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Antiradical action of flavonoid–ascorbate mixtures

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ABSTRACT

Flavonoids and ascorbic acid are antioxidants usually consumed together in foods, taking this into account, the antiradical capacity of mixtures of ascorbic acid with some representative flavonoids (flavanones, chalcones and flavonols) against the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) was evaluated. Antiradical capacities of naringin, naringenin, hesperidin, hesperetin, naringin chalcone, naringin dihydrochalcone, rutin and quercetin were measured alone and in different combinations with ascorbic acid. Experimental and theoretical values of antiradical activities for these mixtures as well as the values obtained in sequential reactions were compared in order to determine synergistic or antagonistic effects. Among the different ascorbic acid–flavonoid combinations analyzed only the mixture with naringin or quercetin exhibited synergistic effects, the mixture activity being 33 and 18% higher than the theoretical value, respectively. On the contrary, antagonistic effects were found in the cases of rutin, hesperidin, naringin chalcone, naringin dihydrochalcone and naringenin. Moreover, only hesperetin showed an additive effect. These behaviours were ascribed to the regeneration of the oxidized species on the basis of the structural features of each flavonoid and their different reactivity against DPPH• These results show that the antiradical activity can be modified by the interaction among the mixture constituents and this may be considered to improve antioxidant formulations.

1. Introduction

Epidemiological studies have shown that a diet rich in fruit and vegetables has an important role in reducing the incidence of serious diseases. These preventive actions have been related to the presence of bioactive substances such as vitamins, carotenoids and polyphenols (Hertog et al., 1995). These phytochemicals have as a common feature an active role as antioxidants (AO). Moreover, the use of these purified substances obtained from natural sources as nutraceuticals is world-wide spread.

The antioxidant activities of the crude extracts as well as the isolated compounds have extensively been studied during the last decade. However, in most of the cases, the efficiency of single compounds is different from the action of the AO occurring in a complex mixture as in foods. Several studies have shown that the interactions among these compounds are able to produce variations in the total antioxidant activity (Liu, Shi, Colina Ibarra, Kakuda, & Jun Xue, 2008; Pinelo, Manzocco, Nuñez, & Nicoli, 2004).

The interactions between AO have been studied combining different compounds as α-tocopherol, ascorbate, β-carotene and polyphenols (Mukai, Mitani, Ohara, & Nagaoka, 2005; Zhou et al., 2000; Zhou, Wu, Yang, & Liu, 2005; Zhu, Huang, & Chen, 2000). This phenomenon which can take place in homogeneous as well as in organized media has frequently been ascribed to the capacity of a moderately active AO to regenerate a more potent one by electron or hydrogen transfers to the corresponding radical produced from the latter.

Among phenolic compounds, flavonoids (FlOH) belong to a large group widely distributed in edible plants. The global interest in FlOH is due to their antioxidant properties and bioactivity (Burda & Oleszek, 2001; Pannala, Chan, O'Brien, & Rice-Evans, 2001; Pietta, 2000). The interaction between some FlOH and ascorbic acid (AA) has received a great attention by researchers (Bors, Michel, & Shikora, 1995) as an analogy of that previously observed between ascorbate ion and tocopherol (Chen, 1989). Moreover, the synergistic effects between some FlOH (eriocitrin and eriodictyol) and vitamin C have been mentioned in a patent of an AO agent used in food or drinks (Fukumoto, Tsuruhami, & Mori, 2007). Some FlOH have been described as AA protectors or co-antioxidants (Skaper, Fabris, Ferrari, Carbonare, & Leon, 1997); while the reverse protective effect can also be found in the literature (Kaack & Austed, 1998; Miller & Rice-Evans, 1997). However, despite clearly knowing the antiradical mechanisms of the individual constituents against DPPH radical (DPPH•), there are still controversies about the behaviour of

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the mixtures and information about their possible interactions between FlOH and other food constituent as AA is still scarce.

In order to study this controversial behaviour, a series of FlOH and their derivatives were selected for this study as follows: a glycosilated flavonol rutin and its aglycone quercetin, the glycosilated flavanones naringin and hesperidin, their respective aglycones, naringenin and hesperetin, as well as two naringin derivatives, the chalcone and the dihydrochalcone. The flavonols selected for this study, quercetin and rutin, are ubiquitous in plants (Herrmann, 1976) and present antioxidant as well as therapeutic properties (Yang, Guo, & Yuan, 2008); therefore, they are extensively used in the food and cosmetic industry (Palmer, Ohta, Watanabe, & Suzuki, 2002). The flavanones have a more restricted distribution and the major source of this subgroup of FlOH is citrus species. Hesperidin is known to reduce vascular permeability (Garg, Garg, Zaneveld, & Singla, 2001), naringin acts as antimutagenic agent as well as an AO protecting against lipid peroxidation (Chen, Zheng, Jia, & Ju, 1990). The naringin derivatives were chosen due to the industrial interest because the remarkably sweet taste of the dihydrochalcone gives it a potential use as a sweetener and the chalcone being its synthetic precursor. All the selected FlOH have a great interest for pharmacological and food industry (Benavente-García, Castillo, Marin, Ortuño, & Del Río, 1997) and they coexist with AA in many natural foods and formulations. The assesment of the interactions between AO in mixtures would allow designing much more effective dietary supplements or nutraceuticals.

On the basis of these considerations the aim of this paper was to study the antiradical activity of a series of combinations of these FlOH with AA against DPPH• in order to compare them to the individual behaviour searching for synergistic, additive or antagonistic effects. Antiradical activities were also measured in sequential reactions to evaluate the regeneration capabilities between the mixture constituents.

2. Materials and methods

2.1. Materials

All reagents used for this study were pro-analysis grade. Hesperidin (Hesp), quercetin (Quer), rutin (Rut), naringin (Nar) and ascorbic acid (AA) were provided by Parafarm, Buenos Aires, Argentina, and used without further purification. Aglycones naringenin (Ng) and hesperetin (Ht) were synthesized following the procedure of acid hydrolysis of their corresponding glicosylated flavonoids described by Mabry, Markham, and Thomas (1970, pp. 35–230). Naringin chalcone (NCh) was obtained according to Shimokoriyama (1957) method with minor modifications as described in a previous report (González, Nazareno, & Borsarelli, 2002). Naringin dihydrochalcone (NDCh) was prepared by catalytic hydrogenation of NCh ($P_{H2} = 202.6$ kPa using PtO (IV) as catalyser). Finally, the hydrogenation product was purified by re-crystallization and its purity was checked by HPLC, using a Konik Liquid Chromatograph, model KNK 500 Series A, equipped with a Konik ODS-2 column (250 × 46 mm i.d.; 5 μm) operating at 25 °C. The mobile phase was water–acetonitrile–acetic acid (79.5:20:0.5, v/v) at a flow rate of 1.2 mL min^{-1}. The detection was performed at 284 nm and 366 nm using a Konik UV–Vis 200 detector). The purity of the NCh and NDCh synthetized were higher than 98% after purification procedures. Radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) was purchased from Sigma–Aldrich, Buenos Aires, Argentina.

2.2. DPPH free radical-scavenging capacity assay

Experiments were performed by measuring the consumption of the DPPH• by the action of individual compounds (FlOH or AA) as well as their mixtures by UV–vis spectrophotometry (Brand-Williams, Cuvelier, & Berset, 1995).

The typical procedure consists of the addition of a methanolic solution aliquot of the AO solution to a cuvette containing 3 ml of c.a. 60 μmol/L DPPH• solution. Radical solution absorbance was adjusted to 0.75 AU at 517 nm. At least four different concentrations were tested for each AO, according to its reactivity in order to consume between 20 and 80% in respect of the initial radical concentration. Stock solutions of the different AO were daily prepared in methanol and 25–100 μL aliquots were taken and diluted to obtain final concentrations in the following ranges (μmol/L): Quer: 3.8–65, Rut: 4.1–67, Ht: 4.6–312, Hesp: 5.3–400, Ng: 6.0–672, Nar: 370–4600, NCh: 6.2–50.2, NDCh: 16.8–69.1 and AA 2.90–27.6. The adequate concentration of each compound in the mixture was selected depending on the individual ability in order to reach significant radical consumption. Each combined solutions of AA (2.9 μmol/L) with FlOH were prepared adjusting to a final concentration (μmol/L) of: 3.8 for Quer, 4.1 for Rut, 312 for Ht, 400 for Hesp, 25.9 for NCh, 29.2 for NDCh, 672 for Ng and 606 for Nar. The reaction progress was followed by measuring the absorbance at 517 nm in cycles for different time periods in a range of 1–180 min depending on the reactivities of the compounds analyzed. All assays were performed by triplicate at room temperature (27 ± 1 °C).

2.3. Calculation of ARA parameters

The percentage of the DPPH• fraction consumed by the samples, expressed as antiradical activity (ARA), was calculated according to the Eq. (1) suggested by Burda and Oleszek (2001):

$$\%ARA = 100 \times [1 - (A_{SS}/A_0)] \quad (1)$$

Where A_{SS} is the absorbance of the solution in the steady state (SS) and A_0 is the absorbance of DPPH• solution before adding the AO. Absorbance decrease by dilution effect was corrected. The A_{SS} value was estimated by exponential fitting of kinetic curves using Microcal Origin 7.0 ® software.

In the case of the single compounds, from these %ARA values obtained, other parameters were calculated, as EC_{50}, corresponding to the amount of antioxidant necessary to decrease the initial DPPH• concentration by 50%, antiradical power (ARP) defined as the inverse of the EC_{50}, and the stoichiometric factor (n) as the number of free radicals consumed per molecule of additive calculated according to Eq. (2) (Brand-Williams et al., 1995).

$$n = 1/(EC_{50} \times 2) \quad (2)$$

2.4. Synergistic effects and sequential reactions

In the case of the mixtures, possible synergistic effects (SE) were evaluated based on the ratio of the experimental and theoretical scavenging activity values using Eq. (3).

$$SE = ESA/TSA \quad (3)$$

Where ESA (experimental scavenging activity) corresponds to the % ARA value determined in the prepared *in situ* mixture and TSA (theoretical scavenging activity) was calculated as the sum of the individual %ARA values for the combined compounds shown in Eq. (4) (Liu et al., 2008).

$$TSA = \%ARA_{FlOH} + \%ARA_{AA} \quad (4)$$

Synergistic effects correspond to SE values higher than 1 ($SE > 1$) while antagonistic effects SE correspond to those lower than 1. In order to consider an effect relevant enough, the difference

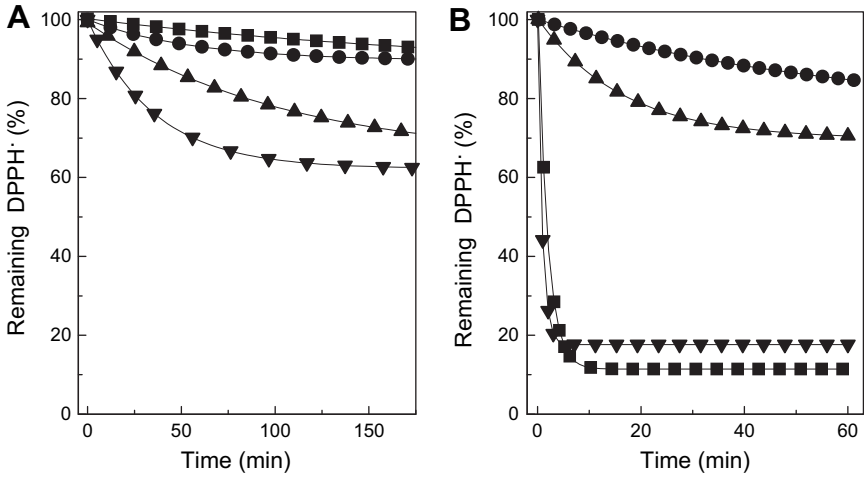

Fig. 1. Kinetic profiles of DPPH• consumption by addition of: (A) Flavanone 50 μmol/L solutions (flavonoids/DPPH• mole ratio = 1). ■ Nar: naringin, ● Ng: naringenin, ▲ Hesp: hesperidin, ▼ Ht: hesperetin, and (B) Chalcone and flavonol 17 μmol/L solutions (flavonoids/DPPH• mole ratio = 0.3). ● NDCh: naringin dihydrochalcone, ▲ NCh: naringin chalcone, ■ Rut: rutin and ▼ Quer: quercetin.

between theoretical and experimental values was statistically evaluated.

To evaluate the reaction pathway sequence, a modification of the previously mentioned procedure was incorporated. The reaction was carried out in two steps with a first addition of ascorbate (Asc^-) solution and, after the SS was reached, a subsequent addition of FlOH solution was done. This experiment was also performed in the inverse order. These results were also calculated as SE according to Eq. (3) and named SQ1SE and SQ2SE, respectively.

2.5. Statistical analysis

All the experiments were performed in triplicate. Student's *t*-test was used for comparison between means (ESA and TSA). A difference was considered statistically significant at $P < 0.05$.

3. Results and discussions

3.1. Antiradical activity of single compounds

The DPPH• bleaching method is a fast and sensitive measurement of the intrinsical ability of pure compounds or complex mixtures to scavenge radicals in homogeneous systems. It is one of the most frequently chosen by its simplicity. It requires small amounts of sample and can be applied to both lipophilic and hydrophilic substances (Kulisic, Radonic, Katalinic, & Milos, 2004).

Ascorbic acid concentration in a typical citrus juice is 2.7 mmol/L; however, the experiments were performed in a range of 2.9–30 μmol AA/L (3 orders of magnitude lower than the natural level). The concentrations chosen for this study were strongly limited by the methodology because if the reactivity of the antioxidant is high, only very diluted solutions can be measured. Therefore, it is not possible to evaluate using the DPPH• method the AA antiradical ability in physiological levels. On the contrary, if the reactivity of the antioxidant is low, higher concentration is needed to assess its behaviour against DPPH• as in the case of flavanones. The concentration of Hesp in a typical orange juice is 82 μmol/L, while Nar concentration in grapefruit juice is about 6 μmol/L.

Fig. 1 shows the kinetic profiles of the radical consumption obtained. Different behaviours toward DPPH• were found for the different FlOH analyzed with a wide range of reactivities from extremely slow to fast kinetic reactions.

Slow DPPH• bleaching rates were observed for the flavanones Nar, Ng, Ht and Hesp (Fig. 1A) while fast rates were found for the flavonols Quer and Rut (Fig. 1B). These results are in good agreement with those previously reported in the literature, which indicate the distinct reactivity toward DPPH• of flavonols compared with flavanones. Lindberg Madsen, Andersen, Jørgensen, and Skibsted (2000) measured the half-life times ($t_{1/2}$) in pseudo first-order conditions for DPPH• consumption reactions by several dietary FlOH. They reported a $t_{1/2}$ higher than 1000 for Nar, although for Ht, $t_{1/2}$ was about 74 s and for Quer it was 1.1 s. Besides, Butković, Klasinc, and Bors (2004) determined a second-order rate constant for Quer 119 times higher than that of Ng for the DPPH• scavenging reaction.

However, in order to compare the antiradical efficiency of FlOH, as pure compounds and the possible effect of their mixtures, the final consumption of the radical should be considered rather than the scavenging reaction rate. This efficiency was calculated and expressed as different parameters as *n*, ARP, and EC_{50}; Table 1 shows the obtained values for the compounds studied.

According to these results, the flavonol Quer and its glycosilated derivative, Rut, presented the highest stoichiometric factors ($n > 3$) and were the most powerful scavengers among the studied FlOH, followed by AA whose calculated *n* was 1.76. This indicates that 1 mol of AA is able to scavenge almost 2 mol of radical. In the cases of NCh and NDCh, intermediate *n* were found, with values close to 1. In the case of flavanones, Hesp, Ht, Nar and Ng very low *n* values were found. These results are in good agreement with structure–activity relationship reports (Pannala et al., 2001; Rice-Evans, Miller, & Paganga, 1996).

Table 1
Antiradical activity parameters. Stoichiometric factor *n*, antiradical power ARP and EC_{50} value of some flavonoids toward DPPH• in methanolic solution.

Compounds	*n*	ARP	EC_{50}
Rutin	3.85 ± 0.20	7.69 ± 0.50	0.13 ± 0.01
Quercetin	3.13 ± 0.26	6.25 ± 0.60	0.16 ± 0.01
Ascorbate	1.79 ± 0.06	3.57 ± 0.13	0.28 ± 0.01
Naringin chalcone	1.06 ± 0.12	2.13 ± 0.23	0.47 ± 0.05
Naringin dihydrochalcone	0.78 ± 0.05	1.56 ± 0.07	0.64 ± 0.04
Hesperidin	0.38 ± 0.05	0.76 ± 0.10	1.32 ± 0.17
Hesperetin	0.31 ± 0.03	0.62 ± 0.06	1.61 ± 0.16
Naringenin	0.13 ± 0.02	0.26 ± 0.03	3.85 ± 0.50
Naringin	0.02 ± 0.01	0.04 ± 0.02	25.0 ± 12.5

The reaction of the FlOH with DPPH• generates a relatively stable aroxyl radical (FlO•) represented in Eq. (5a) (Pietta, 2000). The FlOH ability to scavenge free radicals is related to the stability of FlO• and therefore, it depends on the number and arrangement of active functional groups present in its molecule (Bors, Michel, & Stettmaier, 2001).

$$\mathrm{FlOH} + \mathrm{DPPH}^{\bullet} \rightarrow \mathrm{FlO}^{\bullet} + \mathrm{DPPH} - \mathrm{H} \quad (5a)$$

In the particular case of a FlOH with a catechol group, it undergoes a subsequent further reaction by scavenging an additional DPPH• and leading to the formation of a quinone (Fl=O) according to Eq. (5b) Therefore, their n values should be close to 2.

$$\mathrm{FlO}^{\bullet} + \mathrm{DPPH}^{\bullet} \rightarrow \mathrm{Fl} = \mathrm{O} + \mathrm{DPPH} - \mathrm{H} \quad (5b)$$

Some authors ascribed the n value higher than 3 to the fast disproportion of radicals FlO• into FlOH and a Fl=O. Besides, in alcoholic solvents, subsequent additions of solvent molecules to the Fl=O can take place leading to adducts which are able to undergo further scavenging reactions toward DPPH• (Dangles, Fargeix, & Dufour, 1999; Goupy, Dufour, Loonis, & Dangles, 2003).

The flavanones Nar and Ng present only one hydroxyl group in B ring and a C2-C3 single bond. They show a low reactivity due to the slow formation of FlO• and the reaction requires high FlOH concentrations to consume more than 50% of DPPH• (about 25 mol of Nar/mole of DPPH•). The increase of the activity observed for Hesp and its aglycone Ht is an evidence of the effect of a methoxyl group in C4′ in the ring B which stabilizes the FlO• due to the proximity to the C3′hydroxyl group (Hudson & Lewis, 1983). NCh, the structural open-chain isomer of Nar, presents higher reactivity associated to the presence of a C2-C3 double bond which favours the conjugation between the aromatic rings beside the presence of an additional hydroxyl group in C2′ susceptible of donating hydrogen atoms. The decrease of activity for NDCh is a consequence of the loss of the C2-C3 double bond positive effect.

Quer exerts high efficiency and reactivity to scavenge free radicals as Fig. 1.B shows. This is a flavonol whose chemical structure present the most active antiradical features, that is (a) an *o*-dihydroxyl system in ring B that stabilizes the FlO• formed by an intramolecular hydrogen bond, (b) a double bond C2-C3 that allows the conjugation between the B and C rings and (c) an hydroxyl in C3 that enhances the coplanarity diminishing the torsion angle of ring B with the rest of the molecule (Chaillou & Nazareno, 2006). Its glycoside with C3-hydroxyl group blocked, Rut presented also high efficiency and reactivity. However, the role of a free C3-hydroxyl group in the antioxidant action has frequently been discussed in the literature taking Quer and Rut as a model pair to evaluate this single effect. However, some discrepancies about Quer and Rut ARA were found in the literature. It is generally assumed that aglycones are more active than their glycosides, in this sense, Sánchez-Moreno, Larrauri, and Saura-Calixto (1998) found for Quer a lower EC_{50} value than that of Rut. However, Burda and Oleszek (2001) observed no significant differences between the ARA of these compounds. Moreover, there are also some reports indicating a higher ARA for Rut than for Quer (Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2009; Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007). Besides, further studies about the scavenging ability against other radicals as superoxide anion agree with this last trend (Huguet, Máñez, & Alcaraz, 1990; Yuting, Rongliang, Zhongjian, & Yong, 1990). As Table 1 shows, EC_{50} values were 0.16 mol Quer/mole DPPH• and 0.13 mol Rut/mole DPPH• indicating the highest antiradical activity for Rut among the FlOH studied in this system.

On the other hand, when AA solutions were prepared, the main species in the solution bulk was its monoanion ascorbate since its pK_a is equal to 4.74. Ascorbate (Asc^-) showed a very fast kinetic behaviour toward DPPH&middot. This reaction took less than 30 s to reach the SS and produced dehydroascorbate (Brand-Williams et al., 1995) as the oxidized form of AA Eqs. (6a) and (6b).

$$\mathrm{Asc}^- + \mathrm{DPPH}^{\bullet} \rightarrow \mathrm{SAsc}^{\bullet} + \mathrm{DPPH} - \mathrm{H} \quad (6a)$$

$$\mathrm{SAsc}^{\bullet} + \mathrm{DPPH}^{\bullet} \rightarrow \mathrm{DHA} + \mathrm{DPPH} - \mathrm{H} \quad (6b)$$

Where SAsc• and DHA denote semiascorbyl radical and dehydroascorbate, respectively.

3.2. Antiradical activity of mixtures

Table 2 shows the DPPH• consumption percentages according to Eq. (1) for the single compounds and for their mixtures with Asc^-. Concerning the results obtained in the FlOH-Asc^- mixtures, three different situations were observed: I) an antagonistic effect in the mixtures with Hesp, Rut, NCh, NDCh and Ng, respectively; II) an additive effect in the mixtures with Ht and III) a synergistic effect in the mixtures with Quer and Nar respectively. These different behaviours can be explained in most of cases on the basis of chemical nature and reactivity of the considered compounds (Pinelo et al., 2004). Although, it is not clear the reason for the different behaviour observed between Hesp and Ht or Nar and Ng since the glucosilated hydroxyl is located in C7 and this group is not the determining factor for the aroxyl radical stability or redox potential for the mentioned molecules. In general terms, synergistic or antagonistic effects arise when the mixture of two or more AO react among them instead of reacting with DPPH•. Synergism is observed in the interaction between two AO when the more effective compound is regenerated by the less active one. In order to get a better understanding of the interaction between the components of the mixture, it should be established the nature of the main species involved in each reaction.

In these FlOH-Asc^- mixtures, several reactions can take place depending on the different species present in the reaction medium. The reaction of Asc^- and DPPH• was very fast and the steady state was reached in less than 30 s. Therefore, DHA as the oxidized form of Asc^- was the only species present after this time; hence, one of the possible reactions that can occur is shown in Eq. (7) and constitutes one of the possible reactions of SAsc• regeneration.

Table 2
Antiradical activity (ARA) against DPPH• of the single compounds and the mixture of each flavonoid with ascorbate (2.9 μmol/L). TSA: theoretical scavenging activity; ESA: experimental scavenging activity. SE: synergistic effect.

Compounds	%ARA of single compounds	%ARA of flavonoids + ascorbate mixtures		
		TSA[a]	ESA[a]	SE[b]
Quercetin	32.1 ± 0.2	40.3 ± 0.3	47.4 ± 0.5	1.18 ± 0.02*
Rutin	14.6 ± 0.1	22.8 ± 0.1	20.1 ± 0.4	0.88 ± 0.03*
Hesperetin	76.0 ± 0.5	84.2 ± 0.6	82.2 ± 0.9	0.98 ± 0.02
Hesperidin	87.9 ± 0.2	96.1 ± 0.3	81.3 ± 0.7	0.85 ± 0.01*
Naringin chalcone	62.1 ± 0.2	70.3 ± 0.3	61.5 ± 0.5	0.87 ± 0.01*
Naringin dihydrochalcone	44.2 ± 0.4	52.4 ± 0.5	42.2 ± 0.3	0.81 ± 0.02*
Naringenin	48.5 ± 0.1	56.7 ± 0.2	47.8 ± 0.1	0.84 ± 0.01*
Naringin	9.8 ± 0.2	18.0 ± 0.3	23.9 ± 0.1	1.33 ± 0.02*
Ascorbate	8.2 ± 0.1	–	–	

[a] Values are the mean ± SD ($n = 3$).
[b] SE > 1: synergistic effect; SE = 1: additive effect, SE < 1: antagonistic effect. Asterisks (*) denote a significant difference between TSA and ESA values (*$P < 0.05$). Concentration values for each compound are described in experimental section.

$$FlOH + DHA \rightarrow FlO^{\bullet} + SAsc^{\bullet} \quad (7)$$

This species is able to go on scavenging one more DPPH• according to Eq. (6b). The regeneration of Asc^- from DHA is a process that occurs *in vivo* via enzymes and NADPH or GSH as cofactors (Rose & Bode, 1993). The regeneration of an AO by another is a process that depends on several factors as the reduction potentials of the couples involved as well as their reactivities, i.e., reactions thermodynamically possible may not be kinetically feasible (Buettner, 1993). In the case of Nar, whose reactivity is very low, there is a greater proportion of FlOH than that of the FlO• in the system in the time scale of these experiments.

The synergism observed for the Nar-Asc^- combination can be attributed to the regeneration of SAsc• from the reaction between DHA and Nar in its reduced form as shown in Eq. (7). There is no information about Nar reduction potential in this experimental condition but taking into account that its ring B has only one hydroxyl group, it can be assumed that, the value may be close to a phenol reduction potential of 900 mV according to Buettner (1993) and Jovanovic, Steenken, Tosic, Marjanovic, and Simic (1994). DHA reduction corresponds to −174 mV and so equation (7) should be thermodynamically favoured. However, the reaction feasibility not only depends on the redox potential but also on the reactivity of the different species and their concentration in the reaction medium.

In the case of a high reactivity FlOH, its oxidation products may coexist with intermediate oxidation products of Asc^-, so the reaction shown in Eq. (8) is plausible. For example for Quer, both, the rate and the efficiency to scavenge radicals are higher than those of the flavanone Nar and the reactivity is close to Asc^-. Therefore, when mixed Quer, Asc^- and DPPH•, the main species coexisting in solution with SAsc• or DHA are FlO• radicals and Fl=O.

$$Fl = O + SAsc^{\bullet} \rightleftarrows FlO^{\bullet} + DHA \quad (8)$$

The reaction in Eq. (8) is reversible according to Bors et al. (1995) report, thus, it could explain the synergism observed since the predominant direction of this step determines if the regeneration of the FlO• Eq. (8) or SAsc• Eq. (8) radical will take place.

In the case of Quer, both, the rate and the efficiency to scavenge radicals are higher than those of the flavanone Nar. When Quer and Asc^- are added together to DPPH•, FlO• and SAsc• as well as Fl=O may coexist as oxidized intermediates. Synergistic effects between Quer and Asc^- have previously been investigated in different systems. Takahama (1986) demonstrated that FlOH intermediates proposed as an *ortho*-quinone derivative formed in the oxidation induced by horseradish peroxidase-H_2O_2 can also be reduced by Asc^-. Moreover, Sorata, Takahama, and Kimura (1988) reported the promoter effect of Asc^- in the inhibition of the photosensitized hemolysis in human erythrocytes by Querc suggesting that this effect can be ascribed to the regeneration of Quer from its FlO• by Asc^-. Besides, Jan, Takahama, and Kimura (1991) found that Quer antioxidant activity against α-tocopherol photooxidation is improved in Asc^- presence. Bors et al. (1995) studied in pulsed radiolysis experiments the interaction between FlOH and Asc^- and reported their redox potential. From these data, they concluded that the catecholic compounds have an oxidation potential higher than Asc^-, and therefore, they are able to oxidize SAsc• and thus, to be reduced to and intermediate reduction product.

3.3. Antiradical activity of mixtures: sequential reactions

Sequential experiments were carried out to validate the mentioned hypothesis and assess possible interactions between the species present in the system. These experiments were made in two stages and the consumption of DPPH• was monitored during the whole process. In a typical assay, the first AO was added and the system was allowed to react until the SS was reached. Thus, after reacting with DPPH•, the oxidized form of this first AO was produced and a subsequent addition of the second AO was done. The reactions carried out in several stages involved the interaction between species different from those existing in the mixtures prepared *in situ* and allowed getting a better understanding of the reactions that could take place in this system. Two different situations were analyzed as follows: In case 1, namely SQ1SE, after Asc^- reaction with DPPH• according to Eqs. (6a) and (6b), the complete DHA formation took place. If FlOH was added afterward, its reaction with the remaining DPPH• was expected Eqs. (5a) and (5b) as well as a reaction with the DHA formed would be also feasible (Eq. (7)). In case 2, namely SQ2SE, the FlOH was added to the DPPH• solution and, after reaching the SS, Asc^- was added. After FlOH and DPPH• reaction, the species present in solution at the SS mainly corresponded to FlO•, Fl=O depending on the nature of the FlOH (Eqs. (5a) and (5b)), the remaining DPPH• and its reduced form. If Asc^- was added at this stage, the possible subsequent reactions were not only with the DPPH•, according to Eq. (5a) and (5b), but also with FlO• (Eq. (9)) or the Fl=O (Eq. (10)) depending on the nature of the FlOH.

$$FlO^{\bullet} + Asc^- \rightleftarrows FlOH + SAsc^{\bullet} \quad (9)$$

$$Fl = O + Asc^- \rightleftarrows FlO^{\bullet} + SAsc^{\bullet} \quad (10)$$

Fig. 2 shows SE values for *in situ* FlOH-Asc^- mixtures compared with the results obtained in sequential reactions (SQ1SE and SQ2SE). For most of the FlOH studied (Rut, Ht, NCh and NDCh), SE values were similar to those obtained in the SQ1SE experiments although they were different from those found in SQ2SE ones. This clearly indicates that different species were involved depending on the order of reagent additions. Rut, Ht and NCh presented SE values lower than 1 although a SQ2SE values > 1 indicating a favourable interaction between the oxidized form of FlOH (FlO• or Fl=O) and Asc^- according to Eqs. (9) and (10). By contrast, no significant

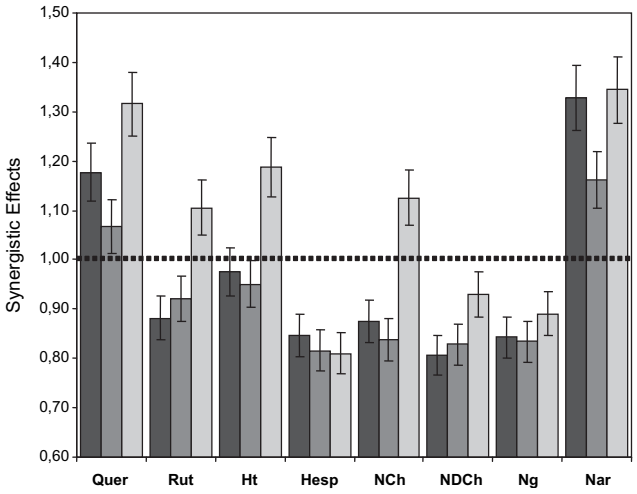

Fig. 2. Comparison of synergistic effects for *in situ* and sequential mixtures of ascorbate and a flavonoid (**Quer:** quercetin, **Rut:** rutin, **Ht:** hesperetin, **Hesp:** hesperidin, **NCh:** naringin chalcone, **NDCh:** naringin dihydrochalcone, **Ng:** naringenin, **Nar:** naringin). ■ SE values as the ratio of experimental and theoretical for *in situ* mixtures. ■ Synergistic effect for sequential mixtures with first addition of ascorbate solution (SQ1SE). ■ Synergistic effect for sequential mixtures with first addition of flavonoid solution (SQ2SE).

differences were observed for the flavanones Hesp and Ng among SE and sequential reactions values being lower than 1. Quer and Nar were the only cases where SE, SQ1SE an SQ2SE were all higher than 1. Nar presented an SE value similar to SQ2SE but different to SQ1SE. Due to its low reactivity, a negligible FlO• concentration was formed even in SQ2SE experiments and thus, the flavanone was the main species in the bulk solution and its interaction with Asc^- was the responsible of the positive effect. The different behaviour in SQ1SE assays was due to the complete formation of DHA and therefore the interaction Nar- Asc^- was not possible in this case. For Quer, the decreasing order SQ2SE > SE > SQ1SE indicated that the most favourable interaction occurred between the Quer oxidized forms (FlO• or Fl=O) and Asc^-. In most of the cases, the radical consumptions in these step by step experiments were higher than those observed when the mixtures were prepared by adding together both AO. This positive effect shown in Fig. 2, indicates that the interaction between the oxidized species of FlOH and Asc^- is favourable, excepted for Hesp and Ng whose consumptions are similar to that of *in situ* mixture. Sequential additions showed that the reaction was mainly controlled by the AO reactivity, the scavenging reaction rate against DPPH• and the reduction potential of the different species involved in the process.

As a conclusion, a significant synergistic effect was exclusively observed in this study for the flavanone Nar and the flavonol Quer when combined with Asc^-. On the contrary, antagonistic effects were found in the cases of Rut, Hesp, NCh, NDCh and Ng. Moreover, only Ht showed an additive effect. These results demonstrated that the ARA may be modified by the interaction among the mixture constituents and this may be taken into account to improve AO formulations.

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Abbreviations

AA	ascorbic acid
AO	antioxidant
ARA	antiradical activity
Asc^-	ascorbate anion
DHA	dehydroascorbic acid
DPPH•	2,2-diphenyl-1-picrylhydrazyl radical
ESA	experimental scavenging activity of mixture
FlO	flavonoid quinone
FlO•	aroxyl radical
FlOH	flavonoid
Hesp	hesperidin
Ht	hesperetin
Nar	naringin
NCh	naringin chalcone
NDCh	naringin dihydrochalcone
Ng	naringenin
Quer	quercetin
Rut	rutin
SAsc•	semiascorbyl radical
SE	synergistic effect
SQ1SE	synergistic effect for sequential mixtures with first addition of Asc^-
SQ2SE	synergistic effect for sequential mixtures with first addition of FlOH
SS	steady state
TSA	theoretical scavenging activity of mixture
UV-vis	ultraviolet- visible

References

Benavente-García, O., Castillo, J., Marin, F., Ortuño, A., & Del Río, J. (1997). Uses and properties of citrus flavonoids. *Journal of Agricultural and Food Chemistry, 45*(12), 4505–4515.

Bors, W., Michel, C., & Shikora, S. (1995). Interaction of flavonoids with ascorbate and determination of their univalent redox potentials: a pulse radiolysis study. *Free Radical Biology & Medicine, 19*, 45–52.

Bors, W., Michel, C., & Stettmaier, K. (2001). Structure–activity relationships governing antioxidant capacities of plant polyphenols. *Methods in Enzymology, 335*, 166–180.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Lebensmittel-Wissenschaft und Technologie, 28*, 25–30.

Buettner, G. R. (1993). The pecking order of free radicals and antioxidants: lipid peroxidation, α-tocopherol, and ascorbate. *Archives of Biochemistry and Biophysics, 300*, 535–543.

Burda, S., & Oleszek, W. (2001). Antioxidant and antiradical activities of flavonoids. *Journal of Agricultural and Food Chemistry, 49*, 2774–2779.

Butković, V., Klasinc, L., & Bors, W. (2004). Kinetic study of flavonoid reactions with stable radicals. *Journal of Agricultural and Food Chemistry, 52*, 2816–2820.

Chaillou, L. L., & Nazareno, M. (2006). New method to determine antioxidant activity of polyphenols. *Journal of Agricultural and Food Chemistry, 54*, 8397–8402.

Chen, L. H. (1989). Interaction of vitamin E and ascorbic acid. *In Vivo, 3*, 199–210.

Chen, Y. T., Zheng, R. L., Jia, Z. J., & Ju, Y. (1990). Flavonoids as superoxide scavengers and antioxidants. *Free Radical Biology & Medicine, 9*, 19–21.

Dangles, O., Fargeix, G., & Dufour, C. (1999). One-electron oxidation of quercetin and quercetin derivatives in protic and non protic media. *Journal of Chemical Society, Perkin Transactions, 2*, 1387–1395.

Fukumoto, S. K., Tsuruhami, K., & Mori, S. (2007). *Antioxidant material, anti-deterioration agent and food or beverage*. European Patent Application. EP 1 783 193 A1.

Garg, A., Garg, S., Zaneveld, L. J., & Singla, A. K. (2001). Chemistry and pharmacology of the citrus bioflavonoid hesperidin. *Phytotherapy Research, 15*, 655–669.

González, E. A., Nazareno, M. A., & Borsarelli, C. D. (2002). Enthalpy-entropy compensation effect in the chalcone formation from naringin in water-ethanol mixtures. *Journal of Chemical Society, Perkin Transactions, 2*, 2052–2056.

Goupy, P., Dufour, C., Loonis, M., & Dangles, O. (2003). Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical. *Journal of Agricultural and Food Chemistry, 51*, 615–622.

Herrmann, K. (1976). Flavonols and flavones in food plants: a review. *Journal Food Technology, 11*, 433–438.

Hertog, G. L., Kromhout, D., Aravanis, C., Blackburn, H., Buzina, R., & Fidanza, F. (1995). Flavonoid intake and long term risk of coronary heart disease and cancer in the seven country study. *Archives of Internal Medicine, 155*, 381–386.

Hudson, B. J. F., & Lewis, J. L. (1983). Polyhydroxy flavonoids antioxidants for edible oils. Structural criteria for activity. *Food Chemistry, 10*, 47–55.

Huguet, A., Máñez, S., & Alcaraz, M. J. (1990). Superoxide scavenging properties of flavonoids in a non-enzymic system. *Journal of Biosciences, 45*, 19–24, Zeitschrift für Naturforschung. C.

Jan, C. Y., Takahama, U., & Kimura, M. (1991). Inhibition of photooxidation of α-tocopherol by quercetin in human blood cell membranes in the presence of hematoporphyrin as a photosensitizer? *Biochimica et Biophysica Acta, 1086*, 7–14.

Jovanovic, S. V., Steenken, S., Tosic, M., Marjanovic, B., & Simic, M. G. (1994). Flavonoids as antioxidants. *Journal of the American Chemical Society, 116*, 4846–4851.

Kaack, K., & Austed, T. (1998). Interaction of vitamin C and flavonoids in elderberry (*Sambucus nigra L.*) during juice processing. *Plant Foods for Human Nutrition, 52*, 187–198.

Kulisic, T., Radonic, A., Katalinic, V., & Milos, M. (2004). Use of different methods for testing antioxidative activity of oregano essential oil. *Food Chemistry, 85*, 633–640.

Lindberg Madsen, H., Andersen, C. M., Jørgensen, L. V., & Skibsted, L. H. (2000). Radical scavenging by dietary flavonoids. A kinetic study of antioxidant efficiencies. *European Food Research and Technology, 211*, 240–246.

Liu, D., Shi, J., Colina Ibarra, A., Kakuda, Y., & Jun Xue, S. (2008). The scavenging capacity and synergistic effects of lycopene, vitamin E, vitamin C, and β-carotene mixtures on the DPPH free radical. *LWT – Food Science and Technology, 41*, 1344–1349.

Mabry, T., Markham, K., & Thomas, M. (1970). *The systematic identification of flavonoids*. New York, USA: Springer-Verlag.

Miller, N., & Rice-Evans, C. (1997). The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink. *Food Chemistry, 60*, 331–337.

Mukai, K., Mitani, S., Ohara, K., & Nagaoka, S. (2005). Structure–activity relationship of the tocopherol-regeneration reaction by catechins. *Free Radical Biology & Medicine, 38*, 1243–1256.

Palmer, H., Ohta, M., Watanabe, M., & Suzuki, T. (2002). Oxidative stress-induced cellular damage caused by UV and methyl viologen in Euglena gracilis and its suppression with rutin. *Journal of Photochemistry and Photobiology B: Biology, 67*, 116–129.

Pannala, A. S., Chan, T. S., O'Brien, P. J., & Rice-Evans, C. A. (2001). Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics. *Biochemical and Biophysical Research Communications, 282*, 1161–1168.

Pietta, P. G. (2000). Flavonoids as antioxidants. *Journal of Natural Products, 63*, 1035–1042.

Pinelo, M., Manzocco, L., Nuñez, M. J., & Nicoli, M. C. (2004). Interaction among phenols in food fortification: Negative synergism on antioxidant capacity. *Journal of Agricultural and Food Chemistry, 52*, 1177–1180.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure–antioxidant activity relationships of flavonoides and phenolic acids. *Free Radical Biology & Medicine, 20*, 933–956.

Rose, R. C., & Bode, A. M. (1993). Biology of free radical scavengers: an evaluation of ascorbate. *The FASEB Journal, 7*, 1135–1142.

Sánchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1998). A procedure to measure the antiradical efficiency of polyphenols. *Journal of Science Food and Agriculture, 76*, 270–276.

Shimokoriyama, M. (1957). Interconversion of chalcones and flavanones of a phloroglucinol-type structure. *Journal of the American Chemical Society, 79*, 4199–4202.

Skaper, S., Fabris, M., Ferrari, V., Carbonare, M., & Leon, A. (1997). Quercetin protects cutaneous tissue-associated cell types including sensory neurons from oxidative stress induced by glutathione depletion: cooperative effects of ascorbic acid. *Free Radical Biology & Medicine, 22*, 669–678.

Sorata, Y., Takahama, U., & Kimura, M. (1988). Cooperation of quercetin with ascorbate in the protection of photosensitized lysis of human erythrocytes in the presence of hematoporphyrin. *Photochemistry and Photobiology, 48*, 95–199.

Tabart, J., Kevers, C., Pincemail, J., Defraigne, J. O., & Dommes, J. (2009). Comparative antioxidant capacities of phenolic compounds measured by various tests. *Food Chemistry, 113*, 1226–1233.

Takahama, U. (1986). Spectrophotometric study on the oxidation of rutin by horseradish peroxidase and characteristics of the oxidized products. *Biochimica et Biophysica Acta, 832*, 445–451.

Villaño, D., Fernández-Pachón, M. S., Moyá, M. L., Troncoso, A. M., & García-Parrilla, M. C. (2007). Radical scavenging ability of polyphenolic compounds towards DPPH free radical. *Talanta, 71*, 230–235.

Yang, J., Guo, J., & Yuan, J. (2008). In vitro antioxidant properties of rutin. *LWT – Food Science and Technology, 41*, 1060–1066.

Yuting, C., Rongliang, Z., Zhongjian, J., & Yong, J. (1990). Flavonoids as superoxide scavengers and antioxidants. *Free Radical Biology and Medicine, 9*, 19–21.

Zhou, B., Jia, Z. S., Chen, Z. H., Yang, L., Wu, L. M., & Liu, Z. L. (2000). Synergistic antioxidant effect of green tea polyphenols with α-tocopherol on free radical-initiated peroxidation of linoleic acid in micelles. *Journal of Chemical Society, Perkin Transactions, 2*, 785–791.

Zhou, B., Wu, L. M., Yang, L., & Liu, Z. L. (2005). Evidence for α-tocopherol regeneration reaction of green tea polyphenols in SDS micelles. *Free Radical Biology & Medicine, 38*, 78–84.

Zhu, Q. Y., Huang, Y., & Chen, Z. Y. (2000). Interaction between flavonoids and α- tocopherol in human low density lipoprotein. *Journal of Nutritional Biochemistry, 11*, 14–21.