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Binary mixtures of natural polyphenolic antioxidants with ascorbic acid: impact of interactions on the antiradical activity

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Abstract: Selected natural polyphenols, including ferulic acid (FA) and hesperetin (Hp) were tested for their antiradical activity using the stable radical DPPH•, as a first step to rank them according to their potency. Ranking also included quercetin (Qt), a very well-studied natural, polyphenolic antioxidant, and ascorbic acid (AA). All phenolics considered were also tested in binary mixtures with AA, to illustrate possible mixture effects. By employing a simple linear regression approach, combinations of AA / Qt, AA / FA and AA / Hp were shown to result in antagonism. The results were discussed on the ground of regeneration reactions, based on the redox potentials.

Keywords: Ascorbic acid, antagonism, antiradical activity, ferulic acid, hesperetin, quercetin

Introduction

Consumer demands for healthier foods with functional properties, as well as the strong evidence provided for plausible toxicity of synthetic additives, has shifted industrial interest into antioxidants of natural origin, including polyphenolic substances (Pokorný, 2007). Phenomena embracing synergism and antagonism among various forms of polyphenolic substances are rather very common in real food matrices (Choe and Min, 2009) and therefore the antioxidant manifestations emerging by enhancing a given food in specific antioxidant additives might not be the anticipated ones.

The "deactivation" of oxidant species by polyphenolic antioxidants (POH) is based, with regard to food systems that are deteriorated by peroxyl radicals (R•), on the donation of hydrogen, which interrupts chain reactions:

$$R^{\bullet} + POH \rightarrow R\text{-}H + PO^{\bullet}$$

Phenoxyl radicals (PO•) generated according to this reaction may be stabilized through resonance and/or intramolecular hydrogen bonding, as proposed for quercetin (Bors *et al*., 1990), or combine to yield dimerisation products, thus terminating the chain reaction:

$$PO^{\bullet} + PO^{\bullet} \rightarrow PO\text{-}OP$$

As pointed out by previous investigations (Brand-Williams *et al*., 1995; Bondet *et al*., 1997), the efficiency of an antioxidant component to reduce R• largely depends on its hydrogen-donating ability. DPPH• is an artificial, stable, model organic radical, which owed to its properties has been used in numerous studies as a valid means of rapidly assaying pure antioxidants, antioxidant mixtures and extracts. Thus DPPH• has become the tool of preference for studies pertaining to the evaluation of radical scavenging activity.

In this line, this investigation was undertaken to obtain an insight into the antiradical behaviour of two phenolic antioxidants, ferulic acid and hesperetin, in relation with their interactions with a well-studied, non-phenolic antioxidant, ascorbic acid (Figure 1). Ferulic acid and hesperetin were chosen because, apart from their abundance in food matrices of plant origin (Clifford, 2000; Tomás-Barberán and Clifford, 2000) both compounds can potentially be produced from cheap sources, such as cereal (Benoit *et al*., 2006; Tilay *et al*., 2008) and citrus (Di Mauro *et al*., 2000; Londoño-Londoño *et al*., 2010) by-products and wastes, respectively. For comparison reasons, parallel experiments were also run with quercetin, which is a very powerful, polyphenolic antioxidant, with well-established properties.

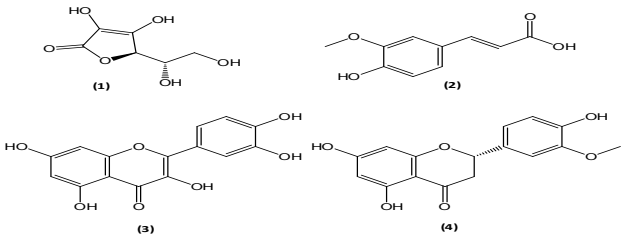

Figure 1. Chemical structures of the natural antioxidants used in this study

Materials and Methods

Chemicals

Quercetin, hesperetin, ferulic acid, ascorbic acid and 2,2-diphenyl-β-picrylhydrazyl (DPPH•) radical

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were from Sigma (St. Louis, MO, U.S.A.).

Determinations

Antiradical Activity (A_{AR}): A previously established methodology was used (Makris *et al.*, 2007). In an Eppendorf tube of 1.5 mL, 0.975 mL of DPPH solution (100 μM in MeOH) and 0.025 mL of sample solution (pure antioxidant or mixtures of an antioxidant and ascorbic acid) were placed and vortexed. The absorbance of this mixture at 515 nm was obtained immediately after vortexing ($A_{515}^{t=0}$). The mixture was left to react at room temperature in the dark, and the absorbance was obtained again after exactly 30 min ($A_{515}^{t=30}$). The antiradical activity was calculated as % decrease in A515 as follows:

$$\%\Delta A_{515} = \left(\frac{A_{515}^{t=0} - A_{515}^{t=30}}{A_{515}^{t=0}}\right) \times 100 \qquad (1)$$

Determination of Mixture Effect (ME)

According to Peyrat-Maillard *et al.*, 2003, as mixture effect (ME) of two antioxidants could be defined the experimental value, divided by the calculated value, which is the sum of the effects of the two antioxidants obtained individually. If this ratio is > 1, then it can be said that synergism is observed, whereas a ratio < 1 would reveal antagonism. In the case of the A_{AR} assay, this could be mathematically expressed as:

$$ME = \frac{\%\Delta A_{515}^{AA+PA}}{\%\Delta A_{515}^{AA} + \%\Delta A_{515}^{PA}} \qquad (2)$$

Where PA, is the polyphenolic antioxidant.

Implementation of linear regression analyses

For single-antioxidant solutions, the response ($\%\Delta A_{515}$) was plotted against concentration of pure antioxidants (Figures 2 and 3), and the linear equation, as well as the square correlation coefficient (R^2) drawn from simple linear regression analyses were calculated (Table 1). For the solutions of antioxidant mixtures, responses were plotted against the total antioxidant concentration of the solutions, which consisted of equimolar amounts of ascorbic acid and either ferulic acid, quercetin or hesperetin (Table 2). In all cases, the concentration ranges used were those within which linearity was best maintained (R^2 > 0.99).

Table 1. Concentration ranges and statistical data generated after implementing simple linear regression of $\%\Delta A_{515}$ against concentration of single-antioxidant solutions

Compound	Concentration range (mM)	Equation	R^2
Ascorbic acid	0.10 – 1.60	y = 54.77x + 0.79	0.995
Quercetin	0.05 – 0.60	y = 116.21x + 3.79	0.999
Ferulic acid	0.10 – 1.60	y = 30.77x + 1.13	0.999
Hesperetin	0.05 – 0.80	y = 10.99x + 5.31	0.996

Table 2. Concentration ranges and statistical data generated after implementing simple linear regression of $\%\Delta A_{515}$ against concentration of solutions of antioxidant mixtures

Compound	Concentration range (mM)	Equation	R^2
AA + FA	0.10 – 3.20	y = 18.83x + 2.03	0.999
AA + HP	0.10 – 3.20	y = 14.47x + 2.08	0.999
AA + QT	0.10 – 1.20	y = 45.74x + 1.78	0.999

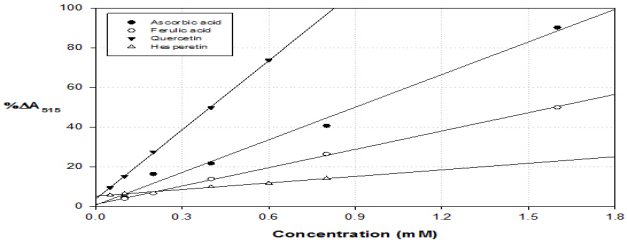

Figure 2. Plots illustrating the implementation of linear regression between the response ($\%\Delta A_{515}$) and the concentration of single-antioxidant solutions

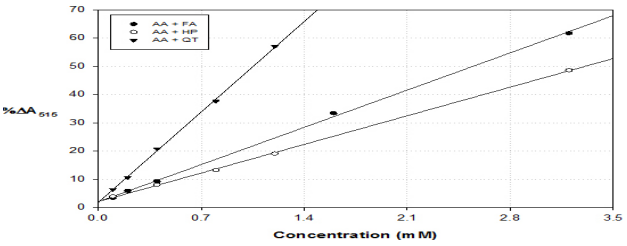

Figure 3. Plots illustrating the implementation of linear regression between the response ($\%\Delta A_{515}$) and the concentration of solutions of antioxidant mixtures. Values on x – axis represent the total concentration of the equimolar amounts of the antioxidants in the solution (e.g. concentration of 0.5 mM corresponds to a solution containing 0.25 mM AA and 0.25 mM of a polyphenolic antioxidant).

Statistical analyses

All determinations were carried out at least in triplicate and values were averaged. For all statistics, SigmaPlot™ 11 and Microsoft Excel™ were used.

Results and Discussion

In studying the interactions of the antioxidants chosen, the first step was to rank them with regard to their antiradical potency, using the assay determining the A_{AR}. As can be seen in Figure 2, and taking into account the slopes of the linear relationships produced (Table 1), in both assays the order was as follows:

Qt > AA > FA > Hp

For reasons of obtaining a picture of the relationships underlying interactions of the polyphenolic antioxidants with AA, a hypothesis was set up. It could be calculated that a mixture of 0.5 mM AA and 0.5 mM Qt (total antioxidant concentration 1 mM) would give, based on the corresponding equation in Table 2, a $\%\Delta A_{515}$ = 47.5. Nevertheless, based on the equations for AA and Qt in Table 1, 0.5 mM AA would give $\%\Delta A_{515}$ = 28.18 and 0.5 mM Qt would give $\%\Delta A_{515}$ = 61.90; in total $\%\Delta A_{515}$ =

90.1. Therefore ME = 0.53, which clearly points to an antagonistic affect. Likewise, the corresponding ME values for the mixtures FA / AA and Hp / AA would be 0.47 and 0.42. Thus it can be argued that the interactions in all mixtures tested resulted in antagonism.

It has been proposed that in binary mixtures of antioxidants coupled reactions of regeneration could be taken into consideration to explain the ME observed (Peyrat-Maillard *et al*., 2003). In this regard, the results anticipated could include (i) a synergistic effect if the less efficient antioxidant regenerates the more efficient one, (ii) an antagonistic effect if the more efficient molecule regenerates the less efficient one or (iii) no ME if both antioxidants have the same efficiency. Thus in a given antioxidant assay it is important to rank the substances used, to obtain an order of efficiency, as assumptions on their interactions are based on their relative antioxidant strength.

This theory can be rationalised by the concession that, as mentioned above, the more efficient molecule regenerates the less efficient one. Taking into account the oxidation potentials, antagonism can be considered as the regeneration of a compound with higher oxidation potential, to the expense of another with lower oxidation potential, by donating H atoms. Hence regeneration of AA by Qt and AA by Hp resulting in antagonism could occur if Qt had lower oxidation potential than AA. A strong background to support such a theory are studies carried out with cyclic voltametry, where it has been demonstrated that Qt possess indeed lower oxidation potential (91 mV) than AA (127 mV), whereas FA (350 mV) and Hp (434 mV) significantly higher (Roleira *et al*., 2010; Abou Samra *et al*., 2011).

Thus indeed in all three combinations tested, the data obtained provided sound evidence that the most efficient antioxidant is probably consumed at the expense of the regeneration of the less efficient one, hence the manifestation of antagonistic phenomena.

Conclusions

A simple linear regression approach disclosed that Qt, FA and Hp exhibit antagonism when combined with AA. This fact was ascribed to the regenerating ability of the most efficient antioxidant at the expense of the less efficient one. The evidence emerged from the investigations indicated that antagonism might be manifested because of regeneration of a compound with higher oxidation potential, to the expense of another with lower oxidation potential, by donating H atoms. This is particularly crucial for antioxidants that are destined to be added in food matrices, where maximal antioxidant protection is always sought.

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