Current Topics in Biophysics 2000, 24(2), 89–95

CHEMILUMINESCENCE DETECTION OF PEROXYL RADICALS AND COMPARISON OF ANTIOXIDANT ACTIVITY OF PHENOLIC COMPOUNDS

ANNA KRASOWSKA, DAWID ROSIAK, KATARZYNA SZKAPIAK, MARCIN ŁUKASZEWICZ*

Institute of Microbiology, Wroclaw University, Przybyszewskiego 63–77, 51–148, Wroclaw, Poland

The aim of this work was to examine the chemiluminescent (CL) method for quick comparison of antioxidant properties of new compounds and biological samples. 2,2'-Azobis(2-amidinopropane)dihydrochloride (AAPH) was used as a source of free radicals and luminol to obtain high long lasting CL. The CL increased with the pH. Two exemplary compounds were compared: Trolox, a water soluble homologue of vitamin E and butylated hydroxytoluene (BHT), a commercially used antioxidant. Trolox quenched CL transiently but almost completely, and at the concentration about 100 times lower (10 nM) than BHT. The duration of quenching, called "the induction time" by other authors, was linearly related to Trolox concentration. On the contrary, BHT quenched CL only partially, depending on its concentration. In our experimental conditions 8.5 μM BHT quenched 50% of CL. Relationships between structure and activity of the tested compounds are discussed.

INTRODUCTION

Compounds with antioxidant properties have been in common use in industry, especially food industry for many years. Lots of these compounds were found to be carcinogenic or toxic, which excluded them at least from the usage in food industry. On the other hand, human health is dependent on an efficient control of free radicals in the organism, as shown by several diseases directly or indirectly involving excessive levels of free radicals. Therefore the extensive search has been launched for new potentially useful antioxidants for food industry and human health management.

The first target of reactive oxygen species (ROS) from exogenous sources are lipids and proteins in the plasma membrane. Lipid peroxidation is a particularly dangerous process. It does not stop with the oxidation of the first substrates but continues as a chain reaction resulting in the formation of conjugated dienes, lipid peroxyl radicals and hydroperoxides (Smirnoff, 1995; Blokhina, Fagerstedt & Chirkova, 1999). Peroxyl radicals in turn can react with new lipid molecules, thus propagating the process (Frankel, 1985). Decomposition of lipid hydroperoxides results in different reactive secondary products such as free radicals (Aoshima, Kadoya, Taniguchi, Satoh & Hatanaka, 1999) or 4-hydroxy-2-nonenal (Uchida & Stadtman, 1992). Lipid peroxidation affects also the activities of different membrane-bound proteins (Uchida & Stadtman, 1992). Eventually, it leads to changes in membrane permeability and to the destruction of the whole cell (Rauchova, Ledvinkova, Kalous & Drahota, 1995).

The determination of formation, propagation and action of free radicals in cells is complicated. Therefore *in vitro* study in a simplified model systems is often required. A variety of methods have been developed to assess the oxidation or autoxidation of lipids. Different methods are studied for the effective rough screening of antioxidant potential of various compounds. Some of them are already commercially available. However, lots of these methods have a limited sensitivity or specificity, others are either too invasive or not adapt-

*Author for correspondence: Marcin Łukaszewicz, Institute of Microbiology, Wroclaw University, Przybyszewskiego 63–77, 51-148 Wroclaw, Poland; lukasz@microb.uni.wroc.pl, tel. (71) 3247250, fax (71) 3252151

Abbreviations: AAPH – 2,2'-azobis(2-amidinopropane)dihydrochloride; ABAP – 2,2'-azobis(2-amidinopropane)hydrochloride; BHT – butylated hydroxytoluene; CL – chemiluminescence; MDA – malondialdehyde; PUFA – polyunsaturated fatty acids; RLU – relative light units; TBARS – thiobarbituric acid-reactive substances

able for human cells. The standard methods for detection lipid peroxidation are: electron spin resonance (ESR); spin trapping; assay for thiobarbituric acid reactive substances (TBARS); detection of malondialdehyde (MDA) by direct methods (such as HPLC); detection of other oxidation products from polyunsaturated fatty acids (PUFA) such as 4-hydroxy-2-nonenal or diene conjugation; quantification of lipid hydroperoxides; detection of oxidation products from lipids other than PUFA (e.g. cholesterol); and chemiluminescence methods (Rauchova, Drahota. & Koudelova, 1999; Pyron, 1989).

Chemiluminescence (CL) is a potentially sensitive method. It permits not only the evaluation of the end products of reaction of cell constituents with free radicals, but also the observation of the reaction kinetics. Most CL methods use only a few chemical components which are excited by the reaction with free radicals, and have a high quantum yield of photon emission. Luminol is often used as a light source after the excitation by different kinds of free radicals, including peroxyl radicals.

Because of the simplicity and accuracy, thermally decomposited peroxides, hyponitrites and azo compounds are used as free radical initiators. Azo compounds undergo thermal decomposition without either enzymes or biotransformation. This yields molecular nitrogen and two carbon centered radicals R$^{\cdot}$ (Fig 1). The carbon radicals may form pairs or recombine to more stable products but lots of them react rapidly with oxygen and give peroxyl radicals $RO_2^{\cdot}$ (Halliwel & Gutterige, 1999). 2,2'-Azobis(2-amidinopropane)dihydrochloride (AAPH) is often used as a source of hydrophilic radicals (Halliwel & Gutterige, 1999; Niki, 1990; Zanocco, Pavez, Videla & Lissi, 1989; Lissi & Clavero, 1990). At 37°C in neutral water, the half-life of AAPH is about 175 h and the generation rate of radicals is constant for the first few hours (Halliwel & Gutterige, 1999). The rate of free radical generation (R_i) from AAPH at 37°C equals 1.36×10^{-6} mol $l^{-1}s^{-1}$.

Luminol luminescence induced by AAPH under different conditions and after the addition of various enzymatic and non-enzymatic antioxidants was extensively investigated (Niki, 1987). The present study aims to examine the AAPH-luminol system as a fast and sensitive method to compare an antioxidant potential of new compounds with the commercially used lipid protector BHT (butylated hydroxytoluene) and Trolox (a water soluble homologue of vitamin E), a widely used reference compound (Fig. 1).

MATERIALS AND METHODS

Photons were counted in a EG&G Berthold LB96P microplate luminometer at 30°C. The experiments were performed in a final volume of 250 µl on white microplates in 0.1 M Tris buffer, pH 9.0. Determination of the pH dependence of luminescence intensity was done in the Britton and Robinson buffer (0.04 M acetic acid, 0.04 M phosphoric acid, 0.04 M boric acid and 0.2 M sodium hydroxide). 25 µl of freshly prepared AAPH (2,2'-azobis(2-amidinopropane)dihydrochloride) was pipetted into a microplate well. 1 mM stock solution of luminol was diluted four times in distilled water. 100 µl of the diluted solution was automatically injected into the sample at the beginning of the measurement, the tested compound being added 60 s later.

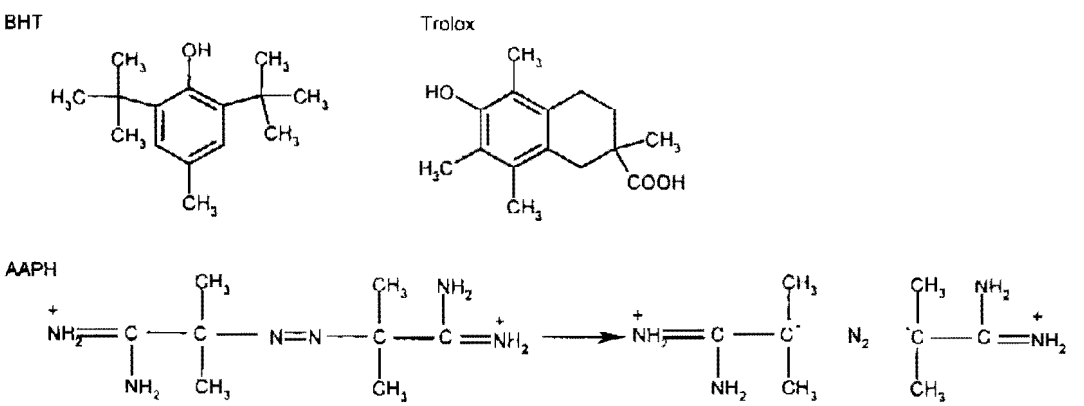

Fig. 1 Chemical structures BHT, Trolox and AAPH. BHT, Butylated hydroxytoluene. AAPH (2,2'-azobis(2-amidinopropane)dihydrochloride) undergoes thermolysis with generation of two alkyl radicals and nitrogen

40 mM stock solution of AAPH (Polyscience, USA) was prepared in distilled water. 1 mM stock solution of luminol (Aldrich, Poland) was obtained by solubilisation in 0.1 M NaOH. 10 mM stock solution of Trolox (Aldrich, Poland) and 20 mM stock solution of BHT (Aldrich, Poland) were prepared in ethanol.

RESULTS

AAPH (2,2'-azobis(2-amidinopropane)dihydrochloride) with luminol generates strong long lasting luminescence (Fig. 2). During the first 3 h the luminescence rapidly decreases to 2500 relative light units (RLU). Afterwards the rate of decrease slows down gradually with the average slope of −41 RLU/h for the next 13 h. The CL declines to the background level after two weeks (data not shown).

The CL intensity increases with the pH (Fig. 3). Below pH 7.0 CL is at the background level. From pH 7.0 to 10.5 the light intensity increases linearly ($t^{-} 20.8^{**}$), afterwards it grows more rapidly. It is known that the CL of luminol-H_2O_2 is remarkably high under alkaline conditions (pH 10–11; Lissi, Pascual & del Castillo, 1992). We decided to perform our experiments at pH 9.0 which is still in the range of the buffering power of Tris. The luminescence in these conditions is approximately 2500–3000 RLU (Fig. 3) which is about 100 times higher than the background, thus giving highly reproducible results. The steady state luminol CL intensity was decreased by antioxidants like Trolox or BHT. However, the light profile of the CL after Trolox and BHT addition was different (Fig. 4A, B). Addition of Trolox (in our experimental conditions over 10 nM) transiently quenched the luminescence. This period of time was earlier called the "induction time" (Tsugakoshi, Sumiyama, Nakajima, Nakayama & Maeda, 1998), after which CL intensity returns to previous steady state probably due to the Trolox consumption. The induction time (Fig. 4A) starts with the almost

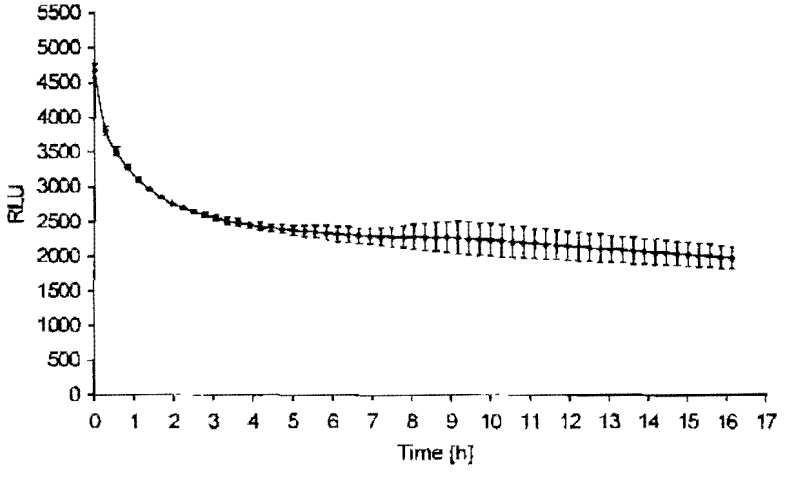

Fig. 2. The time profile of luminol (100 μM) CL induced by AAPH (4 mM) at 30°C. Light intensity measured in relative light units (RLU), SD ± 2

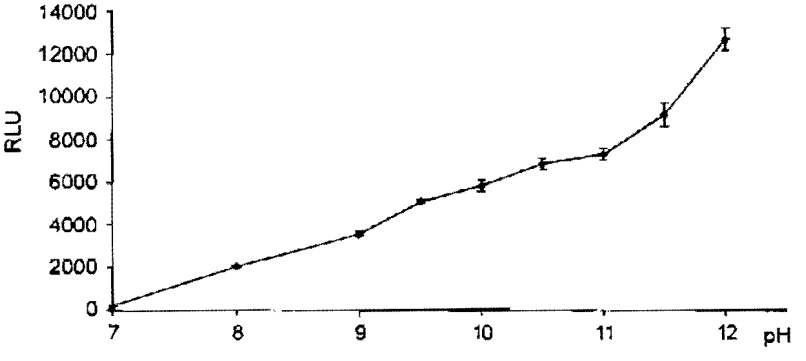

Fig. 3. Dependence of the luminol CL intensity on pH. Luminol 100 μM, AAPH 4 mM; SD ± 2

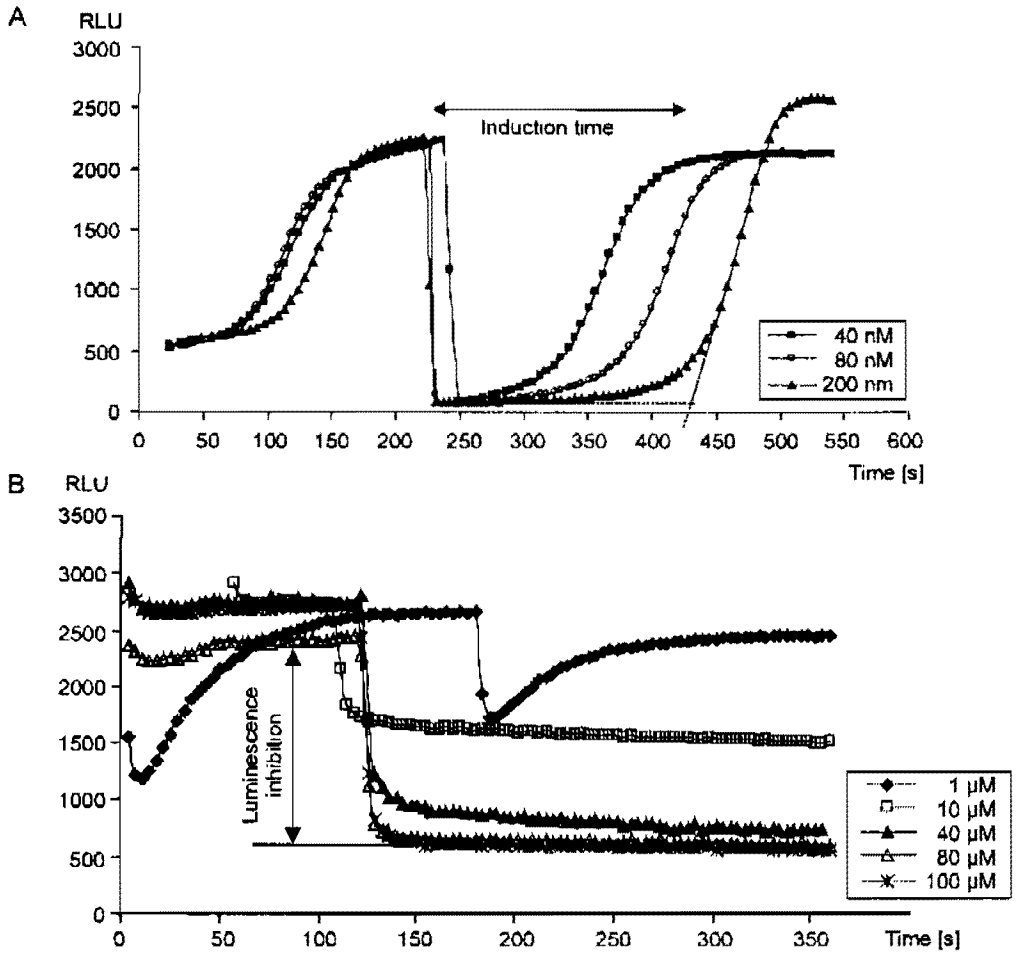

Fig. 4. The profile of luminol luminescence intensity after Trolox (A) and BHT (B) addition. Luminol, 100 μM; AAPH, 4 mM; Trolox, 40, 80 and 200 nM, BHT, 1, 10, 40, 80 and 100 μM. Arrows indicate Trolox and BHT addition. Induction time was evaluated by extrapolation the maximum slope of Trolox consumption to zero light intensity. CL inhibition was calculated from the subtraction of C_0 (the RLU value for the light intensity in the absence of BHT) and C_1 (the minimal RLU value for the light intensity after BHT addition)

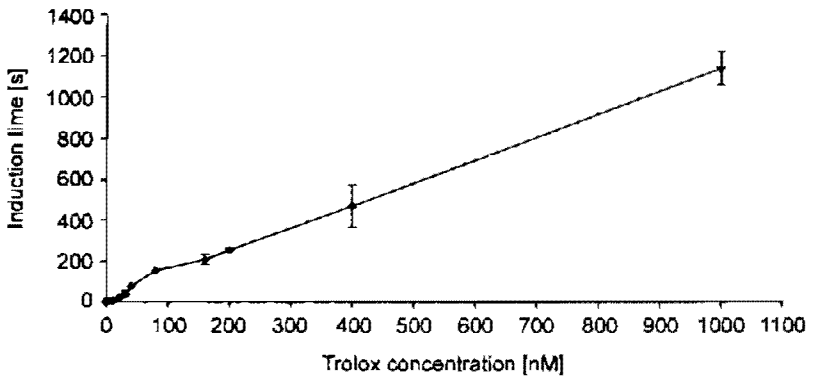

Fig. 5. Diagram of the induction time as a function of Trolox concentration. Luminol, 100 μM; AAPH, 4 mM

complete suppression of CL by the addition of Trolox. The end of the induction time is the point where the line approximating the curve (plot of the RLU in time) in its steepest position crosses the horizontal line approximating the curve position at the beginning of the induction time (just after Trolox addition). Data in Fig. 5 show a linear relationship (t - 56.8**) between the induction

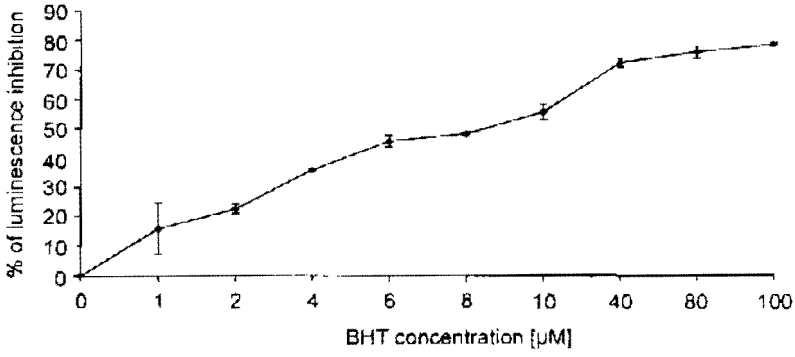

Fig. 6. Diagram of the CL inhibition as a function of BHT concentration. Luminol, 100 μM, AAPH, 4 mM

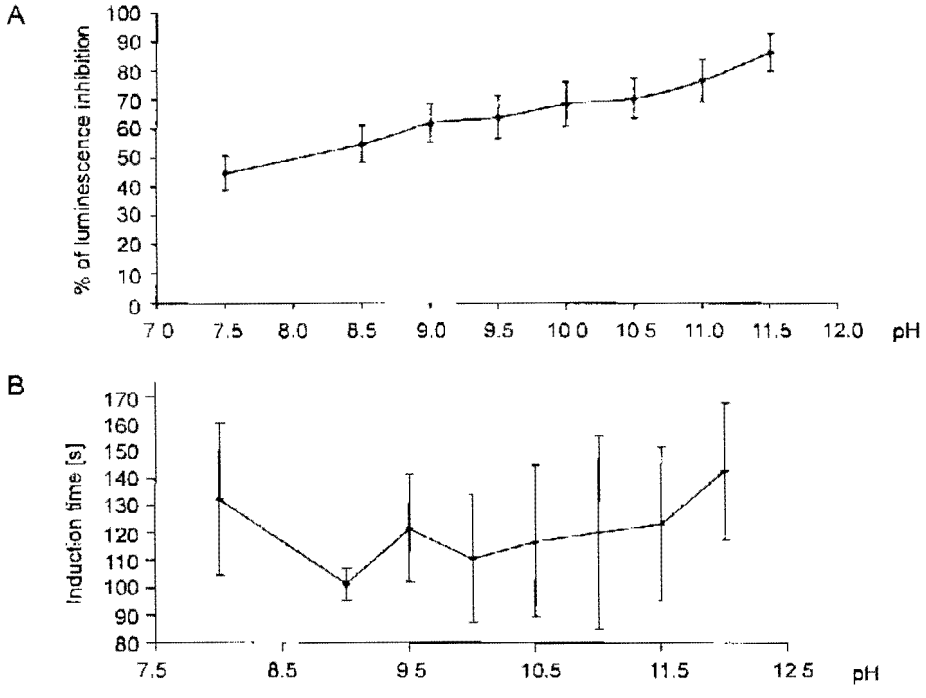

Fig. 7. Dependence of the CL inhibition by BHT (A) and the induction time of Trolox (B) on pH. Luminol, 100 μM; AAPH, 4 mM; BHT, 10 μM; Trolox, 80 nM

time and Trolox concentration within the range from 10 to 1000 nM.

The antioxidant properties of BHT are different from Trolox (Fig. 4B). Depending on BHT concentration, the level of the CL decrease is different. The relationship between BHT concentration (ranging from 1 to 100 μM) and CL inhibition is presented in Fig. 6. A good parameter to compare the antioxidant activity of various compounds could be the concentration inhibiting 50% of chemiluminescence (IC50) which is about 8.5 μM for BHT in our experimental conditions.

The CL inhibition caused by BHT is directly depended on pH (Fig. 7A). In the pH 8, 10 μM BHT inhibits 41.7% of CL, while in the pH 12 – 86.3%. On the other hand the pH of samples has not influenced the induction time of Trolox (Fig. 7B). The relationship between the CL inhibition and pH can be caused by the compound ionisation.

DISCUSSION

The lipid peroxidation process is accompanied by CL that coincides with the decomposition of hydroperoxides, rather than the formation of secondary products. This CL is extremely weak at photon fluxes below 10^4 photons cm^{-1} s^{-1}. Therefore it is important to use an appropriate CL enhancer. The properties of luminol are characterised well

enough. In the presence of luminol, peroxyl radicals undergo trapping causing the light release. This process can be used for determination of the active radicals, for example radicals formed in peroxidation of lipids. The main drawback of this compound is the necessity of use of non-physiological pH i.e. above 8.0 (Fig. 3). Surprisingly in the *in vivo* experiments on neutrophils or other cells, pH as low as 7.4 was successfully used (Mueller & Arnhold, 1995; Jakubowski, Ertel, Biliński, Kędziora & Bartosz, 1998). However, these systems are very complicated and luminol can be active in alkaline compartments of cells such as mitochondria.

The luminol-AAPH systems can be used to test various antioxidants. Lissi (Lissi *et al.*, 1992; Lissi, Salim-Hanna, Pascual & del Castillo, 1995; Escobar, Cardenas & Lissi, 1997) attempted to explain the mechanism of luminol chemiluminescence induced by ABAP (2,2'-azobis(2-amidinopropane)hydrochloride) decomposition to peroxyl radicals. In this particular system the luminescence intensity rapidly reaches a maximum value and then remains almost constant for several minutes (Lissi *et al.*, 1992). We obtained similar results using AAPH instead of ABAP.

Fig. 4A and 4B show that important differences in the quenching activity can occur even between compounds with similar antioxidant groups. Both Trolox and BHT have a phenol ring with an OH substituent (Fig. 1). The OH bond dissociation enthalpy, which is a very good parameter to predict the antioxidant activity, is smaller for α-tocopherol (78.23) than for BHT (81.02) (Lucarini, Pedrielli & Pedulli, 1996). The exceptional antioxidant activity of α-tocopherol, which is due to minimized OH bond strength (Wayner, Lusztyk & Ingold, 1996) could be even stronger for Trolox, which differs from α-tocopherol by the carboxyl group. Trolox is active at extremely low concentrations (Fig. 5) quenching most of CL. On the contrary in the case of BHT addition, the long lasting partial CL inhibition takes place (Fig. 4B). The observed differences between Trolox and BHT could be also due to the charge of the molecules. In the pH range tested the great majority of Trolox molecules probably has a negative charge, due to the dissociation of the carboxyl group. Homolytic decay of AAPH generates two positively charged peroxyl radicals (Fig 1). In contrast, at pH 9 the majority of BHT molecules is not charged. With the increase of pH, the OH group starts to ionise thus facilitating the reaction with positively charged AAPH-derived free radicals. This could explain the more efficient CL inhibition by BHT at higher pH (Fig. 7A) and no impact of pH on the Trolox induction time. Therefore the antioxidant properties of BHT can strongly depend on the pH.

REFERENCES

Aoshima H., Kadoya K., Taniguchi H., Satoh T. & Hatanaka H. (1999). Generation of free radicals during the death of *Saccharomyces cerevisiae* caused by lipid hydroperoxide. *Biosci. Biotechnol. Biochem.*, **63(6)**, 1025–1031

Blokhina O. B., Fagerstedt K. V & Chirkova T. V. (1999). Relationships between lipid peroxidation and anoxia tolerance in a range of species during post-anoxic reaeration. *Physiol. Plant.*, **105**, 625–632

Escobar J., Cardenas G. & Lissi E. (1997). Evaluation of the total content of lipid-soluble antioxidants in blood plasma samples employing a simple chemiluminescence quencing procedure. *J. Biochem. Biophys. Meth.*, **35**, 57–60

Frankel E. N. (1985). Chemistry of free radical and singlet oxidation of lipids. *Progr. Lipid Res.*, **23**, 197–221

Halliwel B. & Gutterige J. M. C. (1999). Detection of free radicals and other reactive species: trapping and fingerprinting; [In:] *Free Radicals in Biology and Medicine* (Oxford University Press), p. 351

Jakubowski W., Ertel D., Biliński T., Kędziora J. & Bartosz G. (1998). Luminol luminescence induced by antioxidants in antioxidant-deficient yeast *Saccharomyces cerevisiae Biochem. Mol Biol. Int.*, **45**, 191–203

Lissi E. A. & Clavero N. (1990) Inactivation of lysosyme by alkylperoxyl radicals. *Free Radic. Res. Commun.*, **10**, 177–184

Lissi E., Pascual C. & del Castillo M. D. (1992). Luminol luminecence induced by 2,2'-azo-bis(2-amidinopropane) thermolysis. *Free Radic. Res. Commun.*, **17**, 299–311

Lissi E., Salim-Hanna M., Pascual C. & del Castillo M. D. (1995). Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements. *Free Radic. Biol. Med.*, **18**, 153–158

Lucarini M., Pedrielli P. & Pedulli G. F. (1996). Bond dissociation energies of O-H bonds in substituted phenols from equilibration studies. *J. Org. Chem.*, **61**, 9259–9263

Mueller S. & Arnhold J. (1995). Fast and sensitive chemiluminescence determination of H_2O_2 concentration in stimulated human neutrophils. *J. Biolumin Chemilumin.*, **10**, 229–237

Niki E. (1987). Antioxidants in relation to lipid peroxidation. *Chem.Phys. Lipids*, **44**, 227–253

Niki E. (1990). Free radical initiators as source of water- or lipid-soluble peroxyl radicals. *Methods Enzymol.*, **186**, 100–108

Pyron W. (1989). On the detection of lipid hydroperoxides in biological samples. *Free Radic. Biol. Med.*, 7, 177–178

Rauchova H., Drahota Z. & Koudelova J. (1999). The role of membrane fluidity changes and cerebral cortex Na^+/K^+-ATPase activity. *Physiol. Res.*, **48**, 73–78

Rauchova H., Ledvinkova J., Kalous M. & Drahota Z. (1995). The effect of lipid peroxidation on the activity of various membrane-bound ATPases in rat kidney *Int. J. Biochem. Cell Biol.*, **27(3)**, 251–255

Smirnoff N. (1995) *Environment and Plant Metabolism*, Bios. Scientific Publishers, Oxford, 217–243

Tilbury R. N. & Miller H. (2000). *Chemiluminescence from the oxidation of model lipid systems*. http://www.photobiology.com/photobiology99/contrib/tilbury/index.htm

Tsugakoshi K., Sumiyama M., Nakajima R., Nakayama M. & Maeda M. (1998). Chemiluminescence property of the luminol-hydrogen peroxide-copper (II) system in the presence of surface-carboxylated microspheres. *Anal. Sci.*, **14**, 409–412

Uchida K. & Stadtman E. R. (1992). Modification of histidine residues in proteins by reaction with 4-hydroxynonenal. *Proc. Natl. Acad. Sci. USA*, **89**, 4544–4548

Wayner D. D. M., Lusztyk E. & Ingold K. U. (1996). Application of photoacustic calorimetry to the measurement of the O-H bond strength in vitamin E (α- and δ-tocopherol) and related phenolic antioxidants. *J. Org. Chem.*, **61**, 6430–6433.

Zanocco A. L., Pavez R., Videla L. A. & Lissi E. A. (1989). Antioxidant capacity of diethyldithylocarbamate in a metal independent lipid peroxidative process. *Free Radic. Biol.Med.*, 7, 151–156