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Antioxidant capacity of herbal infusions and tea extracts: A comparison of ORAC-fluorescein and ORAC-pyrogallol red methodologies

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Abstract

Oxygen radical absorbance capacity (ORAC) values have been obtained for a series of teas and herbal infusions employing 2,2′-azo-bis(2-amidinopropane) as free radical source, and fluorescein and pyrogallol red as target molecules. The amounts of phenols in the extracts were evaluated by Folin's methodology. ORAC values are extremely dependent upon the employed target molecule. Even more, relative ORAC values measured for different infusions depend upon the employed methodology. For example, ORAC-fluorescein value of *Aloysia citriodora* is larger than that of green tea, while if pyrogallol red is employed as target molecule green tea appears as nearly nine times more efficient. Similarly, for extracts with comparable amounts of phenols, herbal infusions are more efficient than teas by ORAC-fluorescein, while opposite conclusions are obtained if ORAC-pyrogallol red values are considered. Extreme care must then be taken for conclusions obtained from ORAC values estimated by employing a single target molecule.

Keywords: Antioxidant capacity; ORAC; Herbal teas; Teas

1. Introduction

Plant phenolic compounds can reduce the deleterious effects of reactive oxygen species (ROS) on a number of biological and pathological processes. Scavenging of ROS by plant phenolics may be the basis of the purported human health benefits of plants (Sawa, Nakao, Akaike, Ono, & Maeda, 1999). Beverages such as herbal infusions and teas that do not have any particular nutritional value, also constitute an important source of antioxidants (Warren, 1999). Then, herbal infusions and teas could be taken as a good complement of the antioxidants intake in the human diet. Thereby, the antioxidant capacity of herbal infusions and teas have been studied in different *in vitro* systems, comprising stable free radicals (such as DPPH and ABTS), following the oxygen concentration in presence of 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH), chemiluminescence, protection of erythrocyte lysis AAPH mediated, oxygen radical absorbance capacity (ORAC), and ferric reducing antioxidant potential (FRAP) methodologies (Caldwell, 2001; Campos & Lissi, 1995; Cao, Sofic, & Prior, 1996; Chen et al., 2006; Jimenez, Garrido, Bannach, Gotteland, & Speisky, 2000; Maulik et al., 1997; O'Brien, Carrasco-Pozo, & Speisky, 2006; Rechner et al., 2002; Roginsky & Barsukova, 2001; Roginsky & Lissi, 2005; Schmeda-Hirschmann et al., 2003; Wojcikowski, Stevenson, Leach, Wohlmuth, & Gobe, 2007; Yokozawa, Cho, Hara, & Kitani, 2000).

The ORAC-index has been widely employed in the antioxidant capacity evaluation of beverages (Cao et al., 1996; Prior & Cao, 1999). This methodology measures the protection afforded by an antioxidant to a target molecule

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that is being oxidized by peroxyl radicals, estimating the changes of area under curve of the kinetics profiles of the target molecule decay in fluorescence or absorbance. ORAC assay was proposed originally by Cao, Alessio, and Cutler (1993) using phycoerythrin as target molecule, but at present, fluorescein is the target molecule most employed (Ou, Hampsch-Woodill, & Prior, 2001). However, we have shown that ORAC-fluorescein for single antioxidants and/or complex mixtures including very reactive compounds is estimated generally from kinetics profiles showing a neat induction time. Thus, the ORAC-fluorescein index would be more related with stoichiometric factors than the reactivity of antioxidants towards peroxyl radicals AAPH derived (López-Alarcón & Lissi, 2006). In a recent report we have proposed that the use of pyrogallol red as target molecule gives ORAC-index more related to the reactivity of the antioxidants towards peroxyl radicals (López-Alarcón & Lissi, 2006; López-Alarcón & Lissi, 2005).

Therefore, this study was undertaken in order to evaluate the ORAC-index of herbal infusions and tea extracts using fluorescein and pyrogallol red as target molecules. Also, a comparison of ORAC-fluorescein and ORAC-pyrogallol red with the total phenolic content is included.

2. Experimental

2.1. Chemicals

2,2′-Azo-bis(2-amidinopropane) dihydrochloride (AAPH), was used as peroxyl radical source. Pyrogallol red (PGR), Trolox (6-hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid), fluorescein, and AAPH were purchased from Sigma–Aldrich (St. Louis, MO). Folin–Ciocalteau reactive and sodium carbonate were supplied by Merck (Darmstadt, Germany). All compounds were employed as received.

2.2. Herbal materials

Herbs and teas (blacks, green, red and white) bags were Chilean commercial products. The following herbs were studied: bailahuen (*Haplopappus baylahuen,* Remy), boldo (*Peumus boldus*, Mol), chamomile (*Matricaria chamomilla*, L.), matico (*Buddleia globosa*, Hope), cedrón (*Aloysia citriodora*, Ort.), and paico (*Chenopodium ambrosioides*, L.). Infusions were prepared by adding 150 mL of distilled water (95–100 °C) to the bags (each containing 2 g of herbal or tea material). The infusions were brewed for 5 min. Upon withdrawing the bags, the resulting solutions were cooled to 20 °C and immediately used to assess both their total phenolic content and antioxidant properties.

2.3. Solutions

Stock solutions of pyrogallol red (1×10^{-4} M) or fluorescein (1×10^{-5} M) were prepared daily in phosphate buffer 75 mM, pH 7.4. A reaction mixture containing AAPH (10 mM), pyrogallol red (5 μM) with or without the tested infusions was incubated in phosphate buffer (75 mM, pH 7.4) at 37 °C. Pyrogallol red consumption was evaluated from the progressive absorbance decrease measured at 540 nm in the thermostatized cuvette of an Agilent 8453 (Palo Alto, CA, USA) UV–visible spectrophotometer. A similar procedure was carried out employing fluorescein (70 nM), but its consumption was assessed from the decrease in the sample fluorescence intensity (excitation: 493 nm; emission 515 nm). Fluorescence measurements were carried out using a Perkin Elmer LS-55 spectrofluorimeter (Beaconstield, U.K).

2.4. ORAC determinations

The consumption of the probe molecules, fluorescein or pyrogallol red, associated to its incubation with AAPH, was estimated from fluorescence (F) and absorbance (A) measurements, respectively. Values of (F/F_0) or (A/A_0) were plotted as a function of time. Integration of the area under the curve (AUC) was performed up to a time such that (F/F_0) or (A/A_0) reached a value of 0.2. These areas were employed to obtain ORAC values, according to Eq. (1). All experiments were carried out in triplicate.

$$\mathrm{ORAC} = \frac{[\mathrm{AUC\_AUC^0}]}{[\mathrm{AUC_{Gallic\ acid}} - \mathrm{AUC^0}]} f[\text{Gallic acid}], \qquad (1)$$

where AUC is area under curve in presence of the tested extracts, integrated between time zero and that corresponding to 80% of the probe consumption; $\mathrm{AUC^o}$ is area under curve for the control; $\mathrm{AUC_{Gallic\ acid}}$ is area under curve for gallic acid; f is dilution factor, equal to the ratio between the total volume of the AAPH-pyrogallol red or AAPH-fluorescein solution and the added infusion volume; [Gallic acid] is Gallic acid molar concentration.

2.5. Total phenolics

Total phenol content in infusions was determined according to the Folin–Ciocalteau colorimetric method (Singleton & Rossi, 1965), using gallic acid as a standard. Briefly, appropriate dilutions of the samples (1 mL) were added to 0.2 N Folin–Ciocalteau reagent (5 mL) (2 N, diluted ten-fold). After 5 min, sodium carbonate (75 g/L) was added. The mixtures were incubated for 2 h and the absorbance of the resulting blue colour was measured at 740 nm using an ultraviolet–visible Agilent 8453 spectrophotometer. Quantification was carried out on the basis of the standard curve of gallic acid, and the results were expressed as mg of gallic acid per litre of infusion.

3. Results and discussion

3.1. Determination of total phenols content in herbal infusions and teas

Folin–Ciocalteau method (Singleton & Rossi, 1965) was employed to evaluate the total amount of phenolic groups

in the infusions. As shown in Table 1, among the herbal teas, the lowest total phenols content (expressed as mg gallic acid equivalents/L) was found in *Chenopodium ambrosoides* (36 ± 2), while the highest was found in *Aloysia citriodora* (378 ± 5). The order of total phenols content was: *Aloysia citriodora* ≈ *Peumus boldus* > *Haplopappus baylahuen* > *Buddleia globosa* > *Matricaria chamomilla* > *Chenopodium ambrosoides*. Table 1 also shows the total phenols content of tea samples. As can be seen, tea samples showed a higher total phenols content than herb infusions. The lowest and highest total phenols content in teas was white tea (445 ± 5) and black tea-1 (677 ± 16), respectively. The order of total phenols content values was: black tea-1 > black tea-2 > green tea > white tea.

3.2. Antioxidant capacity of herbal infusions and teas, using the ORAC methodology

ORAC-fluorescein is a convenient method that is extensively employed for the estimation of the antioxidant capacity of pure compounds or complex mixtures, such as herbal infusions and teas. The ORAC-index has previously shown to depend on the target molecule used (López-Alarcón & Lissi, 2006). In order to test the magnitude of this difference, and how the ORAC-index correlates with the total content of phenolic compounds, we evaluated the antioxidant capacity of several infusions using the ORAC methodology with fluorescein and pyrogallol red as target molecules.

3.2.1. ORAC-fluorescein values

Fig. 1 shows the results of AAPH mediated fluorescein oxidation in the absence and presence of *Peumus boldus*extract in phosphate buffer. In the absence of the additive, a progressive decrease of fluorescein fluorescence was observed, with almost total consumption after 13 minutes

Table 1
ORAC-fluorescein (ORAC-FL) and ORAC-pyrogallol red (ORAC-PGR) values of herbal infusions and tea extracts

Herb or tea	Total[a] phenols	ORAC-FL[b]	ORAC-PGR[b]
Buddleia globosa	165 ± 5	2175 ± 62	25 ± 1
Matricaria chamomilla	65 ± 1	431 ± 11	9 ± 0.5
Aloysia citriodora	378 ± 5	3368 ± 107	38 ± 1
Chenopodium ambrosioides	36 ± 2	395 ± 13	3.3 ± 0.2
Peumus boldus	376 ± 4	2728 ± 122	53 ± 3
Haplopappus baylahuen	245 ± 11	2250 ± 71	47 ± 2
Green tea	517 ± 7	2086 ± 43	325 ± 23
Black tea-1	677 ± 16	2957 ± 250	301 ± 28
Black tea-2	553 ± 20	2329 ± 286	324 ± 21
White tea	445 ± 5	1721 ± 207	293 ± 23

All experiments were carried out in triplicate, and the data represent the mean values (±S.D.).

[a] Expressed as gallic acid equivalents (mg gallic acid equivalents/L).

[b] Evaluated according to experimental section. Values represent the concentration (mg/L) of a gallic acid solution that produces the same effect than the tested extracts.

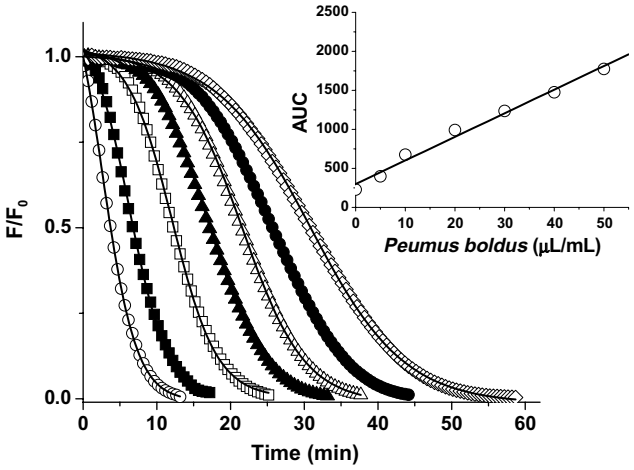

Fig. 1. Effect of *Peumus boldus* extract on fluorescein consumption induced by AAPH. Fluorescein (70 nM) was incubated in presence of AAPH (10 mM) and *Peumus boldus* extract. Control (○), *Peumus boldus*: 0.05 μL/mL (■); 0.1 μL/mL (□); 0.2 μL/mL (▲); 0.3 μL/mL (△); 0.4 μL/mL (●); 0.5 μL/mL (◇). The reaction was followed by the decrease in fluorescein fluorescence intensity (excitation at 493 nm, emission at 515 nm) in phosphate buffer 75 mM, pH 7.4, at 37 °C. Insert: dependence of the area under curve after 80% of reaction versus *Peumus boldus* concentration.

incubation. The addition of *Peumus boldus* extract aliquot produces a lower rate of fluorescein consumption, and an induction time dependent of the dose added. The area under curve of the kinetic profiles was linearly related to the amount of added *Peumus boldus* extract (Fig. 1, insert). This behaviour was observed for all herbal teas studied. From the AUC (area under curve), ORAC values were obtained by employing Eq. (1). The ORAC-fluorescein values obtained (expressed as mg of gallic acid equivalents/L) are shown in Table 1. Among the six herbal teas studied, the highest ORAC-fluorescein value was found for *Aloysia citriodora* extract (3368 ± 107), and the lowest for *Chenopodium ambrosoides* (395 ± 13). The order of ORAC-fluorescein values was

Aloysia citriodora > *Peumus boldus* > *Haplopappus baylahuen* ⩾ *Buddleia globosa* > *Matricaria chamomilla* > *Chenopodium ambrosoides*.

Fig. 2 shows the kinetic profiles of fluorescein consumption (closed symbols) in absence and presence of a black tea-2 extract. As can be seen in this figure, fluorescein was efficiently protected by black tea-2, and an induction time was observed. A similar behaviour was observed for all tea samples. As can be seen in Table 1, black tea-1 showed the highest ORAC-fluorescein value (2957 ± 250 mg of gallic acid equivalents/L) and white tea was the tea with the lowest ORAC-fluorescein value (1721 ± 207). The order in ORAC-fluorescein values was:

black tea-1 > black tea-2 > green tea > white tea

In any case, the differences between teas are rather small, less than a factor 2 between the most and least efficient

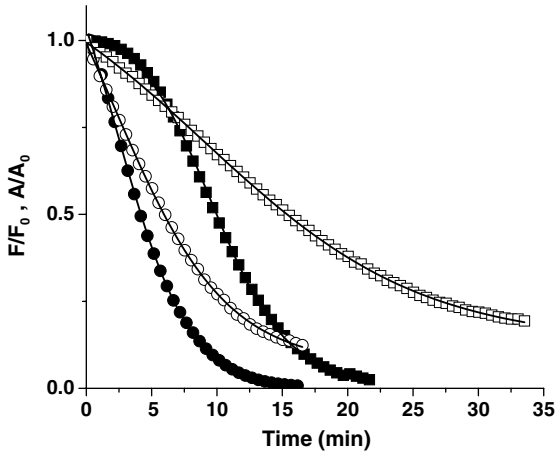

Fig. 2. Kinetic profiles of fluorescein and pyrogallol red consumption AAPH mediated in presence of black tea-2 extract. Fluorescein (70 nM) or pyrogallol red (5 μM) were incubated in presence of AAPH (10 mM) and black tea-2 in phosphate buffer (75 mM, pH 7.4) at 37 °C. Closed symbols correspond to fluorescein experiments: Control (●), and black tea-2, 0.1 μL/mL (■). Open symbols correspond to pyrogallol red experiments: Control (○), and black tea-2, 2.5 μL/mL (□).

infusions. In agreement with this conclusion, Cao et al. (1996) reported, employing an ORAC-phycoerythrin methodology, that black teas contain more antioxidants than green teas.

3.2.2. ORAC-pyrogallol red values

When pyrogallol red was incubated with AAPH in phosphate buffer (75 mM, pH 7.4) at 37 °C, a decrease of the absorption at 540 nm was observed (Fig. 3, control experiment). A reduced consumption rate of pyrogallol red was observed when an extract of *Haplopappus baylahuen* was added to the solution, as shown in Fig. 3. In this figure, the extract was found to retard pyrogallol red consumption in a concentration dependent manner. In contrast with the data obtained employing fluorescein as probe, no induction times were observed for all herbal infusions and teas studied (data not shown). The different behaviour of fluorescein and pyrogallol red is stressed when data for both systems is plotted together (see Fig. 2).

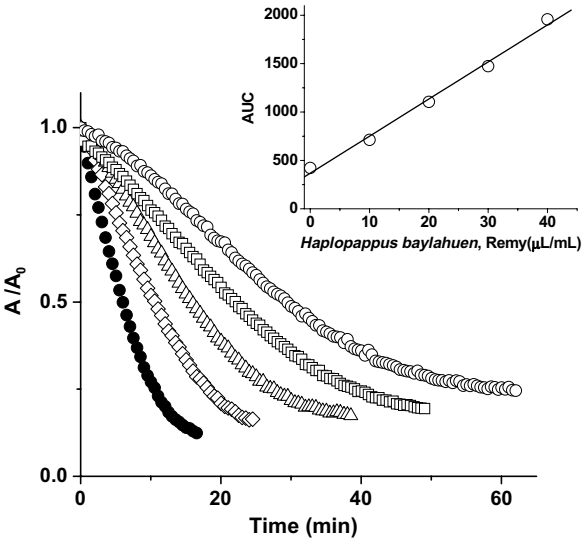

Fig. 3. Effect of *Haplopappus baylahuen* extract on pyrogallol red bleaching mediated by AAPH. Pyrogallol red (5 μM) was incubated in presence of AAPH (10 mM) and *Haplopappus baylahuen* extract in phosphate buffer (75 mM, pH 7.4) at 37 °C. Control (●). *Haplopappus baylahuen*: 10 μL/mL (◇); 20 μL/mL (△); 30 μL/mL (□); 40 μL/mL (○). Insert: dependence of the area under curve after 80% of reaction versus *Haplopappus baylahuen* concentration.

The area under curve was linearly related to the size of the infusion aliquot (Fig. 3, insert). ORAC-pyrogallol red values, included in Table 1, show that *Peumus boldus* was the herbal infusion with highest ORAC-pyrogallol red value. The order of ORAC-pyrogallol red values was

Peumus boldus > *Haplopappus baylahuen* > *Aloysia citriodora* > *Buddleia globosa* > *Matricaria chamomilla* > *Chenopodium ambrosoides*.

Table 1 are also included ORAC-pyrogallol red values obtained for tea samples. The values obtained were very similar for the four tested teas, ranging from 293 ± 23 mg of gallic acid equivalents/L (white tea) to 325 ± 23 (green tea).

3.3. Correlations between ORAC-indexes and Folin's total phenol content of the infusions

ORAC-fluorescein and ORAC-pyrogallol red values are plotted against the total phenol content of the infusions in Fig. 4. If all data are considered, no relationship among ORAC values and phenol content is observed. On the other hand, there is a good correlation between ORAC-fluorescein and phenol content in herbal infusions ($r = 0.935$) and teas ($r = 0.999$) (Fig. 4a). Similarly, there is a good correlation between ORAC-pyrogallol red and phenol content in herbal infusions (0.956) (Fig. 4b). On the contrary, ORAC-pyrogallol red of teas appear as unrelated to the phenol content of the infusion (Fig. 4b).

A remarkable feature of the data given in Fig. 4 is the different ORAC values when herbal infusions and teas of similar content of phenols are compared. In fact, the data show that:

(i) ORAC-fluorescein values of herbal infusions are considerably larger (by a factor near two) than those measured for tea extracts.
(ii) ORAC-pyrogallol red values of herbal infusions are considerably smaller (by a factor six) than those measured in tea extracts.

This different behaviour is stressed when ORAC-pyrogallol red values are plotted against ORAC-fluorescein values (Fig. 5). This figure shows that:

(i) When all data are considered, there is not correlation between both indexes.

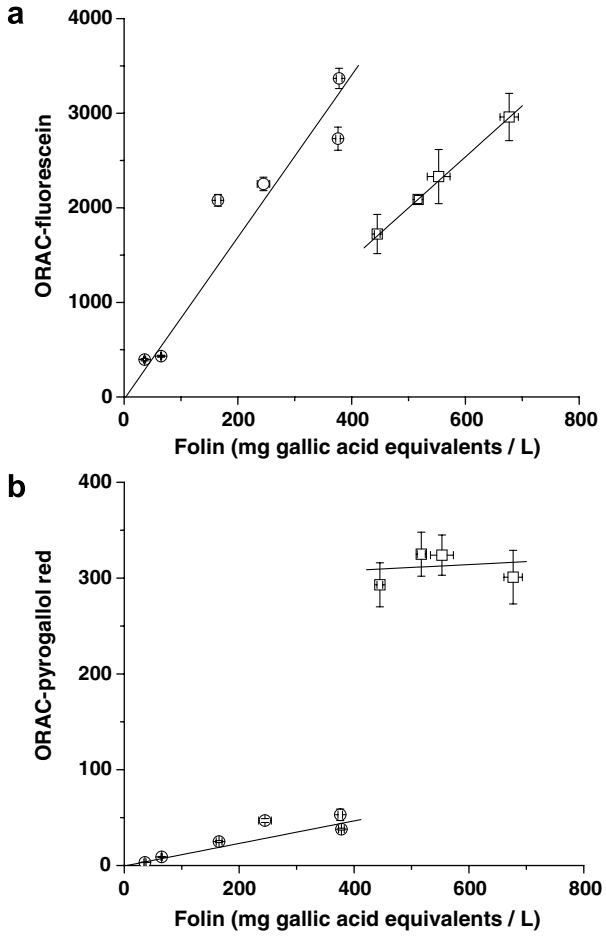

Fig. 4. ORAC-index *versus* total phenolic content plots. Correlation between ORAC-fluorescein (graphic a) and ORAC-pyrogallol red (graphic b) with total phenolic content of medicinal herbs (○) and teas (□).

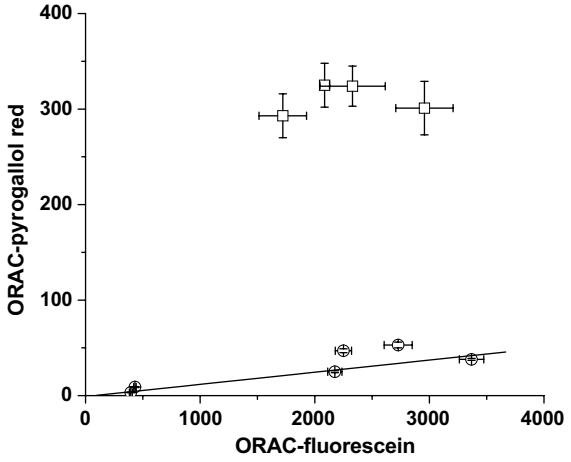

Fig. 5. Correlation between ORAC-fluorescein with ORAC-pyrogallol red of medicinal herbs (○) and teas (□).

(ii) For teas, there is not correlation between both indexes.

(iii) For herbal infusions, there is a good correlation between both indexes ($r = 0.944$). However, the linear correlation has a slope of 0.013, indicating that fluorescein renders ORAC values nearly 80 times larger than those obtained employing pyrogallol red as target molecule.

On the other hand, herbal infusions show a good correlation ($r = 0.9438$) between ORAC-fluorescein and ORAC-pyrogallol red (Fig. 5). However, no correlation was observed for tea samples (Fig. 5).

It is difficult to establish the reasons of these differences. However, it is remarkable that even relative ORAC values strongly depend upon the methodology employed. For example, ORAC-fluorescein for *Aloysia citriodora* is 1.6 times larger than the green tea value. On the other hand, regarding ORAC-pyrogallol red, *Aloysia citriodora* is nearly 8.6 times less efficient than green tea. These large differences cast doubts in values derived from a single ORAC determination.

In order to assess if the differences in ORAC values of complex mixtures employing fluorescein and pyrogallol red as target molecules are also observed in simpler systems, we compare data obtained for a series of pure compounds. The type of correlation observed is presented in Fig. 6. These data conclusively show large differences in both indexes even for pure compounds. This would indicate that, for pure compounds, it is difficult to establish even relative "antioxidant capacities" from a single ORAC experiment. This is particularly worrying since there are not conclusive arguments to validate one of the several possible methodologies.

Since all methodologies are considered to determine the capacity of the tested sample to trap peroxyl radicals, it is not easy to establish why ORAC values are so dependent upon the employed target molecule. Several explanations has been advanced to explain these dependence. Ou et al. (2001) explained differences obtained employing phycoerythrin and fluorescein, in terms of adsorption of the tested compounds on the protein. Pino, Campos, and Lissi

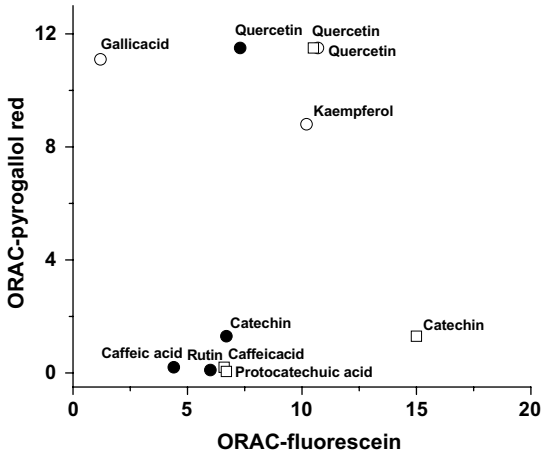

Fig. 6. Correlation between ORAC-fluorescein with ORAC-pyrogallol red measured for pure compounds. Data taken from Ou et al. (2001) (●), López-Alarcón and Lissi (2006) (□), and Dávalos et al. (2004) (○).

(2003) explained anomalous results obtained by employing pyranine as a target molecule in terms of a dominant role of repair mechanisms in the protection of the target molecule by phenols. López-Alarcón and Lissi (2006) explained differences between ORAC values obtained employing pyrogallol red and fluorescein as target molecules in terms of differences in their reactivities. This would lead to ORAC-fluorescein values conditioned by stoichiometric factors, while ORAC-pyrogallol red values would be more dependent upon the antioxidants reactivity. This multiplicity of factors make difficult to establish the "ideal" target molecule without a detailed study of the mechanism of the protection afforded for different compounds to a given target molecule.

4. Conclusions

ORAC values are extremely dependent upon the employed target molecule. Even more, relative ORAC values measured for different infusions depend upon the employed methodology. For example, ORAC-fluorescein value of *Aloysia citriodora* is larger than that of green tea, while if pyrogallol red is employed as target molecule, green tea appears as nearly nine times more efficient. Similarly, for extracts with similar amounts of phenols, herbal infusions are more efficient than teas by ORAC-fluorescein, while the opposite conclusions are obtained if ORAC-pyrogallol red values are considered. Extreme care must then be given to conclusions obtained from ORAC values estimated employing a single target molecule.

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