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Antioxidant capacity of human blood plasma and human urine: Simultaneous evaluation of the ORAC index and ascorbic acid concentration employing pyrogallol red as probe

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ABSTRACT

The oxygen radical absorbance capacity (ORAC) methodology has been employed to estimate the antioxidant capacity of human blood plasma and human urine using pyrogallol red (ORAC-PGR) as target molecule. Uric acid, reduced glutathione, human serum albumin, and ascorbic acid (ASC) inhibited the consumption of pyrogallol red, but only ASC generated an induction time. Human blood plasma and human urine protected efficiently pyrogallol red. In these assays, both biological fluids generated neat induction times that were removed by ascorbate oxidase. From these results, ORAC-PGR method could be proposed as a simple alternative to evaluate an ORAC index and, simultaneously, to estimate the concentration of ascorbic acid in human blood plasma or human urine.

1. Introduction

Today there is strong evidence that free radical damage is implicated in aging, and in the pathological conditions of a number of diseases, such as atherosclerosis, cancer, and rheumatoid arthritis.[1–6] Our defences against the free radical damage are the antioxidants synthesized in our body and the antioxidants consumed in our diet.[1] To establish the relevance of the human diet in the development of oxidative stress conditions, different studies have been aimed to estimate—using different methods—the antioxidant capacity of human blood plasma.[7–11] Among the methodologies used, the ORAC assay (oxygen radical absorbance capacity) has been widely employed to evaluate the changes of the antioxidant capacity of human blood plasma during a rich diet in antioxidants.[12–17]

The ORAC assay was originally proposed by Cao et al. using β-phycoerythrin as target molecule.[18] Afterwards, other molecules, such as C-phycocyanin, were employed as targets.[19] At present, fluorescein is the target molecule most used in ORAC methodologies (ORAC-FL).[20] This methodology has been applied to a large variety of pure compounds, infusions, foods and biological samples.[21] Nevertheless, we have shown that ORAC-FL for single antioxidants and/or complex mixtures including very reactive compounds is estimated generally from kinetics profiles showing a neat induction time. Then, ORAC-FL values would be more related to stoichiometric factors than the efficiency of the antioxidants.[22] Recently, we have proposed that ORAC values obtained employing pyrogallol red as probe (ORAC-PGR) would be more related to the reactivity of the additives towards peroxyl radicals, than to the stoichiometry of the reaction.[22–24] Pyrogallol red does not produce induction times, even with very reactive polyphenols. In particular, ascorbic acid was the only antioxidant, among the tested compounds, that generated a clear induction time.[23]

ORAC-PGR has been applied to tea infusions,[25] and wines.[23,24] In all cases, protection by the additives takes place without observable induction times. However, in rich ascorbic acid samples such as raspberry, a neat induction time related exclusively to the presence of ascorbic acid has been observed.[26] This lag time was used to estimate the ascorbic acid concentration, and the results were similar to those determined by HPLC (high performance liquid chromatography).[26] From these results, we have suggested that ORAC-PGR methodology could be considered as a simple procedure to estimate ascorbic acid and the ORAC index (ORAC-PGR) of ascorbic acid rich samples.[26]

Most methodologies employed to estimate the antioxidant potential of biological fluids lump together different types of compounds,[27,28] including uric acid, albumins, polyphenols and ascorbic acid. Furthermore, the relative contribution of each type of compound is notably influenced by the chosen methodology.[29]

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Therefore, it is important to develop methodologies able to discriminate among the different categories of antioxidants present in biological samples. In the present work we applied ORAC-PGR method to human blood plasma and human urine samples. We show that the use of pyrogallol red as probe allows to evaluate an antioxidant capacity index (ORAC-PGR), and to estimate the ascorbic acid concentration and its contribution to the ORAC index. Interestingly, the evaluation of ascorbic acid by ORAC-PGR method is not affected by the presence of human serum albumin, uric acid, and reduced glutathione.

2. Results and discussion

2.1. Protection of pyrogallol red by reduced glutathione (GSH), ascorbic acid (ASC), uric acid (UA), and human serum albumin (HSA)

Figure 1 shows the protection of pyrogallol red (PGR) by UA, GSH, and HSA. As can be seen in this figure, all additives inhibited the consumption of PGR without an induction time. Interestingly, high concentrations of HSA and UA were necessaries to protect PGR (Fig. 1A). The protection given by the additives was dose-dependent, as shown in Figure 1B for HSA. When ASC was added to a solution containing PGR plus 2,2′-Azo-bis(2-amidinopropane) dihydrochloride (AAPH) a clear induction time was observed (Fig. 2).[26] The generation of this induction time implies that ASC inhibited the consumption of PGR more efficiently than UA, GSH, and HSA. The inset of Figure 2 shows the dependence of the induction time with ascorbic acid concentration (data taken from Ref. 26). The induction time was not lineal with a noticeable downward curvature. This behavior has been interpreted in terms of changes in the reaction stoichiometry and/or the presence of a small chain consumption of the additive.[26,30] From the data presented in Figures 1 and 2 were obtained ORAC-PGR values of the additives (Table 1). ORAC-PGR index of ASC was nearly 1.7 and 485 times higher than GSH and UA, respectively. The order in ORAC-PGR values of the additives was:

ASC > GSH >> HSA > UA

In Table 1 we have included ORAC values obtained employing fluorescein as target molecule. It is noticeable the different hierarchy of the tested antioxidants. In particular, it is remarkable the low efficiency of ASC and the high efficiency of HSA when fluorescein is employed as target molecule. Considering that fluorescein was highly protected by HSA (Table 1), the ORAC-FL value of human blood plasma would be mostly related to the presence of HSA in the sample. In this context, Otaolaurruchi et al.[16] have recently described a good correlation between ORAC-FL values and thiol concentration in human blood plasma after wine consumption.

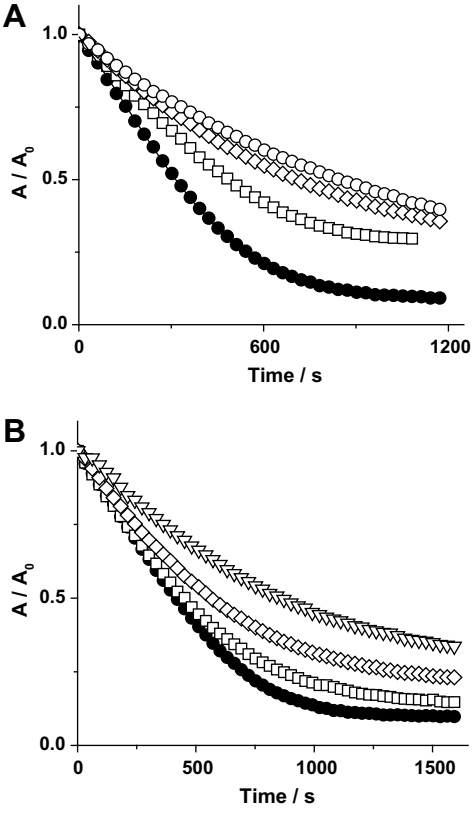

Figure 1. Kinetic profiles of AAPH mediated pyrogallol red consumption in presence of uric acid (UA), reduced glutathione (GSH), and human serum albumin (HSA). Pyrogallol red (5 μM) was incubated in presence of AAPH (10 mM) and the additives in phosphate buffer (75 mM, pH 7.4) at 37 °C. (A) Protection of pyrogallol red by UA (1 mM, □), HSA (100 μM, ◇), and GSH (10 μM, ○). (B) Protection of pyrogallol red by HSA at: 25 μM (□); 75 μM (◇); 150 μM (▽). Control experiments (without additives, ●).

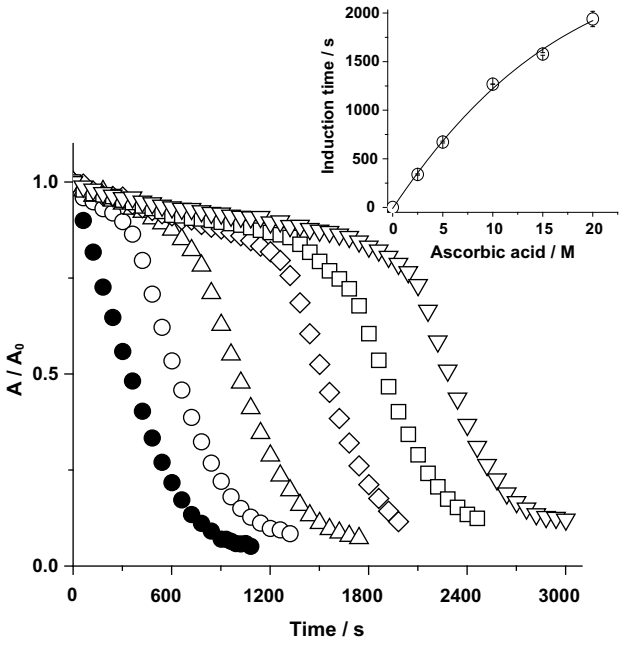

Figure 2. Effect of ascorbic acid on pyrogallol red consumption induced by AAPH. Pyrogallol red (5 μM) was incubated in presence of AAPH (10 mM), and different ascorbic acid concentrations in phosphate buffer (75 mM, pH 7.4) at 37 °C. Control (●); ascorbic acid: 2.5 μM (○); 5 μM (Δ); 10 μM (◇); 15 μM (□); 20 μM (▽). Data taken from Ref. 26. Inset: plot of the induction time versus ascorbic acid concentration.

Table 1
ORAC-pyrogallol red (ORAC-PGR) values of pure compounds

	ORAC-FL	ORAC-PGR
ASC	0.95 ± 0.02[a] 0.3[b]	9.7 ± 0.7[c,d]
UA	0.72[b]	0.02 ± 0.01
GSH	0.62 ± 0.02[a]	5.6 ± 0.4[e]
HSA	9.5 ± 0.9[c]	0.34 ± 0.01

[a] Taken from Ref. 20.
[b] Taken from Ref. 36.
[c] Unpublished result.
[d] Value obtained with ASC = 5 μM.
[e] Value obtained with GSH = 10 μM.

The results obtained by the ORAC-PGR methodology (Table 1) would imply that an ORAC index of a mixture of additives such as UA, GSH, ASC, and HSA, would be highly influenced by ASC. To test this assumption we used a solution containing GSH, UA, ASC, and HSA, named 'simulated human blood plasma'. This simulated human plasma was added to a PGR-AAPH solution and incubated at 37 °C. Figure 3 shows the effect of the addition of simulated human blood plasma on the consumption of PGR induced by peroxyl radicals. As can be seen in this figure, the addition of simulated human blood plasma generated a neat lag time in the kinetic profiles of PGR consumption. This lag time (inset of Fig. 3) is determined by the ascorbic acid concentration, and correlates with the induction time estimated from the data of Figure 2. Pre-treatment of simulated human blood plasma with ascorbate oxidase completely removes the induction time, without affecting the slope of PGR consumption after the lag time. The ORAC-PGR value of simulated blood human plasma was 0.66 mM Trolox equivalents, (Table 2).

2.2. Inhibition of pyrogallol red consumption by human blood plasma and human urine

The consumption of PGR (Fig. 4) induced by peroxyl radicals was inhibited by the presence of human blood plasma and human urine. The addition of these biological fluids generated a neat induction time that was removed completely by ascorbate oxidase, but was not changed by uricase (data not shown). Then, the presence of these induction times is related exclusively to the presence of ASC in the samples. From these lag times, an ascorbic acid concentration of 21 μM, and 60 μM were estimated for the employed human blood plasma and human urine samples, respectively. These values are inside the physiological range.[31,32]

From the kinetic profiles showed in Figure 4, ORAC-PGR values of 0.53, and 3.2 mM Trolox equivalents were estimated for human blood plasma, and human urine, respectively. These results are shown in Table 2. As can be seen in this table, 40%, 53%, and 75%

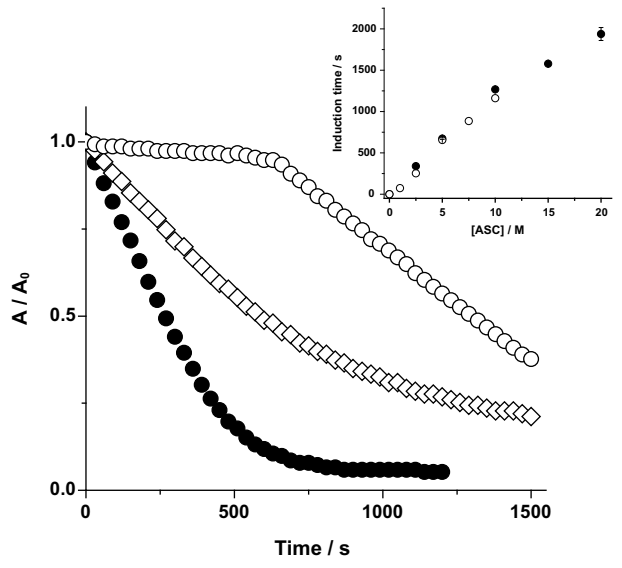

Figure 3. Kinetic profiles of AAPH mediated pyrogallol red consumption in presence of 'simulated human blood plasma'. Pyrogallol red (5 μM) was incubated in presence of AAPH (10 mM), and 'simulated human blood plasma' (with ASC 5 μM) in phosphate buffer (75 mM, pH 7.4) at 37 °C. In presence of simulated human blood plasma (○). In presence of simulated human blood plasma pre-incubated with ascorbate oxidase (0.09 U/mL) (◇). Control experiment (●). Inset: dependence of induction time with added ascorbic acid concentration. Pure ascorbic acid (data taken from Ref 26, ●). In presence of simulated human blood plasma (○).

Table 2
ORAC-pyrogallol red (ORAC-PGR) values of human blood plasma, and human urine, expressed as milimolar Trolox equivalents

	ORAC-PGR[a]	ASC contribution to ORAC-PGR[b] (%)
Simulated human blood plasma	0.66 ± 0.05[c]	40
Human blood plasma	0.51 ± 0.08	53
Human urine	3.2 ± 0.1	75

[a] Values represent the concentration (mM) of a Trolox solution that produces the same effect than the tested sample.
[b] Percentage of ascorbic acid contribution to ORAC-PGR values.
[c] ORAC-PGR value estimated for simulated human blood plasma with ASC at 2.5 μM concentration.

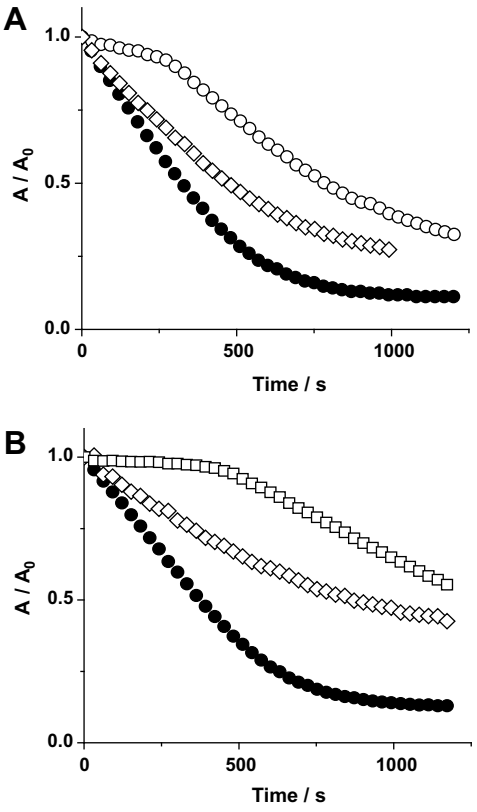

Figure 4. Effect of human blood plasma and human urine on pyrogallol red consumption induced by peroxyl radicals. Pyrogallol red (5 μM) was incubated in presence of AAPH (10 mM), and the biological fluids in phosphate buffer (75 mM, pH 7.4) at 37 °C. (A) Human blood plasma (100 μL/mL) (○); human blood plasma (100 μL/mL) pre-incubated with ascorbate oxidase (0.09 U/mL) (◇); Control (●). (B) Human urine (50 μL/mL) (□); human urine (50 μL/mL) pre-incubated with ascorbate oxidase (0.09 U/mL) (◇). Control (●).

of the ORAC-PGR value of simulated blood human plasma, human plasma, and human urine can be ascribed to the presence of ascorbic acid in the samples. The ASC contribution to the ORAC value of the tested sample is considerably larger than that reported employing a FRAP methodology (15%).[33] This difference can be attributed to the large ASC value when PGR is employed as target molecule (Table 1) and emphasizes the dominant role of this compound when ORAC-PGR values are estimated in ASC containing complex samples. This large ascorbic acid contribution contrasts with results obtained employing fluorescein as target molecule since ORAC-FL values of blood plasma and urine are almost unaffected by pre-incubation of the samples with ascorbate oxidase (data not shown).

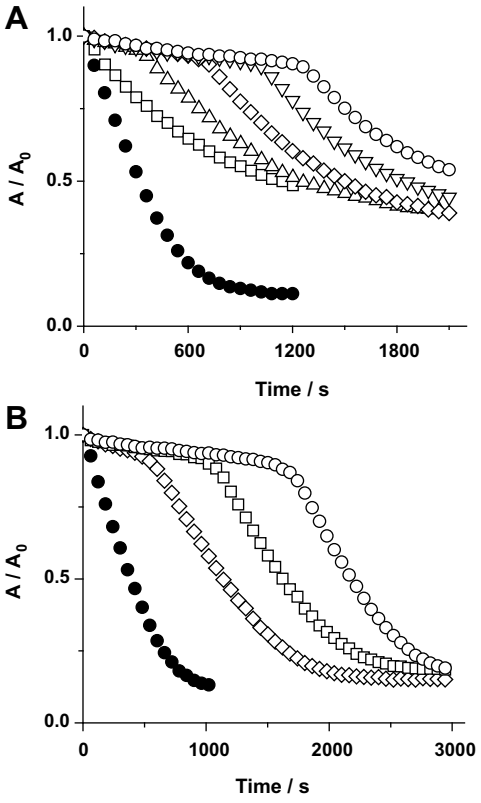

Figure 5. Protective effect of aged human blood plasma and human urine in presence of ascorbic acid concentration on the pyrogallol red consumption. Pyrogallol red (5 μM) was incubated with AAPH (10 mM) phosphate buffer (75 mM, pH 7.4) at 37 °C. (A) In presence of aged human blood plasma (100 μL/mL) (□); in presence of aged human blood plasma (100 μL/mL) and ascorbic acid at the following concentrations: 2.5 μM (Δ); 5 μM (◇); 7.5 μM (▽); 10 μM (○). Control (●). (B) In presence of human urine, 25 μL/mL (◇); in presence of human urine (25 μL/mL) plus ascorbic acid at the following concentrations: 5 μM (□); 10 μM (○). Control (●).

The addition of ascorbic acid to a human blood plasma previously stored at −20 °C (aged human blood plasma, devoid of ascorbic acid) or human urine generates and induction time that correlates with the added amount. These data are given in Figure 5. The induction times generated agree with those expected from the added ascorbic acid concentration, implying that the sample matrix does not affect the estimation of ascorbic acid. This is particularly relevant regarding the lack of albumin effect, given the dominance of this compound in determining ORAC indexes evaluated employing fluorescein as target molecule.

The use of a PGR based ORAC methodology applied to biological fluids presents several advantages: use of absorption spectroscopy,[22,23] ORAC contributions proportional to the reactivity of the different compounds[22] and the possibility of estimating total antioxidant potential and the contribution of ascorbic acid in a single assay. All these characteristics, makes ORAC-PGR methodology a valuable complement to other techniques currently employed to estimate the antioxidant capacity of complex biological mixtures.

3. Conclusions

The results above presented strongly support the proposal that the induction times observed in the protection of pyrogallol red can be employed to estimate the amount of ascorbic acid present in the tested sample. Furthermore, they imply that ORAC-PGR values calculated by integration of decay curves after the induction time can be considered as a measure of the amount of other antioxidants. Therefore, ORAC-PGR methodology could be considered as a simple procedure to evaluate an antioxidant capacity index, and to estimate the concentration of ascorbic acid present in human blood plasma and human urine samples.

4. Materials and methods

4.1. Chemicals

2,2′-Azo-bis(2-amidinopropane) dihydrochloride (AAPH), was used as peroxyl radical source.[34] Pyrogallol red (PGR), Trolox (6-hydroxy-2,5,8-tetramethyl chroman-2-carboxylic acid), AAPH, ascorbic acid (ASC), reduced glutathione (GSH), uric acid (UA), human serum albumin (HSA, fatty acid free), and ascorbate oxidase were purchased from Sigma–Aldrich (St. Louis, MO). All compounds were employed as received.

4.2. Human blood plasma samples

Human blood plasma was obtained from fresh heparinized blood. Fresh blood was centrifuged at 3000 rpm for 15 min, and the supernatant was analyzed immediately. Undiluted aliquots of human blood plasma were added directly in the ORAC-PGR assays.In some experiments aged human blood plasma (devoid of ascorbic acid) and simulated human plasma were used. Aged human blood plasma corresponds to human blood plasma stored at −20 °C for three months. Before the assays, aged human blood plasma was kept at room temperature for 30 min. Simulated human blood plasma corresponds to a mixture of GSH (6 μM), UA (360 μM), ASC (10–100 μM), and HSA (750 μM). Simulated human blood plasma was assayed identically to fresh human blood plasma.

4.3. Human urine samples

Human urine was collected and used immediately. For ORAC-PGR assays, human urine samples were used without previous dilution.

4.4. Solutions

Stock solutions of PGR, UA, GSH, and HSA 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 sample 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 either a Hewlett Packard 8453 (Palo Alto, CA, USA) or a Unicam Helios-α (Cambridge, England) UV–visible spectrophotometer.

4.5. ORAC determinations

The consumption PGR, associated to its incubation in presence of AAPH, was estimated from absorbance (A) measurements. Integration of the area under the curve (AUC) was performed up to a time such that (A/A_0) reached a value of 0.2 or 0.4. These areas were employed to obtain ORAC values, according to Eqs. 1 and 2, for fluids and pure compounds, respectively:

$$\mathrm{ORAC} = \frac{(\mathrm{AUC} - \mathrm{AUC}^0)}{(\mathrm{AUC}_{\mathrm{Trolox}} - \mathrm{AUC}^0)} f[\mathrm{Trolox}] \quad (1)$$

$$\mathrm{ORAC} = \frac{(\mathrm{AUC}_{\mathrm{ad}} - \mathrm{AUC}^0)}{(\mathrm{AUC}_{\mathrm{Trolox}} - \mathrm{AUC}^0)} \frac{[\mathrm{Trolox}]}{[\mathrm{additive}]} \quad (2)$$

where AUC = area under curve in presence of the tested fluid sample, integrated between time zero and that corresponding to 60%

or 80% of the probe consumption; AUC_{ad} = area under curve in presence of the additives, integrated between time zero and that corresponding to 60% or 80% of the probe consumption; AUC^0 = area under curve for the control (target molecule plus AAPH solution); AUC_{Trolox} = area under curve for Trolox; f = Dilution factor, equal to the ratio between the total volume of the working solution (target molecule plus AAPH plus the sample aliquot) and the added sample volume. [Trolox] = Trolox molar concentration; [additive] = Additive molar concentration.

All experiments were carried out in triplicate.

4.6. Ascorbic acid determination

Stock solutions of ascorbic acid at 1 mM concentration were prepared daily in ethanol. Working solutions of ascorbic acid were prepared diluting a stock solution in phosphate buffer, 75 mM, pH 7.4. The final concentration was estimated using a molar absorptivity of 13,800 M^{-1} cm^{-1} at 270 nm.[35] Ascorbic acid was directly added to solutions containing PGR plus AAPH with or without pure compounds or biological samples (human blood plasma, and human urine). The induction times generated by ascorbic acid were defined as the time at which intercept the straight lines drawn to data corresponding to the slow and fastest consumption rates. This criterion is somehow arbitrary but it was chosen for its simplicity.[27] In some experiments, solutions containing an aliquot of human blood plasma sample, simulated human blood plasma, or human urine were pre-incubated with ascorbate oxidase. Briefly, ascorbate oxidase was reconstituted with phosphate buffer 4 mM, pH 6.5 (2 mL). The activity was estimated by both UV–visible spectrophotometry, and reflectometry (RQflex 10, Merck). A mixture of ascorbate oxidase (final concentration = 0.09 U/mL), the sample and/or ascorbic acid in buffer phosphate was incubated at room temperature during 40 min. After this pre-incubation, the solution was thermostatized at 37 °C, and PGR and AAPH were added. The course of the reaction was followed by UV–visible spectroscopy at 540 nm, respectively.

Acknowledgments

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References and notes

1. Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*, 3rd ed.; University Press: Oxford, 2000.
2. Jurgens, G.; Hoff, H. F.; Chisolm, G. M., III; Esterbauer, H. *Chem. Phys. Lipids* **1987**, *45*, 315.
3. Steinberg, D. *Circulation* **1991**, *84*, 420.
4. Darlington, L. G.; Stone, T. W. *Br. J. Nutr.* **2001**, *85*, 251.
5. Ames, B. N. *Science* **1983**, *221*, 1256.
6. Ames, B. N.; Shigena, M. K.; Hegen, T. M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7915.
7. Tubaro, F.; Ghiselli, A.; Rapuzzi, P.; Maiorino, M.; Ursini, F. *Free Radic. Biol. Med.* **1998**, *24*, 1228.
8. Kampa, M.; Nistikaki, A.; Tsaousis, V.; Maliaraki, N.; Notas, G.; Castanas, E. *BMC Clin. Pathol.* **2002**, *2*, 3.
9. Natella, F.; Nardini, M.; Giannetti, I.; Dattilo, C.; Scaccini, C. *J. Agric. Food Chem.* **2002**, *50*, 6211.
10. Apak, R.; Güçlü, K.; Ozyürek, M.; Karademir, S. E.; Altun, M. *Free Radic. Res.* **2005**, *39*, 949.
11. Lotito, S.; Frei, B. *Free Radic. Biol. Med.* **2004**, *36*, 201.
12. Fernández-Pachón, M. S.; Villaño, D.; Troncoso, A. M.; García-Parrilla, M. C. *J. Agric. Food Chem.* **2005**, *53*, 5024.
13. Devaraja, S.; Vega-López, S.; Kaula, N.; Schönlaub, F.; Rohdewaldb, P.; Jialala, I. *Lipids* **2002**, *37*, 931.
14. Cao, G.; Booth, S. L.; Sadowski, J. A.; Prior, R. L. *Am. J. Clin. Nutr.* **1998**, *68*, 1081.
15. Prior, R. L.; Cao, G. *Free Radic. Biol. Med.* **1999**, *27*, 1173.
16. Otaolaurruchi, E.; Fernández-Pachón, M. S.; Gonzalez, A. G.; Troncoso, A. M.; García-Parrilla, M. C. *J. Agric. Food Chem.* **2007**, *55*, 9713.
17. Prior, R. L.; Gu, L.; Wu, X.; Jacob, R. A.; Sotoudeh, G.; Kader, A. A.; Cook, R. A. *J. Am. Coll. Nutr.* **2007**, *26*, 170.
18. Cao, G.; Alessio, H. M.; Cutler, R. G. *Free Radic. Biol. Med.* **1993**, *14*, 303.
19. Pino, E.; Campos, A. M.; López-Alarcón, C.; Aspée, A.; Lissi, E. *J. Phys. Org. Chem.* **2006**, *19*, 759.
20. Ou, B.; Hampsch-Woodill, M.; Prior, R. L. *J. Agric. Food Chem.* **2001**, *49*, 4619.
21. Prior, R. L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsch-Woodill, M.; Huang, D.; Ou, B.; Jacob, R. *J. Agric. Food Chem.* **2003**, *51*, 3273.
22. López-Alarcón, C.; Lissi, E. *Free Radic. Res.* **2006**, *40*, 979.
23. López-Alarcón, C.; Lissi, E. *Free Radic. Res.* **2005**, *39*, 729.
24. Omata, Y.; Saito, Y.; Yoshida, Y.; Niki, E. *J. Agric. Food Chem.* **2008**, *56*, 3386.
25. Alarcón, E.; Campos, A. M.; Edwards, A. M.; Lissi, E.; López-Alarcón, C. *Food Chem.* **2008**, *107*, 1114.
26. Atala, E.; Vásquez, L.; Speisky, H.; Lissi, E.; López-Alarcón, C. Food Chem. **2008**, doi:10.1016/j.foodchem.2008.07.063.
27. Roginsky, V.; Lissi, E. *Food Chem.* **2005**, *92*, 235.
28. Lissi, E.; Salim-Hanna, M.; Pascual, C.; Del Castillo, M. D. *Free Radic. Biol. Med.* **1995**, *18*, 153.
29. Janaszewska, A.; Bartosz, G. *Scand. J. Clin. Lab. Invest.* **2002**, *62*, 231.
30. Niki, E. *World Rev. Nutr. Diet* **1991**, *64*, 1.
31. Graumlich, J. F.; Ludden, T.; Conry-Cantilena, C.; Cantinela, L. R.; Wang, Y.; Levine, M. *Pharm. Res.* **1997**, *14*, 1133.
32. Roe, J.; Hall, J. *J. Biol. Chem.* **1939**, *128*, 329.
33. Benzie, I.; Strain, J. *Anal. Biochem.* **1996**, *230*, 70.
34. Niki, E. *Methods Enzymol.* **1990**, *186*, 100.
35. Karayannis, M. I.; Samios, D. N.; Gousetis, C. H. *Anal. Chim. Acta* **1977**, *93*, 275.
36. Nenadis, N.; Lazaridou, O.; Tsimidou, M. *J. Agric. Food Chem.* **2007**, *55*, 452.