OXYGEN RADICAL ANTIOXIDANT CAPACITY (ORAC) VALUES OF HERBAL TEAS OBTAINED EMPLOYING DIFFERENT METHODOLOGIES CAN PROVIDE COMPLEMENTARY DATA.

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ABSTRACT

The antioxidant capacity of six herbal tea infusions (*Mentha piperita*, *Erythroxylum coca*, *Rosa moschata*, *Tilia spp*, *Plantago major*, and *Aloysia citriodora*) was measured by ORAC type methodologies employing fluorescein (ORAC-FL) and pyrogallol red (ORAC-PGR) as target compounds. Probe consumption profiles were widely different, with neat induction times when FL is employed as target. Relative ORAC values of different herbal infusions depend upon the test molecule employed. Relative ORAC-PGR values follow the order:

Rosa moschata > Mentha piperita > Tilia spp > Plantago major > Aloysia citriodora >Erythroxylum coca, while ORAC-Fl values order is: *Mentha piperita > Aloysia citriodora > Erythroxylum coca >Rosa moschata >Tilia spp > Plantago major.* These differences are related to the different relevance of the parameters (amount of phenols and reactivity) that determine ORAC values for different target molecules. In particular, ORAC-FL values are associated with the polyphenolic levels present in the sample and, hence, correlate with the Folin index of the infusion. On the other hand, ORAC-PGR values are determined by the quantity and average quality of the antioxidants present in the tested sample. From these results, it is proposed that the ratio ORAC-PGR/ORAC-FL could be employed as a rough measure of the antioxidant average quality of the phenols present in the tested infusion.

Key Words: herbal teas, ORAC, pyrogallol red, fluorescein, antioxidant capacity.

INTRODUCTION

There exist a large number of methodologies aimed to evaluate the antioxidant capacity of complex mixtures.[1] ORAC type methodologies stand among the most employed procedures [2-11]. However, a drawback of this type of assays is that the measured values are extremely dependent of the target molecule employed as sensor of the added compounds capacity to avoid their oxidative degradation. Alarcón et al.[12] have shown that not only the absolute ORAC values depend on the target molecule, but that also relative values are strongly dependent of the employed methodology. In particular, they report striking differences in the behavior of medicinal herb extracts and teas when fluorescein or pyrogallol red are employed as target molecules.[12] On the other hand, a fair correlation was observed between both ORAC indexes of each set of infusions (teas or herbal extracts). The differences observed were related to noticeable differences in the factors determining ORAC values. ORAC-fluorescein (ORAC-FL) values are primarily determined by the concentration of phenol-group containing compounds, while ORAC-pyrogallol red (ORAC-PGR) values are more influenced by the reactivity of the phenols present in the infusion. In the present communication we analyze a wider set of herbal infusions and show that, even for a series of medicinal herb extracts, relative antioxidant capacities strongly depends upon the chosen target molecule. The results allow concluding that ORAC-PGR values give more information regarding the capacity of a given infusion to reduce the oxidative damage to valuable molecules elicited by peroxyl radicals. Furthermore, we propose that both methodologies can provide complementary results regarding the antioxidant capacity of a complex mixture. In particular, the ratio ORAC-PGR/ORAC-FL could be considered as an index of the average quality of the antioxidants present in a given infusion.

MATERIALS AND METHODS

Chemicals

2,2'-Azo-bis(2-amidinopropane) dihydrochloride, (AAPH), was used as peroxyl radical source.[13] Pyrogallol red (PGR), trolox (6-hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid), fluorescein disodium salt (FL), and AAPH were purchased from Sigma-Aldrich (St. Louis, MO). Folin-Ciocalteau reactive and sodium carbonate were supplied by Merck. All compounds were employed as received.

Herbal materials

Herb bags were chilean commercial products. The following herbs were studied: *Mentha piperita* (mentha), *Rosa moschata (*mosqueta), *Aloysia citriodora* (cedrón), *Tilia spp* (tilo), *Plantago major* (llantén), and *Erythroxylum coca* (coca). Infusions were prepared by adding 150 mL of distilled water (95–100 °C) to the bags (each containing 2 g of dry herbs). The infusions were brewed for 5 min, with gentle stirring every 45 s. Upon withdrawing the bags, the resulting solutions were cooled to 20°C, centrifuged and immediately used to assess both their total phenolic content and antioxidant properties.

Solutions

Stock solutions of PGR ($1x10^{-4}$M) or FL ($1x10^{-5}$M) were prepared daily in phosphate buffer 75 mM, pH 7.4. A reaction mixture containing AAPH (10 mM), PGR (5 μM) with or without the tested infusion was incubated in phosphate buffer (75 mM, pH 7.4) at 37°C. PGR consumption was evaluated from the progressive absorbance decrease measured at 540 nm in the thermostatized cuvette of a Shimadsu UV- 160 spectrophotometer. A similar procedure was carried out employing FL (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 in an Aminco-Bowman Series 2 spectrofluorimeter.

ORAC determinations

The consumption of the probe molecules, FL or PGR, associated to its incubation in presence of AAPH, was estimated from fluorescence (F) and absorbance (A) measurements, respectively.[12] 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 **Eqn [1]**. All experiments were carried out in triplicate.

$$ORAC = \frac{[AUC - AUC^{o}]}{[AUC_{reference} - AUC^{o}]} f\,[reference] \qquad (1)$$

where:

AUC = Area under curve in presence of the tested infusions, integrated between time zero and that corresponding to 80 % of the probe consumption;

AUC^{o} = Area under curve for the control.

$AUC_{reference}$ = Area under curve for the reference compound.

f = dilution factor, equal to the ratio between the total volume of the AAPH-pyrogallol red or AAPH-FL solution and the added infusion volume.

[reference] = Trolox or gallic acid milimolar concentration.

This formula provides ORAC values in terms of the reference milimolar equivalents.

Total phenolics

Total phenol content in infusions was determined according to the Folin–Ciocalteau colorimetric method.[15] Briefly, appropriate dilutions of the samples were added to 0.2 N Folin-Ciocalteau reagent (Merck Darmstadt,

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Germany - 2N, diluted ten-fold). After 5 minutes, sodium carbonate (75 g/L) was added. The mixtures were incubated for 2 hours and the absorbance of the resulting blue color was measured at 740 nm using a Shimadzu UV-160 spectrophotometer. Quantification was carried out on the basis of the standard curve of gallic acid, and the results were expressed as milimolar equivalents of gallic acid per liter of infusion.

RESULTS

Addition of herbal tea infusions delay the rate of FL (**Fig. 1A**) or PGR (**Fig. 1B**) consumption elicited by their incubation in presence of AAPH. A comparison of the area under the curve, resulting of the integration of these plots from t = 0 to the time require to bleach 80 % of FL fluorescence, relative to that elicited by a reference compound (trolox or gallic acid) allows an evaluation of the ORAC-FL index employing **Eqn [1]**.

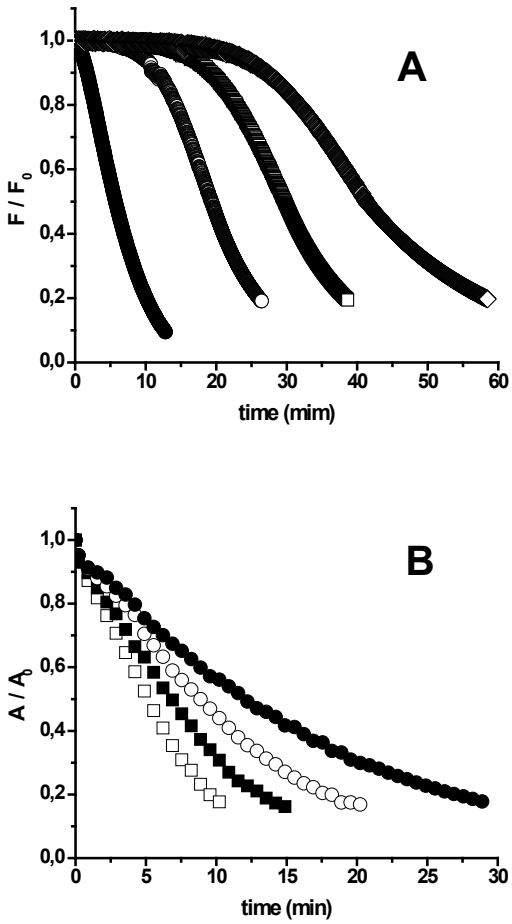

Figure 1: Kinetic profiles of FL (A) or PGR (B) consumption in presence of *Aloysia citriodora* infusions. FL (70 nM) or PGR (5 µM) were incubated in presence of AAPH (10 mM) and different amounts of a *Aloysia citriodora* infusion in phosphate buffer (75 mM, pH 7.4) at 37ºC. (A): Control (●), *Aloysia citriodora* infusion: 0.33 µL/mL (○); 0.66 µL/mL (□); 1 µL/mL (◇). (B): Control (□), *Aloysia citriodora*: 5 µL/mL (■); 17 µL/mL (○); 27 µL/mL (●).

Among the factors that can be modified in ORAC measurements stand the reference compound[16]. Trolox and gallic acid are most frequently employed for these purposes. For a given methodology, changing the reference compound only shifts the ORAC scale. However, the shift observed strongly depends upon the employed methodology. In the ORAC-FL technique, changing the reference from gallic acid to trolox renders almost unmodified ORAC values. On the other hand, ORAC-PGR values expressed in trolox equivalents are almost eleven times higher than those obtained employing gallic acid as reference.

Values obtained, employing gallic acid as reference, are included in **Table 1** and **Table 2**. In **Table 1** are also included the Folin index of the infusions considered in the present work. These data allow concluding that:

1. ORAC-FL values are much higher than ORAC-PGR values.
2. ORAC-PGR/ORAC-FL ratios are extremely dependent upon the tested sample. This leads to a very poor correlation between ORAC-PGR and ORAC-FL values (r = 0.08; p = 0.88; **Fig. 2**). This implies that the relative antioxidant capacity of two samples depends of the methodology employed. For example, the data show that mentha has more activity (14.7 mM gallic acid equivalents) than mosqueta (7.2 mM gallic acid equivalents) when FL is employed as sensor, while the opposite conclusion is reached when PGR is employed as target molecule (0.37 and 0.7 mM gallic acid equivalents for mentha and mosqueta, respectively).
3. There is a fair correlation between ORAC-FL values and the Folin index of the tested infusion (r = 0.84; p = 0.04; **Fig. 3**). This correlation is not observed when ORAC-PGR values are considered (r = 0.25; p = 0.63; **Fig. 4**).

Table 1.- ORAC-PGR, ORAC-FL and Folin values of different infusions. Data expressed as gallic acid equivalents (mM).

Herbal tea	ORAC-PGR	ORAC-FL	FOLIN
Rosa moschata	0.72 ± 0.03	7.23 ± 0.20	2.43 ± 0.02
Mentha piperita	0.37 ± 0.02	14.71 ± 0,38	3.33 ± 0.01
Tilia spp	0.34 ± 0.01	6.09 ± 0.16	0.87 ± 0.02
Plantago major	0.21 ± 0.01	4.60 ± 0.12	1.59 ± 0.01
Aloysia citriodora	0.20 ± 0.01	9.45 ± 0.24	2.67 ± 0.01
Erythroxylum coca	0.09 ± 0.01	7.61 ± 0.20	1.77 ± 0.02

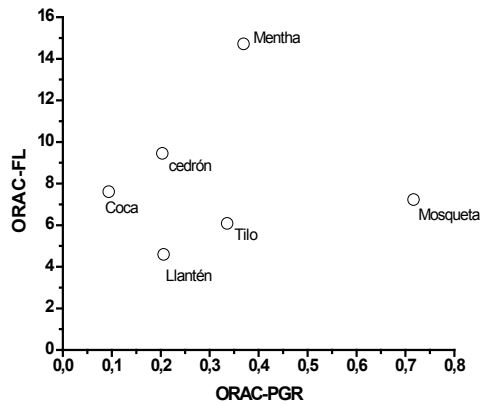

Figure 2: Correlation between ORAC-FL and ORAC-PGR values of herbal tea infusions (r = 0.08 ; p = 0.88)

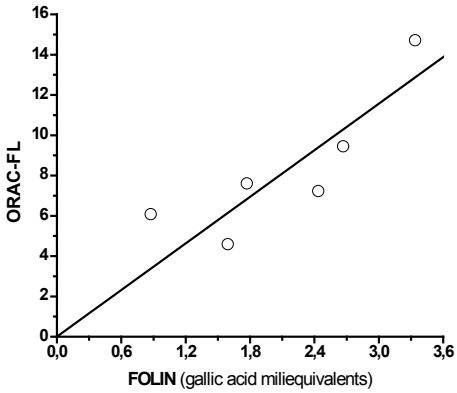

Figure 3: Correlation between ORAC-FL and total phenolic content of herbal tea infusions (r = 0.84; p = 0.035).

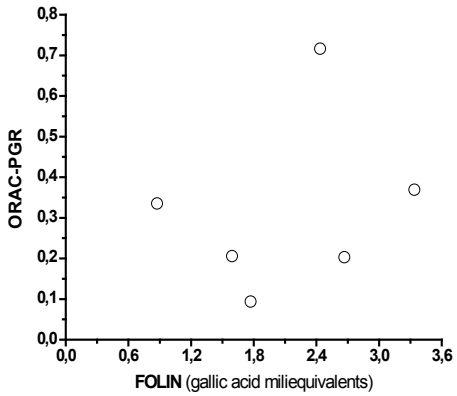

Figure 4: Correlation between ORAC-PGR and total phenolic content of herbal tea infusions (r = 0.25 ; p = 0.63).

DISCUSSION

The differences observed when FL or PGR are employed as target molecules introduce in ORAC type methodologies a large degree of arbitrariness. This probe dependent behavior has been related to differences in the consumption profiles shown in **Fig. 1** [12,14]. In fact, FL consumption shows clear induction times that strongly contribute to the value of the ORAC index. Since the magnitude of the induction time is related to the amount of efficient antioxidants present in the sample, irrespective of their reactivity, ORAC values determined by this methodology are correlated to the number of reactive groups present in the sample. This is supported by the fair correlation observed between ORAC-FL values and Folin indexes (Fig. 3). On the other hand, no correlation is observed between ORAC-PGR values and Folin indexes (Fig. 4). Furthermore, no induction times are observed when PGR is employed as target molecule (**Fig. 1B**).This would imply that, in this system, PGR competes with the added phenols for the peroxyl radicals. However, secondary reactions in which phenol derived radicals react with PGR could contribute to the observed dye bleaching[14]. In fact, cinnamic acid derivatives, even at relatively high concentrations, barely protect PGR from bleaching, giving very low ORAC values. This lack of protection is contrary to that expected from relative reactivities, suggesting that it results from secondary reactions of phenol derived radicals. In any case, the observed index will depend upon the amount and reactivity of the phenols present in the sample and/or the capacity of the phenoxyl radicals to interact with PGR. A good antioxidant must present a high reactivity towards the peroxyl radicals and provide phenol derived radicals unable to damage the test molecule. In this sense, it can be considered that ORAC-PGR values are determined by the quantity and average quality of the antioxidants present in the tested sample.

The above considerations lead to the conclusion that ORAC-FL can be considered to be a measure of the amount of reactive phenols present in the sample. ORAC-FL follows the order:

Mentha > cedrón > coca > mosqueta > tilo > llantén

This is close to the order of their Folin indexes:

Mentha > cedrón > mosqueta > coca > llantén > tilo

On the other hand, the order of their capacities to protect PGR is widely different:

Mosqueta > mentha > tilo > llantén > cedrón > coca

This difference can be attributed to the fact that ORAC-PGR values are influenced by the average "quality" of the antioxidants present in the sample. In this sense, it is interesting to note that *Rosa moschata* infusions were among the most active in the removal of stable radicals (ABTS)[17,18] and reactive oxygen species (hypochlorite and peroxynitrite).[17] Furthermore, *Rosa moschata* is rich in ascorbic acid,[19] a compound that presents particularly high values of the ORAC-PGR index.[20]

The above considerations suggest that the ratio ORAC-PGR/ORAC-FL can be considered as a measure of the average quality of the antioxidants present in a given infusion. The values obtained are collected in **Table 2**, where are also included data previously reported.[12] The calculated values show a wide variability, ranging from 0.008 (*Chenopodium ambrosioides*) to 0.17 (white tea), allowing to establish an order of relative antioxidant quality. In any case, it is interesting to note that all the values are considerably smaller than one. This implies that, on the average, the titrated compounds present in the infusions are less able to protect PGR than the reference compound employed (gallic acid). Further studies employing families of compounds of known reactivity[21,22] will be necessary to establish the role of the derived phenol radicals upon the index (ORAC-PGR/ORAC-Fl) proposed as a measure of the quality of the tested antioxidants.

Table 2.- Values of ORAC-PGR/ORAC-FL ratio for different infusions.

Infusion	ORAC-PGR/ORAC-FL
Chenopodium ambrosioides	0.008[a]
Buddleia globosa	0.011[a]
Erythroxylum coca	0.012
Aloysia citriodora	0.021 (0.011[a])
Matricaria chamomilla	0.021[a]
Peumus boldus	0.019[a]
Haplopappus baylahuen	0.020[a]
Mentha piperita	0.025
Plantago major	0.045
Tilia spp	0.055
Rosa moschata	0.10
Black tea (1)	0.10[a]
Black tea (2)	0.14[a]
Green tea	0.15[a]
White tea	0.17[a]

[a] Taken from reference 12.

CONCLUSIONS

Relative ORAC values of different herbal infusions depend upon the target molecule employed. In particular, ORAC-FL values are determined by the amount of phenols present in the sample, while ORAC-PGR values are determined by their quantity and average quality. From these results, it is proposed that the ratio ORAC-PGR/ORAC-FL could be employed as a rough measure of the average quality of the phenols present in the tested infusion.

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