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Eriochrome Blue Black R and Fluorescein degradation by hydrogen peroxide oxidation with horseradish peroxidase and hematin as biocatalysts

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ABSTRACT

We describe an UV–vis study of Eriochrome Blue Black R and Fluorescein elimination by chemical transformation and oligo/polymerization using the horseradish peroxidase enzyme (HRP) and the biomimetic hematin as catalysts in aqueous solution. Using HRP/H_2O_2 and Eriochrome Blue Black R a decolorization of 92.5% was achieved at the highest dye concentration (150 mg/L). In the case of the hematin/H_2O_2 system, the decolorization of Eriochrome Blue Black R solutions decreased with the increase of the dye concentration reaching only a removal of 30.2% at the highest dye concentration tested. Fluorescein decolorization in the presence of HRP was very good at 25 mg/L (near 85%) but decreased to 35.7% as the dye concentration increased to 150 mg/L. On the other hand, hematin was poorly active as catalyst for Fluorescein elimination. The use of hematin as catalyst instead of HRP has advantages from the technological and economical points of view for Eriochrome Blue Black R. Hematin can be considered an adequate alternative to the use of enzymatic catalysts based on peroxidases.

1. Introduction

The importance of dyes to civilization is evident and well documented. However, dye pollutants produced from the textile industries are becoming a major source of environmental contaminations [1]. Approximately 150 tons of dyes daily are released into the aquatic environment all over the world [2]. A survey of the literature shows that the oxidative degradation of these dyes seems the most convenient solution and some promising systems have been disclosed [3]. Concerning environment protection, hydrogen peroxide is a desirable oxidant but its activation to generate an efficient oxidant species is still a challenge [4].

Peroxidases from horseradish (HRP) or soybean (Glycine max, SBP) are typical oxidoreductases. They are able to catalyze different reactions using H_2O_2 as an H acceptor. Peroxidases have been used in the oxidative polymerization of aromatic compounds to generate polyaromatics and in phenol elimination as a clean alternative for detoxifying wastewater [5–7]. Another option is the use of biomimetic compounds instead of enzymes. Peroxidases include a heme group, needed cofactor for the reaction to take place. In HRP, iron is a five coordinated species with oxidation state +3. Heme is the protoporphyrin IX complex of the ferrous ion (Fe^{2+}). Hemin is the protoporphyrin IX complex of the ferric ion (Fe^{3+}). Hematin is the hydroxylated hemin [8]. This biomimetic compound would avoid the problems associated to the protein stability. Besides, a biomimetic would be more resistant, for practical purposes, to the known strong inactivation suffered by peroxidases (HRP and SBP) at intermediate H_2O_2 concentrations [7–12]. For example, reference 10 shows that a biomimetic presented a maximum degradation activity at $[H_2O_2] = 0.1$ M, a concentration of oxidant where HRP suffers strong inhibition at similar conditions of substrate concentration.

Peroxidases in the presence of H_2O_2 catalyze the oxidation of a wide variety of phenols, biphenyls, anilines, benzidines and other aromatic compounds [9]. The mechanism has been studied carefully and is described in detail elsewhere [10,11]. In the absence of the usual reducing substrates (AH_2), H_2O_2 can perform a dual role, as oxidant and as a typical one-electron donor (reducing) substrate for peroxidase. It is worth to mention that a number of additional complexities with peroxidases have been identified: a rather slow final step [13–15]; a catalase-like activity which releases oxygen [16–19] and a progressive inactivation of the enzyme producing a verdohaemoprotein [18–22]. There is a narrow optimum concentration range for hydrogen peroxide. If the concentration of hydrogen peroxide used is too low, there is low activity. If the peroxide concentration is too high, competitive substrate inhibition takes place. In addition, a large excess of hydrogen peroxide results in an irreversible enzyme inactivation [12].

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The use of metalloporphyrins as biomimetics is being increasingly studied, especially for remediation purposes [6,7]. Metalloporphyrins are unstable in the presence of oxidants at high concentration. Their lability is assigned to self-destruction or to the formation of inactive complexes [23,24]. Several authors studied metalloporphine immobilization. The coordination between modified solid supports and the metal ions complexed within the porphine macrocycle is the most fruitful approach. Different immobilized systems have been used including Fe(III)-5,10,15,20-tetrakis(pentafluorophenyl)porphine supported on pyridyl-functionalized and crosslinked poly(vinyl alcohol) as a biomimetic versatile-peroxidase-like catalyst [25]. Iron porphyrins, phtalocyanins and salen complexes have been reported as active catalysts for the elimination of phenol and phenol derivatives in the presence of hydrogen peroxide [26–28]. Besides, other iron complexes have been explored in different oxidative degradation processes [28]. However, there are few systematic studies and there is a need of further research on the ability of biomimetics for the efficient substitution of some costly and H_2O_2-sensitive enzymes used for wastewater treatment and for effluent remediation of textile and paper industries.

The goal of this study was to perform a comparative analysis of HRP and hematin as catalysts for the elimination of two different commercially available textile dyes from aqueous solutions. Eriochrome Blue Black R (EBBR) and Fluorescein were selected as model dyes. The reactions were carried out in the presence of excess of hydrogen peroxide. The conditions were selected to establish comparison terms with the hematin, since studies designed in terms of the potential applications of this biomimetic are scarce in the literature. The dyes were chosen because of their structures, which are commonly present in textile industry dyes. The effect of the temperature and the dye concentration in the elimination efficiency were studied by using UV/Visible techniques. The spectroscopic data were analyzed by multivariate regression methods to obtain information on the kinetic aspects of the catalyst-dye-H_2O_2 reactions. Besides, in order to characterize some kinetic and mechanistic differences between the HRP and the biomimetic, the concentration profiles of dissolved oxygen were measured for both dyes using each catalyst at room temperature.

2. Experimental

2.1. Materials

All the reagents used in this study were of analytical grade. Horseradish peroxidase was kindly provided by Amano Inc. (EEUU) and was used without further purification. The enzyme has a molecular weight of 41 000 Da, isolectric points of 6.2, 7.2 and 8.8, a stable activity pH range from 6 to 10, with an activity > 180 Units/mg for the 4 aminoantipyrine/phenol reaction in the presence of H_2O_2 (from datasheet available from Amano Inc). Hematin from Sigma Chemical Co. was employed as provided.

Two different commercially available textile dyes were used in the study: (i) an azo dye, EBBR, with a λ_{max} = 527 nm, and (ii) a triarylmethane dye, Fluorescein, with a λ_{max} = 491 nm. EBBR was supplied by The G. Frederick Smith Chemical Co., Columbus, Ohio; and Fluorescein was supplied by Riedel-De Haën AG. Seelze-Hannover, Germany. Chemical structures of the dyes used are shown in Fig. 1.

2.2. Methods

2.2.1. Decolorization efficiency studies

The experiments were carried out using different dye concentrations (25, 50, 75, 100 and 150 mg/L). We used 0.25 mg of free HRP or hematin, 80 μL of hydrogen peroxide 30% (v/v) (stepwise addition of 20 μL at 5, 10, 15 and 20 min after the addition of HRP or hematin; $[H_2O_2]_{total}$ = 0.021 M), and the necessary amount of pH 7 buffer (KH_2PO_4/NaOH, pH = 7.0 ± 0.1) to reach a final volume of 10 mL. The latter conditions were selected in order to compare the biomimetic with the biocatalyst, but no attempts to minimize deactivation in the case of HRP were done. The solution was magnetically stirred at 25 °C. After 2.5 h, 200 μL of HCl 37% were added as flocculant to change the pH and to precipitate the resulting oligomeric/polymeric products. UV–vis analyses were carried out using a UV-Visible Spectroscopy System Agilent 8453 from Hewlett-Packard. Spectra were recorded in the 300–900 nm range. The evaluation of the treatment efficiency for each dye was carried out through absorbance readings, at their respective maximum wavelengths, before hydrogen peroxide addition, after 2 h – before HCl addition – and after 24 h. The decolorization percentage efficiency – or dye conversion – was estimated as $(C_0 - C_f) \times 100/C_0$ being C_0 the initial concentration and C_f the final concentration of the dye at 2 h. The λ_{max} values and the optimal conditions to assess the removal efficiency were obtained from a detailed UV/Visible study using each dye at different concentrations and pH values. The absorption coefficient of Fluorescein at 491 nm and pH = 11 is 0.1918 L/cm × mg, whereas absorption coefficient of EBBR at 527 nm and pH = 7 is 0.037 L/cm × mg. The average standard deviation for data obtained by this UV/Visible methodology is in the 1.9–2.3% range (tested through duplicate experiments for each dye). It should be pointed out that, in order to avoid linearity losses in the case of Fluorescein, samples with absorbance values higher than 2 at the selected wavelengths were diluted and their corresponding absorbance readings were used for a proper evaluation of decolorization percentages. Even when it is known that

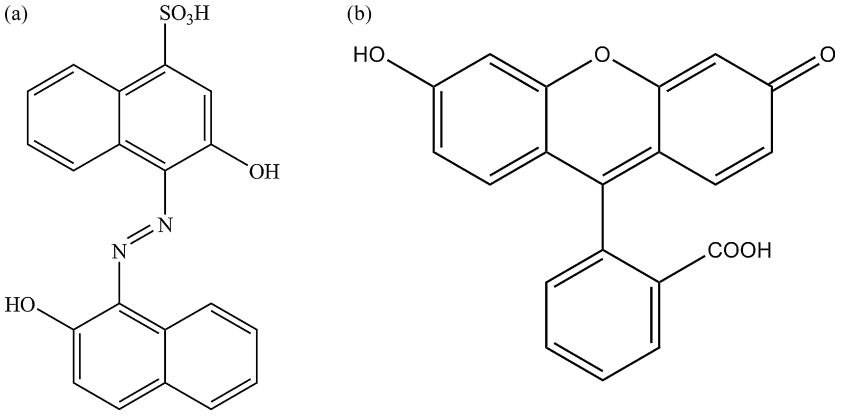

Fig. 1. Structures of the dyes used (neutral forms): (a) Eriochrome Blue Black R and (b) Fluorescein.

polymerization reaction products may have some radiation absorption at the wavelengths used for the calculation of the removal efficiencies, the main bands of the reaction products appear in other spectrum zones. This assumption is common when dyes are being analyzed with HRP/biomimetics in the presence of H_2O_2 [11].

Although liquid chromatography may be used to analyze different by-products formed in the studied systems, we discarded the use of this technique due to the required step of enzyme separation and because of the impossibility to know beforehand the by-products identity. In addition, analytical techniques such as Total Organic Carbon (TOC) or Chemical Oxygen Demand (COD) were not implemented because the standard conditions involved in these techniques could introduce changes masking the results in the studied systems. Therefore, taking into account the literature on the topic [29–32], we choose the UV/Visible technique as a particularly simple experimental method for analyzing the total degradation of the dyes.

2.2.2. Kinetics studies

These studies were performed at pH 7 using the UV/Visible Spectrophotometer in continuous measurement mode for 4 h. Substrate concentrations were 50 mg/L for EBBR and 25 mg/L for Fluorescein. Dye concentrations were selected in order to keep absorbance values below 2 and to avoid aggregation phenomena frequently observed at high concentrations. The H_2O_2 concentration was 0.021 M and the hematin or peroxidase concentration was 25 mg/L. The molar ratio hydrogen peroxide/dye concentration ranged between 34 and 335. The experimental conditions were selected to assure comparative terms with hematin that requires hypothetically more hydrogen peroxide than the HRP.

2.2.3. Effect of temperature

Free HRP and hematin were tested at 25, 45 and 65 °C. These studies were carried out with the following conditions: EBBR 25 mg/L and Fluorescein 25 mg/L.

2.2.4. Oxygen concentration profiles

The oxygen concentration profiles were recorded with a Heavy Duty Dissolved Oxygen Meter, Model 407510 Extech instrument. The reactions in the presence of hematin (25 mg/L) were carried out at 25 °C mixing 20 mL of 100 mg/L dye solution with 400 μL of hydrogen peroxide 30% (v/v) (added at reaction times of 5, 10, 15 and 20 min, total concentration of 0.021 M) and the necessary amount of pH 7 buffer (KH_2PO_4/NaOH, pH = 7.0 ± 0.1) to reach a final volume of 50 mL with magnetic stirring.

Experiments in the presence of HRP were performed by using H_2O_2 concentrations ranging from 2.14×10^{-3} M to 2.14×10^{-2} M, dye concentrations ranging from 10 to 100 mg/L and catalyst concentrations ranging from 2.5 to 25 mg/L. The data presented and analyzed are the best results found in terms of oxygen generation.

2.2.5. Multivariate regression analysis of the UV/Visible spectra

Time resolved spectra were analyzed with the aim of constructing a simple model for comparing the main kinetic and mechanistic features associated to each catalyst. Multivariate self-modeling curve resolution (MCR) can be applied to bilinear spectroscopic-kinetic data from a chemical reaction to provide information about composition changes in an evolving system [33]. In the present work we choose one of the most widely used algorithms, the alternating least-squares (ALS), that can help to simultaneously estimate concentration and spectral profiles [34]. ALS extracts useful information from the experimental data matrix $\mathbf{A}(t \times w)$ by the iterative application of the following matrix product:

$$\mathbf{A} = \mathbf{C}\mathbf{S}^{\mathrm{T}} + \mathbf{E}$$

Table 1
Decolorization percentages obtained with HRP or hematin for different initial dye concentrations at pH 7, $[H_2O_2] = 0.021$ M and 25 °C.

Condition	Percentage removed at 2 h (without HCl addition)				
	Dye concentration (mg/L)				
	25	50	75	100	150
HRP					
Eriochrome Blue Black R	86.4	87.0	86.3	86.4	92.5
Fluorescein	84.8	85.2	77.4	50.6	35.7
Hematin					
Eriochrome Blue Black R	62.0	58.2	53.6	38.4	30.2
Fluorescein	12	12.1	–	–	–

where $\mathbf{C}(t \times n)$ is the matrix of the kinetic profiles; $\mathbf{S}^{\mathrm{T}}(n \times w)$ is that containing the spectral profiles and $\mathbf{E}(t \times w)$ represents the error matrix. The numbers t, n and w denote the sampling times, absorbing species and recorded wavelengths, respectively. Resolving matrix **A** is no easy task [35] since on the one hand n is usually unknown and on the other hand curve resolution methods cannot deliver a single solution because of the rotational and scale ambiguities. We applied Factor Analysis and Singular Value Decomposition for the estimation of n. In order to reduce rotational and scale ambiguities we used matrix augmentation strategy [36] and some chemically relevant constraints [33] such as non-negativity (for both spectra and concentrations) and uni-modality (for the kinetic profiles). The software including standard algorithms for multivariate analysis of the UV/Visible spectra was developed in our laboratory.

3. Results and discussion

3.1. Effect of initial dye concentration in the amount of dye removed for the enzymatic catalyst (HRP) and for hematin

Table 1 shows the percentage of EBBR and Fluorescein removed by peroxidase and hematin for different initial dye concentrations at 25 °C and pH 7.

In the case of Fluorescein the percentage of dye removed by HRP indicates that at concentrations higher than 50 mg/L the amount of dye removed decreased. However, with EBBR removal efficiencies were almost independent of initial dye concentration. In addition, HRP shows a better efficiency to degrade EBBR than Fluorescein.

Using hematin as catalyst the amount of EBBR removed decreased as the initial dye concentration increased, the removal efficiency being a 50% lower for 150 mg/L than for 25 mg/L. On the other hand, due to the limited activity of hematin for Fluorescein elimination, only the lower concentrations were considered.

3.2. Analysis of UV–vis spectra

3.2.1. Eriochrome Blue Black R (EBBR)

Fig. 2(a) and (b) shows the UV–vis spectra for EBBR 150 mg/L with hematin and peroxidase, respectively. It is worth mentioning that nor HRP neither hematin solutions in pH 7 buffer show peaks in the 300–900 nm range (results not shown). Fig. 2(a) shows the emergence a new band at higher wavelengths (≈760 nm) together with partial absorption decrease and a slight displacement to higher wavelengths (batochromic shift) of the reaction mixture absorption in the wavelength region of the principal dye band. These spectral changes, observed for dye concentrations from 75 to 150 mg/L, suggest the possible formation of compounds with higher molecular weight [37–41] when hematin is used. For dye concentrations below 75 mg/L, the 760 nm band was not detected

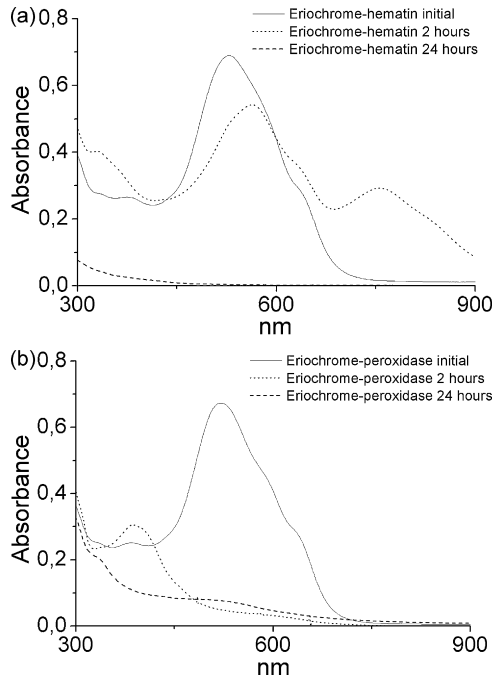

Fig. 2. Scanning spectra of Eriochrome Blue Black R 150 mg/L: (a) with hematin, and (b) with peroxidase. Before hydrogen peroxide addition, after 2 h and after 24 h.

and significant lower absorbances were observed in the spectral region of the principal dye band (data not shown). This result is in line with the higher decolorization efficiencies recorded for the low dye concentrations (Table 1).

In the presence of peroxidase, the main dye band of EBBR practically disappeared and a new band appeared at 390 nm after 2 h. This different behavior between peroxidase and hematin suggests a different reaction pathway for both catalysts: condensation reaction for hematin and degradation for peroxidase. In addition, peroxidase exhibits a higher efficiency than hematin to remove EBBR.

3.2.2. Fluorescein

Fig. 3(a) shows the result of the blank test of Fluorescein transformation in the absence of hematin or peroxidase. The effects of hematin and peroxidase are shown in Fig. 3(b) and (c), respectively. The efficiency of Fluorescein removal by hematin at 2 h of reaction was very low (Fig. 3b). Hematin did not show a significant effect in the removal of Fluorescein even at low concentrations of this dye (Table 1). Fluorescein-hematin system may be considered not active according to the UV–vis analysis.

When the peroxidase is present, a strong decrease in the principal dye band with a slight shift to higher wavelengths is observed (Fig. 3c). Peroxidase is more useful to remove Fluorescein than hematin although its efficiency decreases as the dye concentration increases.

The comparison of the results obtained in the presence of hematin with the ones obtained in the absence of catalyst (Fig. 3(b) and (a), respectively) shows that the addition of HCl results in an hypsochromic shift and causes an intensity decrease with respect to the Fluorescein band (spectra registered after 24 h). In addition, since the spectra recorded in Fig. 3(a) and (b) are very similar; the formation of by-products with high absorption at 437 nm cannot be assigned to a catalytic effect. In the presence of HRP this peak only appears at dye concentrations higher than 75 mg/L (results not shown) suggesting an increase in the amount of non-catalyzed transformation of Fluorescein.

Fluorescein was less decolorized in both systems when compared with the EBBR. This lack of reactivity is probably related to the high stability of the aromatic moieties in the Fluorescein. The peak remaining after 24 h may be assigned to both soluble species of low molecular weight (that cannot be precipitated by the pH change) or to residual Fluorescein.

3.3. Effect of temperature

Table 2 shows the effect of the temperature in the removal of both dyes by hematin or HRP.

In the EBBR-peroxidase system a slight decrease in the activity is observed for the experiment performed at 65 °C. This effect is

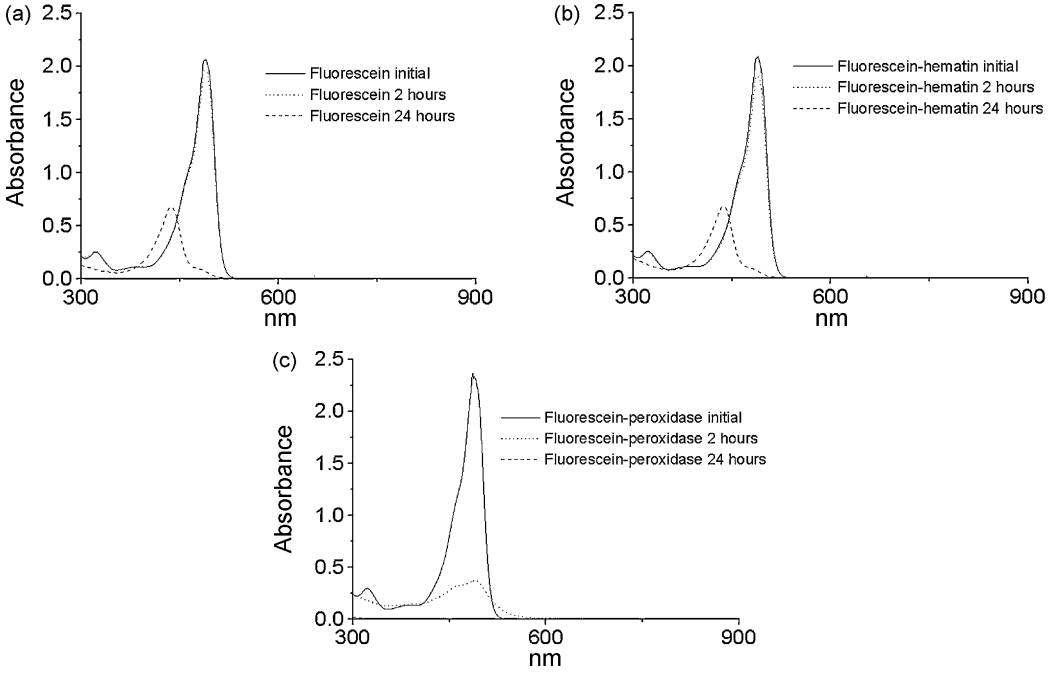

Fig. 3. Scanning spectra of Fluorescein 50 mg/L: (a) in the absence of hematin or peroxidase, (b) with hematin, and (c) with peroxidase. Before hydrogen peroxide addition, after 2 h and after 24 h (all initial spectra are shown without dilution).

Table 2
Decolorization percentages obtained at 2 h using hematin and peroxidase at different temperatures for an initial dye concentration of 25 mg/L at pH 7. [H_2O_2] = 0.021 M.

Condition	Percentage removed at 2 h		
	Temperature (°C)		
	25	45	65
Eriochrome Blue Black R-hematin	62.0	77.2	80.5
Eriochrome Blue Black R-peroxidase	86.4	87.8	78.9
Fluorescein-hematin	12.0	12.0	12.0
Fluorescein-peroxidase	84.8	74.6	59.1

more important in the Fluorescein-peroxidase system where the optimum in the activity is reached at 25 °C. The catalytic effect of hematin increases with temperature in the case of EBBR elimination. For EBBR both, the enzyme and the biomimetic, are active in the temperature range studied. This finding has potential consequences from practical and technological viewpoints. A higher deactivation with the temperature is observed in the Fluorescein-HRP system.

3.4. Oxygen evolution/consumption measurements

The simplified mechanism for HRP is well known and it has been published elsewhere [10–21]. There are several additional steps looking at the enzyme secondary reactions [42]. AH• or R• (alkyl) radicals from the dyes may be generated and decomposed spontaneously or react with O_2 to produce ROO• and $O_2^{\bullet -}$. In addition, a reaction between superoxide radical and hydrogen peroxide has been proposed [8]. The latter process, known as the Haber–Weiss reaction, produces the hydroxyl free radical (OH•) which is one of the most reactive species known. It should be taken into account that, although its rate constant is rather small, the Haber–Weiss is known to be catalyzed by chelated iron. Hydroxyl radicals formed may also be scavenged by H_2O_2, generating the radical $HO_2^{\bullet}$. This situation is applicable to our case because of the high molar ratio of hydrogen peroxide to HRP or hematin used.

Kalyanaraman et al. [42] reported several reactions of H_2O_2 and O_2 with hematin. Hematin is known to catalyze the decomposition of hydroperoxides via the same redox reactions as reported for transition metal ions and the classical Fenton reaction. It is thought that, in addition to OH•, other oxidizing species may be found in systems containing $Fe^{3+/2+}$, $O_2^{\bullet -}$ and H_2O_2.

Tappel [43] has proposed an alternative scheme for hematin catalyzed reactions which does not involve changes in the iron valence, the net reaction being:

$$2HOOH \rightarrow OH^{\bullet} + HOO^{\bullet} + H_2O$$

There are three types of reactions of the OH radicals with organic compounds: abstraction of H atoms, electrophilic addition to multiple bonds and electron transfer processes. In the case of an addition to C=C bonds the final adduct is the radical $R_2C(OH)\text{-}CR_2^{\bullet}$. This addition may also take place in the case of the –N=N– of EBBR.

Fig. 4 shows the oxygen concentration profiles obtained using the best decolorization conditions for EBBR and Fluorescein in the presence of HRP or hematin.

Using hematin as catalyst, for EBBR and Fluorescein a step of decrease and after an increase of the evolution of oxygen are evident, especially for the EBBR. Thus, the behavior observed in Fig. 4 can be partially attributed to the steps related to the peroxyl radical reactions [8]:

(a) An initial step involving peroxyl radicals whose polymerization is faster than decomposition, and therefore consumption of O_2 is found.
(b) A second step involving peroxyl radicals whose decomposition is faster than polymerization, and therefore evolution of O_2 is found.

In addition, the catalase-like activity of peroxidase and hematin may play a decisive role in the concentration profiles recorded for dissolved oxygen:

$$2H_2O_2 \rightarrow O_2 + 2H_2O$$

Taking into consideration the species that may be formed in the reaction media, we separated the reactions in two groups: group I (reactions consuming O_2) and group II (reactions evolving O_2). Table 3 lists the reactions that consume/evolve O_2. EBBR shows a biphasic behavior considering the first 20 min of reaction (first step of O_2 consumption, second step of O_2 evolution) [44,45]. With Fluorescein, the group II of reactions is favored for hematin. However, with HRP the group II of reactions is predominant within the first 5 min, and then the relative contribution of this group of reactions decreases and the group I of reactions becomes important. Again the correlation of higher removal of the dyes with reactions of group I is evident in the O_2 measurements. In the case of EBBR, the group II of reactions is predominant during the first 40 min and the group I is favored in the next 80 min when HRP is used. In the case of hematin the group I of reactions is favored within the first 10 min, afterwards the O_2 amount begins to increase. The low activity in elimination of the dye in the system Fluorescein-hematin-H_2O_2 seems to indicate that the evolution of O_2 in this case is related to catalase-like activity of hematin. The catalatic activity should be considered as a secondary reaction for hematin and for peroxidase when measurements of O_2 concentration are done in presence of these catalysts, phenolic aromatic compounds and hydrogen peroxide.

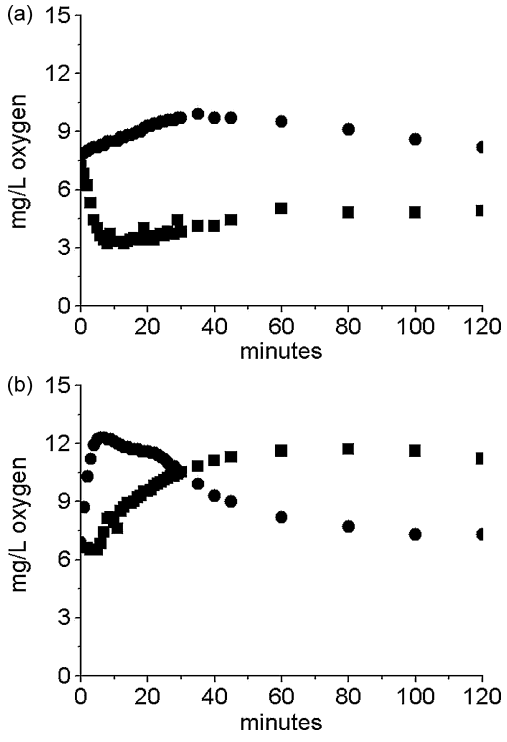

Fig. 4. Oxygen evolution for: (a) Eriochrome Blue Black R, and (b) Fluorescein: (■) hematin, (●) peroxidase. [Dye] = 100 mg/L. [Catalyst] = 25 mg/L. [H_2O_2] = 0.021 M.

Table 3
Reactions that consume/evolve O_2.

Group I: reactions consuming O_2	Group II: reactions evolving O_2
Formation of compound III (Ferrous enzyme + O_2 → Compound III)	Superoxide oxidation by organic radicals ($AH^{\bullet} + O_2^{\bullet -} \rightarrow AH^- + O_2$)
Formation of peroxyl radicals[a] ($R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$)	Superoxide oxidation by Ferric ions ($Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$)
Superoxide anion formation ($A^{\bullet -} + O_2 \rightarrow A + O_2^{\bullet -}$)	Decomposition of hydroperoxide radical ($2HO_2^{\bullet} \rightarrow H_2O_2 + O_2$)
Hematin-Fe^{2+} oxidation	Catalase-like activity[a] ($2H_2O_2 \rightarrow O_2 + 2H_2O$)
Dye degradation	Haber–Weiss reaction ($H_2O_2 + O_2^- \rightarrow O_2 + OH^- + OH^{\bullet}$)
Other enzyme reactions	Other radical reactions

[a] Considered very important.

3.5. Kinetic study

The results of the kinetic study are clearly different when hematin/H_2O_2 and peroxidase/H_2O_2 systems are compared.

In the case of Fluorescein, negligible changes in the absorption spectra were observed within the first 2 h of reaction time in the presence of hematin whereas a very significant decrease in the overall absorption was observed in the presence of HRP (data not shown).

Time resolved spectra obtained for EBBR using both catalysts are shown in Fig. 6. The arrows show the trends of the different bands as reaction time increased. Depending on the catalyst used, batochromic or hypsochromic shifts are observed for the spectral evolution of the reaction mixtures. In agreement with the results presented in Section 3.2, in the hematin/H_2O_2 system a new band with a maximum around 760 nm emerges whereas in the peroxidase/H_2O_2 system the solution spectra show an initial increase and a subsequent decrease of absorption at 390 nm. In addition, the timescales associated to each catalyst are clearly different; the bleaching in the peroxidase/H_2O_2 system being practically instantaneous after H_2O_2 addition.

In order to compare with more detail the results presented in Fig. 5 we have used numerical techniques such as Singular Value Decomposition, Factor Analysis and Constrained Alternating Least Squares as described in the experimental section. The inspection of the singular values of the augmented experimental matrix (i.e. a matrix constructed appending the time resolved spectra obtained in the presence of each catalyst) showed that considering only three contributing factors, the augmented experimental matrix can be reproduced within a 20% of error. Although the consideration of 4 and 5 contributing factors improves the experimental matrix reproduction, we choose 3 contributing factors since our objective was to construct the simplest mathematical model capable of describing the main features of the spectral changes observed.

Fig. 6 shows the spectroscopic and kinetic results obtained using 3 contributing factors and Constrained Alternating Least Squares Regression Analysis for the spectra recorded during EBBR transformation in the presence of hematin or peroxidase.

The spectral features of the 3 principal contributions show that the first factor is closely related to the dye spectrum. The second factor can be assigned to the intermediate product observed in the presence of HRP whereas the third factor is mainly associated to the batochromic shift observed in the presence of hematin. The kinetic profiles corresponding to the experiments performed in the presence of each catalyst show that factors 1 and 2 are observed with both HRP and hematin. The group of chemical species represented

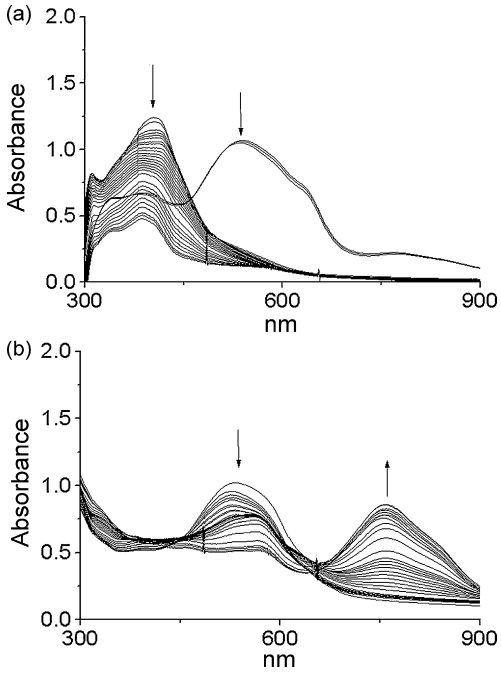

Fig. 5. Eriochrome Blue Black R degradation using (a) peroxidase, and (b) hematin at pH 7 for 4 h. [Eriochrome Blue Black R] = 50 mg/L. [Catalyst] = 25 mg/L. [H_2O_2] = 0.021 M.

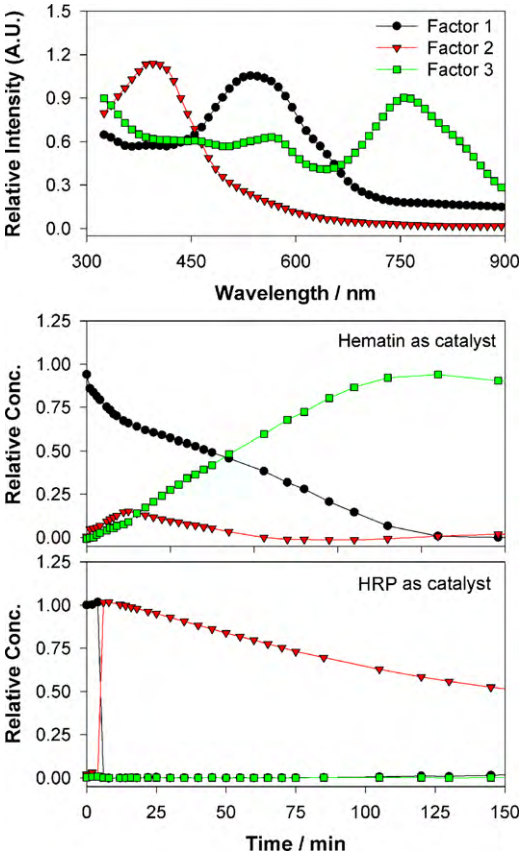

Fig. 6. Results of the multivariate regression analysis of experiments presented in Fig. 5.

by factor 3 shows a significant contribution only in the presence of hematin. In addition, the timescale associated to the emergence of factor 2 is much shorter in the presence of HRP than in the presence of hematin.

Although this rough analysis does not allow drawing categorical conclusions, it is possible that the EBBR degradation follows similar **initial** paths in the presence of both catalysts. During the initial stages, intermediaries that have lost conjugation are likely formed. Then the reaction pathways show important differences. In the presence of HRP after the fast formation of species strongly absorbing at 490 nm, the overall absorption steadily decreases. On the other hand, in the presence of hematin a slow initial increase in concentration of 490 nm-absorbing species is followed by the formation and decay of secondary products with higher conjugation as evidenced by the absorption evolution of absorbance peak at 760 nm.

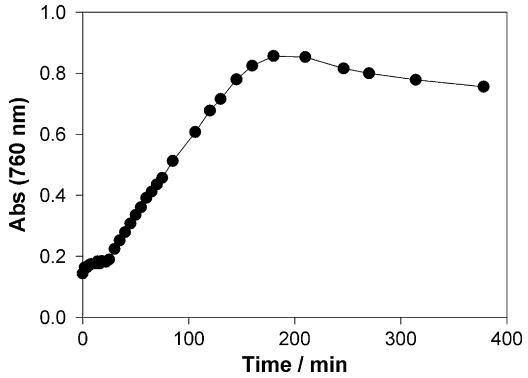

Fig. 7. Absorbance evolution at 760 nm found in Eriochrome Blue Black R kinetic study at pH 7.

3.6. -Comparative analysis of UV/Visible spectra and oxygen evolution results

3.6.1. Hematin

In the case of Fluorescein only catalase-like activity is present, since hematin cannot catalyze the H_2O_2-mediated dye oxidation. On the other hand, there is a high rate of oxygen consumption at the beginning of EBBR transformation but after a little recovering the dissolved oxygen concentration remains almost constant. This biphasic behavior is also observed for the evolution of the absorbance at 760 nm during the kinetic study presented in Section 3.5, but at different timescales since reaction conditions were not the same. Moreover, the plot of the absorbance at 760 nm against reaction time shows a trend (Fig. 7) which looks like the mirror image of the O_2 profile in the assay of EBBR degradation using hematin.

3.6.2. Peroxidase

From the published literature, it is expected partial peroxidase inactivation due to the high hydrogen peroxide concentrations used [12]. However, this inactivation would be present in all assays. With both dyes there is an initial release of O_2 and then a prepon-

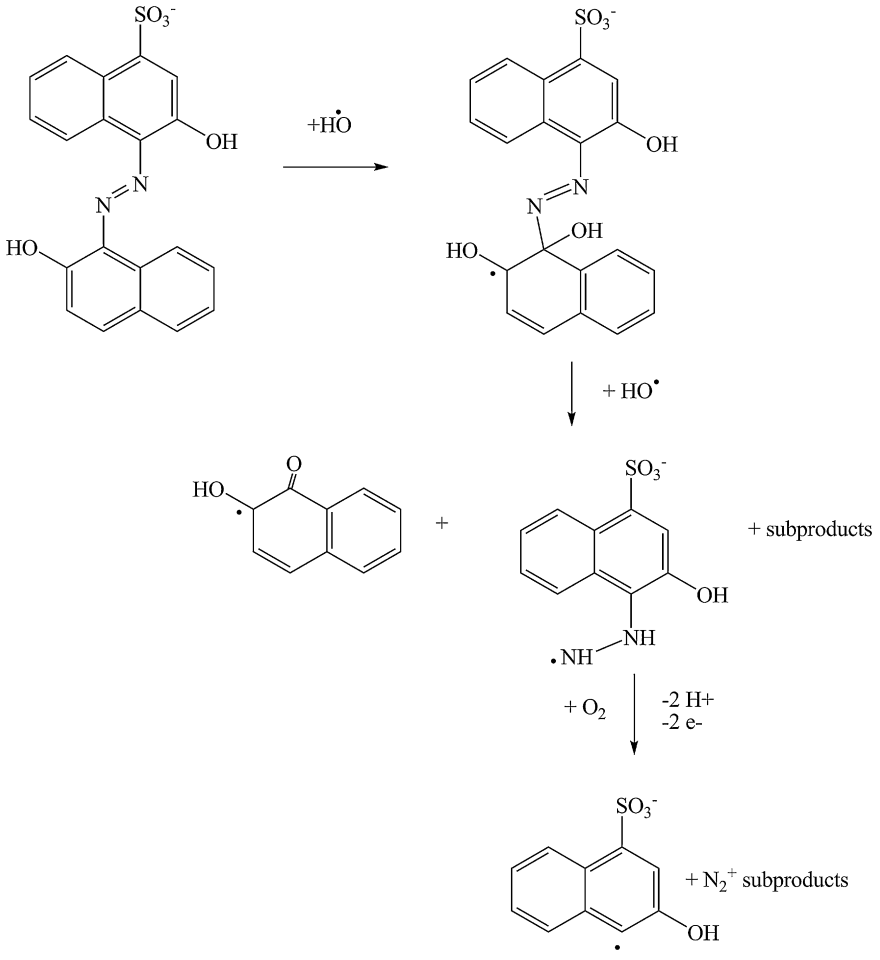

Fig. 8. A possible pathway of decomposition of Eriochrome Blue Black R to give N_2.

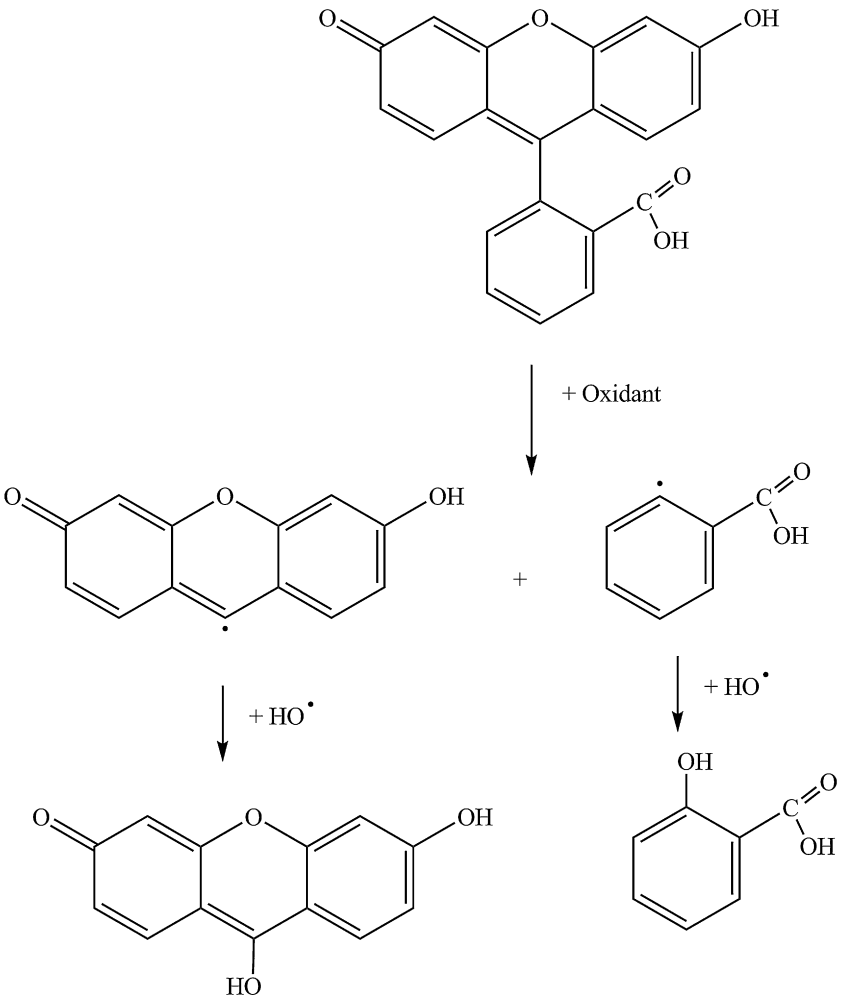

Fig. 9. A possible pathway of decomposition for Fluorescein.

derance of the group I of reactions is observed. These trends can be mainly assigned to an initial predominance of catalase-like activity (H_2O_2 decomposition) due to the high hydrogen peroxide concentration used. After this, depending on the dye structure, the results are different. The second stage of O_2 evolution could be correlated with the nature of the "intermediaries" present in EBBR and Fluorescein at pH 7. During the last stage, the O_2 concentration profiles are almost flat. The latter behavior may be assigned to biocatalyst inactivation, limited hydrogen peroxide availability or both. Once the peroxidase is partially inactivated, the catalatic activity is not entirely lost, but probably the peroxidatic activity is.

Considering all the information presented in the preceding sections not only polymerization of the radicals but also degradation should be taken into account. The presence of the COOH group in Fluorescein makes the evolution of O_2 and CO_2 probable because of redox reactions. In the presence of oxygen, the attack by OH radicals initiates a complex cascade of oxidative reactions that may lead to the mineralization of the organic compound. The exact routes of these reactions are still not quite clear [42]. We cannot discard the presence of these reactions in our systems.

In the case of EBBR, it is known that the breakage of the azo group is one of the first stages of the Fenton processes. However, only hydroxyl radicals contribute in the oxidation of some phenolic compounds during that process. Following Bedoui et al. [46], huge amounts of H_2O_2 (3000 mg/L) are required for obtaining the maximum organic carbon removal using Fenton oxidation. Besides, the reaction of the sulfonic group with the hydroxyl radical should be taken into account [47].

$$R\text{-}SO_3^- + OH^\bullet \rightarrow R\text{-}OH + SO_3^{\bullet -}$$

$$SO_3^{\bullet -} + OH^\bullet \rightarrow SO_4^- + H^\bullet$$

With peroxidase/hematin other radicals may be in-situ generated. EBBR may generate N_2 because of the radical OH attack, such as Fig. 8 shows. In addition, radicals generated during dye degradation may easily participate in polymerization reactions.

For the selected dyes, reactions of radical polymerization (formation of products of increased conjugation) and of degradation (formation of species with decreased conjugation) are proposed to take place. Besides, there are different radicals and anions generated as secondary reactions because of the presence of the hydrogen peroxide. Even the polymerized adducts or the degraded compounds with lower conjugation may also suffer radical attacks and further degradations. Depending on the biocatalyst used, hematin or peroxidase, these reactions may have different importance. Figs. 8 and 9 show the possible decomposition pathways for EBBR and Fluorescein, respectively.

4. Conclusions

The activities of HRP and hematin at intermediate H_2O_2 concentrations, but in excess for peroxidase, are clearly different. Not only the efficiencies in the final elimination of the dyes are dissimilar,

there are also clear differences in the reaction pathways. Both catalysts are efficient for EBBR elimination with different mechanisms. Fluorescein is recalcitrant in the presence of hematin and is oxidized in the presence of HRP although with low efficiency at high dye concentrations.

The comparative analysis of the decolorization efficiencies, the profiles of O_2 evolution at selected conditions and the multivariate regression of the UV/Visible spectra, allowed us to present a feasible explanation of the different trends observed in the presence of each catalyst.

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References

[1] E.J. Weber, R.L. Adams, Environ. Sci. Technol. 29 (1995) 1163–1170.
[2] H. Zollinger, Color Chemistry: Syntheses, Properties and Applications of Organic and Pigments, VCH, New York, 1987.
[3] D. Mantzavinos, E. Psillakis, J. Chem. Technol. Biotechnol. 79 (2004) 431–454.
[4] B.C. Gilbert, J.R. Lindsay Smith, M.S. Newton, J. Oakes, R. Pons i Prats, Org. Biomol. Chem. 1 (2003) 1568–1577.
[5] G. Champetier, L. Monnerie, Introducción a la Química Macromolecular, Espasa Calpe, Madrid, 1973.
[6] N. Durán, E. Esposito, Appl. Catal. B: Environ. 28 (2000) 83–99.
[7] S. Saidman, E.H. Rueda, M.L. Ferreira, Biochem. Eng. J. 28 (2006) 177–186.
[8] H.B. Dunford, Heme Peroxidases, John Wiley, VCH, USA, 1999.
[9] S. Venkata Mohan, J. Karthikeyan, in: P.K. Goel (Ed.), Advances in Wastewater Treatment Technologies, Technoscience Publications, Jaipur, India, 1999, pp. 272–289.
[10] G. Granados-Oliveros, E.A. Páez-Mozo, F. Martínez Ortega, C. Ferronato, J.M. Chovelon, Appl. Catal. B: Environ. 89 (2009) 448–454.
[11] H.B. Dunford, J.S. Stillman, Coord. Chem. Rev. 19 (1976) 187–251.
[12] O. Ryan, M.R. Smyth, C.Ó. Fágáin, in: K.F. Tipton (Ed.), Essays in Biochemistry, Portland Press, London, UK, 1994, pp. 129–144.
[13] B. Chance, Arch. Biochem. Biophys. 41 (1952) 416–424.
[14] R. Nakajima, I. Yamazaki, J. Biol. Chem. 262 (1987) 2576–2581.
[15] S.A. Adediran, A. Lambeir, Eur. J. Biochem. 186 (1989) 571–576.
[16] D. Keilin, E.F. Hartree, Biochem. J. 49 (1951) 88–106.
[17] M. Acosta, M.B. Arnao, J. Hernández-Ruíz, F. García-Cánovas, in: K.G. Welinder, S.K. Rasmussen, C. Penel, H. Greppin (Eds.), Plant Peroxidases: Biochemistry and Physiology, University of Geneva, Geneva, 1993, pp. 201–205.
[18] I. Weinryb, Biochemistry 5 (1966) 2003–2008.
[19] S. Bagger, R.J.P. Williams, Acta Chem. Scand. 25 (1971) 976–982.
[20] S. Marklund, Arch. Biochem. Biophys. 154 (1973) 614–622.
[21] R. Nakajima, I. Yamazaki, J. Biol. Chem. 255 (1980) 2067–2071.
[22] J. Hernández-Ruíz, M.B. Arnao, A.N.P. Hiner, F. García-Cánovas, M. Acosta, Biochem. J. 354 (2001) 107–114.
[23] B. Meunier, in: F. Montanari, L. Casella (Eds.), Metalloporphyrin Catalyzed Oxidations, Kluwer Academic Publishers, Dordrecht, 1994, pp. 11–19.
[24] R.A. Sheldon, in: M. Dekker (Ed.), Metalloporphyrins in Catalytic Oxidations, Inc., New York, 1994, pp. 1–27.
[25] P. Zucca, F. Sollai, A. Garau, A. Rescigno, E. Sanjust, J. Mol. Catal. A: Chem. 306 (2009) 89–96.
[26] A. Haikarainen, J. Sipila, P. Pietikäinen, A. Pajunen, I. Mutikainen, Bioorg. Med. Chem. 9 (2001) 1633–1638.
[27] S. Kobayashi, H. Higashimura, Prog. Polym. Sci. 28 (2003) 1015–1048.
[28] C. Hemmert, M. Renz, B. Meunier, J. Mol. Catal. A: Chem. 137 (1999) 205–212.
[29] G. Liu, T. Wu, J. Zhao, Environ. Sci. Technol. 33 (1999) 2081–2087.
[30] M. Panizza, G. Cerisola, Water Res. 43 (2009) 339–344.
[31] V.L. Maddhinni, H.B. Vurimindi, A. Yerramilli, J. Indian Inst. Sci. 86 (2006) 507–514.
[32] A.C. Serra, C. Docal, A.M.d'A. Rocha Gonsalves, J. Mol. Catal. A: Chem. 238 (2005) 192–198.
[33] M. Garrido, M.S. Larrechi, F.X. Rius, R. Tauler, Chemom. Intell. Lab. Syst. 76 (2005) 111–120.
[34] M. Garrido, I. Lázaro, M.S. Larrechi, F.X. Rius, Anal. Chim. Acta 515 (2004) 65–73.
[35] M. Blanco, A.C. Peinado, J. Mas, Anal. Chim. Acta 544 (2005) 199–205.
[36] A. de Juan, R. Tauler, Anal. Chim. Acta 500 (2003) 195–210.
[37] M.L. Ferreira, Macromol. Biosci. 3 (2003) 179–188.
[38] K. Maksymiuk, Electroanalysis 18 (2006) 1537–1551.
[39] V.A. González, R.C. Cabriales, I. Moggio, E. Arias, Polym. Bull. 58 (2007) 627–634.
[40] M. Andjelkovic, J.V. Camp, B. De Meulenaer, G. Depaemelaere, C. Socaciu, M. Verloo, R. Verhe, Food Chem. 98 (2006) 23–31.
[41] S. González-Manzano, N. Mateus, V. De Freitas, C. Santos-Buelga, Eur. Food Res. Technol. 27 (2008) 83–92.
[42] B. Kalyanaraman, C. Mottley, R.P. Mason, J. Biol. Chem. 258 (1983) 3855–3858.
[43] A.L. Tappel, Autoxidation of Antioxidants, Wiley and Sons, New York, 1961.
[44] D.A. Svistunenko, Biochim. Biophys. Acta 1707 (2005) 127–155.
[45] R. Munter, Proc. Estonian Acad. Sci. Chem. 50 (2001) 59–80.
[46] A. Bedoui, M.F. Ahmadi, N. Bensalah, A. Gadri, Chem. Eng. J. 146 (2009) 98–104.
[47] H. Lachheb, E. Puzenat, A. Houas, M. Ksibi, E. Elaloui, C. uillard, J.M. Herrmann, Appl. Catal. B: Environ. 39 (2002) 75–90.