Free Radical Research, July 2005; 39(7): 729–736

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Interaction of pyrogallol red with peroxyl radicals. A basis for a simple methodology for the evaluation of antioxidant capabilities

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Accepted by Professor E. Niki

(Received 17 January 2005; in revised form 16 April 2005)

Abstract
A competitive method to evaluate the reactivity of highly reactive antioxidants is reported. Pyrogallol red (PGR) and AAPH (2,2′-azo-bis-(2-amidinopropane) dihydrochloride) were employed as target-molecule and peroxyl radical source, respectively. In the zero-order kinetic limit in PGR, the dependence of the ratio R°/R (where R° is the rate of the process in the absence of additive and R is the rate of the process in the presence of additive) upon the additive concentration (Stern-Volmer like plots) was studied. Various polyphenols ($n = 10$) and ascorbic acid (AA) were tested as additives. In PGR protection by AA, was observed a neat induction time, associated to the total protection of the target molecule. On the other hand, the experiments that were carried out in presence of phenolic compounds allowed a relative evaluation of their reactivity towards peroxyl radicals. This reactivity follows the order quercetin > gallic acid > Trolox > kaempferol. Data obtained employing quercetin and Trolox are compatible with a competitive protection by these antioxidants. Due to the high reactivity of PGR towards peroxyl radicals and its high extinction coefficient at long wavelengths, it is a very suitable molecule to be employed as target in the evaluation of the free radical scavenging capability of very reactive phenolic compounds.

Keywords: *Pyrogallol red, peroxyl radicals, antioxidants, flavonoids*

Introduction

Competitive techniques are widely employed to test the reactivity of antioxidants (XH) and/or free-radical scavengers towards free radicals of biological relevance. In these methods it is evaluated how the added substrate protects a reference compound from being degraded by exposure to a controlled free-radical source. The following prerequisites are required for a simple and meaningful application of these procedures:

(1) A controlled free-radical source whose free-radical production rate is determined only by the temperature and the initiator concentration, and
(2) A target molecule whose degradation by free-radicals can be easily evaluated.

AAPH (2,2′-azo-bis(2-amidinopropane) dihydrochloride) has been the most frequently employed free-radical source in aqueous solution [1,2], while a variety of molecules have been employed as reactive targets [2–4]. In most studies, Trolox (6-hydroxy-2,5,8-tetramethylchroman-2-carboxylic acid) is employed as reference free-radical scavenger. In these techniques the rate of the target molecule consumption in absence and presence of the tested antioxidant (R^0 and R, respectively) is usually determined. This kinetic information is obtained from the initial slope of the target molecule consumption [5] and/or from integration procedures, such as ORAC type methodologies [6–8]. However, both procedures do not provide precise kinetic information when the reactivity of the tested antioxidant is high enough to produce induction times. This type of behavior is frequently observed when pure flavonoids

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis Group Ltd
DOI: 10.1080/10715760500143452

or their mixtures are tested [8]. The probability of producing induction times decrease when the concentration and reactivity of the target molecule increases. In this regard, for the evaluation of very reactive antioxidants, it is necessary to employ target molecules whose rate of reaction with peroxyl radicals is high enough to avoid the occurrence of induction times in presence of the tested antioxidant.

Pyrogallol red (PGR) is a colored reagent, which is readily oxidized by bromate, iodate and hydrogen peroxide [9–11]. This oxidation can be easily monitored by changes in the absorption spectra, and has been used for the determination of periodate and iodate [12,13] and for the determination of Palladium II in water [11]. The PGR complex with lead (II), copper (II) and molybdenum (VI) have been used for metal determinations [14–16]. In addition, the PGR-molibdate complex has been widely used in clinical tests for protein determination in urine and biologic fluids [17,18]. Furthermore, PGR oxidation by peroxynitrite has been used for the study of the scavenging properties of different antioxidants [19–21]. In the present work, we report results that show that PGR could also be employed with advantages as target-molecule in the testing of the reactivity of efficient free radical scavengers.

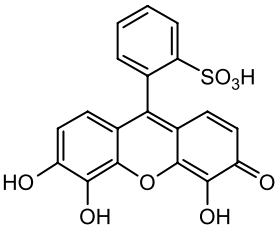
Materials and methods

Chemicals

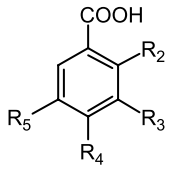
AAPH (2,2′-azo-bis(2-amidinopropane) dihydrochloride), was used as peroxyl radical source. Pyrogallol red (Figure 1), Trolox (6-hydroxy-2,5,8 -tetramethylchroman-2-carboxylic acid), ascorbic acid (AA), all tested polyphenols (Figure 1) and AAPH were purchased from Sigma-Aldrich (St. Louis, MO) and employed as received.

Solutions

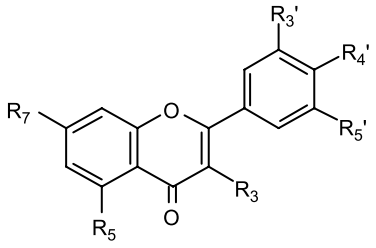
Stock solutions of PGR (1 mM) were prepared daily in phosphate buffer/ethanol, 70/30 at an apparent pH 7.0. Stock solutions of the polyphenols were prepared in ethanol immediately before their use. In some experiments, wine (Cabernet Sauvignon, Santa Emiliana) was added (5–50 μl) directly to the cubette (final volume 3 ml).



Pyrogallol red (PGR)



Compounds	R_2	R_3	R_4	R_5
Salicylic acid	–OH	–H	–H	–H
Gallic acid	–H	–OH	–OH	–OH
Protocatechuic acid	–H	–OH	–OH	–H



Compounds	R_3	R_5	R_7	R_3'	R_4'	R_5'
Flavone	–H	–H	–H	–H	–H	–H
3-Hydroxy-flavone	–OH	–H	–H	–H	–H	–H
Galangin	–OH	–OH	–OH	–H	–H	–H
Kaempferol	–OH	–OH	–OH	–H	–OH	–H
Quercetin	–OH	–OH	–OH	–OH	–OH	–H
Apigenin	–H	–OH	–OH	–H	–OH	–H
Luteolin	–H	–OH	–OH	–OH	–OH	–H

Figure 1. Structures of PGR and tested polyphenols.

The reaction mixture containing AAPH (1–80 mM), PGR (4–160 μM) with or without the tested polyphenols (or wine) in phosphate buffer (10 mM)/ethanol, 70/30 at pH 7.0, was incubated at 37°C in the thermostatized cubette of a Hewlett Packard 8453 (Palo Alto, CA, USA) UV–visible spectrophotometer. Incubation of this mixture produces a progressive decrease of PGR absorbance at 540 nm. The addition of Chelex-100 (1 g/l for 12 h) to the buffer has no effect on the rate of PGR bleaching. Therefore, no special precautions were taken against transition metal ion contamination in the water and salts used for buffer preparation.

Kinetics analysis

Assuming a simple competitive oxidation of a target molecule (PGR) and a given antioxidant, XH (polyphenol) by a radical ROO·, a kinetic model must consider reactions (1)–(4)

$$\mathrm{AAPH} + \mathrm{O_2} \rightarrow 2\mathrm{ROO}\cdot + \mathrm{N_2} \quad (1)$$

$$\mathrm{ROO}\cdot + \mathrm{PGR} \rightarrow \text{bleaching} \quad (2)$$

$$\mathrm{ROO}\cdot + \mathrm{XH} \rightarrow \mathrm{X}\cdot + \mathrm{ROOH} \quad (3)$$

$$2\mathrm{ROO}\cdot \rightarrow \text{non radicals products} \quad (4)$$

and all the self-reactions and cross-reactions of the radicals produced in steps (2) and (3). In this scheme, we are not considering the formation of alcoxyl radicals in reaction (4) for simplicity. This oversimplified scheme predicts that the ratio (R^0/R), where R^0 is the rate of consumption of the target molecule (PGR) in absence of antioxidant, and R is the rate of consumption of PGR in presence of antioxidant (XH), will depend upon the relative rates of processes (2) and (4). If $R_4 \gg R_2$, as expected at low concentrations of the target molecule, the consumption of PGR follows a first-order kinetics [5] and

$$R^0/R = 2(R_1 k_4)^{0.5} / \{-k_3[\mathrm{XH}] + ((k_3[\mathrm{XH}])^2 + 4k_4 R_1)^{0.5}\} \quad (5)$$

On the other hand, at high PGR concentrations, $R_2 \gg R_4$, the consumption of the target molecule follows a zero-order kinetics and

$$R^0/R = 1 + a(k_3/k_2)[\mathrm{XH}]/[\mathrm{PGR}] \quad (6)$$

where a is a parameter whose value, between 0.5 and 2.0, depends upon the relative importance of cross-terminations involving ROO· and PGR and XH derived radicals.

The above analysis shows that a simple relationship between R^0/R and the [XH]/[PGR] concentration over the entire [XH] range can be expected only when the concentration of the target molecule is high enough as to reach the zero-order kinetics limit [5]. However, irrespective of the substrate concentration, a linear relationship between R^0/R and the [XH] concentration can be expected when $R^0/R \gg 1$.

Results and discussion

Interaction between PGR and AAPH derived peroxyl radicals in absence of antioxidants

In order to carry out a quantitative interpretation of the data obtained in presence of antioxidants, we carried out a study of the kinetics of PGR bleaching promoted by peroxyl radicals over a wide range of AAPH and PGR concentrations.

AAPH addition to a PGR solution kept at 37°C results in a progressive bleaching of PGR, as shown in Figure 2. The data given in this figure show that the absorbance at $\lambda = 540$ nm decreases, while a product with a smaller absorbance at $\lambda = 380$ nm appears (Figure 2). The existence of a well-defined isosbestic point at $\lambda = 435$ nm indicates that PGR was oxidized to stable final products, without significant intermediates accumulation. The data given in Figure 2 point to a very efficient trapping of peroxyl radicals by PGR. In fact, its consumption follows a nearly zero-order kinetics up to more than 65% conversion (50 min of reaction). This indicates that most of the AAPH derived radicals are trapped by the additive [5].

Values obtained over a wide range of PGR and AAPH concentrations are represented as log–log plots in Figure 3A and B. These data show that, as expected, at low target molecule concentrations the process follows a kinetics that is first-order in PGR and 0.5 order in AAPH. At high PGR concentrations, the kinetics of the process becomes zero-order in PGR and nearly first-order in AAPH. In the concentration range of zero-order kinetics (over 30 μM PGR), the rate of the reaction is 0.46 μM/min. If this value is compared to the rate of free radical production under our experimental conditions (0.82 μM/min), it can be concluded that ca. 1.8 peroxyl radicals are scavenged by each consumed PGR. This value is fully compatible with the proposed reaction scheme if the main free radicals elimination step is a cross reaction between a ROO· radical and a PGR derived radical. It is interesting to point out that, although the pKa of PGR is close to 6.3 [22], its rate of consumption (at 30 μM) is nearly pH independent in the pH 6–9 range. This is compatible with the zero-order of the reaction under the employed conditions.

Interaction between PGR and peroxyl radicals in presence of antioxidants

The rate of PGR consumption is reduced by the addition of free radicals scavengers. The factors

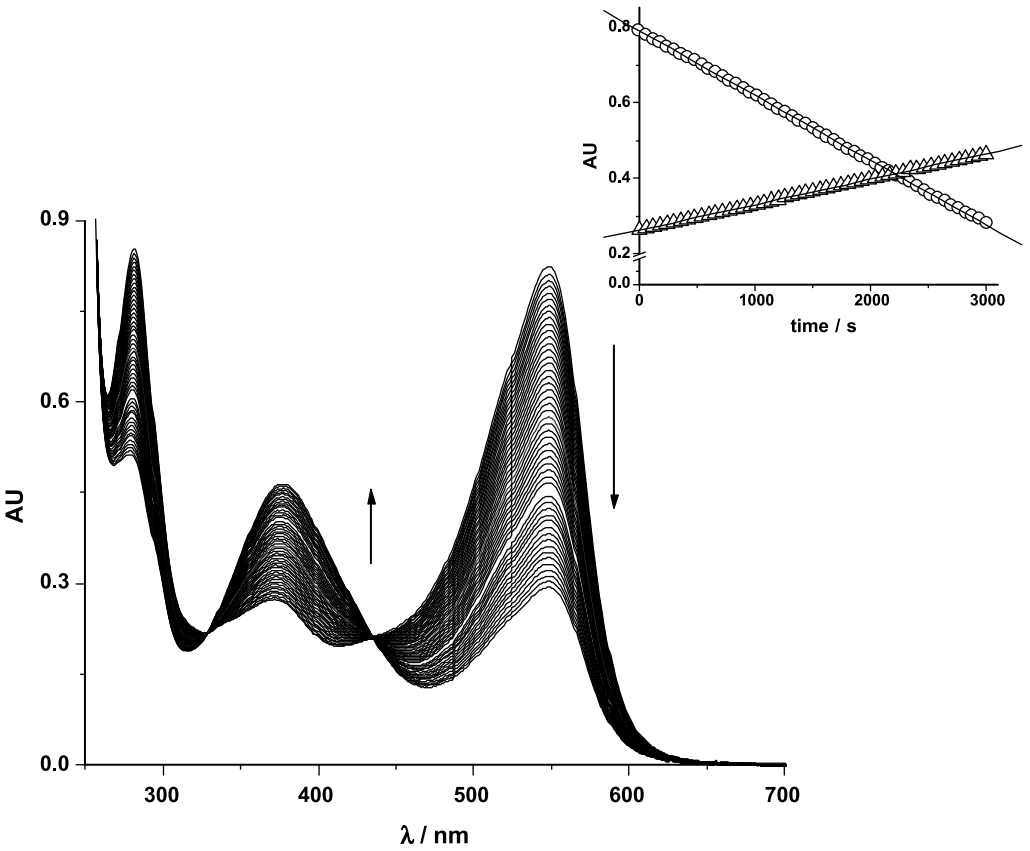


Figure 2. Beaching of PGR (30 μM) by 10 mM AAPH between 0 and 50 min of reaction. Insert: Time course of absorption units (AU) at (○) 540 nm and (△) 380 nm. Phosphate buffer (10 mM) / ethanol, 70/30, pH 7.0, 37°C.

affecting the magnitude of the protection afforded by a given additive depend upon the target molecule concentration. We discuss this point employing quercetin as model antioxidant.

Protection by quercetin when PGR consumption is in the first-order kinetics limit. At low PGR and high (XH) concentrations, the ratio R^0/R given by Equation (5) reduces to that expression given by Equation (7) [5].

$$R^0/R = b(R_1k_4)^{-0.5}k_3[\mathrm{XH}] \qquad (7)$$

where b is a parameter whose value, between 1.0 and 2.0, is determined by the relevance of cross terminations between ROO· and XH derived radicals.

According to Equation (7), a plot of R^0/R against XH concentration must converge to a straight line whose slope is given by $(bk_3/(R_1k_4)^{0.5})$. The data given in Figures 4 and 5 are compatible with this proposal. Figure 4, shows R^0/R vs. [Quercetin] plots obtained at 10 and 40 mM AAPH. The slope of the plot decreases when AAPH concentration increases. The ratio of the slopes is 2.8 ± 0.4, a value not far from the theoretical value (2 .0). Equation (7) predicts that R^0/R values obtained at a given XH and AAPH concentration must be independent of PGR concentration. This prediction is supported by data given in Figure 5, showing that, at low PGR concentrations (4 and 8 μM), R^0/R values become independent of the target molecule concentration.

From all these data we concluded that low target molecule concentrations presents several drawbacks for their use in testing antioxidant capacities. In particular, the fact that the effect of the added scavenger depends on the rate of free radical production precludes a direct comparison of values obtained under different experimental conditions. Furthermore, the lack of a lineal dependence of R^0/R with the additive concentration at low R^0/R values, predicted by Equation (5) and evident in the data shown in Figure 4, makes it more difficult to obtain meaningful results.

Protection by quercetin when the consumption of the target molecule follows a zero-order kinetics. At PGR concentrations (≥30 μM), the consumption of the target molecules follows a zero-order kinetics (Figure 5). In this limit, the protection afforded by an additive can be quantitatively interpreted in terms of Equation (6). In agreement with this prediction, linear Stern-Volmer like plots of R^0/R vs. [XH] were observed (Figures 5 and 6) [5]. The data shown in

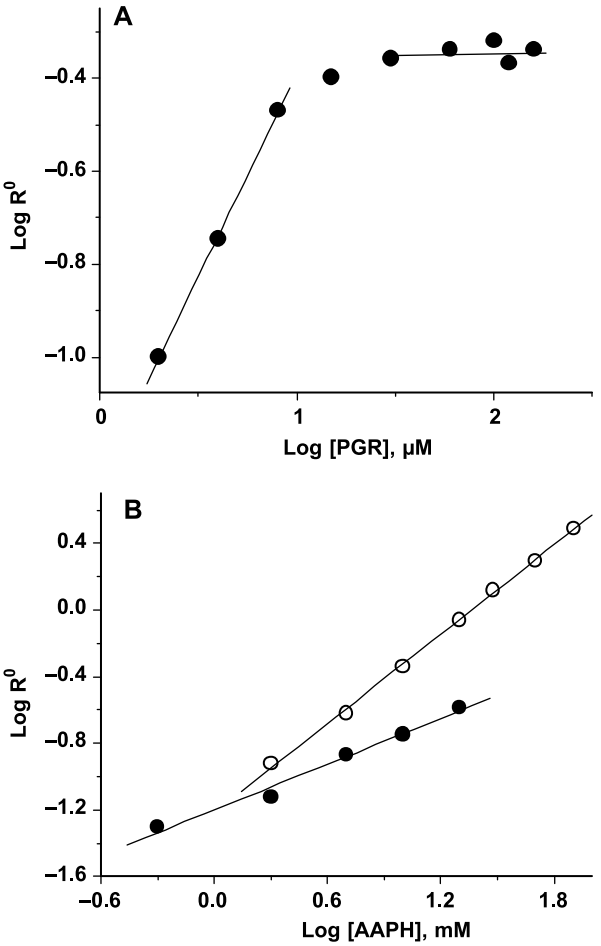


Figure 3. Reactions orders in PGR and AAPH. (A) $\log R^0$ vs. log [PGR]. (B) $\log R^0$ vs. log [AAPH] at PGR (○) 60 μM and (•) 4 μM.

these figures are fully compatible with the behavior predicted by Equation (6). In particular, the data of Figure 5 show that, at high PGR concentrations, the slope of the Stern-Volmer like plot becomes

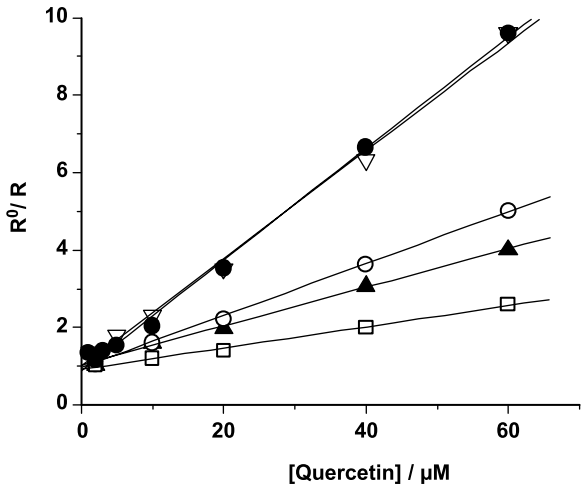


Figure 5. Plot of R^0/R vs. quercetin concentration (μM) at different PGR concentrations. (•) 4 μM, (▽) 8 μM, (○) 30 μM, (▲) 60 μM and (□) 120 μM. AAPH 10 mM, phosphate buffer (10 mM)/ethanol, 70/30, pH 7.0, 37°C.

inversely proportional to the PGR concentration (slope = 0.050 μM^{-1} at 60 μM and 0.026 μM^{-1} at PGR 120 μM). Similarly, the data shown in Figure 6 show that R^0/R values are nearly independent of the free radical source concentration, as predicted by Equation (6) (slope = 0.050 μM^{-1} and slope = 0.046 μM^{-1} at AAPH 10 and 5 mM, respectively).

The linearity of the Stern-Volmer like plots (Figure 5), the independence with the rate of peroxyl radical production (Figure 6) and the inverse dependence with the target molecule concentration (that allows to test the proposed mechanism) make the reaction in this regime particularly suitable for the evaluation of kinetic parameters from competitive experiments.

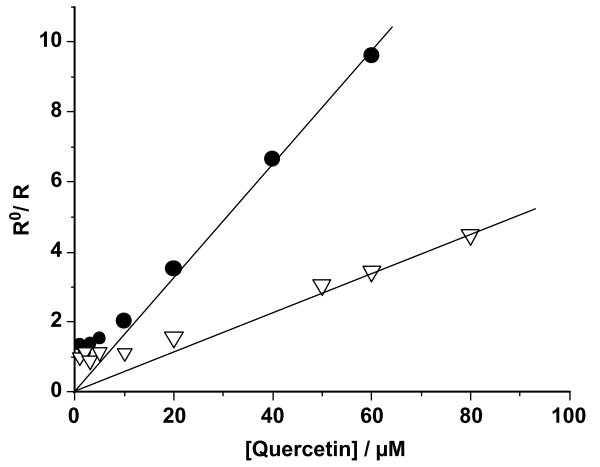


Figure 4. Stern-Volmer like plots (R^0/R vs. [Quercetin]) at PGR 4 μM and two AAPH concentrations: (•) 10 mM; (▽) 40 mM. Phosphate buffer (10 mM)/ethanol, 70/30, pH 7.0, 37°C.

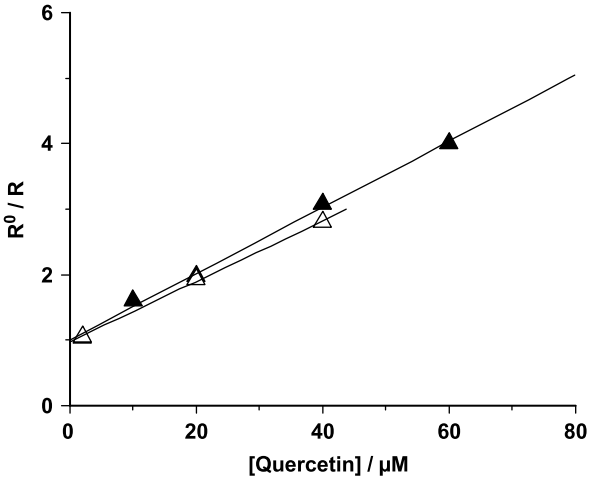


Figure 6. Ratio R^0/R vs. quercetin concentration (μM) at different AAPH concentrations, (△) 5 and (▲) 10 mM. PGR 60 μM, phosphate buffer (10 mM)/ethanol, 70/30, pH 7.0, 37°C.

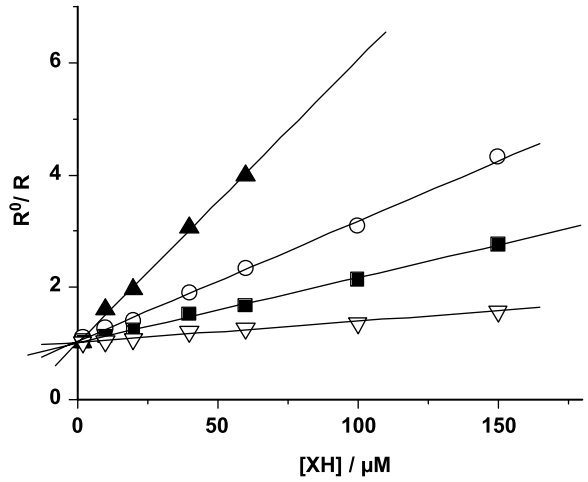


Figure 7. Stern-Volmer like plots (R^0/R vs. [XH]) at PGR 60 μM. (▲)quercetin, (○) gallic acid, (■) Trolox and (▽) kaempferol. Phosphate buffer (10 mM)/ethanol, 70/30, pH 7.0, 37°C.

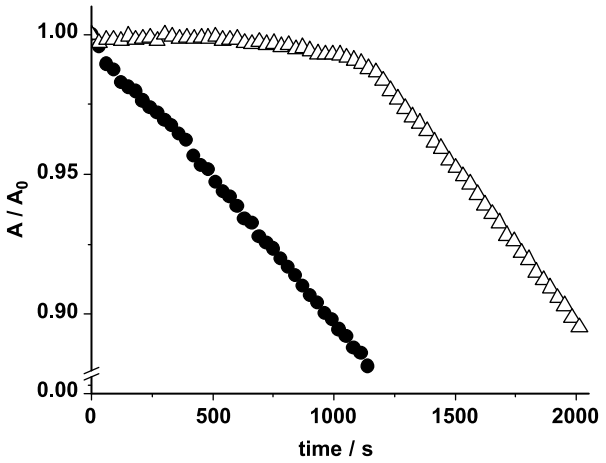


Figure 8. Time-course of PGR-peroxyl radicals reaction in presence of (△) ascorbic acid (10 μM). (•) control. PGR 60 μM, AAPH 10 mM, phosphate buffer (10 mM)/ethanol, 70/30, pH 7.0, 37°C.

Protection by different scavengers when the consumption of the target molecule follows a zero-order kinetics. From the slope of Stern-Volmer like plots (Figure 7), we can estimate the reactivity of the tested compound. Relative rate constants, obtained assuming a value of one for the parameter *a*, are collected in Table I. Salicylic acid, protocatechuic acid, luteolin, apigenin, galangin, and 3-hydroxy-flavone did not protect the target molecule under the present experimental conditions. This emphasizes the particular reactivity of the flavonoids bearing hydroxyl groups in the 3, 3′ and/or 4′ positions [23,24].

The antioxidant capacity of flavonoids and polyphenols has been studied by TEAC and ORAC methodologies [25,26]. For example, the reported order in TEAC values is: Quercetin > gallic acid > kaempferol [25]. On the other hand, Cao et al. [26] used an ORAC methodology to estimate the antioxidant capacity of different polyphenols towards peroxyl radicals ($ORAC_{ROO\cdot}$). In contrast with the present data, it was found to have similar ORAC values for luteolin and quercetin. However, a simple comparison between reactivity data obtained by competitive kinetic techniques and TEAC or ORAC values is not warranted. TEAC values are dominated by stochiometric factors, while in the ORAC assay compounds with totally different consumption profiles (and hence reactivity) can yield the same value of the measured parameter [27].

In order to have a direct estimation of the relative reactivity of the tested polyphenols and that of an important hydro-soluble antioxidant, we evaluated the PGR protection by AA. Typical data are shown in Figure 8. These data show a neat induction time, followed by a probe consumption similar to that observed in absence of additives. This implies that AA reacts much faster than all tested phenols, and/or that AA is particularly efficient in repairing the PGR derived radicals produced in reaction (2). The present data do not allow deciding between these two possibilities.

Protection by a mixture of antioxidants. Since phenols will be present in mixtures, it is interesting to evaluate if the protection afforded by them is additive. To test this, we measured the initial rate of PGR bleaching in presence of two free radicals scavengers

Table I. Parameters of Stern-Volmer like plots. PGR 60 μM and AAPH 10 mM. Phosphate buffer 10 mM/ethanol, 70/30, pH 7.0, 37°C.

Compound	R^0/R vs. [XH (μM)]*	(k_3/k_2)	Rel. Trolox (k_3/k_{TROLOX})
Quercetin	$y = 1.04 \pm 0.06 + 0.050 \pm 0.004x$ $r = 0.996 \pm 0.002$	3.0	4.3
Gallic acid	$y = 1.04 \pm 0.02 + 0.021 \pm 0.002x$ $r = 0.995 \pm 0.003$	1.3	1.9
Kaempferol	$y = 1.01 \pm 0.03 + 0.004 \pm 0.0005x$ $r = 0.992 \pm 0.004$	0.2	0.3
Trolox	$y = 1.03 \pm 0.03 + 0.011 \pm 0.001x$ $r = 0.999 \pm 0.0004$	0.7	1.0
PGR	–	–	1.4

* Experiments carried out in triplicate ($n = 3$).

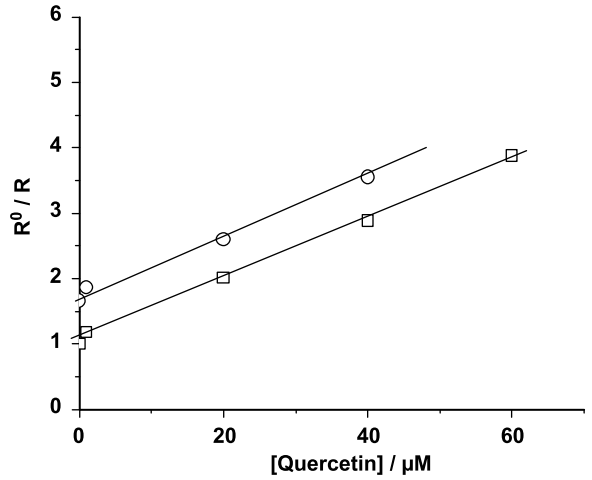


Figure 9. Ratio R^0/R vs. quercetin concentration (μM) with (○) 40 μM Trolox and (□) without Trolox. PGR 60 μM, AAPH 10 mM, phosphate buffer (10 mM)/ethanol, 70/30, pH 7.0, 37°C.

(XH and XH_b). Thus, to the reaction scheme (reactions (1)–(4)) we added reaction (8).

$$ROO\cdot + XH_b \rightarrow X\cdot_b + ROOH \qquad (8)$$

At high substrate concentrations, the ratio R^0/R in presence of XH and XH_b will be given by

$$R^0/R = 1 + (k_8/k_2)[XH_b]/[PGR] + (k_3/k_2)[XH]/[PGR] \qquad (9)$$

This equation predicts that, at a fixed XH_b concentration, a plot of R^0/R vs. [XH] must have an intercept different from one, but with a slope as that obtained when the experiment is carried out in absence of XH_b. Data obtained employing Trolox and quercetin, given in Figure 9, fully agree with these predictions, showing that both antioxidants are competitive and that the fate of XH and XH_b derived radicals are not significantly modified by their joint addition. It is interesting to note that, in this situation, the percentage of inhibition afforded by the addition of a given antioxidant decrease in the presence of other competitive scavengers [28]. This prediction is in full agreement with the results shown in Figure 9.

Protection by a complex mixture of antioxidants. In order to assess the applicability of the proposed methodology to complex mixtures of antioxidants, we evaluated the effect of red wine aliquots upon the rate of PGR consumption. Typical results are shown in (Figure 10). The data presented in this figure show a strong reduction in the rate of PGR consumption, that is proportional to the size of the added wine aliquot. Furthermore, the data show a downward curvature, with an ill-defined induction time. This behavior is that expected when a complex mixture of antioxidants of

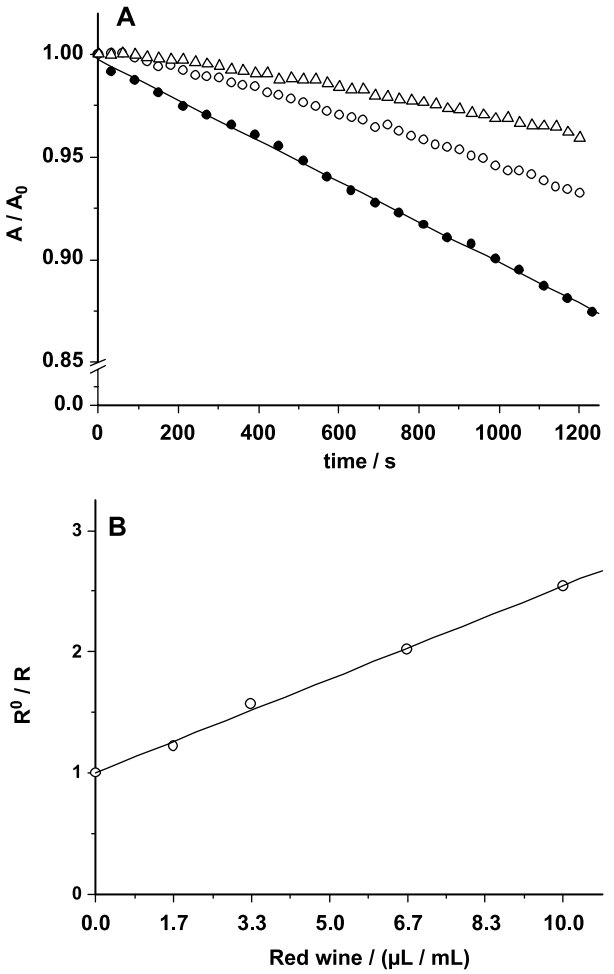


Figure 10. (A) Effect of wine addition upon the rate of PGR concumption. (•) Control; (○) 3.3 μl/ml added wine; (△) 10 μl/ml added wine. (B) Ratio R^0/R vs. wine concentration (μl/ml). PGR 60 μM, AAPH 10 mM, phosphate buffer (10 mM)/ethanol, 70/30, pH 7.0, 37°C.

different reactivity is employed. However, it must be noticed that:

– The amount of compounds consumed at the early stages of the process is very small. A simple calculation indicates that, if each reactive group traps two peroxyl radicals, the total amount of very reactive groups is ca. 0.3 mM, a figure considerably smaller than the total amount of phenols present in the wine sample [29].
– After a few minutes, the consumption of PGR in the presence of wine becomes almost lineal. A plot of R^0/R as a function of the wine concentration is shown in Figure 10B. A comparison of the slope of this plot with that obtained employing Trolox renders that, in the wine sample, there are 16 mM Trolox equivalents. This figure is somehow smaller than the 30 mM reported in the same type of wine for the total amount of antioxidants [29]. This difference would indicate that, on the average,

the main phenols present in the wine sample are almost twice less reactive than Trolox.

In conclusion, PGR presents several advantages as target molecule for the evaluation of the antioxidant capabilities of reactive phenolic compounds:

–given its high extinction coefficient at long wavelengths, its consumption can be easily followed by visible light absorption spectroscopy;
–its consumption by peroxyl radicals takes place without formation of interfering products and follows simple kinetic laws;
–its high reactivity makes possible to obtain quantitative information regarding the relative reactivity of very reactive antioxidants.

Acknowledgements

This work was supported by FONDECYT (Projects no. 3040002 and 1030033).

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