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A novel and simple ORAC methodology based on the interaction of Pyrogallol Red with peroxyl radicals

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Abstract
Oxygen radicals absorbance capacities (ORAC) indexes are frequently employed to characterize the radical trapping capacity of pure compounds and their complex mixtures. A drawback of ORAC values obtained using phycoerythrin, fluorescein (FL) or c-phycocyanin as targets, makes it possible to conclude that for very reactive compounds they are much more related to stoichiometric factors than to the reactivity of the tested compound. In the present paper, we propose a simple methodology, based on the bleaching of Pyrogallol Red (PGR) absorbance that provides ORAC indexes that are almost exclusively determined by the reactivity of the tested compounds. This difference is due to the high reactivity of PGR and the high concentrations of this compound employed in the experiments.

Keywords: *ORAC, Pyrogallol Red, peroxyl radicals, absorbance bleaching*

Introduction

Reactive oxygen species could be important causative agents of a number of human diseases, including cancer and atherosclerosis, and the aging process [1,2]. The antioxidant activity of dietary phytochemicals has been linked to reductions in human degenerative diseases in populations that consume high amounts of fruits and vegetables. In fact, there is a particular interest in phenol and polyphenol compounds due to their ability to scavenge various oxygen and nitrogen free radicals [3,4].

A large number of methodologies have been proposed to determine the free radical scavenging capacity of antioxidants and their complex mixtures [5–8], based on the protection that the tested compound (or mixture) affords to a target molecule being degraded by a free radical process. They differ then in the free radical source, the target molecule and the rationale to evaluate the protection degree. Among these techniques, a procedure developed by Cao et al. is widely employed [6,9]. This approach renders an index, oxygen radicals absorbance capacities (ORAC), related to the capacity of the additive to delay the target molecule (phycoerythrin, FL and c-phycocyanin) consumption evaluated from its fluorescence decrease when it is incubated in presence of a free radical source (generally 2,2′-azo-bis(2-amininopropane)dihydrochloride) [6,9–12]. A shortcoming of the methodology is that it is influenced both by the reactivity of the additive and/or the number of radicals that each additive molecule can remove. The relative importance of these factors can depend upon the target molecule employed, rendering ORAC values that are strongly dependent on the employed methodology.

Pyrogallol Red (PGR) is a colored reagent, which is readily oxidized by bromate, iodate and hydrogen peroxide [13–15]. This oxidation can be easily monitored by changes in the absorption spectra, and has been used for the determination of periodate and iodate [16,17]. The PGR complexes with lead (II), copper (II) and molibdate have been also used for metal determinations [18–20] and for protein determination in urine and biologic fluids [21,22]. Furthermore, PGR oxidation by peroxynitrite has

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been used for the study of the scavenging properties of different antioxidants [23–25].

In a recent paper, we have shown that the use of PGR as a target molecule for peroxyl radicals is a good choice for the evaluation of the reactivity of very reactive phenolic compounds through competitive reactions [26]. In the present work, we discuss the use of PGR as a target molecule in the evaluation of the scavenging capabilities of single antioxidants or their complex mixtures through an ORAC-like methodology. It is concluded that the use of PGR at relatively high concentrations as reference presents two advantages regarding the classical ORAC methodologies: (i) results are better correlated with the reactivity of the tested compounds and (ii) it allows the use of visible spectroscopy to follow the consumption of the target molecule.

Materials and methods

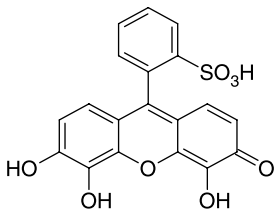
Chemicals

2,2′-Azo-bis(2-amidinopropane)dihydrochloride, (AAPH), was used as peroxyl radical source. PGR (Figure. 1), fluorescein (FL), Trolox (6-hydroxy-2, 5, 8-tetramethylchroman-2-carboxylic acid), quercetin, kaempferol, gallic acid, catechin, caffeic acid, rutin, protocatechuic acid and AAPH were purchased from Sigma–Aldrich (St Louis, MO) and employed as received.

Sample preparation

Stock solutions of the polyphenols were prepared in ethanol immediately before their use. Black tea leaves were processed according to Ou et al. [10]. The dry leaves were ground until a fine powder was obtained. Then, 0.5 g of the powder were accurately weighed and extracted with 20 ml of an acetone/water mixture (50:50, v:v) by gently shaking for 1 h at room temperature. The extracts were centrifuged at 10,000 rpm for 15 min, and the supernatant was analysed after dilution with buffer solution.

Red wine (Cabernet Sauvignon, Santa Emiliana) diluted with phosphate buffer (75 mM) at pH 7.4, (1:10) or undiluted white wine (Sauvignon blanc, Santa Emiliana) were added directly to the cuvette (final volume 3 ml).



Pyrogallol red (PGR)

Figure 1. Structure of PGR.

Solutions

Stock solutions of PGR (1×10^{-4} M) or FL (1×10^{-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 polyphenols in phosphate buffer (75 mM) at pH 7.4, was incubated at 37°C in the thermostatized cuvette of a Hewlett Packard 8453 (Palo Alto, CA, USA) UV–visible spectrophotometer. PGR consumption was evaluated from the progressive absorbance decrease measured at 540 nm. A similar procedure was carried out employing FL (70 nM), but in this case, the 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 the presence of 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. All experiments were carried out in triplicate ($n = 3$).

Results and discussion

ORAC values of pure compounds

Figure 2A shows the consumption of FL (70 nM) elicited by its incubation in presence of AAPH, both in absence (control) and presence of increasing concentrations of Trolox. Similar data, obtained by following the decrease of PGR absorbance at 540 nm are shown in Figure 2B. Three aspects of these results deserve consideration: (i) consumption profiles of FL and PGR in absence of Trolox are fairly similar, in spite of their widely different concentrations; (ii) the efficiency of the additive is considerably higher in the protection of FL than in the protection of PGR and (iii) clear induction times are generated by Trolox in the protection of FL, while only a progressive decrease in rate is observed in PGR protection. These three sets of results are closely related. Under our experimental conditions, the rate of free radical generation by AAPH is 0.82 μM min^{-1} [27]. The rate of FL consumption, derived from the initial slope of the plot shown in Figure 2A is 0.009 μM min^{-1}. This low value, which is due to the low concentration of the target molecule, implies that most of the radicals react by self-reactions and, hence, have a fairly long lifetime. These radicals are then efficiently trapped by the

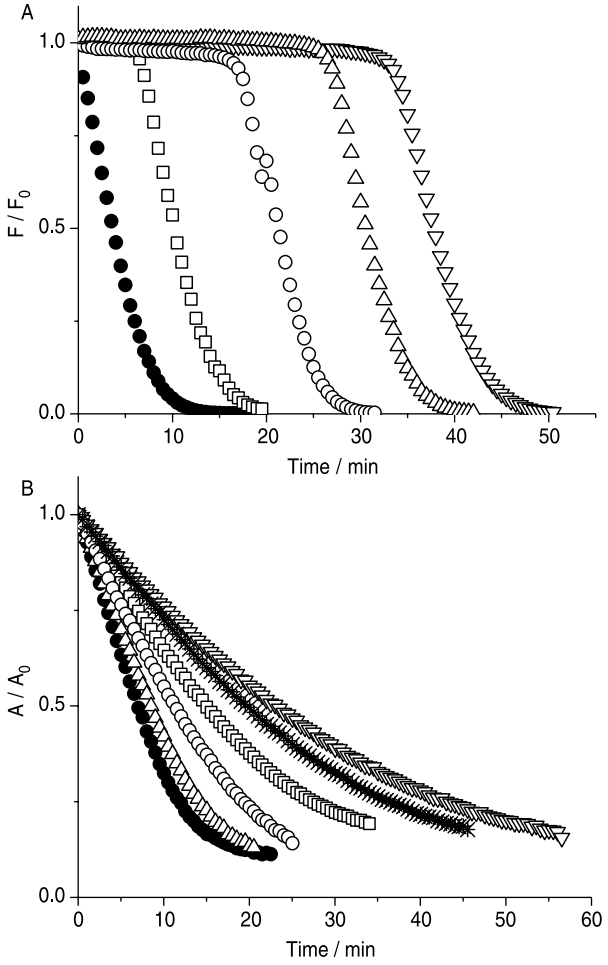


Figure 2. Time-course of the consumption of PGR (5μM) or FL (70 nM) incubated at 37°C in the presence of 10 mM AAPH. (A) FL fluorescence (493 and 515 nm, for the excitation and emission wavelengths, respectively) decay curve induced by AAPH in the presence of different Trolox concentrations. Control (•), Trolox: 1 μM (□); 5 μM (○); 7.5 μM (△); 10 μM (▽). (B) PGR absorbance decay (measured at 540 nm) induced by AAPH in the presence of different Trolox concentrations. Control (•). Trolox: 10 μM (△); 30 μM (○); 50 μM (□); 75 μM (×); 100 μM (▽).

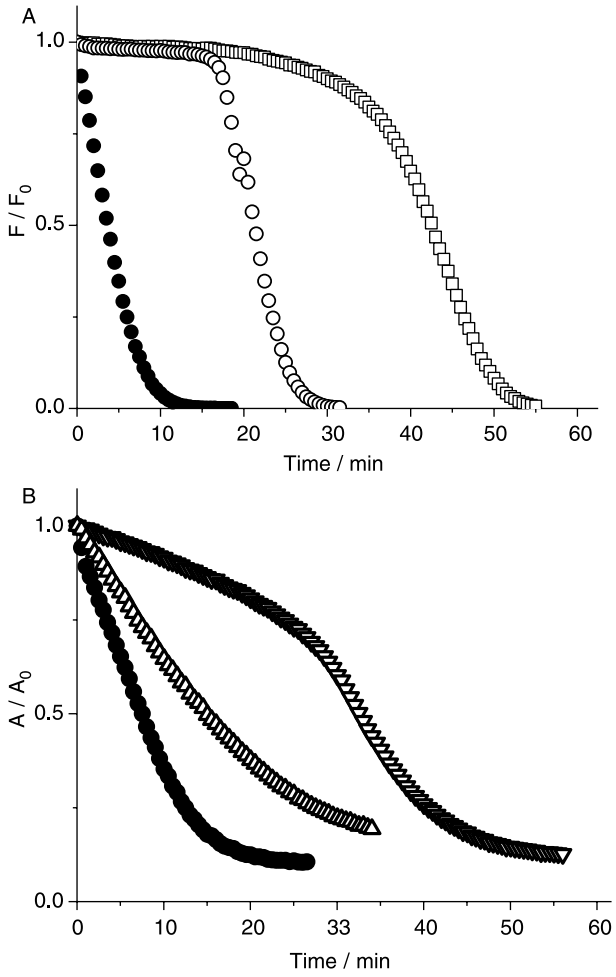


Figure 3. Comparative effects of quercetin and Trolox on the protection of FL and PGR bleaching. (A) Bleaching of FL fluorescence. Control (•); Trolox 5 μM (○); quercetin 1 μM (□). (B) Bleaching of PGR absorbance. Control (•); Trolox 50 μM (△); quercetin 10 μM (▽).

additive, generating clear induction times. On the other hand, the rate of PGR consumption amounts to $0.35\,\mu M\,min^{-1}$, this value suggesting that a large fraction of the peroxyl radicals reacts with the target molecule. Competition for the radicals under these conditions is more difficult and the additive only reduces, in a concentration dependent way, the rate of the target molecule consumption (Figure 2B).

The different profiles elicited by a given additive can have impact on ORAC values and their meaning. This is emphasized by the data shown in Figure 3. These data show that, irrespective of the target molecule employed, the ORAC of quercetin is considerably higher than 1.0 (10.7 employing FL, 11.5 employing PGR). However, the reason for the high ORAC values employing both target molecules is different. When FL is employed, the ORAC of quercetin mainly reflects the larger induction times elicited by this compound, and hence the high ORAC value (relative to Trolox) is due to differences in the stoichiometry of the free radical trapping process. On the other hand, when PGR is employed as target molecule, the high ORAC of quercetin is mainly due to its larger effect on the initial rate of the probe molecule consumption and, hence, it results from a faster interaction with the peroxyl radicals. This difference in reactivity can be estimated from the effect of the additive upon the initial rate of the probe molecule consumption [26,28,29]. In fact, a plot of R°/R (ratio between the initial rate of PGR consumption in absence (R°) or presence (R) of additive) against the additive concentration renders a straight line whose slope is proportional to the rate constant of process (1):

$$ROO^{\cdot} + \text{Additive} \rightarrow \text{inactive radicals} \qquad (1)$$

Table I. ORAC and k_1/k_{Trolox} values of pure compounds.

Compound	ORAC-$_{FL}$	ORAC-$_{PE}$	ORAC-$_{cPc}$ [13]	ORAC-$_{PGR}$	k_1/k_{Trolox}
Quercetin	10.7 ± 0.4 7.28 ± 0.22 [11] 10.5 ± 0.4 [12]	2.7 ± 0.05 [11] 3.29 ± 0.12 [10]	3.5	11.5 ± 0.4	27
Kaempferol	10.2 ± 0.3	2.67 ± 0.13 [10]	–	8.8 ± 0.7	10
Gallic acid	1.2 ± 0.03		1.7	11.1 ± 0.7	11
Catechin	6.76 ± 0.22 [11] 14.9 ± 0.81 [12]	2.57 ± 0.18 [11]	1.5	1.3 ± 0.2	0.56
Caffeic acid	4.37 ± 0.24 [11] 6.63 ± 0.24 [12]	1.4 ± 0.09 [11]	1.9	≈ 0.2	≈ 0.3
Trolox	1	1	1	1	1
Rutin	6.01 ± 0.25 [11]	1.95 ± 0.21 [11]	2.3	≈ 0.1	≈ 0.1
Protocatechuic acid	6.7 ± 0.31 [12]		–	≈ 0.05	≈ 0.3

where inactive radicals represent free radicals without capacity to damage PGR. If this slope is divided by that obtained employing a reference scavenger (Trolox), the quotient provides a value of k_1/k_{Trolox}, where k_{Trolox} is the rate constant of reaction (2):

$$ROO^{\cdot} + \text{Trolox} \rightarrow \text{inactive radicals} \qquad (2)$$

values of ORAC obtained with different target molecules and k_1/k_{Trolox} obtained employing PGR under the present conditions are collected in Table I. It is interesting to note that, for all the compounds considered, the AUC is linearly related to the additive concentration. Typical data obtained employing quercetin are shown in Figure 4. The linearity of AUC vs antioxidant concentration implies that ORAC values, defined by:

$$\text{ORAC} \equiv \frac{(\text{AUC} - \text{AUC}^{\circ})}{(\text{AUC}_{\text{Trolox}} - \text{AUC}^{\circ})} \frac{\text{Trolox}}{\text{Additive}} \qquad (3)$$

are independent of the additive concentration considered. In equation (3):

AUC = area under curve in presence of the tested polyphenol (or complex mixture), integrated between time 0 and 80% of probe consumption;
AUC° = area under curve for the control;
AUC_{Trolox} = area under curve for Trolox.

Several conclusions can be drawn from the data presented in Table I.

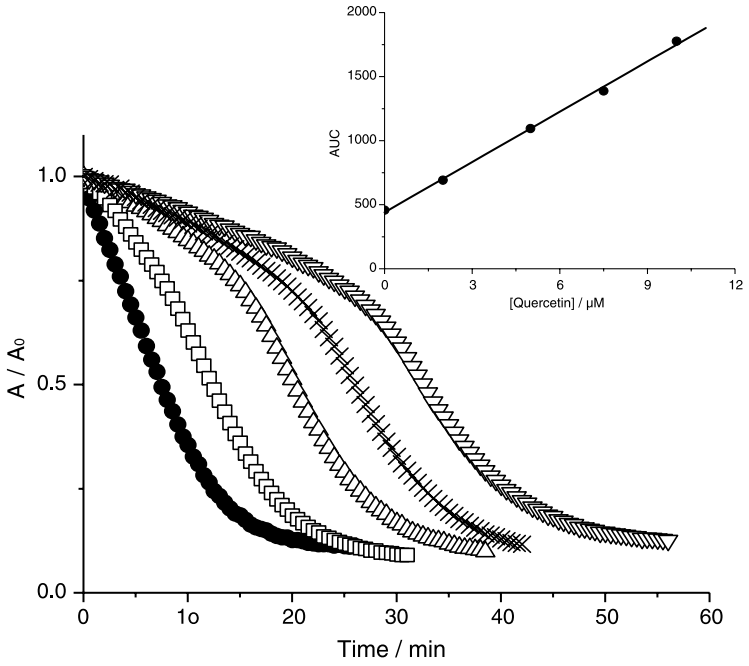


Figure 4. Consumption of PGR (5 μM) elicited by AAPH derived peroxyl radicals in the presence of different quercetin concentrations. Control (•); quercetin: 2 μM (□); 5 μM (△); 7.5 μM (×); 10 μM (▽). Insert: Area under curve (AUC) vs quercetin concentration. The reaction was followed by UV–vis spectroscopy at 540 nm and 37°C.

i) The ORAC value of a given compound strongly depends upon the target molecule employed.
ii) The use of PGR as a target molecule widens the range of ORAC values, increasing the capacity of the methodology to establish differences in free radical removal ability of the tested compounds.
iii) ORAC values obtained employing FL, PE or c-Pc as target molecules are poorly related to the reactivity of the tested compounds, being strongly influenced by stoichiometric factors. On the other hand, $ORAC_{PGR}$ values are mainly determined by the reactivity of the tested compound.

The last point, which *per se* explains the other considerations, is emphasized by a comparison of ORAC and k_1/k_{Trolox} values. This is shown in Figure 5, where $ORAC_{FL}$ (Figure 5A) and $ORAC_{PGR}$ (Figure 5B) values are plotted against k_1/k_{Trolox}

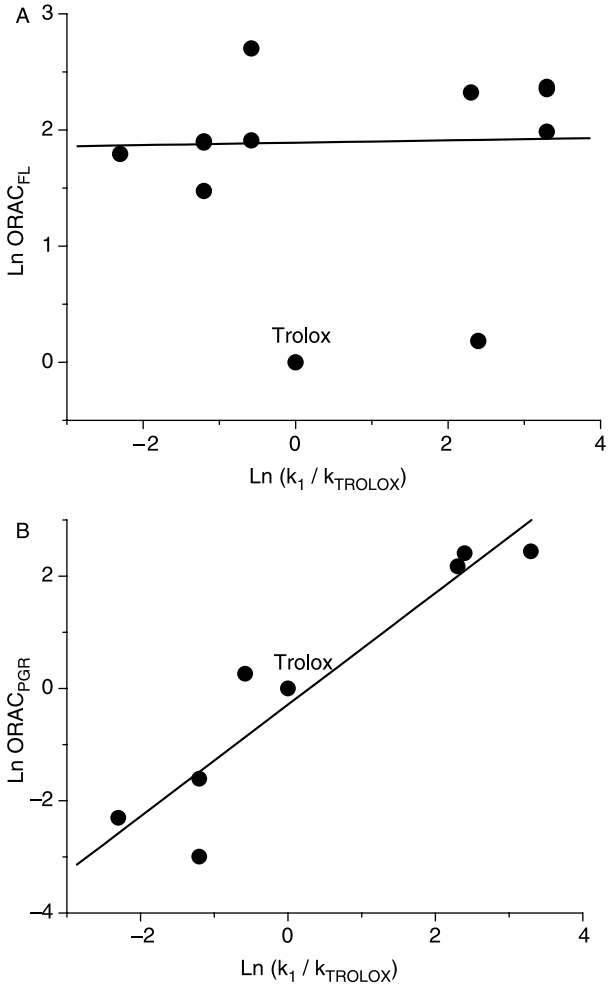


Figure 5. Correlation between ORAC index and reactivity, measured by k_1/k_{Trolox}. (A) Correlation between ln ($ORAC_{FL}$) and ln (k_1/k_{Trolox}). (B) Correlation between ln ($ORAC_{PGR}$) and ln (k_1/k_{Trolox}).

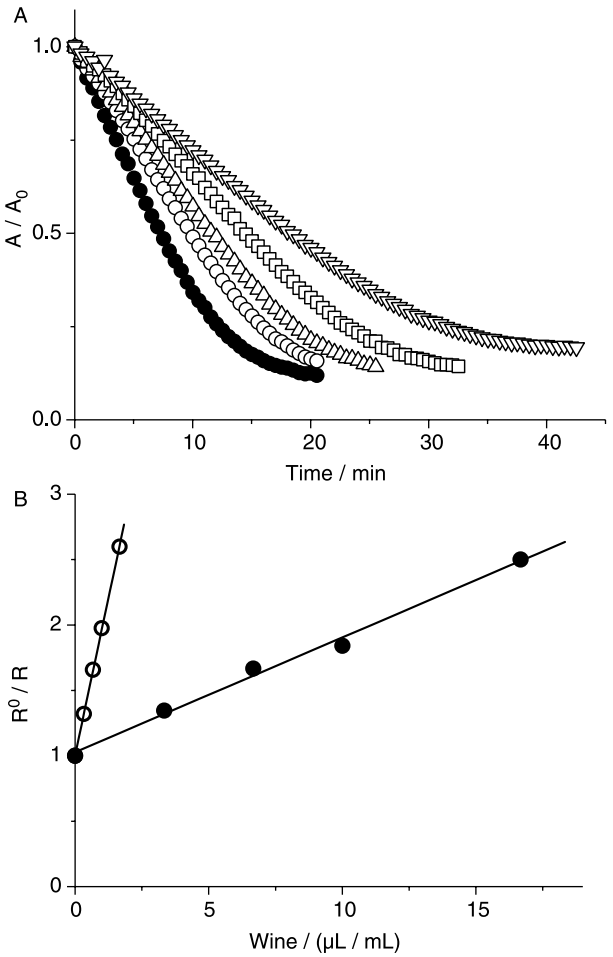


Figure 6. Effect of red wine upon the consumption of PGR (5 µM) elicited by AAPH derived peroxyl radicals. (A) PGR-absorbance decay (540 nm) induced by AAPH (10 mM) in the presence of different aliquots of red wine. Control (•); red wine: 0.33 µl/ml (○); 0.66 µl/ml (△); 1 µl/ml (□); 1.66 µl/ml (▽). Phosphate buffer (75 mM), pH 7.4, 37°C. (B) Plot of R^0/R vs wine concentration (µl/ml). Red wine (○) and white wine (•).

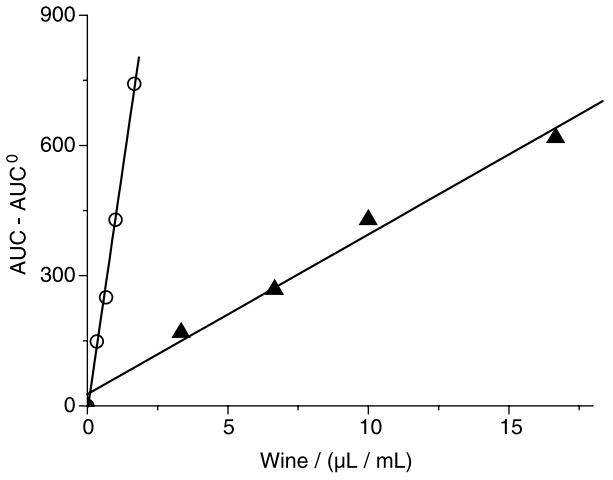


Figure 7. Dependence of the area under curve (AUC − AUC°) vs wine concentration (µl/ml). The reaction was followed by UV–vis spectroscopy at 540 nm at 37°C. Red wine (○) and (▲) white wine.

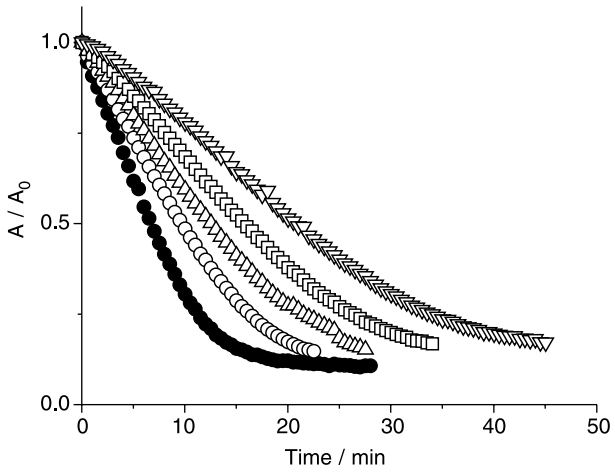


Figure 8. Effect of black tea extract on PGR (5 μM) consumption elicited by AAPH derived peroxyl radicals. Control without tea extract (•); black tea extract: 0.33 μl/ml (○); 0.66 μl/ml (△); 1 μl/ml (□); 1.66 μl/ml (▽). Experiments carried out in phosphate buffer (75 mM), pH 7.4, 37°C.

capacity equivalent to ca. 42 mM of Trolox, while white wine has nearly ten times less. This observed difference between both wines is very close to that obtained by other procedures, suggesting that it is mostly due to differences in the quantity of antioxidants and not to differences in their average reactivities [12].

The results obtained for black tea extracts emphasize the dependence of ORAC values on the employed methodology. It is interesting to note that, for this extract, the Trolox equivalents derived from the initial slopes are somehow higher than the $ORAC_{PGR}$ index. This could be related to the downwards curvature of the A/A_0 vs time plots observed at very short reaction times (Figure 8). This suggests, in this beverage, the presence of small amounts of very reactive compounds. These highly reactive antioxidants would influence more the initial slope than the ORAC index.

Table II. ORAC and k_1/k_{Trolox} values of complex mixtures*.

Mixture	$ORAC_{-FL}$	$ORAC_{-PE}$	$ORAC_{-cPc}$ [13]	$ORAC_{-PGR}$	k_1/k_{Trolox}
Red wine	–	–	11,000	42,400 ± 900	41,800
White wine	–	–	1200	3900 ± 300	3800
Black tea	17,267 ± 44 [11]	8714 ± 213 [11]	–	52,900 ± 1700	78,300

* Values represent the concentration (μM) of a Trolox solution which produces the same effect than the tested beverage.

values. The double logarithmic plots show that only $ORAC_{PGR}$ values are positively correlated ($r = 0.936$, slope 0.99) to the reactivity of the tested compound. This would indicate that this type of index is more suitable in the evaluation of the reactivity of different pure compounds with antioxidant capacity. Furthermore, this implies that it should be better related to the concentration and reactivity of the antioxidants present in complex mixtures.

ORAC values of complex mixtures

The addition of increasing aliquots of red wine, white wine or black tea extracts decreases the rate of PGR consumption in a concentration dependent fashion. Typical data are shown in Figures 6–8. These data show that both the AUC and R°/R-values are, for both wine samples, proportional to the size of the added aliquot. A similar linearity was observed employing black tea extracts (data not shown). The results obtained are summarized in Table II, where values obtained for classical ORAC indexes in similar systems are also included. These data allow us to conclude that the Trolox equivalents evaluated by both procedures (ORAC or initial slopes employing PGR as target molecule) are very similar. In fact, both procedures indicate that red wine has an antioxidant

Conclusions

PGR bleaching, evaluated by the decrease in absorbance measured at 540 nm, can be easily employed to determine ORAC values of pure compounds and complex mixtures. The methodology only requires a visible spectrophotometer and has the advantage that the derived ORAC values correlate with the reactivity of the tested compounds.

Acknowledgements

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