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Antioxidant reactivity evaluated by competitive kinetics: Influence of the target molecule concentration

C. López-Alarcón [a], A. Aspée [b], E. Lissi [b,*]

[a] Departamento de Farmacia, Facultad de Química, Pontificia Universidad Católica de Chile, C.P. 782 0436, Santiago, Chile
[b] Facultad de Química y Biología, Universidad de Santiago de Chile, Casilla 40, Correo-33, Santiago, Chile

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Abstract

In the present work, a competitive kinetic method using c-phycocyanin (from Arthospira maxima species) as target molecule was employed to estimate the reactivity of several phenols and flavonoids present in the human diet towards peroxyl radicals. The results obtained indicate that the protection afforded by a given compound strongly depends upon the experimental conditions employed and, in particular, on the concentration of the target molecule. This dependence is related to increased role of secondary reactions of the phenol-derived radicals initially formed. Secondary reactions can explain the strong downward curvature observed in R^0/R (where R^0 is the rate of the process in the absence of additive, and R is the rate of the process in the presence of additive) vs. additive concentration plots, for compounds such as kaempferol and protocatechuic acid, particularly at high c-phycocyanin concentration. Also, it can explain the prooxidant role played by phenolic compounds of low reactivity, such as 3-hydroxyflavone at low c-phycocyanin concentrations.

Keywords: Flavonoids; Phenols; c-Phycocyanin; Peroxyl radicals; Antioxidants

1. Introduction

There is consensus that free radical reactions are relevant in many physiological and pathological processes. Reactive oxygen species (ROS) could be important causative agents of a number of human diseases, including cancer and atherosclerosis, as well as the aging process itself (Jurgens, Hoff, Chisolm, & Esterbauer, 1987; Steinberg, 1991).

The antioxidant activity of dietary phytochemicals has been linked to a reduction in human degenerative diseases in populations that consume high amounts of fruits and vegetables. In particular, the ability of plant polyphenolic compounds and/or their metabolites to scavenge oxygen and nitrogen free radicals, has been related to the health benefits of diets rich in fruits and vegetables (Sun, Chu, Wu, & Liu, 2002; Huang, Johanning, & O'Dell, 1986).

Competitive techniques have been widely employed to test the reactivity of antioxidants (XH) and/or free radical scavengers towards free radicals. These methods evaluate how the added substrate protects a reference compound from being degraded by peroxyl radicals (Niki, 1990) using a variety of molecules as reactive targets (phycobiliproteins, crocin, pyrogallol red, etc.) (Bhat & Madyastha, 2000; Chatterjee, Poduval, Tilak, & Devasagayam, 2005; Huang, Ou, & Prior, 2005; Lissi, Pascual, & Del Castillo, 1992; Lissi, Pizarro, Aspée, & Romay, 2000; López-Alarcón & Lissi, 2006; López-Alarcón & Lissi, 2005; Pérez, Leighton, Aspée, Aliaga, & Lissi, 2000; Prior & Cao, 1999; Roginsky & Lissi, 2005; Tubaro, Ghiselli, Rapuzzi, Maiorino, & Ursini, 1998; Tubaro, Rapuzzi, & Ursini, 1999).

In spite of the wide use and advantages of competitive kinetics methods, possible secondary reactions of antioxidant derived radicals with the target molecule can preclude

* Corresponding author. Tel.: +56 2 6812575; fax: +56 2 6812108.
E-mail address: elissi@lauca.usach.cl (E. Lissi).

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a straightforward evolution of the tested compound reactivity. In spite of this, the possibility is generally not considered and only a few works have taken this point into account in the kinetic analysis (Galati, Sabzevari, Wilson, & O'Brien, 2002; Pino & Lissi, 2001). In the present work, we employed a competitive kinetic method using c-phycocyanin (c-Pc) as a target molecule for estimating the reactivity of several natural phenols and flavonoids presents in the human diet towards 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH) derived peroxyl radicals. The results obtained indicate that the relative protection afforded by a given compound strongly depends upon the experimental conditions employed, and emphasize the role of secondary reactions of the phenol-derived radicals initially formed.

2. Experimental

2.1. Chemicals

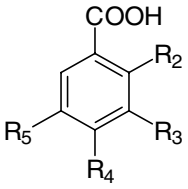
AAPH (2,2′-azo-bis(2-amidinopropane) dihydrochloride) thermolysis in air saturated solutions was used as peroxyl radical source (Niki, 1990). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), flavonoids, phenolic compounds (Fig. 1) and AAPH were purchased from Sigma–Aldrich (St. Louis, MO, USA) and employed as received. Phycocyanin (c-Pc) was obtained from Arthospira maxima species and purified by the method of Neufeld and Riggs (Neufeld & Riggs, 1969). The c-Pc concentration was estimated by considering a mw of ca. 13,000 per each bilin group.

2.2. Solutions

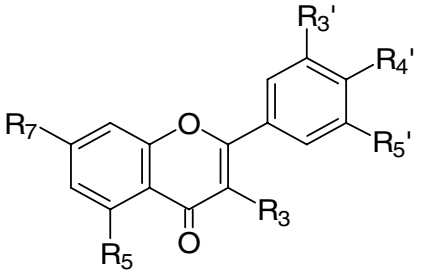
A mixture containing c-Pc (0.8 or 23 μM) with or without the tested compounds (1–50 μM) in phosphate buffer (10 mM) at pH 7.0, was incubated at 37 °C in the thermostated cuvette of either a Perkin Elmer LS-50 (Norwalk, CT, USA) spectrofluorimeter (c-Pc = 0.8 μM) or a Hewlett Packard 8453 (Palo Alto, CA, USA) UV-visible spectrophotometer (c-Pc = 23 μM). The reaction was initiated by adding an aliquot of AAPH (10 mM final concentration). The consumption of the target molecule (c-Pc) was evaluated from the decrease in its fluorescence intensity (excitation: 620 nm; emission: 640 nm) or from the progressive absorbance decrease, measured at 620 nm. Stock solutions of c-Pc (77–230 μM) were prepared daily in phosphate buffer 10 mM, pH 7.0. Stock solutions of the tested phenolic compounds were prepared in ethanol immediately before their use. The final ethanol concentration in the cuvette was below 5%.

2.3. Kinetic analysis

A simple kinetic model for the competitive oxidation of a target molecule (c-Pc) and a given antioxidant (XH) by a radical ($ROO^{\cdot}$) must consider reactions (1)–(4)



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

Fig. 1. Structures of tested polyphenols.

$$AAPH \xrightarrow{O_2} 2ROO^{\cdot} + N_2 \quad (1)$$

$$ROO^{\cdot} + c - Pc \rightarrow \text{bleaching} \quad (2)$$

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

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

and all the self-reactions and cross-reactions of the radicals produced in steps (2) and (3). In this scheme, and for simplicity, we are not considering the formation of alkoxyl radicals in reaction (4). This oversimplified scheme predicts a monotonous increase in the (R^0/R) values (where R^0 is the initial rate of consumption of the target molecule in absence of antioxidant, and R is the initial rate of consumption of c-Pc in presence of antioxidant) with XH concentration, depending upon the relative rates of processes (2)–(4). If $R_4 \gg R_2$ (where R_4 and R_2 are the rates of reactions (4) and (2), respectively), as expected at low c-Pc concentrations, the consumption of c-Pc in absence of XH follows a first-order kinetics (Pino & Lissi, 2001) and

$$R^0/R = 2(R_1k_4)^{0.5}/\{-k_3[\mathrm{XH}] + ((k_3[\mathrm{XH}])^2 + 4k_4R_1)^{0.5}\} \quad (5)$$

where:

R^0 initial rate of c-Pc consumption in absence of antioxidant
R initial rate of c-Pc consumption in presence of antioxidant
R_1 rate of peroxyl radicals production from AAPH
k_3 and k_4 kinetic rate constant of reactions (3) and (4), respectively
[XH] antioxidant concentration.

Eq. (5) at high [XH] values, reduces to

$$\frac{R^0}{R} = a(R_1k_4)^{0.5}k_3[\mathrm{XH}] \quad (6)$$

where a is a parameter whose value, between 1.0 and 2.0, is determined by the relevance of cross terminations between ROO$^{\cdot}$ and XH derived radicals.

On the other hand, at high c-Pc concentrations, $R_2 \gg R_4$, the consumption of the target molecule in absence of XH follows a zero order kinetics and, in the presence of XH:

$$R^0/R = 1 + a(k_3/k_2)[\mathrm{XH}]/[\mathrm{c-Pc}] \quad (7)$$

where a is a parameter whose value, between 0.5 and 2.0, depending upon the relative importance of cross-terminations involving ROO$^{\cdot}$ and c-Pc and XH derived radicals. The value of this parameter, at a given c-Pc concentration, could depend upon the XH structure and concentration.

The above analysis shows that a simple linear relationship between R^0/R and [XH]/[c-Pc] over the entire [XH] range can be expected only when the concentration of the target molecule is high enough to reach the zero order kinetics limit (Pino & Lissi, 2001). On the other hand, irrespective of the substrate concentration, a linear relationship between R^0/R and the [XH] concentration can be expected when $R^0/R \gg 1$. However, it is important to realise that the slopes of the plots are determined by different parameters. In fact, at low target concentration,

$$\mathrm{Slope} = a(R_1/k_4)^{-0.5}k_3 \quad (8)$$

while, at high target concentrations, it is given by:

$$\mathrm{Slope} = a(k_3/k_2)(1/[\mathrm{c-Pc}]) \quad (9)$$

It is relevant to point out that a common feature of the slopes is that both of them are proportional to k_3 and, hence, are determined by the reactivity of the additive towards peroxyl radicals.

3. Results and discussion

Exposure of c-Pc to the free radical source (AAPH) leads to a progressive loss of its visible absorbance and related fluorescence intensity. This bleaching of the target molecule is due to the occurrence of reaction (2). The rate of the process, determined by the initial slope of the A/A^0 or F/F^0 vs. time plots, depending on the concentration of c-Pc (Fig. 2). The data presented in this figure show that the rate of the process is concentration-dependent (first-order limit) up to ≈0.8 μM and concentration-independent (zero order limit) over 11.5 μM of c-Pc. The first-order kinetic limit (at low c-Pc concentrations), implies that most of the peroxyl radicals react by self-reactions, and R^0/R values would be given by Eq. (5). In the zero order kinetic limit (over 11.5 μM c-Pc), most peroxyl radicals interact with c-Pc and R^0/R values would be described by Eq. (7). In this concentration range, the rate of the reaction is 0.51 μM/min. If this values is compared to the rate of free radical production under our experimental conditions (0.82 μM/min) (Niki, 1990), it can be concluded that ca. 1.6 peroxyl radicals are scavenged by each consumed bilin group. This value differs by a factor of near two from that reported by Lissi et al. (2000). Probably the different values can be explained in terms of the different c-Pc sources used. Kinetic data on the c-Pc–AAPH system were obtained, both in the first and zero order kinetic limit, to study the effect of the target concentration on the antioxidant capacity of different phenols and related compounds. In principle, if a simple kinetic scheme holds, Eqs. (6) and (7) can be employed to obtain relative k_3 values. In fact, if the slopes of R^0/R vs. [XH] plots (Slope) are compared to that of a reference inhibitor, such as Trolox, ($\mathrm{Slope_{Trolox}}$), Eqs. (8) and (9) indicate that, irrespective of c-Pc concentration:

$$\mathrm{Slope}/\mathrm{Slope_{Trolox}} = ak_3/k_{\mathrm{Trolox}} \quad (10)$$

where a varies between 0.5 and 2.0, and k_{Trolox} is the specific rate constant for the reaction between peroxyl radicals and Trolox. A remarkable feature of this equation is that the ratio between the slopes is nearly independent of the target molecule employed and its concentration.

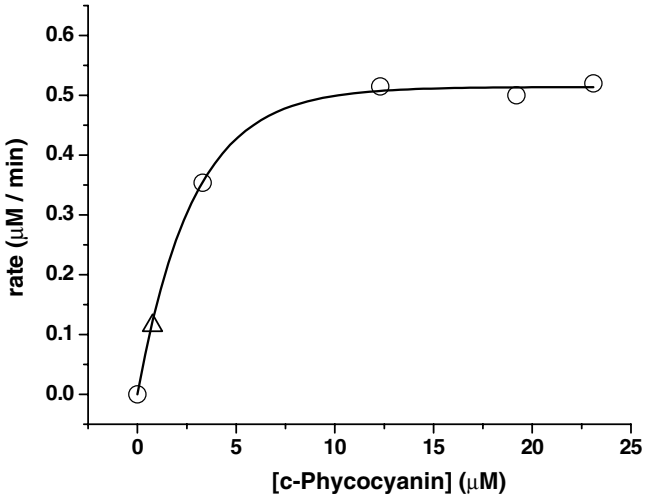


Fig. 2. Dependence of the bleaching rate with c-Phycocyanin (c-Pc) concentration. (○): reaction followed by the decrease of c-Pc absorbance at 620 nm. (△): reaction followed by the decrease of c-Pc fluorescence intensity (excitation: 620 nm; emission: 640 nm). AAPH = 10 mM; temperature = 37 °C.

3.1. Effect of antioxidants on the c-Pc bleaching

Bleaching of c-Pc, both at low (0.8 μM) and high (23 μM) c-Pc concentrations could be influenced by the addition of XH. In fact, the kinetic profiles show different behaviours that depend of the antioxidant added and the initial c-Pc concentration. Fig. 3 shows the effect of protocatechuic acid (Fig. 3a) and quercetin (Fig. 3b) on c-Pc (at 0.8 μM) bleaching promoted by AAPH. In Fig. 3a we can observe that protocatechuic acid (1–50 μM) addition generated a decrease in the bleaching rate of c-Pc (followed by fluorescence). This effect was dependent on the protocatechuic acid concentration and allowed to evaluate the initial rate (R) of cPc consumption as a function of the antioxidant concentration. However, when quercetin was added (Fig. 3b), a clear induction time was noticed. This induction time implies that quercetin is more reactive than c-Pc towards AAPH derived peroxyl radicals. The presence of an induction time makes estimation of the initial rate difficult and, hence, of the R^0/R ratio.

For the XH that do not generate induction times it is possible, from the kinetics profiles (A/A^0 or F/F^0) of c-Pc bleaching, to estimate R^0/R ratios. Figs. 4 and 5 show the dependence of R^0/R values with the antioxidant concentration ([XH]) at low (0.8 μM) and high (23 μM) c-Pc concentrations, respectively. From these figures it is clear that the behaviour is very dependent on the antioxidant added and the c-Pc concentration. In these figures we can distinguish four behaviours: a linear relationship with $R^0/R > 1$, values of $R^0/R > 1$ with downward curvatures,

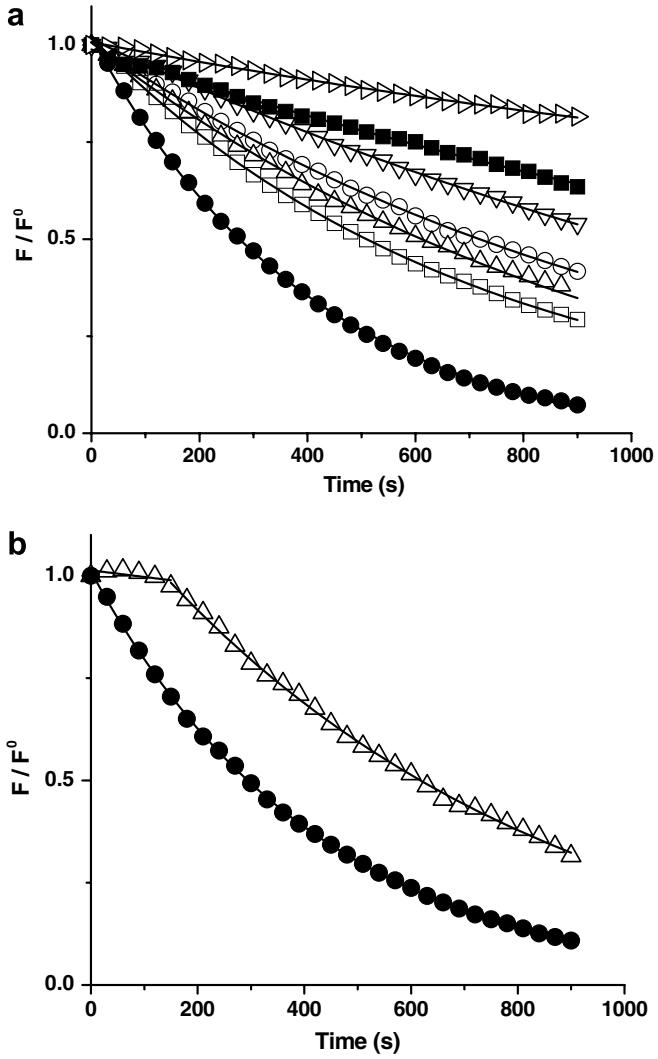


Fig. 3. Bleaching of c-Phycocyanin (0.8 μM) elicited by AAPH (10 mM) in presence of antioxidants. (a) Protocatechuic acid: 1 μM (□); 2 μM (△); 5 μM (○); 10 μM (▽); 20 μM (■); 50 μM (▷) and control (●) and (b) Quercetin: 0.5 μM (△) and control (●). The reaction was followed by the decrease in c-Pc fluorescence intensity (excitation at 620 nm, emission at 640 nm) at 37 °C.

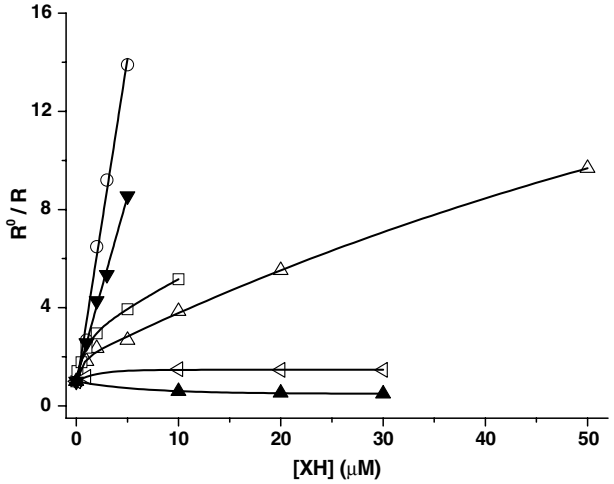


Fig. 4. Protection of c-Pc (0.8 μM) elicited by several phenolic compounds. The data are plotted as the ratio between the initial rate of c-Pc consumption in absence (R^0) and presence (R) of the additive. Gallic acid (○); protocatechuic acid (△); luteolin (□); galangin (▷); Trolox (▼) 3-OH-flavone (▲). Phosphate buffer (10 mM), pH 7.0, 37 °C.

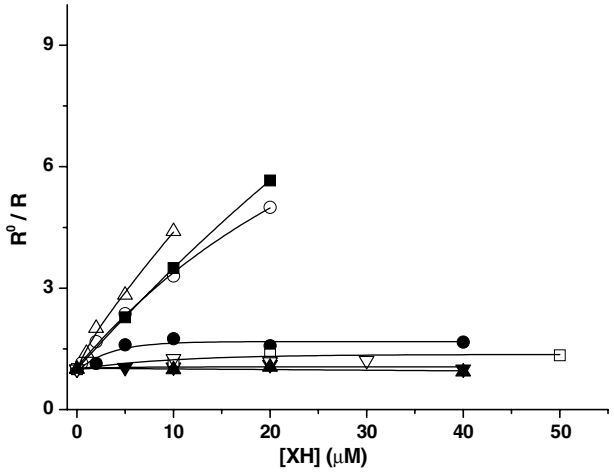


Fig. 5. Protection of c-Pc (23 μM) elicited by phenolic compounds. The data are plotted as the ratio between the initial rate of c-Pc consumption in absence (R^0) and presence (R) of the additive. Kaempferol (○); gallic acid (△); protocatechuic acid (●); luteolin (□); apigenin (▼); Trolox (■); 3-OH-flavone (▽). Phosphate buffer (10 mM), pH 7.0, 37 °C.

values of $R^0/R < 1.0$ and no protection ($R^0/R \approx 1$ over all the [XH] range considered).

3.1.1. Linear relationship

Linear relationship was observed for compounds such as Trolox and gallic acid (Fig. 4). This behaviour is the one predicted by Eqs. (6) and (7) and explained in terms of the simple mechanism depicted by Eqs. (1)–(4).

3.1.2. Downward curvature

Plots of R^0/R vs. [XH] show a noticeable downward curvature (Figs. 4 and 5). This behaviour is incompatible with the simplified mechanism depicted by reactions (1)–(4) and has been attributed to secondary reactions of phenol-derived radicals, such as:

$$XO^{\cdot} + c - Pc \rightarrow \text{bleaching} \quad (11)$$

The maximum attainable R^0/R value (R^0/R_∞) can be considered as a rough indicator of the damaging capacity of the $XO^{\cdot}$ radical. Therefore, a lower value of R^0/R_∞ is related to a higher damaging capacity of a secondary phenol-derived radical. This damaging capacity will be determined by the reactivity of the radical with the target molecule (k_{11}) and the rate of their self reactions:

$$XO^{\cdot} + XO^{\cdot} \rightarrow \text{non} - \text{radicals products} \quad (12)$$

Table 1 shows the parameters obtained from R^0/R vs. [XH] plots at low and high c-Pc concentration. These parameters were the protective effect of the XH tested, evaluated by slope/slope$_{Trolox}$, and the secondary damage, evaluated by R^0/R_∞ values. At low and high c-Pc concentration, gallic acid has a slope/slope$_{Trolox}$ higher than kaempferol. This result is in agreement with previous works that have shown a better protection of pyrogallol red by gallic acid than by kaempferol (López-Alarcón & Lissi, 2005). In Table 1, it is observed that galangin was the antioxidant with the major secondary damage (minor R^0/R_∞ values). This result would suggest a fast rate of reaction (11) for this compound. This is compatible with the reported low reactivity of galangin towards peroxyl radicals (López-Alarcón & Lissi, 2005), and with previous studies where cinammic acid derivatives were tested using c-Pc as target molecule (Pino, Campos, López-Alarcón, Aspée, & Lissi, 2006). In this study, the compound with minor reactivity (coumaric acid, $R^0/R_\infty = 2.7$) showed a secondary damage higher than caffeic and ferulic acids ($R^0/R_\infty = 50$ and 55, respectively). This would be compatible with the naïve assumption that the most stable radical (that more easily produced) is the least reactive one.

Table 1
Efficiency of c-Pc protection, measured by slope/slope$_{Trolox}$ values (Eq. (10)), and secondary damage, evaluated by R^0/R extrapolated at "infinite" substrate concentration

Compound	c-Pc = 0.8 μM		c-Pc = 23 μM	
	slope$_{XH}$/slope$_{Trolox}$	$(R^0/R)_\infty$	slope$_{XH}$/slope$_{Trolox}$	$(R^0/R)_\infty$
Quercetin	Induction time	Induction time	Induction time	Induction time
Kaempferol	0.5	>16	≈1	9.9
Luteolin	≈1	12.2	–[a]	≈1.5
Galangin	0.12	1.5	≈0	–[a]
3-OH-Flavone	−0.027	<1.0	≈0	–[a]
Gallic acid	1.8	∞	1.4	∞
Protocatechuic acid	0.45	19.6	0.53	1.7
Salycilic acid	0.0012	–[a]	≈0	–[a]
Trolox	1	∞	1	∞

Data are given at low (0.8 M) and high (23 M) c-Pc concentration.
[a] Not evaluable due to the low protection extent.

3.1.3. Inverse relation

Inverse relation behaviour was evidenced only for 3-OH-Flavone. This additive shows R^0/R_∞ values minor than one. 3-OH-Flavone derived radicals would provide an extreme example of the importance of secondary reactions of the additive derived radicals. In fact, when all the initial peroxyl radicals are transformed in 3-OH-Flavonoxyl radicals, the rate of c-Pc consumption increases. Probably, this effect is due to the fact that, at the lowest c-Pc concentrations employed, only a fraction of the peroxyl radicals are trapped by c-Pc. If, due to its low rates of radical-radical reactions, a larger fraction of the 3-OH-Flavone derived radicals react with c-Pc, the presence of the "antioxidant" would increase the rate of c-Pc bleaching. Similar results have already been reported employing 2-hydroxynaphtalene as a free radical scavenger (Pino, Aspée, López-Alarcón, & Lissi, 2006).

3.1.4. No protection

No protection was evidenced for Flavone and Apigenin at low and high c-Pc concentrations, and for luteolin, galangin and salicylic acid at high c-Pc concentration. This lack of protection would indicate that, in the present conditions, the XH are not able to compete with c-Pc for peroxyl radicals and/or that replacement of peroxyl radicals by additive derived radicals does not modify the rate of c-Pc consumption. In both situations, this behaviour would be very dependent on the type of target molecule and its concentration.

3.2. Effect of target (c-Pc) concentration on the R^0/R vs. [XH] plots

The values of slope/slope$_{Trolox}$ were similar at low and high c-Pc concentrations (Table 1). In fact, and in agreement with predictions of Eq. (10), we found a difference not more than a factor of 2 (factor a in Eq. (10)) between slope/slope$_{Trolox}$ values measured at low and high c-Pc concentrations. This allows a reliable estimation of k_3/k_{Trolox}, independent of the target molecule and its concentration.

The limiting rate of c-Pc consumption at high antioxidant concentration, determined by R^0/R_∞, is dependent on c-Pc concentration (Figs. 4 and 5). At low c-Pc concentration (0.8 μM), the R^0/R_∞ values were between 1.6 and

11.5 times higher than at 23 μM c-Pc concentration (Table 1). This result is explained in terms of an increase in the fraction of $XO^{\bullet}$ radicals that reacts by reaction (11) when c-Pc concentration increases. On the other hand, it is interesting to note that the prooxidant effect of 3-OH-Flavone observed at low c-Pc concentration disappears at high concentrations of the target molecule (zero order region) (Figs. 4 and 5 and Table 1). Under these conditions, c-Pc trap all the peroxyl radicals produced and 3-hydroxyflavone does not induce further damage.

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