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Interaction among Phenols in Food Fortification: Negative Synergism on Antioxidant Capacity

MANUEL PINELO,*,† LARA MANZOCCO,‡ MARIA JOSÉ NUÑEZ,† AND MARIA CRISTINA NICOLI‡

Escuela Técnica Superior de Ingeniería, Universidad de Santiago de Compostela, Lope Gómez de Marzoa, 15782 Santiago de Compostela, Spain, and Dipartimento di Scienze degli Alimenti, Università degli Studi di Udine, Via Marangoni 97, 33100 Udine, Italy

In this work, a study about the consequences of the interaction among phenol compounds on antioxidant capacity is proposed. The antiradical activity evolution of an ethanol solution containing a mixture of three monophenols (catechin, resveratrol, and quercetin) was compared with the trend followed by each single phenol at three different temperatures (22, 37, and 60 °C). An initial increase and a following decrease in antioxidant activity were observed for all solutions at the three temperatures. The lower antiradical activity values of the mixture in comparison to the controls during the entire period of storage clearly showed that interaction among these polyphenols promotes a negative sinergistic effect on this property.

KEYWORDS: Antiradical activity; phenols; oxidation; negative synergism; temperature

INTRODUCTION

Polyphenols are claimed to promote so many beneficial effects on human health. In the past several years, a large number of scientific reports have shown studies suggesting an important role of these compounds on the prevention of some degenerative diseases and certain carcinogenic processes (*1*−*3*). However, despite the general acceptance of their health protecting capability, a clear correlation between phenol content in foods and their effects in vivo has not been stated yet. This fact has given rise to a strong criticism toward studies dealing with species such as flavonoids and other phenolics. Despite the latter being well established as antioxidants, the assay methodology fails to correlate with any role for these compounds in the human health.

Notwithstanding these considerations, the use of polyphenols in food fortification is a common custom generally carried out to increase its nutritional value. Apple polyphenols, for instance, having a large amount of a catechin oligomer, were reported to be effective inhibitors of cholesterol oxidation in commercial meat products such as pork sausage, raw and roast ham, bacon, and hamburgers (*4*). Similarly, other flavonoids (e.g., quercetin) are successfully employed to inhibit fish oil degradation (*5*). Resveratrol is known to possess intense preservative and pharmacologic characteristics (*6*, *7*).

It is possible to find in the literature abundant studies that try to clarify the antioxidant behavior of these individual compounds. However, very little is known about the outcomes of their interaction on the antioxidant properties and, as a consequence, on food stability. Indeed, interaction among different species may promote changes in overall antioxidant capacity, which are difficult to predict on the basis of their individual antioxidant capacity. Three different effects have been reported to take place in these systems: (1) an additive effect such as that observed for phenolic components from Dalbergis odorifera when mixed with either BHT or α-tocopherol (*8*); (2) a synergic effect as detected by Peyrat-Maillard et al. (*9*) for α-tocopherol added to malt rootlet extracts (a similar result was also obtained among phenols extracted from several fruits and aged wines (*10*, *11*)); (3) possible depletion of the antioxidant capacity upon addition of certain species. This negative synergism was observed when plant extracts like origano or rosemary were mixed with α-tocopherol or when BHA was added to methanolic extracts of peanut hulls (*12*, *13*).

The different behavior could probably be explained on the basis of the chemical nature and reactivity of the compounds considered. It is well-accepted that the radical scavenging capability of phenolic compounds is due to their hydrogen-donating ability: the more the number of hydroxyl groups, the higher the possibility of free radical scavenging activity (*14*, *15*). The availability of the hydroxyl groups closely depends on both chemical structure and spatial conformation, which can modify the reactivity of the molecules. Some flavonoids are reported to exhibit a strong tendency to suffer polymerization reactions that promote important structural changes and, as a consequence, variations in their radical scavenging activity (*16*, *17*). Previous findings show that monophenols present lower radical capacity than corresponding oligomers, probably because of their more significant areas of charge delocalization (*18*, *19*). Nicoli et al. (*20*) have reported an increase higher than 50% of

* Corresponding author (telephone +39.0432.590744; fax +39.0432.590719; e-mail: mpinelo@usc.es).
† Universidad de Santiago de Compostela.
‡ Università degli Studi di Udine.

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the initial antiradical activity of a catechin solution during the 2 first days of storage at 25 °C. Likewise, Espin et al. (*21*) detected major antioxidant activity of the dimer of resveratrol produced by oxidation in comparison to the individual molecule. Anyhow, when the degree of polymerization exceeds a critical value, the increased molecular complexity promotes a decrease in antioxidant capacity, probably because of the steric hindrance, which reduces the availability of the hydroxyl groups. All these considerations make it difficult to anticipate the antioxidant behavior of systems containing different phenolic species.

On the basis of these considerations, this work was designed to study the possible synergism of several polyphenols allowed to concomitantly react in an antioxidant capacity. In particular, the evolution of antioxidant properties of a system containing a mixture of three monophenols (catechin, quercetin, and resveratrol) was compared to the trend followed by each single phenol. Changes in antioxidant capacity were assessed at three different temperatures using the DPPH method.

MATERIALS AND METHODS

Sample Preparation. Ethanol (Carlo Erba, Milano, Italy) solution containing 10^{-4} M quercetin (Q-0125, lot 90K1746, Sigma, St. Louis, MO), 10^{-4} M catechin (C-1251, lot 120K1194, Sigma), and 10^{-4} M resveratrol (R-0456, lot 42K1146, Sigma) was prepared. Control ethanol solutions containing 10^{-4} M of each single phenol were also prepared. Five-hundred milliliter capacity screw capped flasks containing 200 mL of polyphenol solution were mantained in water baths at 22, 37, and 60 °C. At different lengths of time, 5 mL of the solution were removed and immediately analyzed.

Analytical Determinations. *Optical Density*. Chemical oxidation was followed by means of measurement of optical density at 380 nm using a Uvikon 860 spectrophotometer (Kontron Instruments, Milano, Italy). This wavelength is in the spectral region of these phenols' maximum absorption.

Antiradical Activity. The antiradical activity was measured following the methodology described by Brand-Williams et al. (*22*), whereas the bleaching rate of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) is monitored at the characteristic wavelength in the presence of the sample. In its radical form, DPPH• absorbs at 515 nm, decreasing its absorption upon reduction by an antioxidant or a radical species.

A volume of 1.80 mL of 6.1×10^{-5} M DPPH• methanol solution was used. The reaction was started by the addition of 200 μL of samples. The bleaching of DPPH• was followed at 515 nm (Uvikon 860, Kontron Instruments, Milano, Italy) at 25 °C for 20 min. In all cases, the DPPH• bleaching rate was proportional to the sample concentration added to the medium. The following equation was chosen in order to obtain the rate of DPPH• bleaching, k (*20, 23*):

$$\frac{1}{A^3} - \frac{1}{A_0^3} = 3kt \qquad (1)$$

where A_0 is the initial optical density and A is the optical density at increasing time t. The antiradical activity was expressed as k ($-\mathrm{OD}^{-3}$ min^{-1} (mg of dry matter)$^{-1}$).

Statistical Analyses. The results reported in this work are the average of at least three measurements, and the coefficients of variations, expressed as the percentage ratio between standard deviations and the mean values, were found to be less than 7 for antiradical activity and 6 for optical density.

RESULTS AND DISCUSSION

Antiradical activity values of an ethanol solution containing 10^{-4} M quercetin, 10^{-4} M catechin, and 10^{-4} M resveratrol and those corresponding to the control systems containing 10^{-4} M of each single phenol in ethanol are shown in **Table 1**. The DPPH antioxidant capacity assay showed that the combination of the three polyphenols does not only prevent the expected increase in the radical-scavenging capacity but also promote a considerable decrease in this parameter. As can be observed, the order of increasing antioxidant capacity was complex system < quercetin ≤ resveratrol < catechin, with a doubling of the antiradical activity value detected in the phenol mixture for the last one.

Table 1. Antiradical Activity of an Ethanol Solution of Resveratrol, Catechin, and Quercetin and Those Corresponding to the Single Phenol Control Solutions

polyphenol in ethanol solution	antiradical activity[a] ($-\mathrm{OD}^{-3}$ min^{-1} mg^{-1})
10^{-4} M resveratrol	64.9 ± 3.4
10^{-4} M catechin	73.2 ± 2.7
10^{-4} M quercetin	61.8 ± 3.4
10^{-4} M resveratrol + 10^{-4} M catechin + 10^{-4} M quercetin	33.7 ± 2.0

[a] Mean ± SD (n = 3).

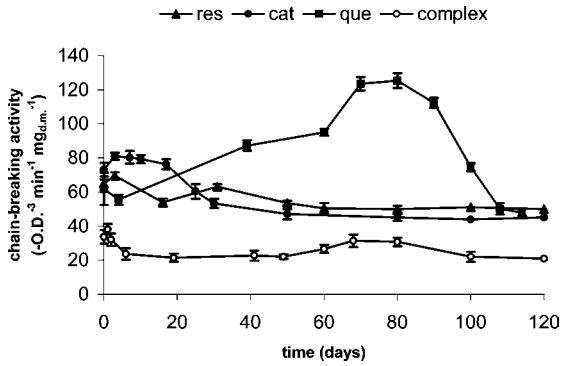

Figure 1. Antiradical activity value of an ethanol solution of 10^{-4} M resveratrol, 10^{-4} M catechin, and 10^{-4} M quercetin and those corresponding to the single phenol control systems at 22 °C.

This acute difference in antioxidant activity can be justified by the interaction among polyphenols. It is well-known that the tendency to combine themselves through polymerization reactions promotes an increasing molecular complexity, thus decreasing the availability of the hidroxyl groups when the degree of polymerization is considerable. This reaction pathway is likely to occur to a higher extent in the complex solution, which is 3 times more concentrated than the simple ones. The higher concentration allows a higher possibility of effective collisions among individual molecules. In this way, the polymerization pathway is favored, reducing the relative extent of the singular molecule's interaction with DPPH radicals.

The effect of polymerization can also be noted in the evolution of antiradical activity during storage of the considered solutions at 22 °C (**Figure 1**). In the case of single phenol solutions, an increase of this parameter was detected in all cases, followed by a progressive decrease on further storage. This is in agreement with the typical behavior of phenols capable of promoting the formation of partially polymerized compounds (*17*, *19*). However, despite a similar trend, the maximum value of antioxidant capacity was reached at very different times. In particular, the antiradical activity of catechin and resveratrol was increased by almost 10% of their initial values during the first days of storage. In the case of quercetin, however, the maximum value of antioxidant capacity was doubled those detected in the other phenolic single systems and was reached after 80-day storage. This can be probably explained through the structural differences existing among the three phenols because availability of hydroxyl groups is strongly affected by

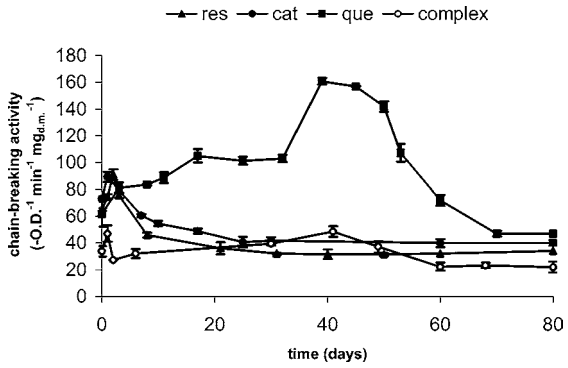

Figure 2. Antiradical activity value of an ethanol solution of 10^{-4} M resveratrol, 10^{-4} M catechin, and 10^{-4} M quercetin and those corresponding to the single phenol control systems at 37 °C.

the tridimensional disposition of the molecule (*14*, *15*). With regard to the solution that contains the mixture of the three polyphenols, the total values of antioxidant capacity reached are always lower than those corresponding to the single systems. This result clearly indicates that interaction among the three polyphenols promotes a negative synergistic effect on the total radical scavenging capacity during the entire period of storage. In addition, these data support the hypothesis that interaction among phenols is responsible for the low antioxidant activity of the complex system in comparison to the simple ones (**Table 1**). Following the antioxidant evolution of the complex system, two peaks of antioxidant capacity were detected. The first one occurs in concommitance with the peaks detected for catechin and resveratrol during the first days of storage, but the descent after reaching the maximum value of antioxidant capacity is faster in the mixture of phenols, probably because of the higher concentration of the latter, which promotes an increase of effective collisions among molecules. While a prevalent contribution of resveratrol and catechin can be noted during the initial stages of storage period, a major role of quercetin has been observed in the more advanced stage. In fact, a peak of antioxidant capacity appears after 60-day storage in both systems.

Equivalent antioxidant capacity behavior of the considered ethanol solutions was detected at 37 °C (**Figure 2**). As was already observed at 22 °C, a peak of maximum antioxidant activity was found in resveratrol and catechin solutions during the first period of storage. In the quercetin case, however, the maximum values of this parameter were reached after 40 storage days, indicating that quercetin reactivity is much more sensitive to temperature than the other phenols assayed. In addition, the values of maximum antiradical activity were observed to be higher than those detected at 22 °C in all cases and the period of maintenance of maximum antiradical activity was longer at lower temperatures. With regard to the solution that contains the mixture of the three polyphenols, the total values of antioxidant capacity were practically always lower than those corresponding to other single systems, again except during the storage days around 40, where the mixture showed a slightly major antiradical activity compared with the catechin and resveratrol cases. Two peaks of antioxidant capacity were also detected in this case.

The trend followed by the antioxidant capacity of the different solutions at 60 °C is shown in **Figure 3**. As expected, a further increase in storage temperature promotes a new acceleration of the reaction rate. In fact, the maximum of antioxidant capacity was gained in shorter times compared to those observed at 22 and 37 °C and the period of maintenance of maximum

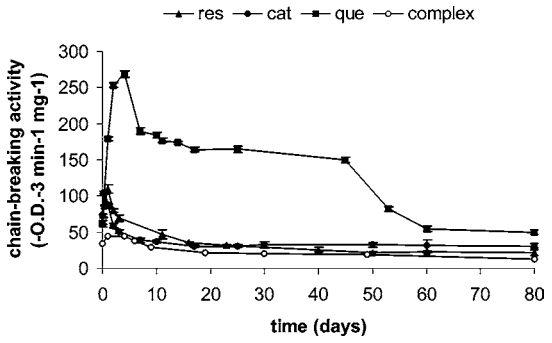

Figure 3. Antiradical activity value of an ethanol solution of 10^{-4} M resveratrol, 10^{-4} M catechin, and 10^{-4} M quercetin and those corresponding to the single phenol control systems at 60 °C.

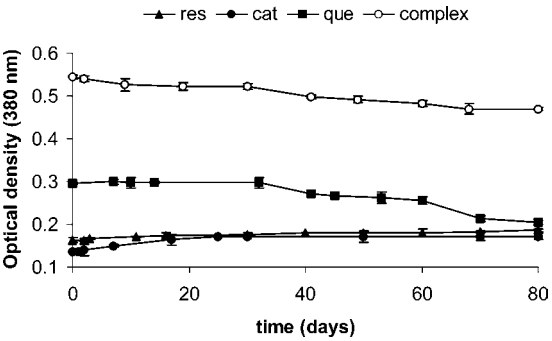

Figure 4. Optical density value of an ethanol solution of 10^{-4} M resveratrol, 10^{-4} M catechin, and 10^{-4} M quercetin and those corresponding to the single phenol control systems at 37 °C.

antioxidant activity was reduced in all cases. The peak of antioxidant activity of quercetin was observed to occur during the first days of storage, approaching in time to those of catechin and resveratrol. As a consequence, only a peak of antioxidant capacity was detected in the mixture of phenols at this temperature. The values of antioxidant capacity were also lower than those observed in the single solutions, confirming the negative synergism reported at previous temperatures.

Figure 4 shows the changes in optical density at 380 nm detected in all systems during storage at 37 °C. Considering the different extinction coefficients of phenol oxidation products, measurements of optical density at 380 nm can give some useful information on the extent of their oxidation. The applicability of this methodology has been previously reported for resveratrol and catechin oxidation. (*6*, *21*, *24*). In fact, by comparison of these data to those relevant to antioxidant capacity reported in **Figure 2**, it can be observed that the initial increase in antioxidant activity did not correspond to any appreciable change in optical density. However, after the maximum value was reached, the behavior of this parameter followed a different trend depending on the considered single phenol system. In fact, the evolution of the ethanol solution optical density containing catechin and resveratrol indicates that polymeric compounds obtained from these monophenols are darker than the original molecule. By contrast, degradation products in quercetin solution are colorless compared with the single molecule. In addition, this decrease in absorbance detected in quercetin was much higher than the corresponding increase observed in the catechin and resveratrol cases and is in agreement with variations in antiradical activity being much higher than those detected in quercetin. Since the optical density of the complex mixture was characterized by a progressive decrease, it can be inferred that polymerization and, as a consequence, variations in antioxidant

capacity mainly occur as affected by the pathway determined by quercetin.

Data not shown reveal an equivalent general behavior for the solutions subjected to storage temperatures of 60 and 22 °C.

In conclusion, it is possible to obtain important information about changes in antioxidant capacity of the solutions. First, changes in optical density could indicate that the storage time corresponding to maximum values of antioxidant activity is over. Besides, being that the optical density is much easier to be assessed compared to the measurement of the antioxidant capacity, the general trend could be useful for indicating whether a specific phenol could exert a dominant role in determining the antioxidant capacity trend of a mixture of phenols.

CONCLUSIONS

Results obtained in this study clearly showed that the addition of a new polyphenol to a complex phenolic system does not always promote a positive effect on its overall antioxidant capacity. In fact, in opposition to what could be expectable, it can cause dramatic consequences because its value can be considerably reduced.

This has important implications in food fortification when it is carried out to increase the stability of foods with time. In light of the results shown in this study, it is obvious that the effect of a new functional ingredient on the antioxidant capacity of a system is not easy to anticipate. Initially, there are two parameters that could seem useful for solving this question. The first one is the initial antioxidant capacity of single phenols. However, as has been observed in this work, the catechin, despite exhibiting the higher initial antioxidant capacity, did not have a crucial role in the overall evolution of this parameter. The other one, the redox potential, could also be useful in order to provide the possible interactions among the different compounds and the implications in the antioxidant capacity; however, the great variety and complexity of compounds present in food matrixes (other antioxidants and polyphenols, oxidative enzymes, metals, ...) make impossible an exhaustive study of their reactivity and their consequences.

In conclusion, the only way to know the implications that the addition of a new phenol can cause on the antioxidant capacity, and as a consequence on the stability of food matrixes, is a specific experimental study of the system considered in each case.′

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