

A Pyranine based Procedure for Evaluation of the Total Antioxidant Potential (TRAP) of Polyphenols. A Comparison with closely related Methodologies

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ABSTRACT

A novel procedure for the evaluation of total reactive antioxidant potentials (TRAP) is described. The method is based on the measurement of the bleaching of pyranine by peroxy radicals. The addition of the antioxidants produces a clear induction time whose magnitude is directly related to the antioxidant concentration. A comparison of the values obtained with those reported employing closely related methodologies shows that the results are significantly affected by the substrate employed to monitor the steady state free radical concentration. Possible sources of this dependence are discussed.

Key words: Peroxy radicals, polyphenols, pyranine, total reactive antioxidant potential.

Abbreviations: AAPH: 2,2'-azo-bis (2-amidinopropane) hydrochloride; ABTS: 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); TRAP: total reactive antioxidant potentials.

INTRODUCTION

There is great interest in the evaluation of the antioxidant capacity of pure compounds and/or complex mixtures, such as tea or wine. A large number of methodologies have been developed for this type of determinations (Llesuy et al., 2001). However, the information obtained from these determinations strongly depends upon the procedure employed (Pérez et al., 2000). This is mainly due to the fact that some of the methodologies evaluate the total amount of antioxidants and/or reactive groups in a given compound, while other techniques evaluate the amount and reactivity of the antioxidants present in the sample (Llesuy et al., 2001; Lissi et al., 1995). The former methodologies are generally based on the measurement of induction times (Wayner et al., 1985; Lissi

et al., 1995) or the bleaching of pre-formed radicals (Campos and Lissi, 1996; Campos and Lissi, 1997), while methodologies that determine amount and reactivity are generally based on the determination of changes in steady state free radical concentrations (Lissi et al., 1995; Pérez et al., 2000). In a previous work (Pérez et al., 2000), we have shown that even when methodologies that measure only the amount of antioxidants are employed, the results can be widely different. In the present communication we propose a new and simple methodology to measure amounts of antioxidants from induction times, and we compare the data with that obtained by similar methodologies. The results indicate that, even in this case, the results are extremely dependent upon the substrate employed to monitor the change in free radical concentration.

MATERIALS AND METHODS

Pyranine (Aldrich), Trolox (Aldrich) and 2,2'-azo-bis(2-amidinopropane) hydrochloride (AAPH) (Wako chemicals) were employed as received. All solutions were freshly prepared in phosphate buffer (10 mM, pH 7.0) employing twice distilled water. The kinetics of pyranine bleaching were evaluated at 37°C by measuring the change in fluorescence intensity (excitation at 460 nm; emission at 510 nm). Kinetic runs were carried out under air employing 10 mM AAPH and 5 μ M pyranine.

RESULTS

Pyranine is a reporter compound whose consumption can be easily measured by fluorescence and/or absorbance determinations. This compound has the advantage of its high reactivity towards

peroxyl radicals (Pino et al., 2003) and a strong absorbance and fluorescence that take place at relatively long wavelengths, minimizing the interference by additives and/or their products. Furthermore, its consumption in the presence of a free radical source follows a simple kinetic law, and can be totally inhibited by the addition of antioxidants. In these cases, a clear induction time, associated to the total consumption of the added antioxidants, is defined. Typical results are shown in Figures 1 and 2 for quercetin and rutin. The data of these figures show that both compounds behave as nearly ideal antioxidants. In fact, irrespective of their initial concentration, the protection is almost total, the induction time is clearly defined, and the slope after the induction time is very similar to that obtained in the absence of additives. Furthermore, there is a fair linearity between the amount of additive and the induction time (Fig 3). The

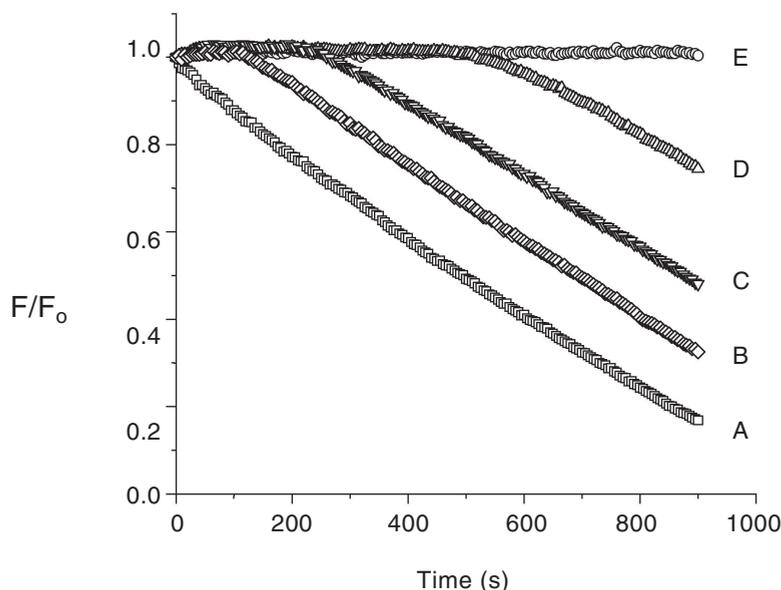


Figure 1. Time course of the bleaching of pyranine fluorescence elicited by peroxyl radicals produced in AAPH thermolysis. Effect of quercetin addition.

- (A) Control (without quercetin)
- (B) 0.5 micromolar quercetin.
- (C) 1 micromolar quercetin.
- (D) 1.77 micromolar quercetin
- (E) 2.5 micromolar quercetin.

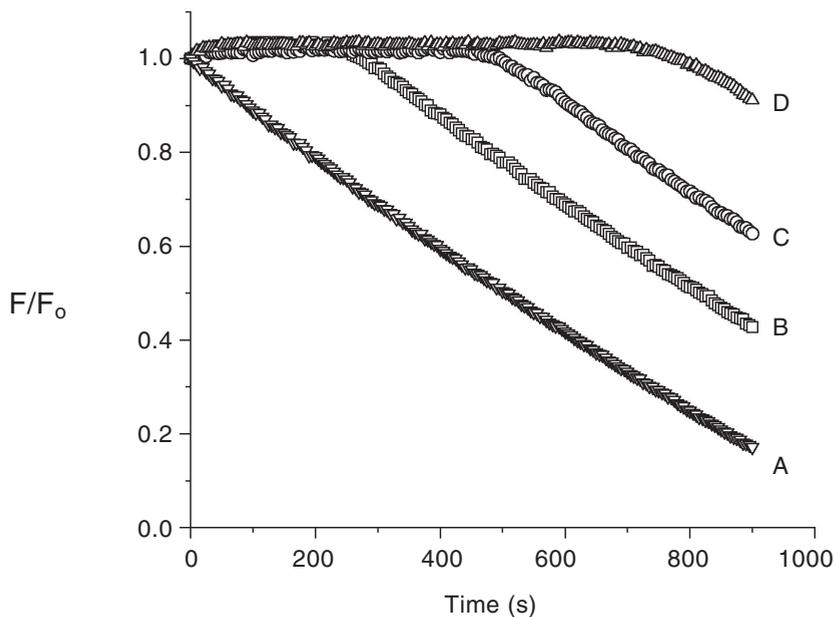


Figure 2. Time course of the bleaching of pyranine fluorescence elicited by peroxy radicals produced in AAPH thermolysis. Effect of rutin addition.

- (A) Control (without rutin)
- (B) 0.48 micromolar rutin.
- (C) 0.96 micromolar rutin
- (D) 1.44 micromolar rutin.

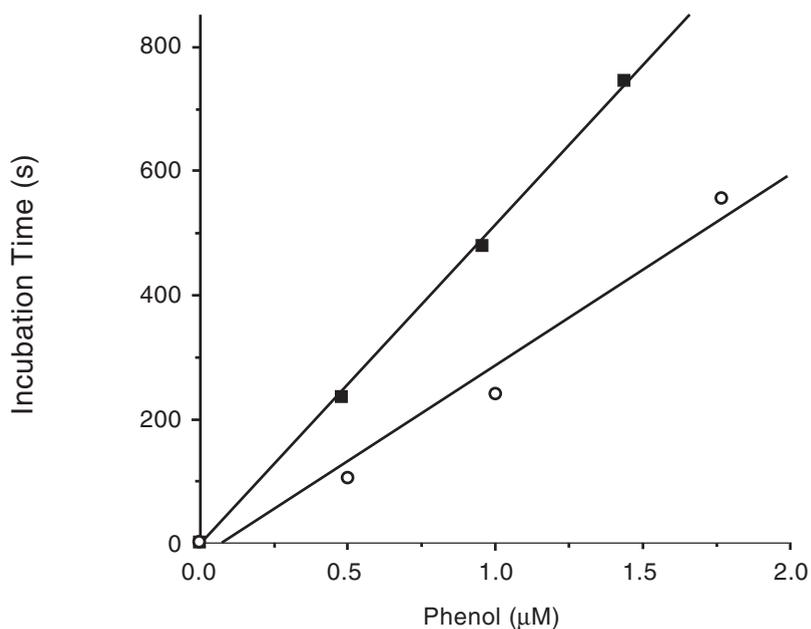


Figure 3. Dependence of the induction time on rutin (■) and quercetin (○) concentration.

same behavior has been observed with other polyphenols (such as catechin, gallic acid and caffeic acid) or complex mixtures, such as red and white wine (data not shown).

The antioxidant capacity of the pure compounds (TRAP), relative to that of a reference compound such as Trolox, can be obtained from:

$$s\text{TRAP} = (\text{slope})/(\text{slope})_{\text{ref}}$$

where (slope) represents the value of the slope of a plot such as that of Fig. 3 for the considered compound, and (slope)_{ref} is that of the reference compound (Trolox). Since each Trolox molecule is able to trap two free radicals (Llesuy et al., 2001), the number of radicals that can trap each molecule of the considered phenols (**n**) is given by

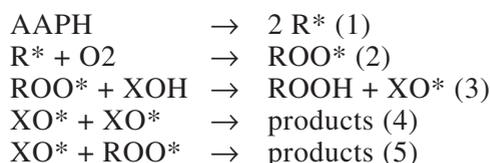
$$n = 2 \text{ TRAP}$$

Values obtained in the present work are summarized in Table I. This table also includes values reported by Pérez et al. (2000) for n values measured in the AAPH-luminol system and the number of ABTS derived radicals that can remove each polyphenol molecule.

DISCUSSION

As previously mentioned (Campodónico et al., 1998; Pérez et al., 2000), there is a fair proportionality between the number of

phenolic groups and the number of ABTS derived radicals that can be bleached (Fig 4). In fact, the data represented in Figure 4 gives a slope of 1.92, a correlation factor of 0.969 and a p value equal to 0.0015. This implies that titration of complex phenolic mixtures with ABTS derived radicals gives an estimation of the total amount of phenolic groups (Campodónico et al., 1998; Lissi et al., 1999). On the other hand, there is a very poor relationship between number of phenolic groups and TRAP values, irrespective of the procedure employed in its evaluation (Table I). The data obtained in the Pyranine/AAPH system, plotted in Figure 4, give a slope of 0.41, a correlation factor of 0.50 and a p value of 0.305. Furthermore, there is almost no correlation between n values evaluated in the luminol/AAPH and Pyranine/AAPH systems. This is most surprising since both methodologies employ AAPH as the free radical source. In fact, if it is assumed that in the inhibited region (prior to the induction time) the reaction scheme can be simply represented by



it can be expected n values between one and two for monofunctional compounds, depending of the relative relevance of reactions (4) and (5). With this scheme

TABLE I

Number of ABTS derived radicals (**n**) that can remove a molecule of the corresponding phenolic compound.

Compound	Number of OH Groups	n * ABTS	n * luminol/AAPH	N Pyranine/AAPH
Trolox	1	2	2	2
Caffeic acid	2	3.8	6.8	3.2
Gallic acid	3	6.6	2.2	2
Catechin	4	6.6	18.6	3.6
Rutin	4	8.6	5.4	5.2
Quercetin	5	9.8	7.0	3.0

(*) Data from Pérez et al., (2000).

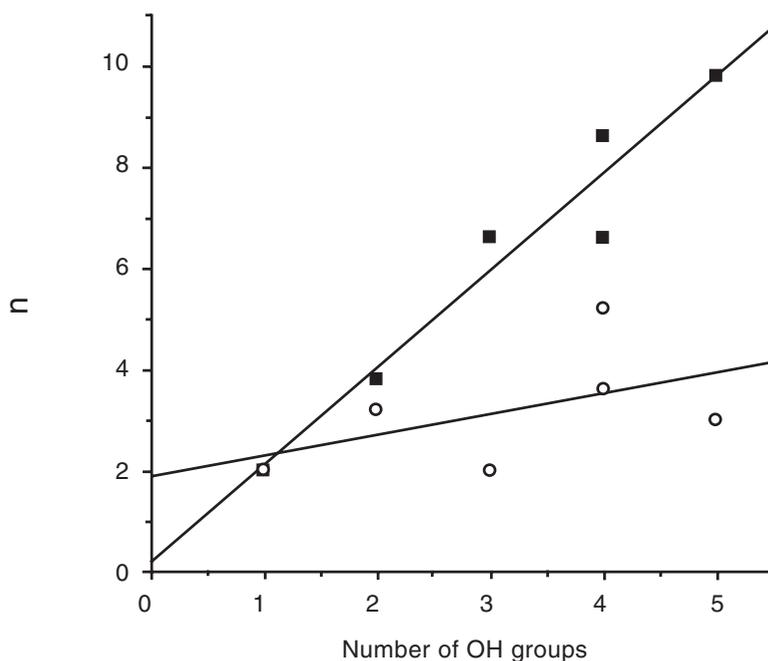


Figure 4. Relationship between number of radicals trapped (n) and number of potentially reactive hydroxyl groups.

(■) values obtained employing ABTS radical cation (Pérez et al., 2000). Parameters of the best fit straight line: slope = 1.92; $r = 0.969$; $p = 0.0015$

(○) values obtained in the present work in the pyranine/AAPH system. Parameters of the best fit straight line: slope 0.41; $r = 0.51$; $p = 0.305$

$n \rightarrow 1$ when $R_4 \gg R_5$

$n \rightarrow 2$ when $R_4 \ll R_5$

In polyfunctional compounds, n can be higher than two due to the presence of potentially reactive groups in the products arising from processes (4) and/or (5).

A noticeable feature of this reaction scheme is that, in the totally inhibited region, the values of n should be independent of the target molecule employed to monitor the steady state free radical concentration. This simple conclusion is in disagreement with the data presented in Table I. Three plausible explanations can be advanced to explain the strong dependence of n values on the employed methodology:

i) Differences in the solution pH

ii) Relevance of luminol derived radicals in the removal of XOH derived radicals (Lissi et al., 1992); and

iii) Relevant role of repair processes.

Unfortunately, possibility i) could not be tested due to the impossibility of carrying out the experiments with pyranine at the high pHs required in the luminol/AAPH system.

Possibility iii) takes into account protection of pyranine by the phenols through the sequence:



as proposed in closely related systems (Pino and Lissi, 2001; Pino et al., 2003). This scheme implies that the value of n will depend upon the capacity of the products

formed in reactions (4) and (5) of reacting with the radical PyO^* and not with the AAPH derived radicals. The different behavior of these radicals could explain the differences in n values reported in Table I.

The preset results indicate that pyranine bleaching can be employed to determine TRAP values. However, care must be taken when these values are compared to those obtained by different methodologies, even if they employ the same free radical source. Secondary free radical-free radical reactions determine the stoichiometric factors included in the TRAP parameter, and they can be widely different when different substrates are employed.

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