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Journal of Photochemistry and Photobiology A: Chemistry 155 (2003) 63–68

Journal of Photochemistry and Photobiology A:Chemistry

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Changes in pyranine absorption and emission spectra arising from its complexation to 2,2′-azo-bis(2-amidinopropane)

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Received 17 June 2002; received in revised form 3 September 2002; accepted 12 September 2002

Abstract

2,2′-Azo-bis(2-amidinopropane) dihydrochlorid (AAPH) is probably the most extensively employed water soluble free radical source. In the present work, we show that it readily forms a 1:1 complex with pyranine. The complex present a red shifted absorption spectra and a reduced fluorescence yield, without significant changes in the fluorescence band shape. These changes are associated to a decreased value of the phenolyc pK_a in the complex. Time resolved fluorescence measurements reported a short lived component (due to the complexed pyranine) and a long lived component, due to free pyranine, whose lifetime decreases when AAPH concentration increases. The complex formation was quantitatively evaluated from the changes elicited in the absorption spectra. The formation of this complex is extremely dependent of the medium ionic strength. In 10 mM phosphate buffer, pH 7.0, the association constant of the process

$$Py^{4-} + AAPH^{2+} \rightarrow (complex)^{-2}$$

is $730 \pm 70\ M^{-1}$.

Keywords: Pyranine; 2,2,Azo-bis(2-amidinopropane); Complex formation; Complex deprotonation; Fluorescence quenching

1. Introduction

,2′-Azo-bis(2-amidinopropane) dihydrochlorid (AAPH) is probably the most extensively employed water soluble free radical source [1–6]. The popularity of AAPH is due to the fact that under mild conditions (10 mM at 37 °C), it produces free radicals at a very convenient rate (0.75 μM min^{-1}), and that this rate is nearly independent of the presence of additives, in particular redox active metals [7,8]. The radicals produced in the thermolysis of AAPH react with a wide range of compounds, in particular phenols, and the mechanism has been represented by a simple reaction scheme such as that given in Scheme 1.

This simple set of reactions constitute the basis of several procedures devoted to evaluate antioxidant capacities that employ AAPH as an “ideal” free radical source [9]. As part of a systematic study on the reactivity of hydroxyaromatic compounds towards peroxyl radicals, we have studied the reaction between AAPH derived radicals and pyranine (see Scheme 2). In the course of this investigation, we concluded that this simple scheme can not be a priory applied to this system, due to a strong interaction between both compounds that leads to complex formation. This complex formation could affect both pyranine photophysics and the free radical production rate and/or behaviour of the primary radicals. In the present work, we present data regarding the effect of AAPH upon the absorption and fluorescence spectra of pyranine and evaluate, from these data, the association constant between both compounds.

2. Experimental

Pyranine (Sigma) and AAPH (Wako) were employed as received. Absorption spectra were recorded in a Hewlett-Packard 8453 diode array spectrometer. Fluorescence spectra and fluorescence intensities were measured in Fluorolog Spex spectrofluorimeter (excitation 460 nm, slits 1.25 mm; emission 510 nm, slits 2.5 mm). Time resolved experiments were carried out in an Edinburgh Instruments OB 900 time-correlated single photon counting fluorimeter employing an hydrogen filled lamp. Analysis of fluorescence decays were carried out by a least-squares iterative

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PII: S1010-6030(02)00356-8

AAPH -------→ 2 R*

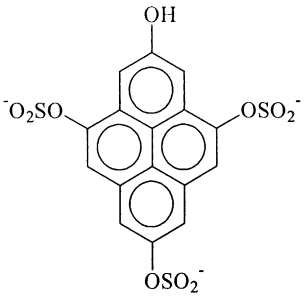
R* + O_2 ----→ ROO*

ROO* + XH ----→ ROOH + X*

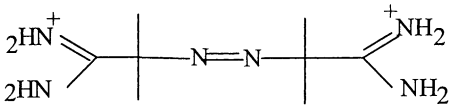
ROO* (or R*) + X* ----→ non-radical products

X* + X* --------→ non-radical products

Scheme 1.



Pyranine



2,2'-azo-bis(2-amidinopropane)

Scheme 2.

convolution method based on the Marquardt algorithm, using the analysis routine provided by Edinburgh Instruments (Edinburgh, UK). All measurements were carried out at room temperature (22 °C) in phosphate buffer, 10 mM, pH 7.0.

3. Results and discussion

3.1. Change in pyranine absorption spectrum with AAPH concentration

Pyranine absorption spectra show bands centred at 370, 400 and 460 nm whose relative importance is pH dependent (Fig. 1). The spectrum shows an isosbestic point at 416 nm that allows a simple two state treatment of the data. In fact, the change in the intensity at 460 nm can be employed to obtain the pyranine pK_a. The type of plot obtained is shown in Fig. 2. From this plot it is obtained a pK_a = 7.4, in good agreement with previously reported values [10,11]. We can

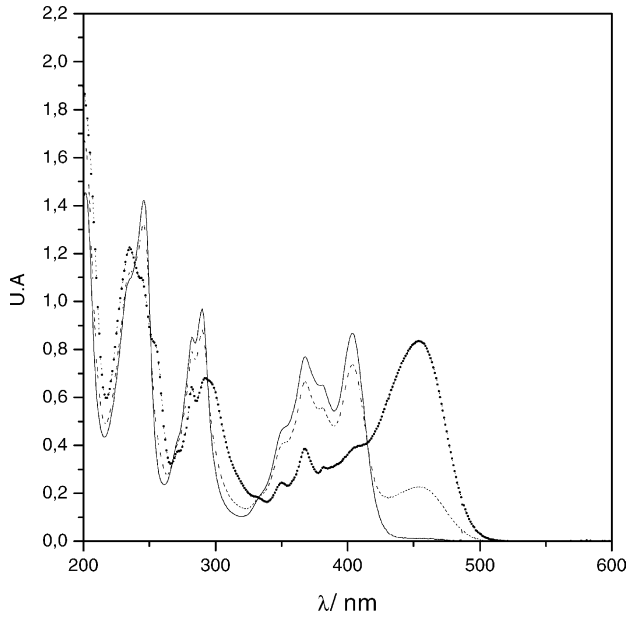


Fig. 1. Pyranine absorption spectra measured at pH 5.7 (—); pH 7.0 (---); pH 8.5 (···).

conclude then that the band centred at 460 nm is due to the deprotonated pyranine, while the bands located at 370 and 400 nm are mainly due to the protonated species [11].

Changes in pyranine photophysical and photochemical properties due to complex formation have been previously reported for alkylpiridinium ions [12,13], methyl viologen [10] and liposome forming amphiphiles [14]. The presence of an excess AAPH (10 mM) also drastically changes the

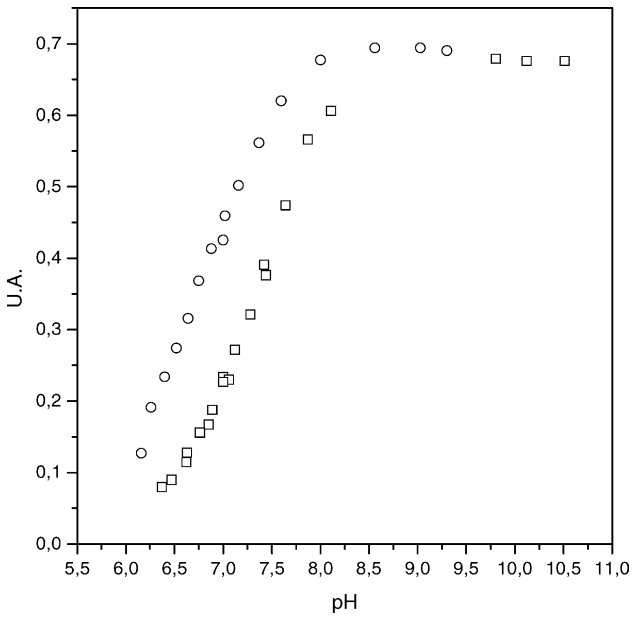


Fig. 2. Effect of AAPH addition (10 mM) upon the pK_a of pyranine (50 μM): (□) absorbance of the sample measured at 460 nm in absence of AAPH; (○) data obtained in presence of AAPH.

absorption spectra of pyranine (50 μM), even after substraction of the absorbance due to the presence of AAPH. This is shown in Fig. 3A, where are shown spectra obtained at pH 7.0 in absence and in presence of AAPH. This figure shows that AAPH addition increases the intensity of the band at 460 and decreases those of the bands located at shorter wavelengths. These data allows to conclude that

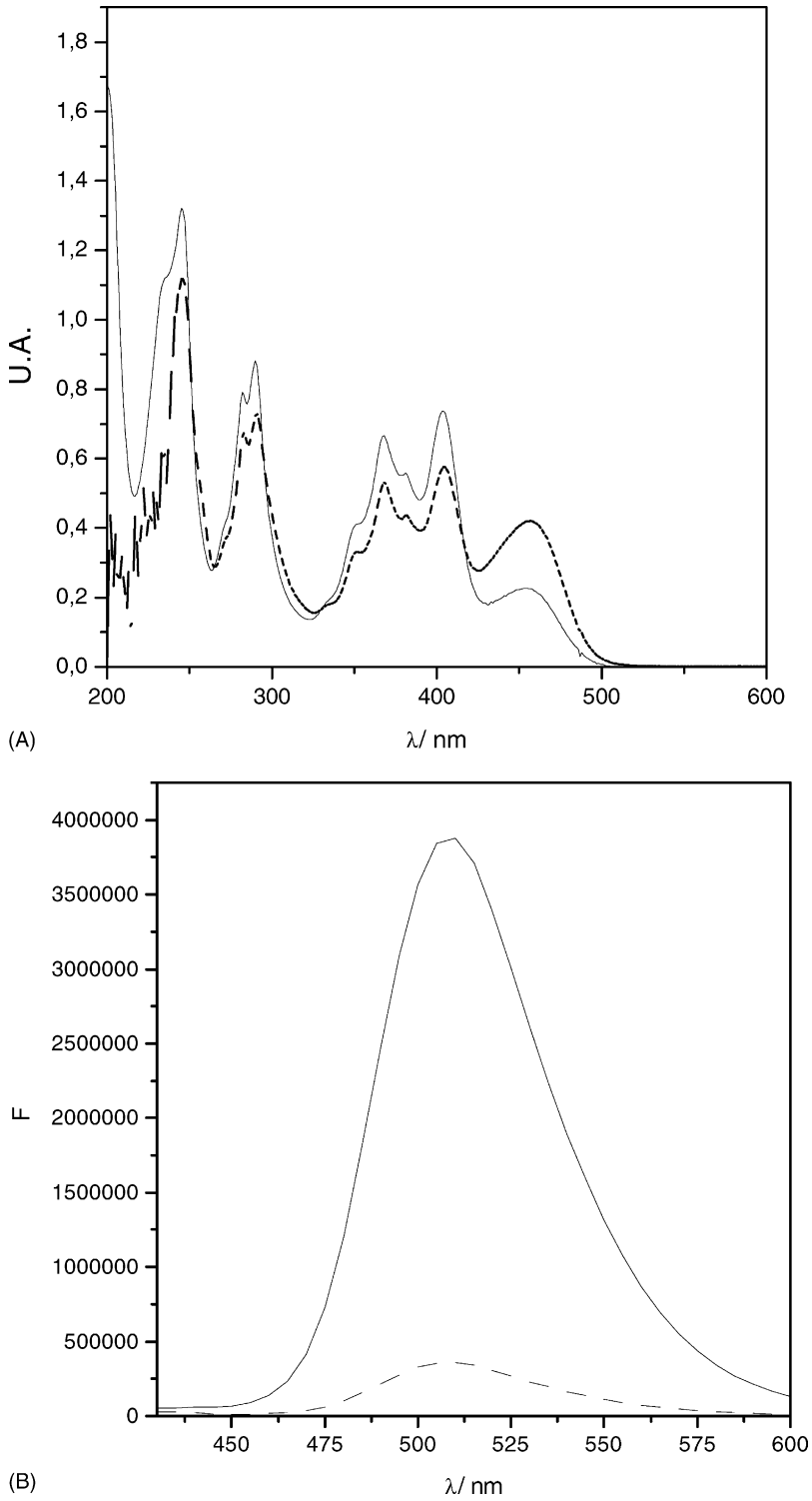


Fig. 3. (A) Effect of AAPH addition (10 mM) upon pyranine absorption spectra at pH 7.0: (—) spectra in absence of pyranine; (---) spectra in presence of AAPH after substraction of the absorbance due to the free AAPH; (B) Effect of AAPH addition (10 mM) upon pyranine (5 μM) fluorescence spectra at pH 7.0: excitation, 416 nm; emission, 510 nm; (—) in absence of AAPH; (---) in presence of AAPH.

(i) there must be an interaction between ground state pyranine and AAPH molecules;
(ii) the effect of AAPH is similar to that observed when the pH of the solution increases.

In order to evaluate the characteristics of the pyranine/AAPH association, spectra were taken at a fixed pH (7.0) and at several AAPH concentrations. The absorbance at 460 nm steadily increases with the AAPH concentration, reaching a plateau at AAPH ca. 6 mM. If these changes are associated to a simple complexation, process such as

$$\text{Pyranine} + \text{AAPH} \leftrightarrow \text{complex} \quad (1)$$

The absorbance at a given wavelength (i.e. 460) can be related to the complex formation through

$$(A - A_0)^{-1} = (A_{\text{complex}} - A_0)^{-1} + (A_{\text{complex}} - A_0)^{-1} K^{-1} [\text{AAPH}]^{-1} \quad (2)$$

where A_0 is the absorbance in absence of AAPH and A_{complex} the absorbance of the totally associated pyranine. A plot of the left hand side of Eq. (2) against the inverse of the AAPH concentration provides the value of K as the ratio between the ordinate and the slope. This type of plot is shown in Fig. 4. The linearity of the plot indicates that the association can be represented by a simple process such as indicated in Eq. (1), and provides an association constant value of $520 \pm 20\ \text{M}^{-1}$. This value implies that half of the pyranine is complexed when the AAPH concentration is ca. 2 mM. Furthermore, it is interesting to note that this association constant is very close to that reported by Rosenbluth and co-workers [12,13]

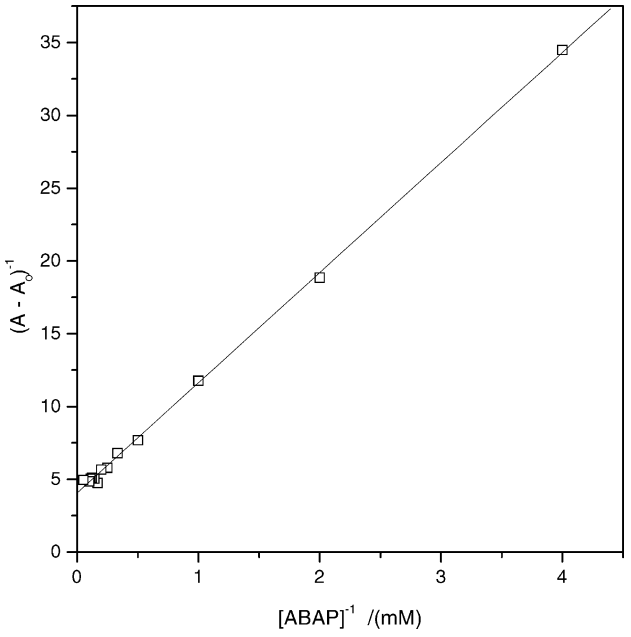


Fig. 4. Change in pyranine absorbance at 460 nm elicited by AAPH addition. Data are plotted as $(A - A_0)^{-1}$ vs. the inverse of AAPH concentration. A is the absorbance of the sample, and A_0 is the absorbance prior to AAPH addition.

for the interaction of pyranine with several short chain alkyl pyridinium ions.

The similarity between the changes observed in presence of AAPH and those elicited by an increase in the solution pH can be explained if the pK_a of the complexed pyranine is lower than that of the free molecule. This is supported by the data shown in Fig. 2. This figure shows that the pK_a of the pyranine is displaced towards lower values in presence of AAPH. Those results would indicate that the main effect of the complexation is to favour the deprotonation of the pyranine molecule. This is compatible with the 2+ charge of the AAPH ion.

The apparent association constant evaluated by the procedure described corresponds to the ratio between the total (3− and 4−) pyranine and the complex (mostly involving deprotonated pyranine (Fig. 2)). In this case

$$K = \frac{\text{complex}(-2)}{[\text{pyranine}(3-) + \text{pyranine}(4-)][\text{AAPH}(2+)]} \quad (3)$$

This association is at least partially promoted by the opposite charge of pyranine and AAPH. This is evidenced by the large effect of increasing the solution ionic strength by NaCl addition. In fact, 100 mM NaCl decreases the apparent association constant value to 180, while at 1 M NaCl the absorption spectra of pyranine barely changes up to 10 mM added AAPH.

From the apparent value of the association constant corresponding to the process represented by Eq. (3) and pyranine pK_a, it can be obtained the equilibrium constant ($K_\#$) corresponding to the process

$$\text{AAPH}(2+) + \text{pyranine}\,(4-) \leftrightarrow \text{complex}\,(2-) \quad (4)$$

The value of $K_\#$ obtained by this procedure is $730 \pm 70\ \text{M}^{-1}$ at the ionic strength of a 10 mM buffer phosphate solution.

3.2. Change in pyranine fluorescence with AAPH concentration

Pyranine fluorescence spectra show a single band located at 510 nm. The position of this band is independent of the pH and the excitation wavelength. Also, the fluorescence yield is also independent of the pH (in the 5–8 range) and of the excitation wavelength. This indicates that a fast equilibration between protonated and deprotonated species takes place in the excited state. A monoexponential decay of the fluorescence intensity takes place with a lifetime of 5.4 ns. This value is close to previously reported data [12,13]. Since the pK_a of phenols in the excited state are usually considerably smaller than that of the ground state species [15], under our experimental conditions only the excited (4−) species has to be considered. This is compatible with the fact that at pH 7.0 the behaviour of excited pyranine is wavelength independent and that, at a given excitation wavelength, the fluorescence spectra is pH independent up to very low pH values (data not shown).

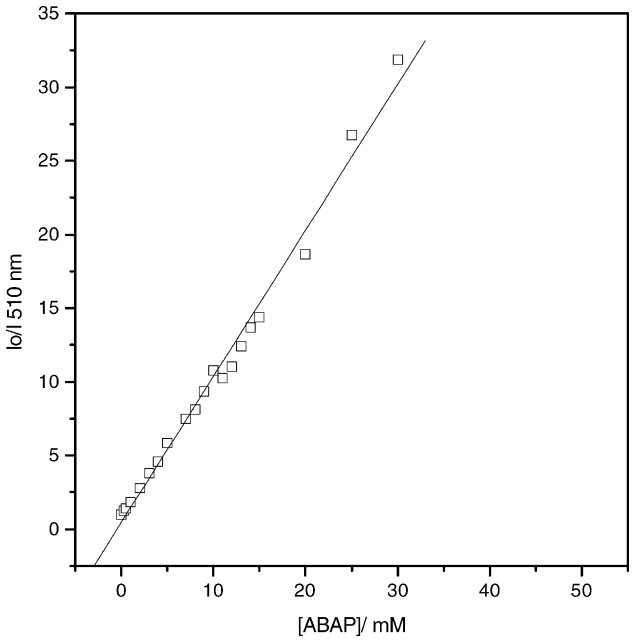


Fig. 5. Stern–Volmer's plot for the quenching of pyranine fluorescence by AAPH at pH 7.0. Pyranine: 5 μM; excitation, 416 nm; emission, 510 nm.

Addition of AAPH readily reduces pyranine fluorescence intensity without changing the fluorescence band shape (Fig. 3B). Data obtained at pH 7.0 are plotted, according to the Stern–Volmer equation, in Fig. 5. These data were obtained by excitation at the isosbestic wavelength to avoid changes in the intensity of the adsorbed light. Up to $F_0/F \approx 20$, the plot is nearly linear, with a slope of 990 M^{-1}.

Table 1
Lifetimes measured in presence of AAPH (5 μM pyranine, pH 7.0)

AAPH (mM)	Component	Lifetime (ns)	Amplitude (%)
0	1	5.4	100
1	1	5.0	93
	2	0.8	7
2	1	4.3	85
	2	0.5	15
4	1	3.6	73
	2	0.54	27
7	1	3.1	55
	2	0.52	45

A linear Stern–Volmer plot can arise if

(i) the ground state complex is frozen after excitation and non-emissive;
(ii) the ground state complex dissociates after excitation and there is a dynamic quenching thereafter;
(iii) there is a compensation of effects. For example, there is an small emission from the complexed pyranines, and dynamic quenching of the excited pyranines that were uncomplexed at the moment of the excitation.

In order to test these possibilities, we have carried out time resolved fluorescence measurements. The results obtained are given in Table 1. These data show that there is a

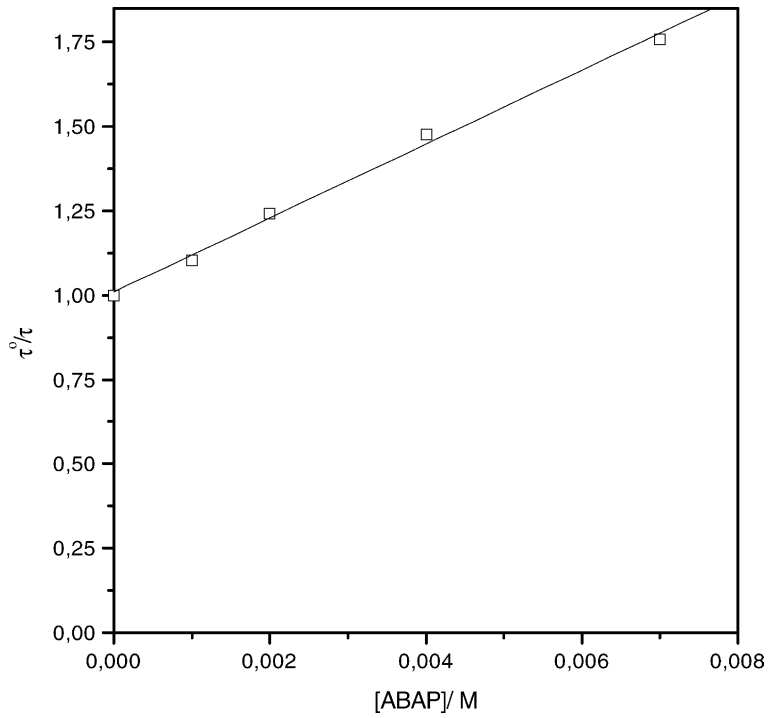


Fig. 6. Change in the lifetime of the long living component of pyranine fluorescence with AAPH concentration. Lifetimes (τ) are plotted as τ_0/τ (where τ_0 is the lifetime in absence of AAPH) against AAPH concentration.

long component whose lifetime and percent contribution decreases when AAPH concentration increases. Furthermore, in presence of AAPH there is a considerably shorter component, of nearly constant lifetime, whose relative contribution increases when AAPH concentration increases. The difference between the value measured at low AAPH concentration (0.8 ns) and the values measured at higher AAPH concentrations (0.5 ns) is not considered significant due to the large error of the former value resulting from its small contribution to the initial intensity (7%).

Possibilities (i) and (ii) can be disregarded under our experimental conditions. If the excited state is frozen and non-emissive, the slope of the Stern–Volmer plot should correspond to the ground state association constant. The difference between both values (990 and 550 M^{-1}, respectively), argues against this possibility. Furthermore, this proposal is incompatible with the lifetime measurements carried out in presence of AAPH (Table 1).

Possibility (ii) can also be disregarded in similar grounds, since it implies a monoexponential decay with $\tau_0/\tau = I_0/I$. This is in complete disagreement with the data given in Table 1. On the other hand, the data given in Table 1 are not incompatible with the proposal (iii). In fact, the short lived component could correspond to the excited complexes, while the reduced long lived component could reflect dynamic quenching of the free excited pyranines. In fact, if the long lived component is treated according to a simple Stern–Volmer formalism (Fig. 6), it is obtained a dynamic quenching constant of $(2.0 \pm 0.1)\ 10^{11}\ M^{-1}\ s^{-1}$. This value, although relatively large, is not incompatible with that of a near diffusion controlled reaction between a (4−) donor and a (2+) quencher.

In conclusion, we have shown that pyranine readily complexes to AAPH dications, and that this complexation leads to significant changes in the aromatic compound photophysics. This complex formation must be taken into account when AAPH is employed as a free radical source in presence of negatively charged polyaromatic compounds.

Acknowledgements

This work was supported by FONDECYT (Project 1000022) and a Ph.D. CONICYT fellowship (A.P.).

References

[1] E. Lissi, M. Salim-Hanna, M. Fauré, L. Videla, Xenobiotica 21 (1991) 955.
[2] A. Valdevenito, E. Lissi, M. Encinas, Macromol. Chem. Phys. 202 (2001) 2581.
[3] E. Lissi, M. Pizarro, A. Aspée, C. Romay, Free Rad. Biol. Med. 28 (2000) 1051.
[4] M. Cubillos, E. Lissi, E. Abuin, Chem. Phys. Lipids 194 (2000) 49.
[5] I.F. Pazos, C. Alvarez, M.E. Lanio, D. Martinez, V. Morera, E. Lissi, A.M. Campos, Toxicon 36 (1998) 1383.
[6] G. Celedón, V. Lips, C. Alvarado, M. Cortés, E. Lissi, G. Gonzalez, Biochem. Mol. Biol. Int. 43 (1997) 1121.
[7] L.A. Videla, T. Cáceres, E. Lissi, Biochem. Int. 16 (1988) 799.
[8] A. Zanocco, R. Pavez, L. Videla, E. Lissi, Free Rad. Biol. Med. 7 (1989) 151.
[9] E. Pino, E. Lissi, Helv. Chim. Acta 84 (2001) 3677.
[10] E.B. de Borba, C. Amaral, M. Politi, R. Villalobos, M. Baptista, Langmuir 16 (2000) 5900.
[11] J. Moorthy, T. Shevchenko, A. Magon, C. Bohne, J. Photochem. Photobiol., A: Chemistry 113 (1998) 189.
[12] H. Rosenbluth, B. Weiss-Lopez, A.F. Olea, Photochem. Photobiol. 66 (1997) 802.
[13] C. Fernandez, M.J. Politi, J. Photochem. Photobiol., A: Chem. 104 (1997) 165.
[14] A.B. Kotlyar, N. Borovok, S. Raviv, L. Zimanyl, M. Gutman, Photochem. Photobiol. 63 (1996) 448.
[15] B. Marcianiak, H. Kozubex, S. Paszyc, J. Chem. Educ. 69 (1992) 247.