Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 108 (2008) 1002–1007
Food Chemistry
www.elsevier.com/locate/foodchem

Effect of antioxidant oxidation potential in the oxygen radical absorption capacity (ORAC) assay

Roger H. Bisby [a,*], Rachel Brooke [a], Suppiah Navaratnam [a,b]

[a] *Biomedical Sciences Research Institute, University of Salford, Salford M5 4WT, UK*
[b] *FRRF, STFC Daresbury Laboratory, Warrington WA4 4AD, UK*

Received 8 June 2007; accepted 4 December 2007

Abstract

The "oxygen radical absorption capacity" (ORAC) assay (Ou, B., Hampsch-Woodill, M., Prior, R.L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry 49*, 4619–4626) is widely employed to determine antioxidant content of foods and uses fluorescein as a probe for oxidation by peroxyl radicals. Kinetic modeling of the ORAC assay suggests that the lag phase for loss of fluorescence results from equilibrium between antioxidant and fluorescein radicals and the value of the equilibrium constant determines the shape of the lag phase. For an efficient antioxidant this constitutes a "repair" reaction for fluoresceinyl radicals and produces a well defined lag phase. The lag phase becomes less marked with increasing oxidation potential of the antioxidant. Pulse radiolysis confirms that fluoresceinyl radicals are rapidly ($k \sim 10^9$ dm^3 mol^{-1} s^{-1}) reduced by Trolox C, a water soluble vitamin E analogue. ORAC assays of phenols with varying oxidation potentials suggest that it might be employed to obtain an estimate of the redox potential of antioxidants within food materials.

Keywords: Antioxidant; Fluorescein; Oxygen; Radical; ORAC; Pulse radiolysis; Reduction potential

1. Introduction

Dietary antioxidants such as vitamin E and a range of plant-derived polyphenols are recognized as protecting against oxidative stress due to both normal processes and those induced by exposure to external agents (Schneider, 2005; Stoclet et al., 2004; Tucker & Townsend, 2005). Vitamin E is a hydrophobic molecule and acts as a chain breaking antioxidant (Burton & Ingold, 1986) in protecting polyunsaturated fatty acyl groups in fats and lipids against peroxidative decomposition. Hydrophilic antioxidants such as vitamin C and some phenolic compounds either provide synergistic support for vitamin E or may directly scavenge damaging free radicals in the biological milieu (Beuttner, 1993; Halliwell, 1996). The activity of such antioxidants depends upon a range of factors, the most important being the bioavailability, the oxidation potential of the antioxidant, the rate of reaction with the radical being scavenged, and the low reactivity and stability of the subsequent antioxidant-derived radical.

Dietary polyphenols are becoming increasingly recognized as having health benefits and are obtained from a wide range of foods such as fruit, vegetables and beverages (Wu et al., 2004). Among the many methods for the *in vitro* analysis of potential antioxidants in foodstuffs (Schleiser, Harwat, Bohm, & Bitsch, 2002) there are those that measure the ability of the antioxidant to break the chain reaction of lipid peroxidation (Roginsky & Lissi, 2005). In these assays a lag phase in the reaction is typically observed during which the antioxidant is consumed and after which more rapid peroxidation is detected by methods such as oxygen uptake or chemiluminescence. The source of oxidizing peroxyl radicals in such assays is usually an "azo-initiator" such as 2,2′azobis(2-amidinopropane)dihydrochloride (AAPH). By thermolysis these initiators generate

* Corresponding author. Tel.: +44 161 295 4912; fax: +44 161 295 5015.
E-mail address: r.h.bisby@salford.ac.uk (R.H. Bisby).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.12.012

carbon-centered radicals which react with oxygen to give the reactive peroxyl radical. Such peroxyl radicals are taken to be representative of those involved in biochemical autoxidations and may be utilized at physiological pH. In a second type of assay the reducing power of the antioxidant is assessed by its ability to reduce a coloured stable free radical such as the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) radical cation ($ABTS^{+}$) (Re et al., 1999) or 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) (Silva et al., 2000) and the antioxidant concentration or equivalent is obtained by titration of the absorbance. Alternatively peroxyl radicals may be reduced by the antioxidant in competition with an indicator molecule such as crocin (Bors, Michel, & Saran, 1984). In such circumstances simple competition kinetics may be used to evaluate the rate constant for reaction of peroxyl radical with the antioxidant (Tubaro, Ghiselli, Rapuzzi, Maiorino, & Ursini, 1998). The "oxygen radical absorbance capacity assay" (ORAC) for the estimation of antioxidants was introduced using β-phycoerythrin as a sensitive fluorescent indicator of peroxyl radical scavenging (Cao, Alessio, & Cutler 1993). More recently Ou et al. (2001) introduced fluorescein as a more reliable indicator in the ORAC assay, and pyrogallol has also been proposed by Lopez-Alarcon and Lissi (2006) as an alternative indicator using absorption measurements.

In the ORAC assay the loss of indicator fluorescence shows a lag phase, during which the kinetics have been described in terms of competition between reaction of peroxyl radical with indicator and antioxidant (Huang, Ou, & Prior, 2005). The lag phase is similar in character to that observed for oxygen uptake during the inhibition of autoxidation reactions by chain breaking antioxidants and contrasts with competitive assay such as that using crocin bleaching (Bors et al., 1984). In the ORAC assay the effectiveness of an antioxidant is evaluated from the area under the curve of fluorescence intensity versus time, suggesting that a stoichiometry for reaction of peroxyl radicals with a defined compound may be assessed, or allowing the evaluation of the total antioxidant capacity of a food extract. The potential advantages of the ORAC assay over other methods include the use of peroxyl radical as reactants with redox potential and mechanism of reaction (i.e. hydrogen atom vs. electron transfer) similar to those of physiological oxidants, and the use of a physiological pH so that the antioxidants react with an overall charge and protonation state similar to that in the body. Whilst the results obtained here with individual compounds suggest a relation between the shape of the curve for bleaching of fluorescence in the ORAC and oxidation potential of the antioxidant, it is anticipated that they may be more generally applied to indicate the overall oxidation potential of a more complex mixture of antioxidants such as in a food or plant extract.

2. Experimental

Chemicals were obtained from Sigma Aldrich and used as received. Solutions were prepared in water purified by deionization and ultrafiltration. ORAC assays were conducted as described by Ou et al. (2001) using a Spex Fluoromax spectrofluorimeter equipped with a thermostatted rotating 4-cell sample holder. Data was collected by the PC-controller and analyzed in Microsoft Excel. The cell was thermostatted to 40 °C and contained final concentrations of fluorescein (40 nmol dm^{-3}) and AAPH (17 mmol dm^{-3}) in phosphate buffered saline (pH 7.4, prepared at one-half normal strength). Fluorescence was collected at 520 nm on excitation at 480 nm with 2.5 nm slit widths, taking measurements from each sample at 60 s intervals.

Pulse radiolysis studies were undertaken using the l2 MeV linear accelerator at the Facility for Free Radical Research (Daresbury, UK) using methods previously described (Holder, Allen, Land, & Navaratnam, 2002). Samples were irradiated in a 2.5 cm optical path-length quartz capillary cell. Kinetic modeling was performed using the FASCIMILE for Windows (Version 3, AEA Technology plc) computer program running on a Pentium 4 PC, in which chemical changes based on a defined reaction scheme are computed by numerical integration.

3. Results and discussion

3.1. ORAC assays of phenolic compounds

ORAC assays were performed as described by Ou et al., 2001 for several phenolic compounds and the results shown in Fig. 1. The ORAC assay, as described in the Introduction, estimates the antioxidant potential of the sample under study from the area under the plotted curve of fluorescence versus time. For the well known water soluble vitamin E analogue, Trolox C [one electron reduction potential at pH 7, E'_0 ($RO^{\cdot}$, H^{+}/ROH), 480 mV] (Steenken and Neta, 1982), the ORAC curves show a distinct lag phase during which essentially no consumption of the fluorescein indicator dye occurs. This persists until the Trolox C is consumed and the fluorescein is then also rapidly oxidized. The length of the lag phase and the total area under the curve is proportional to the concentration of Trolox C (0–20 mol dm^{-3} in the sample cell). In contrast the ORAC curves for ferulate, a plant derived antioxidant [E'_0 ($RO^{\cdot}$, H^{+}/ROH), 595 mV] (Foley et al., 1999) have rather less distinct lag phases. A slow rate of fluorescein oxidation is observed from the start of the assay and increases with time. Finally, the ORAC curves for *N*-acetyl-tyrosine (NAT) [E'_0 ($RO^{\cdot}$, H^{+}/ROH), 930 mV] (Harriman, 1987) show that whilst there is a net increase in area under the curve with increasing NAT concentration, but there is no lag phase and in all cases fluorescence is lost approximately exponentially from the beginning of the reaction. These changes in behaviour are clearly related to the reduction potential of the antioxidant radical, with Trolox C as the most effective antioxidant displaying the most striking lag phase. Examination of data in the work by Ou et al. (2001) shows similar differences between the ORAC curves for Trolox C and other phenolics. For each of these

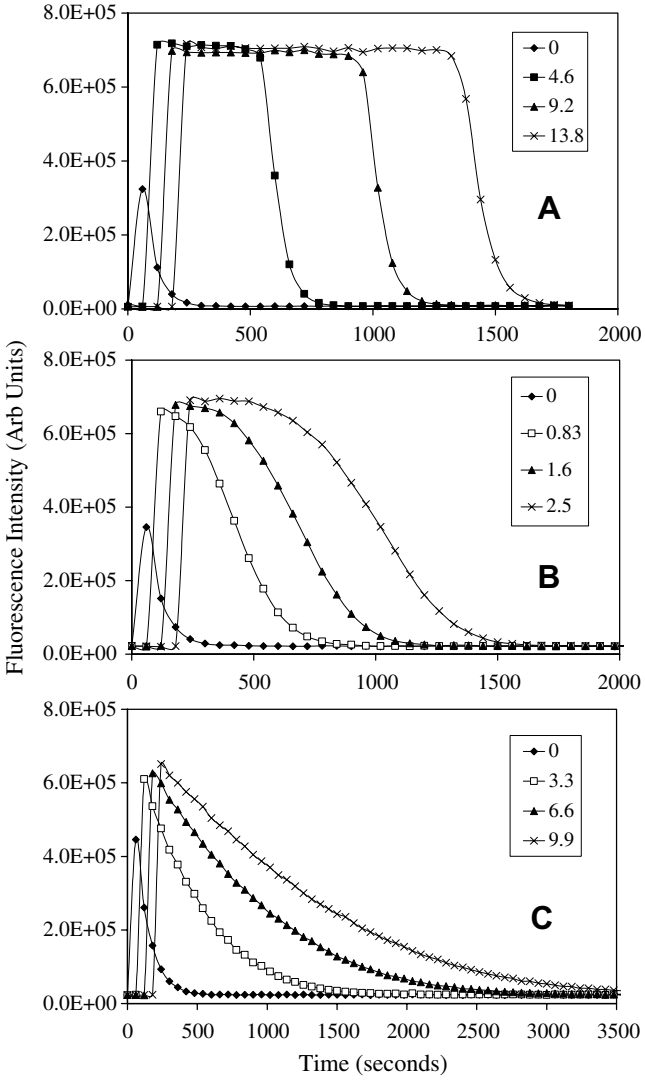


Fig. 1. ORAC assays for Trolox C (A), ferulate (B) and tyrosine (C) at the indicated initial micromolar concentrations, showing the time-dependent loss of fluorescence of solutions of fluorescein (40 nmol dm^{-3}) containing AAPH (17 mmol dm^{-3}) in phosphate-buffered saline (pH 7.4).

phenols the total areas under the curves are linearly related to antioxidant concentration.

3.2. Modeling of the kinetic processes in the ORAC assay

In order to understand the differences in shape of the ORAC curves for antioxidants with differing oxidation potentials, the reactions were modeled in the computer program "FACSIMILE". A proposed scheme of the major reactions involved in the ORAC assay is listed below, showing the reactions of the peroxyl radicals (ROO$^{\cdot}$) with fluorescein (FH) and antioxidant (AH).

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

$$ROO^{\cdot} + AAPH \rightarrow \text{products} \quad (2)$$

$$FH + ROO^{\cdot} \rightarrow F^{\cdot} + ROOH \quad (3)$$

$$F^{\cdot} + F^{\cdot} \rightarrow \text{products} \quad (4)$$

$$AH + ROO^{\cdot} \rightarrow A^{\cdot} + ROOH \quad (5)$$

$$A^{\cdot} + A^{\cdot} \rightarrow \text{products} \quad (6)$$

Decomposition of AAPH is effectively zero order and it is not necessary at present to consider cage effects and the overall yield of peroxyl radical formation in reaction (1). Reaction (2) provides a route for disappearance of peroxyl radicals in the absence of other reactions and might be taken to include other possible routes such as second order termination of peroxyl radicals. Reactions (3) and (5) indicate the one-electron oxidations by peroxyl radicals of fluorescein and antioxidant, respectively. Reactions (4) and (6) represent the second order decay of the corresponding radical species by either dimerization or disproportionation. This scheme essentially describes a simple kinetic competition between FH and AH for reaction with the peroxyl radicals. It suggests that varying the concntration ratio [AH]/[FH] would simply alter the rate of oxidation of each component by analogy with the scheme described by Tubaro et al. (1998) in the assay based on crocin bleaching. Fig. 2A shows the result of kinetic modeling using the FASCIMILE package considering only reactions (1)–(6) with representative values shown in Table 1. The initial rates reflect the ratio k_3[FH]/k_5[AH] and there is no appearance of a significant lag period.

Consideration of the reactions involved in the ORAC assay suggests a scheme involving the reaction between the fluoresceinyl radical and antioxidant (reaction (7)).

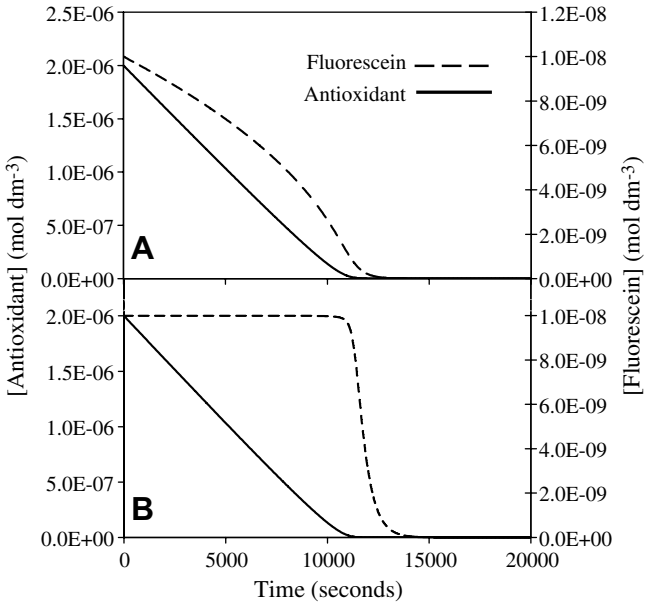


Fig. 2. Simulated curves for the consumption of fluorescein (FH) and antioxidant (AH) computed by FACSMILE. (A) Progress curves using the reactions (1)–(6) and rate constants in Table 1. (B) Progress curves using the reactions (1)–(6) together with (7) assuming $k_{+7} = 10^7$ dm^3 mol^{-1} s^{-1} and $k_{-7} = 10^6$ dm^3 mol^{-1} s^{-1} ($K_7 = 10$). The modelled solution contained initial concentrations of AAPH (10^{-2} mol dm^{-3}), AH (2×10^{-6} mol dm^{-3}) and FH (10^{-8} mol dm^{-3}).

Table 1
Rate constants used in modelling the ORAC assay

Reaction	Rate/rate constant (units)
(1) AAPH + O_2 → ROO·	10^{-8} mol dm^{-3}s^{-1}
(2) ROO· + AAPH → Products	10^2 dm^{3}mol^{-1}s^{-1}
(3) ROO· + FH → ROOH + F·	10^7 dm^{3}mol^{-1}s^{-1}
(4) F· + F· → products	10^8 dm^{3}mol^{-1}s^{-1}
(5) AH + ROO· → A· + ROOH	2×10^7 dm^{3}mol^{-1}s^{-1}
(6) A· + A· → products	10^8 dm^{3}mol^{-1}s^{-1}

Pulse radiolysis studies confirm that for Trolox C *(vide infra)* the forward reaction (7) is very rapid and kinetic modeling studies suggests that the shape of the curves involved in the ORAC assay reflect the magnitude of the equilibrium constant K_7.

$$F^{\cdot} + AH \rightleftharpoons FH + A^{\cdot} \quad (7)$$

Fig. 2B shows the result of modeling in FACSIMILE including reaction (7) with values of the forward second order rate constant $k_{+7} = 10^7$ dm^3 mol^{-1} s^{-1} and the reverse second order rate constant $k_{-7} = 10^6$ dm^3 mol^{-1} s^{-1} (i.e an equilibrium constant $K_7 = 10$). This computed time-course of FH oxidation now displays the characteristic lag phase during which AH is consumed linearly with time. Only when all AH is consumed does the concentration of FH rapidly falls to zero A further characteristic of the curve for FH under these conditions is a very "square" shape with almost complete preservation of FH until the end of the lag phase. The reactions were also modeled for a range of values of values for K_7 (from 2 to 0.05) obtained by fixing the k_7 at and varying the backward second order rate constant $k_{-7} = 10^7$ dm^3 mol^{-1} s^{-1} and varying k_{-7} from 2×10^7 to 5×10^5 dm^3 mol^{-1} s^{-1}. The results in Fig. 3 show a gradual loss of the lag phase with decreasing K_7 until with a value of 0.05 the shape indicates preferential loss of FH and preservation of AH during FH oxidation.

The equilibrium constant of reaction (7) is related to the relative difference in reduction potentials (E'_0) between F**.** and A**.** through the relationship

$$\Delta E'_0 = (RT/nF)\ln(K_7) \quad (8)$$

This model of reactions in the ORAC assay therefore suggests that the shape of the curves reflect the redox potential of the antioxidant. A good antioxidant is expected to have a low value of E'_0(A·,H$^+$/AH) and hence a larger value for K_7. It is therefore to be expected from this analysis that Trolox C will produce a trace in the ORAC assay with a very distinct "square" lag phase, whilst tyrosine with a much larger value of E'_0(A·,H$^+$/AH) and smaller E_0 and K_7 will produce a curve with little or no lag phase. Indeed, it may be possible that an analysis of the shape of curves in the ORAC assay might be used to obtain an estimate of the redox potential for an antioxidant.

3.3. Pulse radiolysis of fluorescein – antioxidant reactions

The one-electron oxidation of fluorescein by the inorganic radical anions $Br_2^{-\cdot}$ and $N_3^{\cdot}$ was studied by pulse radiolysis in N_2O-saturated solution in order to convert radiolytically produced ·OH and e^-_{aq} into the corresponding inorganic radical. The second order rate constant for oxidation of fluorescein by $Br_2^{-\cdot}$ at pH 7.4 was determined as $(4.1 \pm 0.4) \times 10^8$ dm^3 mol^{-1} s^{-1} from the decay of the $Br_2^{-\cdot}$ absorption at 360 nm in solutions containing up to 120 μmol dm^{-3} fluorescein. Pulse radiolysis of solutions containing fluorescein (10^{-4} mol dm^{-3}), sodium azide (10^{-1} mol dm^{-3}) and phosphate buffer (pH 7.4, 10 mmol dm^{-3}) produced the transient spectra shown in Fig. 4. A rate constant for reaction of $N_3^{\cdot}$ with fluorescein of 3.1×10^9 dm^3 mol^{-1} s^{-1} was obtained from the rate of formation of the transient product at 430 nm. An essentially identical transient product spectrum (not shown) was obtained from reaction of $Br_2^{-\cdot}$, suggesting that these inorganic radicals oxidize fluorescein by one-electron transfer

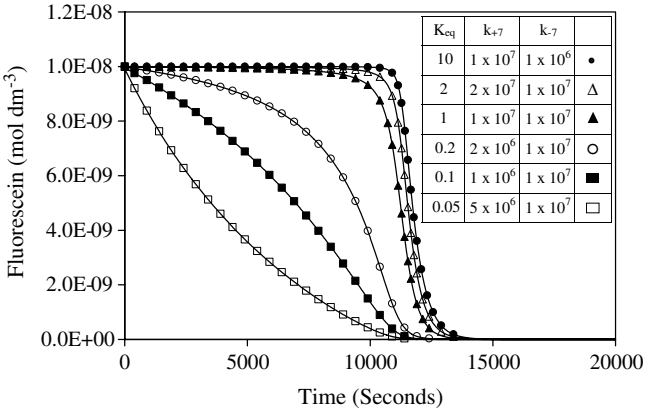


Fig. 3. Simulated curves for the consumption of fluorescein (FH) in an ORAC type assay computed by FACSMILE using the reactions (1)–(6) plus the equilibrium (7). Curves represent K_7 having values of 10 (●), 2 (Δ), 1(▲), 0.2 (○), 0.1 (■) and 0.05 (□) obtained using the indicated second order rate constants (units dm^3 mol^{-1} s^{-1}).

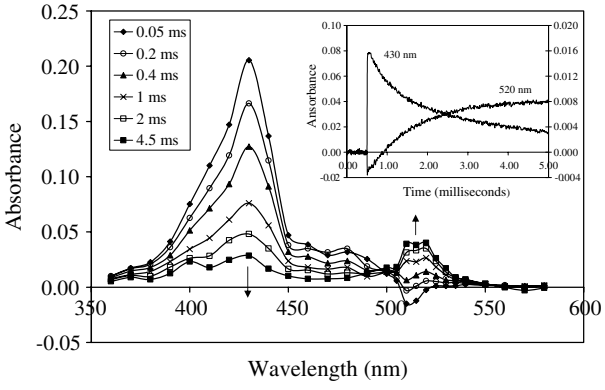
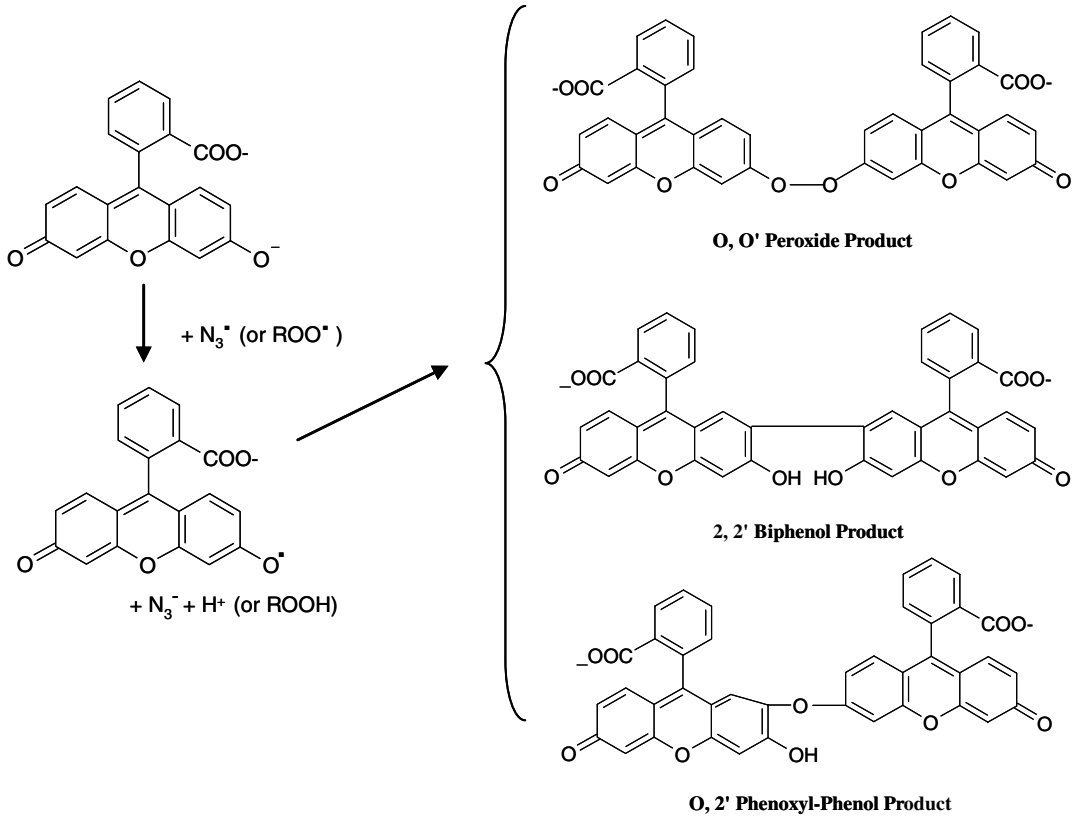


Fig. 4. Transient spectra from pulse radiolysis of N_2O-saturated solutions containing sodium azide (0.1 mol dm^{-3}) and fluorescein (100 μmol dm^{-3}) buffered to pH 7.4 with phosphate (10 mmol dm^{-3}). Spectra were determined at the indicated times after the radiolysis pulse. Dose 4.5 Gy pulse^{-1}. Inset:- absorbance transients measured at 430 nm (left hand scale) and 520 nm (right hand scale).



Scheme 1. One-electron oxidation of fluorescein to fluoresceinyl radical and its dimerisation to products as proposed by Ou et al. [16].

to an oxygen-centered fluoresceinyl radical as indicated in Scheme 1. At pH 7.4 fluorescein exists mainly (90%) as the dianion species since the phenolic group has a pK_a of 6.4 (Sjoback, Nygren, & Kubista, 1995). The spectra in Fig. 4 represent the difference between radical and ground state absorption spectra and due to the high absorption of the ground state at the concentration of fluorescein used are unreliable between 440 and 500 nm. At other wavelengths they are useful in observing reactions of the fluoresceinyl radical. The peak of the difference spectrum at 430 nm decays by second order kinetics with $2k_2 = (1.9 \pm 0.4) \times 10^9$ dm^3 mol^{-1} s^{-1}. The second order decay process corresponds to a dimerisation reaction to one of three possible structures shown in Scheme 1. Ou et al. (2001) observed the formation of the dimer through mass spectrometry and proposed the *O*,*O*′ peroxide structure, although the 2,2′ biphenol and *O*,2′ phenoxyl-phenol products seem more likely on the basis of previous observations (Joschek & Miller, 1966; Prutz, Butler, & Land, 1983). The formation of the dimer product is observed at 520 nm where, following an initial bleach of the ground state fluorescein absorption, the product absorption grows in with a second order process with a rate matching the decay of the radical absorbance at 430 nm (see inset to Fig. 4).

The reaction of the fluorescein radical with Trolox C was studied in N_2O-saturated solutions at pH 7.4 containing sodium azide (0.1 mol dm^{-3}), fluorescein (0.5 mmol dm^{-3}) and Trolox C (0–50 mol dm^{-3}). In these solutions the azidyl radical reacts almost entirely (>90%) with fluorescein, forming the fluoresceinyl radical within 1microsecond and allowing its reaction with other species such as Trolox to be investigated. The results shown in Fig. 5 show that with increasing Trolox C concentration there is an increasing rate of decay of the fluoresceinyl radical absorption at 410 nm, corresponding to the forward reaction (7). The slope of this plot gives $k_{+7} = (1.4 \pm 0.1) \times 10^9$ dm^3 mol^{-1} s^{-1}. The residual absorbance after the decay of the

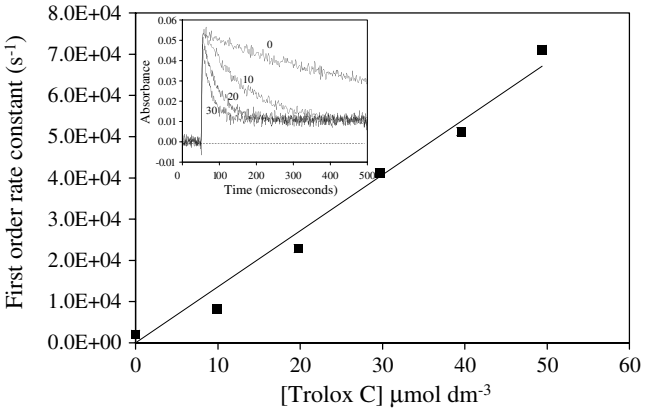


Fig. 5. First order rate constants from pulse radiolysis for decay of the transient at 410 nm plotted against Trolox C concentration in N_2O-saturated solutions containing sodium azide (0.1 mol dm^{-3}), fluorescein (100 µmol dm^{-3}) and phosphate buffer (pH 7.4, 10 mmol dm^{-3}). Inset: Absorbance transients measured at 410 nm and the indicated micromolar concentrations of Trolox C.

fluoresceinyl radical shown in the inset to Fig. 4 is that of the Trolox C radical (λ_{max} 440 nm, 5.4×10^3 dm^3 mol^{-1} cm^{-1} (Bisby, Ahmed, & Cundall, 1984)) and there is no indication of reversibility in reaction (7) for Trolox C under the conditions of this experiment. Clearly at pH 7.4, Trolox C is more reducing than fluorescein and reaction (7) proceeds in the forward direction as proposed in the modeling of the ORAC reactions. Hence the pulse radiolysis experiments provide strong support for the proposed mechanism of the ORAC assay.

4. Conclusions

The shape of the ORAC curved observed for three phenols with varying redox potential has been modeling using a simple kinetic scheme. The computer-generated curves show characteristic lag phases depending on the regeneration of fluorescein from its one-electron oxidized free radical by the antioxidant to an extent depending on the equilibrium constant and difference in one-electron reduction potential. Hence it is possible to reproduce the experimental forms of ORAC curves without the need to consider the second order rate constants for oxidation of fluorescein and antioxidant by the peroxyl radical (k_3 and k_5, respectively). Complementary pulse radiolysis experiments confirm that for Trolox, with a low reduction potential, there is a rapid reaction of the fluoresceinyl radical that regenerates fluorescein and thereby preserving its fluorescence in the ORAC assay. The results suggest that the ORAC assay may have the potential from the shape of the lag phase curve to report not only on the quantity of antioxidant from the area under the curve as presently used, but also on the "quality" of the antioxidant in terms of its redox potential. Therefore those foods and their extracts which produce a well-defined lag phase in the ORAC assay are likely to be the source of the most effective antioxidants.

Acknowledgements

The authors thank STFC Daresbury Laboratory for providing access to the Free Radical Research Facility.

References

Beuttner, G. R. (1993). The pecking order of free radicals and antioxidants: Lipid peroxidation, α-tocopherol and ascorbate. *Archives of Biochemistry and Biophysics, 300*, 535–543.

Bisby, R. H., Ahmed, S., & Cundall, R. B. (1984). Repair of amino acid radicals by a vitamin E analogue. *Biochemical and Biophysical Research Communications, 119*, 245–251.

Bors, W., Michel, C., & Saran, M. (1984). Inhibition of bleaching of the carotenoid crocin, a rapid test for quantifying antioxidant activity. *Biochimica et Biophysica Acta, 796*, 312–319.

Burton, G. W., & Ingold, K. U. (1986). Vitamin E: Application of the principles of physical organic chemistry to the exploration of its structure and function. *Accounts of Chemical Research, 19*, 194–201.

Cao, G. H., Alessio, H. M., & Cutler, R. G. (1993). Oxygen-radical absorbency capacity assay for antioxidants. *Free Radical Biology and Medicine, 14*, 303–311.

Foley, S., Navaratnam, S., McGarvey, D. J., Land, E. J., Truscott, T. G., & Rice-Evans, C. A. (1999). Singlet oxygen quenching and redox properties of hydroxycinnamic acids. *Free Radical Biology and Medicine, 26*, 1202–1208.

Halliwell, B. (1996). Vitamin C: Antioxidant or pro-oxidant in vivo? *Free Radical Research, 25*, 439–454.

Harriman, A. (1987). Further comments on the redox potentials of tryptophan and tyrosine. *Journal of Physical Chemistry, 91*, 6102–6104.

Holder, D. J., Allen, D., Land, E. J., & Navaratnam, S. (2002). Establishment of pulse radiolysis facility on the SRS linac at Daresbury laboratory. In T. Garvey, L. J. Duff, L. P. Roux, J. C. Petit, J. Poole, & L. Riviki (Eds.), *Proceedings of the 8th european particle accelerator conference* (pp. 2804–2806). Paris: European Physical Society.

Huang, D., Ou, B., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry, 53*, 1841–1856.

Joschek, H.-I., & Miller, S. I. (1966). Photoxidation of phenols, cresols and dihydroxybenzenes. *Journal of the American Chemical Society, 88*, 3273–3281.

Lopez-Alarcon, C., & Lissi, E. (2006). A novel and simple ORAC methodology based on the interaction of pyrogallol red with peroxyl radicals. *Free Radical Research, 40*, 979–985.

Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry, 49*, 4619–4626.

Prutz, W. A., Butler, J., & Land, E. J. (1983). Phenol coupling initiated by one-electron oxidation of tyrosine units in peptides and histone. *International Journal of Radiation Biology, 44*, 183–196.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26*, 1231–1237.

Roginsky, V., & Lissi, E. A. (2005). Review of methods to determine chain-breaking antioxidant activity in food. *Food Chemistry, 92*, 235–254.

Schleiser, K., Harwat, M., Bohm, V., & Bitsch, R. (2002). Assessment of antioxidant activity by using different in vitro methods. *Free Radical Research, 36*, 177–187.

Schneider, C. (2005). Chemistry and biology of vitamin E. *Molecular Nutrition and Food Research, 49*, 7–30.

Silva, F. A. M., Borges, F., Guimaraer, C., Lima, J. L. F. C., Matos, C., & Reis, S. (2000). Phenolic acids and derivatives: Studies on the relationship among structure, radical scavenging activity, and physicochemical parameters. *Journal of Agricultural and Food Chemistry, 48*, 2122–2126.

Sjoback, R., Nygren, J., & Kubista, M. (1995). Absorption and fluorescence properties of fluorescein. *Spectrochimica Acta A, 51*, L7–L21.

Steenken, S., & Neta, P. (1982). One-electron redox potentials of phenols, hydroxyl- and aminophenols and related compounds of biological interest. *Journal of Physical Chemistry, 86*, 3661–3667.

Stoclet, J.-C., Chatsigneau, T., Ndiaye, M., Oak, M.-H., Bedoui, J. E., Chataigneau, M., et al. (2004). Vascular protection by dietary ployphenols. *European Journal of Pharmacology, 500*, 299–313.

Tubaro, F., Ghiselli, A., Rapuzzi, P., Maiorino, M., & Ursini, F. (1998). Analysis of plasma antioxidant capacity by competition kinetics. *Free Radical Biology and Medicine, 24*, 1228–1234.

Tucker, J. M., & Townsend, D. M. (2005). Alpha-tocopherol: Roles in prevention and therapy of human disease. *Biomedical Pharmacotherapy, 59*, 380–387.

Wu, W., Gu, L., Holden, J., Haytowitz, D. B., Gebhardt, S. E., Beecher, G., et al. (2004). Development of a database for total antioxidant capacity in foods: A preliminary study. *Journal of Food Composition and Analysis, 17*, 407–422.