.6
	Caffeic acid	9.1	0.22	4.54
	Gentisic acid	11.1	0.18	5.6
	Gallic acid	12.5	0.16	6.25

BHT and BHA reduce two or more DPPH• molecules despite the fact that they only have one hydroxyl group. This finding is, however, in agreement with the results of Kurechi *et al.* (11). It is known that such compounds with a hydroxyl group sterically hindered by a t-butyl group, present a high antioxidative efficiency (12–14).

In a similar manner, one eugenol molecule, one zingerone molecule and one guaiacol molecule each reduce nearly two DPPH• molecules despite the availability of only one hydrogen on a hydroxyl group. Lamaison *et al.* (4) found a similar result for eugenol: it reduces two DPPH• molecules (ARP = 4.6).

We suggest three hypotheses to explain the antiradical efficiencies of the different monophenolic compounds. The first hypothesis involves the donation of a second hydrogen following electron delocalization onto the para-substituted group as shown in reaction [1] of **Fig. 4**. It only applies to eugenol, zingerone and BHT, which possess two or three hydrogens on the carbon in the para-position of the aromatic ring.

The second hypothesis involves a dimerization between two phenoxyl radicals (reaction [2] of **Fig. 4**) as described by Pokorny for phenols with free para- and ortho- positions (14). After the dimerization, two hydroxyl groups would be regenerated by an intramolecular transfer of H• and could again interact with the DPPH•. In the third hypothesis, one DPPH• molecule complexes with one aryl radical as indicated in reaction [3] of **Fig. 4**.

The reactions 2 and 3 apply to those molecules (guaiacol, BHA, zingerone, and eugenol) which have a free ortho or para position. However, isoeugenol and ferulic acid do not participate in either reaction 2 or reaction 3. They each possess a conjugated group in the para position which would enter into resonance with the aromatic ring. The radical would therefore be delocalized outside of the aromatic ring and the chances of a dimerization or complexation would be lower. This could explain the stoichiometry of 1 obtained for isoeugenol and ferulic acid. The poor efficiency of monophenols (a stoichiometry less than 1 for phenol, coumaric acid, vanillin and vanillic acid) may be explained by the presence of an electron withdrawing group (CHO or COOH) or, as is the case with phenol, the absence of any electron donating group. This poor aromatic ring resonance of the phenoxyl radical considerably lowers the antiradical efficiency.

It is known that polyphenols have a higher antioxidant (antiradical) activity than monophenols (1, 14–16). A close look at our other results also supports this finding. For example, caffeic acid is a more efficient antiradical compound than coumaric acid, its monophenol counterpart (ARP = 9.1 and 0.02 respectively). Gallic acid, a triphenol, is more efficient than protocatechuic acid, its diphenol counterpart (ARP = 12.5 and 7.14 respectively).

The position of these second and third hydroxyl groups is important (16). Those compounds whose second hydroxyl group is in the ortho or para position have a higher activity than when it is meta, as we found for γ-resorcylic acid which is much less efficient than protocatechuic acid and gentisic acid. The efficiency of ortho and para diphenols is in part due to the stabilisation of the aryloxyl radical by hydrogen bonding (14) or by regeneration of another diphenol as indicated in **Fig. 5**.

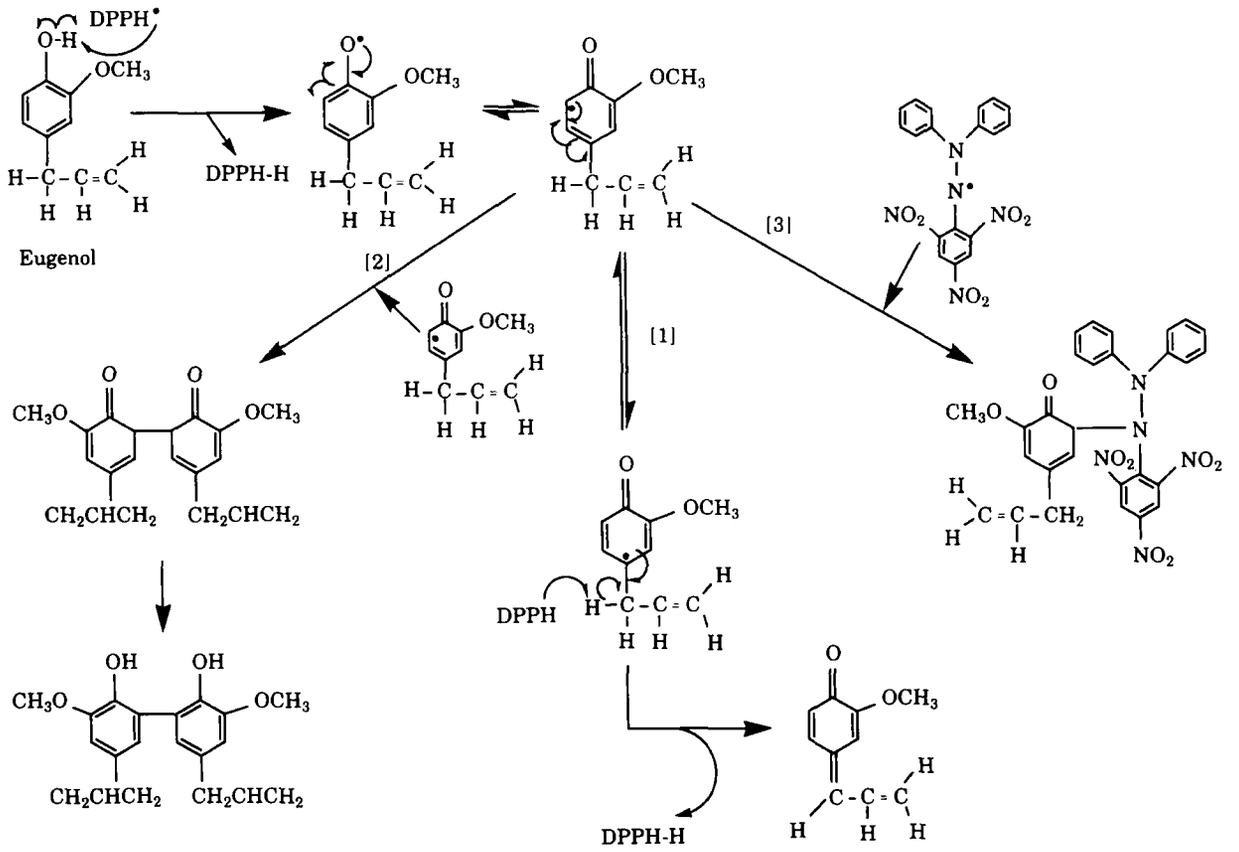


Fig. 4 Potential reactions of DPPH• with eugenol.
Reaction [1], donation of a second hydrogen; Reaction [2], dimerization; Reaction [3], complexation

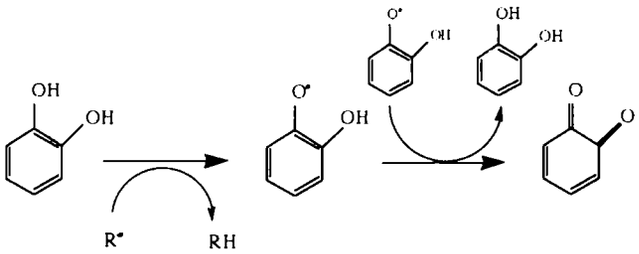


Fig. 5 Mechanism of the regeneration of an ortho-diphenol

Finally, as was discussed by Cuvelier (16) and Shahidi *et al.* (1), the ortho–methoxy substitution also stabilizes the aryloxyl radical by electron donation (18) and therefore increases the antioxidant and antiradical efficiencies. Two examples are guaiacol and ferulic acid which are more active than phenol and p-coumaric acid.

Comparison of ARP with the antioxidant activities determined by the accelerated autoxidation of methyl linoleate

The accelerated autoxidation of methyl linoleate was performed in dodecane, at 110 °C under an oxygen saturated atmosphere. This method was used in an earlier study performed in our laboratory (2,15,16). Antioxidant efficiency was assessed as the percentage increase in the reaction half-life of a control (methyl linoleate without antioxidant). This efficiency varied according to the particular antioxidant tested and its concentration. The antioxidants were compared by determining the efficient quantity of each needed to double the half-life of the control reaction (EQ). The stronger the antioxidant, the smaller the EQ value. Therefore, as was done for the EC_{50} values, the results will be expressed as 1/EQ denoted as the antioxidant power (AOP).

A comparison between AOP and ARP for all the antioxidants tested is shown in **Fig. 6** (only δ-tocopherol was not tested by the two methods). It is important to recall that contrary to the methyl linoleate test, the antiradical test (DPPH•) is performed in a polar medium (methanol) at ambient temperature and without any additional oxygen. It is therefore difficult to compare the quantitative values of the individual compounds determined by either test. However, these compounds (gallic acid, gentisic acid, rosmarinic acid, protocatechuic acid and caffeic acid) with a high antioxidant power also showed a high antiradical activity. Conversely, the compounds with a very low antioxidant activity also had a low antiradical power (for example coumaric acid, vanillic acid, vanillin, phenol, and γ-resorcylic acid). Five exceptions were found: ascorbic acid, isoascorbic acid, zingerone, eugenol and guaiacol.

Ascorbic acid and isoascorbic acid showed no or little antioxidant activity in the methyl linoleate test and yet they react rapidly with DPPH•. One explanation is the temperature (110 °C) and oxygen saturated conditions of the methyl linoleate test. To investigate these effects, the following experiments were performed: ascorbic acid was kept at 110 °C for 0, 20 and 40 min before being mixed with DPPH• and tested. The results showed that this treatment had no effect upon its activity towards the DPPH•. The same experiment was repeated with ascorbic acid placed under oxygen (at ambient temperature). Again this had no effect upon its activity. However, when ascorbic acid was left at 110 °C and under an atmosphere saturated in oxygen for 30 min, its antiradical activity decreased a little. These harsh conditions used for the autoxidation of methyl linoleate combined with a poor affinity for the solvent and the substrate could explain the difference in the results obtained by the two tests.

Eugenol, zingerone and guaiacol were found to be more active than ferulic acid in the antiradicalar test whereas the opposite was found in the accelerated autoxidation test. The only difference in these four compounds is their *z* substituent (**Fig. 3**). The 'polar paradox' described by Porter (19,20) may be illustrated here. According to these authors, lipophilic antioxidants are more active in polar mediums whereas polar antioxidants are more active in lipophilic mediums. However, this paradox does not apply to isoeugenol, which may be explained by its different type of kinetics.

Conclusions

The use of DPPH• provides an easy and rapid way to

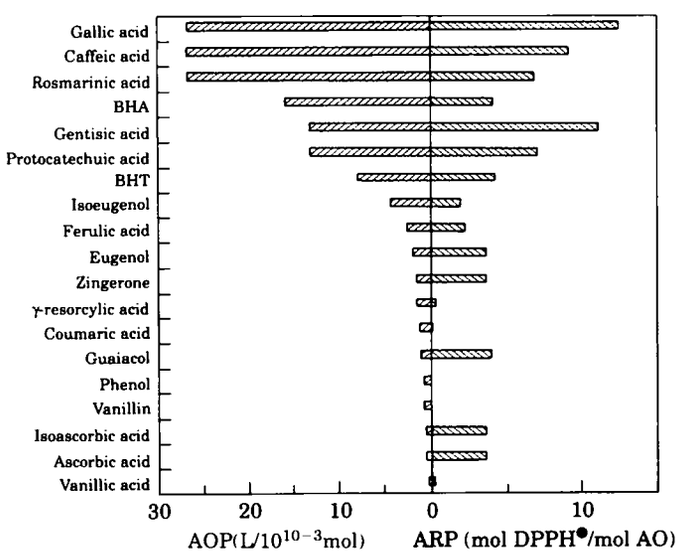


Fig. 6 Comparison of ARP (DPPH•) and AOP (methyl linoleate).

evaluate the antiradical activities of antioxidants, but some caution must be taken when using the method and interpreting the data.

From the results obtained in the present study, it is evident that the interaction of a potential antioxidant with DPPH• depends on its structural conformation. Certain compounds react very rapidly with the DPPH• reducing a number of DPPH• molecules corresponding to the number of available hydroxyl groups. However, for the majority of the compounds tested the mechanism is more complex.

For a better understanding of the mechanisms involving the DPPH• and potential antioxidants, it would be interesting to characterize the reaction intermediates and products. To do this, it is necessary to separate these compounds by chromatography and to identify them. It would also be very useful to build a plausible kinetic model and determine the order of the different reactions and their constants.

Acknowledgement

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