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Organic acids influence on DPPH· scavenging by ascorbic acid

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Abstract

The 2,2-diphenyl-1-picrylhydrazyl (DPPH·) test is largely used in plant or food biochemistry to evaluate the free radical scavenging effect of specific compounds or extracts. In its radical form, DPPH· has a broad absorption band in the visible region at 517 nm, while if it is protonated by an antiradical compound, it loses this property.

This study regarded the effect on DPPH· reduction by ascorbic acid in function of its dissolution in water or ethanol and in addition of acetic, malic and citric acid, which are widely diffused organic acids in the plant kingdom.

The tested acids gave no scavenging activity in the absence of ascorbic acid, while their action was significant when used in the presence of ascorbic acid. They generally enhanced the scavenging activity of ascorbic acid on DPPH· at a steady rate, while malic and citric acid slowed the reaction during the first minute.

Keywords: 2,2-Diphenyl-1-picrylhydrazyl; DPPH; Ascorbic acid; Organic acids; Scavenging activity

1. Introduction

The use of the DPPH· reaction (Fig. 1) has been widely diffused among food technologists and researchers, for the evaluation of free radical scavenging activity on extracts from plant, food material or on single compounds (Kondo, Tsuda, Muto, & Nakatani, 2002; Kondo, Yoshikawa, & Katayama, 2004; Saint-Cricq de Gaulejac, Provost, & Vivas, 1999; Sanchez-Moreno, Larrauri, & Saura-Calixto, 1998; Sanchez-Moreno, 2002; Skupien & Oszmianski, 2004; Von Gadow, Joubert, & Hansmann, 1997; Zhang & Hamauzu, 2003) because of its ease of use and its methodology. The proton transfer reaction of the DPPH· free radical by a scavenger (A-H) causes a decrease in absorbance at 517 nm, which can be followed by a common spectrophotometer set in the visible region.

The scavenging reaction of DPPH· can also be followed in a more direct way, using the presence of an unpaired electron in its radical form, known as the electronic paramagnetic resonance, abbreviated as EPR (Polovka, Brezova, & Staso, 2003).

Ascorbic acid, a well noted antioxidant and free radical scavenger product is widely diffused in the plant kingdom, and it is a DPPH· scavenging agent of medium strength (Schlesier, Harwat, Bohm, & Bitsch, 2002) but its reaction is very fast with respect to other scavenging molecules, such as polyphenols (Brand-Williams, Cuvelier, & Berset, 1995; Zhang & Hamauzu, 2003).

Ascorbic acid is present in fruit and vegetables in different concentrations, ranging from small amounts (5–6 mg 100 g^{-1} f.w. in apples) to about 80-100 mg 100 g^{-1} f.w. in red-pigmented varieties of orange. The measured DPPH· scavenging properties of a plant extract have often been correlated to the amount of ascorbic acid, when it is present at a significant level, but this relation was often not evidenced, because of the presence of other potential scavengers, such as vitamins A, E and polyphenols.

In fruit and vegetables, other molecules are also present in a great amount, such as organic acids, which are not widely considered as potential free radicals scavengers of DPPH·, however previous studies attribute a direct action

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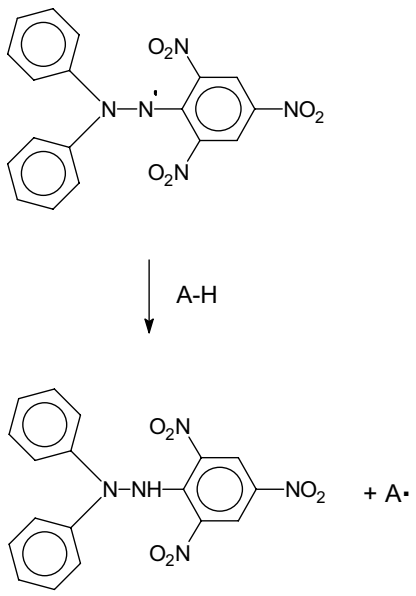

Fig. 1. DPPH· chemical structure and its reaction with a scavenger, indicated by A-H.

on free radicals scavenging to some organic acids (Chang & Chen, 2004; Kayashima & Katayama, 2002). Besides, an effective antioxidant action is well-known as their chelating action, inactivating reducing cations.

To date, no reference data have been found on the possible synergic action of organic acids when mixed with already known DPPH· scavengers molecules.

The present research reports the observations relating to ascorbic acid scavenging action, on DPPH· mediated by the presence of some organic acids widely diffused in plants, such as acetic, malic and citric.

2. Materials and methods

All used reagents were of analytical spectrophotometric grade (Sigma–Aldrich, USA) and all solutions were deaerated with N_2 bubbling before use.

Experimental data were acquired on a spectrophotometer (ATI-Unicam, model UV-4, UK) equipped with a thermostated cell (25 °C), set at 517 nm, measuring the absorbance on a 1 cm optical path of disposable glass cuvettes.

The reaction mixture was made by 2 ml of ethanol 96%, 0.5 ml of DPPH· solution and 0.1 ml of scavenger solution.

The DPPH·, previously dissolved in 96% ethanol (EtOH) and stabilised by sonication, was used at 0.1 mM final concentration.

Ascorbic acid (AA) stock solutions (1.2 mM) were freshly prepared and dissolved in pure water, EtOH and in an EtOH solution of 1.7 N acetic acid (sol. 1), 1.7 N malic acid (sol. 2) and 1.7 N citric acid (sol. 3).

The scavenging reaction solutions of AA (0.1 ml in a total volume of 2.6 ml) had three different final concentrations: 0.045, 0.023 and 0.015 mM. AA was dissolved in pure water, EtOH and in an EtOH solution of 0.067 N final concentration of all the assayed organic acids. The choice of these AA concentrations was mainly due to their correspondence to a common concentration, found in a diluted plant extract effective in scavenging DPPH·.

The absorbance measurements were made by plotting the change in absorbance at 517 nm vs the elapsed time of reaction and each scavenger solution tested was compared to the measurements of the DPPH· stability (blank) by mixing 2 ml of EtOH, 0.5 ml of DPPH· solution and 0.1 ml of pure water, EtOH, sol. 1, 2 and 3 in the absence of AA.

These evaluations were made by recording the scavenging data after 1 min of reaction and at steady state, which was obtained after 30 min of reaction for all the tested solutions.

The efficient concentration of antioxidant which is necessary to decrease the initial concentration of DPPH· by 50% (EC50) was calculated at steady state and expressed as moles AA vs. moles DPPH·, according to Brand-Williams et al. (1995).

Each test was replicated ten times. The data analysis was submitted to analysis of variance and the averages were compared by the Tukey test ($p < 0.05$) using a Statgraphics-5-plus, Manugistics Inc., 2000 software.

3. Results

The stability of DPPH· in the absence of ascorbic acid gave no significant change in absorbance at 517 nm, between all the tested solutions (water, EtOH, sol. 1, 2 and 3). The system showed a total stability within the reaction time (30 min), with some samples assayed for a longer time, 120 min, practically showing the same absorbance as the starting time ($\Delta_{absorbance} = 0.003$ units maximum).

After a 1 min reaction, 0.045 mM ascorbic acid dissolved in water gave a DPPH· reduction of about 80% (remaining DPPH· 20.3%), while the lower concentrations (0.023 and 0.015 mM) gave a residual DPPH· presence of 47.2% and 57.3%, respectively (Table 1).

The scavenging activities after 1 min of AA dissolved in EtOH and sol. 1 were very similar to water-dissolved AA, with no statistical difference, except for 0.023 mM AA dissolved in acetic acid solution.

After 1 min of reaction, the dissolution of AA in sol. 2 and 3 gave significantly higher values of residual DPPH· at all concentrations (Table 1), meaning a lower activity of AA towards DPPH· in a system acidified with malic or citric acid.

At steady state, the percent reduction of DPPH· by water or EtOH dissolved AA remained unaltered with the same values found after 1 min.

In sol. 1, 2 and 3, the amount of remaining DPPH· was significantly lower than that found in water and EtOH at AA concentration of 0.045 mM (average 9%) and 0.023 mM (average 28%), while at 0.015 mM it remained practically unaltered with respect to water and EtOH AA solutions.

Table 1
Average values of percent remaining DPPH· after the reaction with ascorbic acid at different concentrations and in different solutions

Ascorbic acid	Water	EtOH	0.067 N acetic acid sol. 1	0.067 N malic acid sol. 2	0.067 N citric acid sol. 3
After 1 min					
0.045 mM	20.3 a	20.1 a	20.4 a	41.1 b	45.1 b
0.023 mM	47.2 b	47.5 b	37.1 a	64.4 c	65.4 c
0.015 mM	57.3 a	59.0 a	62.3 a	77.6 b	77.2 b
Steady state					
0.045 mM	20.3 b	20.1 b	9.4 a	9.1 a	9.0 a
0.023 mM	47.2 b	47.5 b	27.0 a	29.0 a	29.4 a
0.015 mM	57.7 a	59.0 ab	56.4 a	60.9 b	53.9 a
EC50[a]	0.206 c	0.216 c	0.167 b	0.137 a	0.141a

Different letters mean statistically significant differences in the same row ($p < 0.05$).
[a] The EC50 (moles ascorbic acid/moles DPPH·) values were calculated as described in Section 2.

This means an enhanced activity of 0.045 and 0.023 mM AA at steady state if an organic acid is added to the solution with respect to pure solvent.

The EC50 values (moles AA/moles DPPH·, Table 1) were calculated when the reactions reached their respective plateaus and indicated the antioxidant efficiency of the evaluated systems: the lower the value, the more efficient the antioxidant system. The significantly lowest values were obtained by sol. 2 and 3, followed by sol. 1 and the highest values were found when AA was dissolved in water or EtOH. These results confirm the fact that organic acids added to a solution of ascorbic acid enhance its action on DPPH·.

4. Discussion and conclusion

The DPPH· scavenging activity by AA dissolved in water or EtOH followed the kinetics parametes found by other authors (Brand-Williams et al., 1995; Lamaison, Petitjean-Freytet, Carnat, & Carnat, 1988). The calculation of antiradical power (ARP, 1/EC50) resulted in a higher value than had been found by previous authors: they found 3.7, while the average result for water and EtOH in the present study was 4.7. This difference could probably be due to the different solvent used for the reaction: Brand-Williams et al. used methanol, in this case ethanol was used as the main solvent. However, the calculation of the stoichiometric value (ARP/2) in the present paper and of the consequent number of reduced DPPH· moles by one mole of AA, gave a value of 2.4 which was close to the theoretic value (2.0).

Acetic, malic and citric acid, if added to the DPPH· reaction solution in the absence of ascorbic acid, did not show any effect on the free radical scavenging reaction, giving stable values of absorbance close to those obtained by adding pure water and EtOH.

If AA is dissolved in an ethanol, malic or citric acid solution, the action against DPPH· is significantly slower with respect to pure solvents within 1 min, while at steady state it is enhanced with respect to pure water and EtOH solutions.

As expected, the pH values of the reaction solutions showed different readings, which decrease in the following order: water (5.63), EtOH (5.47), sol. 1 (4.58), sol. 2 (3.38), sol. 3 (3.28).

It could be hypothesised that the decrease in pH increases the AA stability and slows down the transformation:

$$\text{ascorbic acid} \rightleftharpoons \text{semi} - \text{dehydroascorbic acid} \rightleftharpoons \text{dehydroascorbic acid}$$

which is directly involved in the DPPH· scaavenging reaction (Courtland & Sagaut, 1987). Consequently, the action on DPPH· scavenging could be decreased in the first minute but, on the other hand, the low pH could contribute to the slow regeneration of ascorbic acid from dehydroascorbic and semi-dehydroascorbic acid so justifying the activity's enhance in activity of ascorbic acid in the solutions 1, 2 and 3 at steady state.

No literature was found about the action of organic acids on DPPH·, but the interference of organic acids, in the reactive oxygen species scavenging evaluated by EPR, has been demonstrated by other authors (Sentjurc, Nemec, Connor, & Abram, 2003).

However, some previous works affirmed that organic acids possess a biological activity both in reducing reactive oxygen species (van den Berg, Halkes, van Hufford, Hoekstra, & Beukelman, 2003) and in enhancing polyphenols bioavailability (Yamashita et al., 2002).

Concluding, the antioxidant action of ascorbic acid could be influenced by some components not directly involved in the free radical scavenging activity. It is evident that an interaction exists between ascorbic acid and some widely diffused organic acids and that the measurement of an antioxidant could not completely explain the antioxidant properties of a plant extract. Other compounds, not necessarily involved in antioxidant and antiradical action, have to be also considered.

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