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FACULTY OF CHEMISTRY
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DOCTORAL THESIS SUMMARY

KINETICS OF OXIDATIVE DEGRADATION OF SEVERAL PHENOLIC POLLUTANTS IN AQUEOUS MEDIUM

Ph.D Candidate:	Doctoral Coordinator:
Cătălina Florentina Olmazu (Trofoşilă)	Professor Ph.D. Dumitru Oancea

Doctoral Commission:

President: Professor Ph.D. Camelia Bala
Doctoral Coordinator: Profesor Ph.D. Dumitru Oancea
Reviewers:

1. Academician Professor Ph.D. Eugen Segal, University of Bucharest
2. Professor Ph.D. Ing. Dan Geană, University Politehnica of Bucharest
3. Scientific Researcher I Biochem. Ph.D. Radu Albulescu, National Institute for Chemical Pharmaceutical Research and Development, Bucharest

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(The numbering of chapters, subchapters, figures, tables, diagrams, equations and references corresponds to the doctoral thesis.)

INTRODUCTION

The theme *„Kinetics of oxidative degradation of several phenolic pollutants in aqueous medium"* is justified by the fact that the removal of phenolic compounds from oil refining wastes, or resulted from resins and plastics processing, represents a nowadays major problem. Phenolic compounds are harmful to the body, especially chlorinated derivatives, even when they are present in low concentrations (1 µg/ l). The phenolic compounds become inhibitory substrates in certain metabolic changes [1].

Several new technologies based on advanced oxidation processes (AOP) were used in order to convert the pollutants into harmless chemicals. Some major approaches to AOP are under development at this time: homogeneous UV photolysis, dark homogeneous oxidation involving the use of Fenton reaction [2], heterogeneous UV photolysis on TiO_2, radiolysis, wet air oxidation – WAO [3-6].

Certain types of enzymes exist in sub-aquatic plants, such as tyrosinases, peroxidases as well as transition metal ions [10, 11] (copper and iron). Their use can be a cheap method for biodegradation of pollutants in harmful compounds and a feasible alternative to chemical oxidation.

Phenolic pollutants can be transformed into less toxic compounds which have antimicrobial and cytotoxic action. Phenoxazinone derivatives such as xantommatin, actinomycin D or Questiomycin A are compounds exerting strong antitumor effects by intercalating into human DNA and thus inducing enzymatic blocking and reading errors during the replication process [23-25].

The complexity of chemical transformations of phenolic pollutants requires the development of some mathematical models to describe the time evolution of reactant systems. Mathematical models consist of characteristic equations which can be solved by numerical methods using dedicated software.

In this doctoral thesis a kinetic study is conducted by monitoring the oxidative degradation of phenolic pollutants in the presence of catalysts and biocatalysts in almost natural conditions, using batch reactor to simulate real conditions.

The main objectives in this work were:

- Using of several catalysts which exists in natural environment for degradation of several phenolic pollutants from waste waters and kinetic modelling of these processes;
- Using of several biocatalysts for degradation of several phenolic pollutants;
- Accomplishment of a catalytic effeciency study of horseradish peroxidase and tyrosinase from natural and waste waters in order to optimize the oxidative method for phenolic compounds degradation;
- Accomplishment of a inactivation study of horseradish peroxidase for oxidative degradation of phenolic pollutants and estimation of several inactivation constants;
- Conduct a study on the influence of modulators on the kinetics of enzymatic oxidation reactions in aqueous medium;
- Using modern methods of mathematical modelling and data processing methods in order to elucidate the reaction mechanisms of the studied processes.

The doctoral thesis has two parts: the first part is the literature review which describes the current state of knowledge in the studied field, and the second part, the original contributions, is based on the experimental results obtained during the research.

In the literature part are described aspects related to the toxicity of phenols derivatives and their transformation into surface waters (chapter I.1.), the methods of phenols pollutants degradation into less toxic compounds (chapter I.2.), the advantages of using metalloenzymes in waters decontamination (cap. I.3.), kinetic modeling of phenols oxidation to calculate reaction rates and kinetic parameters (cap. I.4.), effects due to the ions presence in solution which appreciably modifies the behavior of the other particles also present in solution (cap. I.5.), the methods used to describe the enzymes inactivation such as Selwyn test and isoconversional method (I.6.), and in the last chapter (I.7.) are described phenols oxidation byproducts which have therapeutic effects.

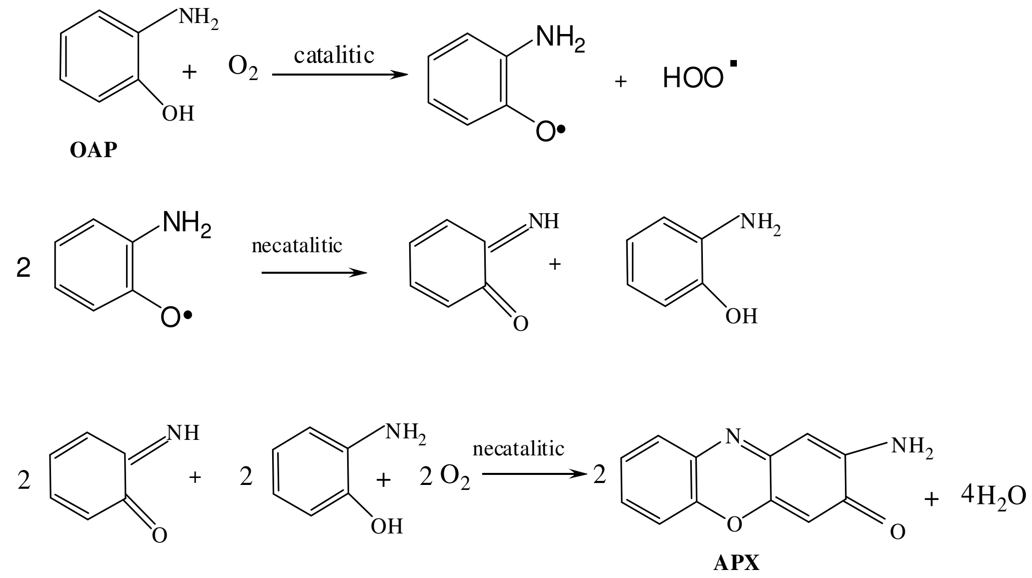
In the original part of the thesis are described kinetic studies on 2-aminophenol and 2-substituted aminophenols (4-chloro-2-aminophenol, 2-amino-*p*-cresol and L-3,4-dihydroxyphenylalanine) oxidation with molecular oxygen in aqueous oxygenated solutions in the presence of transitional metals salts (Cu (II), Co (II), Mn (II)) and in the presence of metalloenzymes: mushroom tyrosinase and horseradish peroxidase.

II. ORIGINAL CONTRIBUTIOS

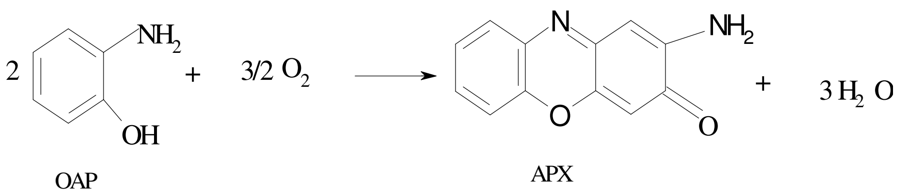
II.2. Kinetics of 2-aminophenol oxidation catalyzed by Cu (II) ions

The main purposes of our work were to use simple copper salts to perform oxidation of OAP in oxygenated aqueous solutions and to develop a reaction scheme model able to explain the low yields in APX along with the operational inactivation of the catalyst. It was demonstrated that the inactivation path can be assigned to formation of the stable bis(*o*-iminosemiquinonato)copper(II) complex [99], a compound reported as a moderate antimicrobial agent.

It is now widely accepted that the generation of aminophenoxyl radical is supposed to occur catalytically or in the absence of the catalyst (auto-oxidation), but the following dismutation, addition, cyclization and oxidation steps do not require catalyst assistance [82] (scheme II.2.1):



Scheme. II.2.1. Simplified model of kinetically significant oxidation steps of OAP



Scheme II.2.2. Overall equation of OAP oxidation to APX

In this work we proposed and developed a reaction sequence for Cu(II) catalyzed oxidation of OAP which includes as plausible active intermediates Cu(II) superoxo species and Cu-OAP complexes, whose occurrence is sustained in literature data [71, 86]. An inactivation step, assigned to the formation of the stable bis(*o*-iminosemiquinonato) copper (II) complex (also described in literature [71]), was added and experimentally checked.

The Cu(II) catalyzed oxidation of OAP being followed in air or dioxygen saturated solutions, the kinetic model took into account the reactions involving dissolved dioxygen according to the overall stoichiometry (Scheme II.2.2.).

The proposed model takes into account the generation of reactive oxygen species (ROS) such as HOO·, HO· and other highly reactive radical species and starts with a set of rate constants reported in literature for 2-aminophenol oxidation [81, 82] and generation of ROS species, others being obtained using an optimization algorithm.

$$SH_2 + Cu^{2+} \xrightarrow{k_1} (CuSH)^+ + H^+ \qquad (II.2.1)$$

$$(CuSH)^+ + O_2 \xrightarrow{k_2} (CuO_2)^+ + SH\cdot \qquad (II.2.2)$$

$$(CuO_2)^+ \xrightarrow{k_3} Cu^{2+} + O_2^{\bullet-} \qquad (II.2.3)$$

$$O_2^{\bullet-} + H^+ \xrightarrow{k_4} HOO\cdot \qquad (II.2.4)$$

$$SH_2 + O_2 \xrightarrow{k_5} SH\cdot + HOO\cdot \qquad (II.2.5)$$

$$2SH\cdot \xrightarrow{k_6} P \qquad (II.2.6)$$

$$2HOO\cdot \xrightarrow{k_7} H_2O_2 + O_2 \qquad (II.2.7)$$

$$SH_2 + H_2O_2 \xrightarrow{k_8} SH\cdot + HO\cdot + H_2O \qquad (II.2.8)$$

$$H_2O_2 + HO\cdot \xrightarrow{k_9} HOO\cdot + H_2O \qquad (II.2.9)$$

$$HOO\cdot + H_2O_2 \xrightarrow{k_{10}} HO\cdot + H_2O + O_2 \qquad (II.2.10)$$

$$(\mathrm{CuSH})^{+} + \mathrm{SH}_2 \xrightarrow{k_{11}} \mathrm{Cu(SH)}_2 + \mathrm{H}^{+} \qquad \text{(II.2.11)}$$

where SH_2 is OAP

Table II.2.2 Rate constants in aqueous medium used in the optimisation procedure

Rate constant	Value/ $M^{-1}\cdot s^{-1}$	Reference
k_7	$1{,}6\cdot 10^{5}$	84, 85
k_9	$2{,}7\cdot 10^{7}$	77
k_{10}	0,50	84

Rate constants (k_5 and k_8) were estimated experimentally following the experimental design and theoretical reasoning depicted in several previous works [81, 82].

Briefly, an apparent first-order rate constant k'_5 was estimated by non-linear regression analysis after fitting an apparent first-order growth function on the progress kinetic curve [APX] vs. time obtained at the oxidation of OAP with dioxygen in dioxygen saturated solution. The second-order rate constant k_5 was obtained as the ratio of k'_5 and $[O_2]$: $k_5 = (1.191\pm0.002)\cdot 10^{-2} M^{-1}s^{-1}$ with a significance level $\alpha=0.05$.

In a similar manner was obtained k_8, using the progress kinetic curve [APX] vs. time obtained during oxidation assay of OAP with hydrogen peroxide in a100-fold excess of H_2O_2: $k_8 = (2.011\pm0.006)\cdot 10^{-3} M^{-1}s^{-1}$ ($\alpha = 0.05$).

The constant k_6 was fixed at a plausible value of $1\cdot 10^{8}$ $M^{-1}\cdot s^{-1}$ because varying this parameter within $1\cdot 10^{6}$-$1\cdot 10^{10}$ $M^{-1}\cdot s^{-1}$ (specific range of rate constant for fast reactions in solution) does not change the values of the optimised parameters.

Estimation of k_{11} the inactivation step of the copper (II) catalyst assigned to formation of the inactive bis(*o*-iminosemiquinonato) copper complex can be pursued using an isoconversional method as described elsewhere for a first-order catalyst inactivation [92].

In isoconversional condition the time evolution of the product (APX) is:

$$\left(\frac{d[\mathrm{APX}]}{dt}\right)_i = f\left([\mathrm{APX}]_{\mathrm{isoconv}}\right)\times[\mathrm{Cu(II)}]^{*}_{0,i} \qquad \text{(II.2.13)}$$

From the catalyst molar balance and from the approximate steady-state conditions was calculated the concentration of the active complex $Cu(SH)^+$:

$$[(CuSH)^+] = \frac{k_1k_3[Cu(II)]_0^*[SH_2]}{k_2k_3[O_2] + [SH_2](k_3k_{11} + k_1k_3 + k_1k_2[O_2])} \tag{II.2.21}$$

The time evolution of the active catalyst was:

$$-\frac{d[Cu(II)]_0^*}{dt} = \frac{k_1k_3k_{11}[SH_2]^2[Cu(II)]_0^*}{k_2k_3[O_2] + (k_1k_3 + k_1k_2[O_2])[SH_2]} \tag{II.2.22}$$

No simple procedure exists to integrate this equation in order to obtain $[Cu(II)]_0^*$ as a function of time when $[SH_2]$ changes in time. However, two simpler limiting cases are obtained when one of the two terms in the denominator can be neglected.

I. If $k_2k_3[O_2] << (k_1k_3 + k_1k_2[O_2])[SH_2]$ equation (II.2.22) becomes:

$$-\frac{d[Cu(II)]_0^*}{dt} = \frac{k_1k_3k_{11}[SH_2]}{(k_1k_3 + k_1k_2[O_2])}[Cu(II)]_0^* \text{ or } -\frac{d[Cu(II)]_0^*}{dt} = \frac{k_{11}[SH_2]}{\left(1 + \frac{k_2}{k_3}[O_2]\right)}[Cu(II)]_0^* \tag{II.2.23}$$

Integration of equation (II.2.23) after separating the variables, and taking into account that the concentration of the dissolved dioxygen was maintained constant during the oxidation assays, gives:

$$-\int_{[Cu(II)]_0}^{Cu(II)_0^*} \frac{d[Cu(II)]_0^*}{[Cu(II)]_0^*} = \frac{k_{11}}{\left(1 + \frac{k_2}{k_3}[O_2]\right)} \int_0^t [SH_2]dt \tag{II.2.24}$$

For I set, from t = 0 to isoconversional time, $t_{isoconv}$ one obtains:

$$[Cu(II)]_{0,i}^* = [Cu(II)]_{0,i} \times e^{-k_{in1}I_1}, \tag{II.2.25}$$

where the inactivation constant k_{in1} and integral I_1 are:

$$k_{in1} = \frac{k_{11}}{1+\frac{k_2}{k_3}[O_2]} \qquad \text{şi} \qquad I_1 = \int_0^{t_{isoconv}} [SH_2]dt \qquad \text{(II.2.26)}$$

II. If $k_2k_3[O_2] >> (k_1k_3+k_1k_2[O_2])[SH_2]$, equation (II.2.22) becomes:

$$-\frac{d[Cu(II)]_0^*}{dt} = \frac{k_1k_{11}[SH_2]^2[Cu(II)]_0^*}{k_2[O_2]} \text{ or } -\frac{d[Cu(II)]_0^*}{dt} = \frac{k_1k_{11}[SH_2]^2}{k_2[O_2]}[Cu(II)]_0^* \qquad \text{(II.2.27)}$$

After integration one obtains:

$$[Cu(II)]_{0,i}^* = [Cu(II)]_{0,i} \times e^{-k_{in2}I_2} \qquad \text{(II.2.28)}$$

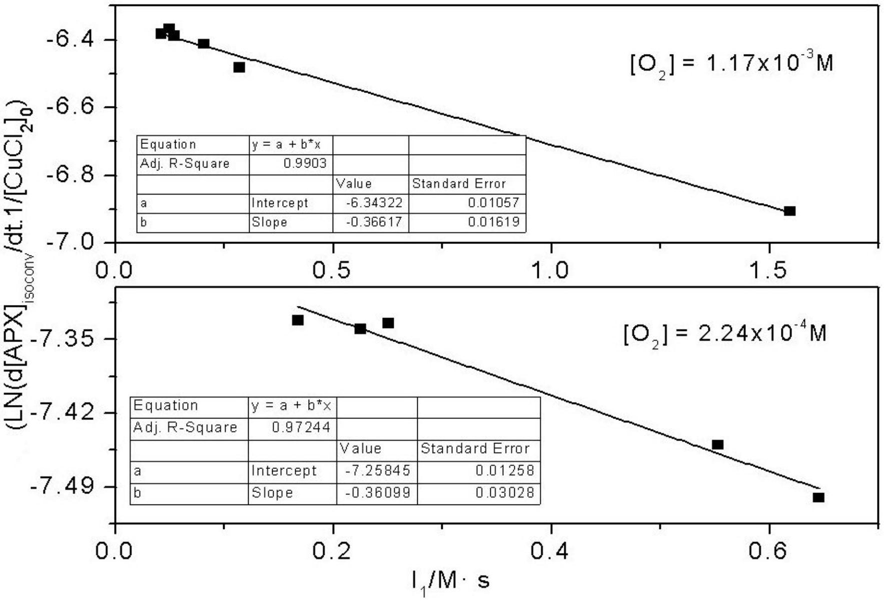
$$k_{in2} = \frac{k_1k_{11}}{k_2[O_2]} \qquad I_2 = \int_0^{t_{isoconv}} [SH_2]^2 dt \qquad \text{(II.2.29)}$$

The integrals *I*$_1$ and *I*$_2$ were calculated numerically as the area under the curves [OAP] or $[OAP]^2$ as functions of time, after fitting smooth functions (polynomials) on the experimental set of data of the form $[OAP] = [OAP]_0 - 2[APX]$.

In the first case replacing the concentration of active catalyst from eq. (II.2.25) in eq. (II.2.13), one obtains:

$$(d[APX]/dt)_{isoconv}/[Cu(II)]_{0,i} = f([APX]_{isoconv}) \cdot e^{-k_{in1}I_1} \qquad \text{(II.2.30)}$$

The inactivation constant k_{in1} was estimated using a nonlinear regression analysis of eq. (II.2.30) with $(d[APX]/dt)_{isoconv}/[Cu(II)]_{0,i}$ as dependent variable, *I*$_1$ as independent variable and $f([APX]_{isoconv})$ and k_{in1} as estimated parameters, or using a linear regression analysis of the logarithmic form of the same equation. The evaluation of the inactivation kinetic constant k_{in1} is illustrated in figure II.2.4:



Equation	y = a + b*x		
Adj. R-Square	0.9903		
		Value	Standard Error
a	Intercept	-6.34322	0.01057
b	Slope	-0.36617	0.01619

Equation	y = a + b*x		
Adj. R-Square	0.97244		
		Value	Standard Error
a	Intercept	-7.25845	0.01258
b	Slope	-0.36099	0.03028

Fig. II.2.4.Estimation of k $_{in1}$ in dioxygen/air saturated solutions according to the first limiting case [equations (II.1.25) and (26)] $[OAP]_0 = 1\cdot10^{-4}M$, T = 30°C, conversion ≈ 20%, $[CuCl_2]_0$ ranging from $5\cdot10^{-7}M$ to $1\cdot10^{-6}M$

The results for k_{in1} [(0.361 ± 0.030) s^{-1} for O_2 and (0.366 ± 0.016) s^{-1} for air] indicate an apparent independence of the inhibition of the dissolved oxygen concentration, but from k_{in1} expression $k_{in1} = \dfrac{k_{11}}{1+\dfrac{k_2}{k_3}[O_2]}$ this results only if the second term of the denominators is much smaller than 1 and can be omitted.

Alternatively, replacing the concentration of the active catalyst from eq. (II.2.28) in eq. (II.2.13), one obtains:

$$(d[APX]/dt)_i/[Cu(II)]_{0,i} = f\left([APX]_{isoconv}\right)\cdot e^{-k_{in2}I_2} \qquad (II.2.31)$$

Following the same procedure, the linear regression analysis of the logarithmic form of eq. (II.2.31) is illustrated in fig. II.2.5:

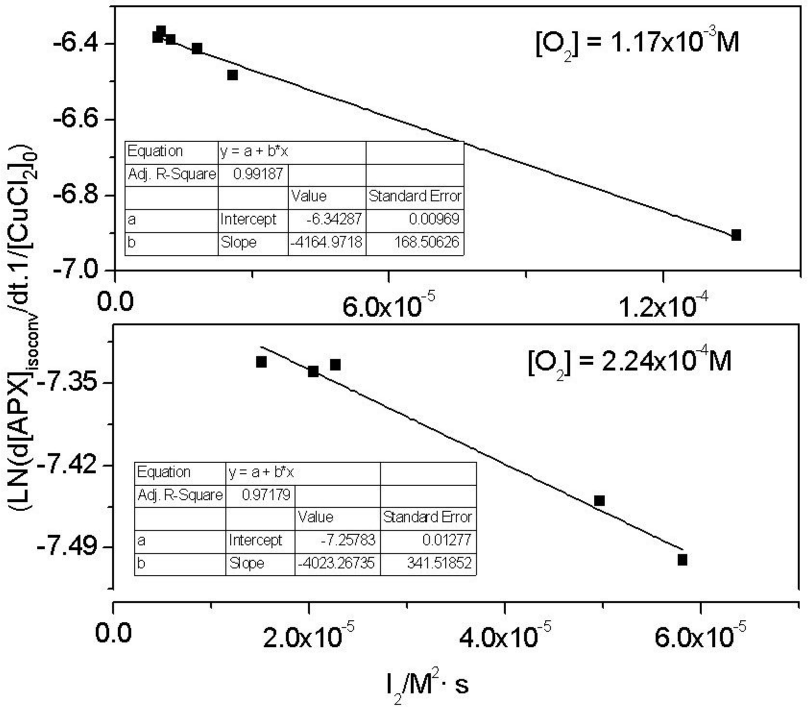


Fig. II.2.5.Estimation of k_{in2} in dioxygen/air saturated solutions according to the second limiting case [equations (II.2.28) and (II.2.29)] $[OAP]_0 = 1\cdot10^{-4}$M, T = 30°C, conversion ≈ 20%, $[CuCl_2]_0$ ranging from $5\cdot10^{-7}$M to $1\cdot10^{-6}$M

The results for k_{in2} [$(4165 \pm 169)\ s^{-1}$ for O_2 and $(4023 \pm 342)\ s^{-1}$ for air] indicate a dependence proportional with the dissolved oxygen concentration, but the values are in disagreement with the equation of k_{in2} $\left(k_{in2} = \dfrac{k_1 k_{11}}{k_2[O_2]}\right)$ predicting an inverse proportionality of the inhibition constant with the oxygen concentration.

The numerical integration and optimization of the rate constants involved in the system of differential equations 1-11 can help to discriminate between these limiting cases and to establish the significance of the inhibition constant. The optimisation process was accomplished with Kintecus [101]

The optimized values are: $k_1 = (58.50\pm 0.04)\ M^{-1}\cdot s^{-1}$, $k_2 = (2.18 \pm 0.04)\ M^{-1}\cdot s^{-1}$, $k_3 = (2.80\pm 0.08)\cdot 10^{2}\ s^{-1}$, $k_4 = (5.100 \pm 0.024)\cdot 10^{6}\ M^{-1}\cdot s^{-1}$and $k_{11}=(4.80\pm0.05)\cdot 10^{-2}\ M^{-1}\cdot s^{-1}$ (with $\alpha = 0.05$).

The sensitivity analysis, accomplished with Atropos [102] at three different time intervals in order to avoid over-parameterization shows that all rate constants do influence the shape of the simulated progress curves and consequently all proposed steps can be considered kinetically significant.

The simulated progress curves for the proposed active and inactive copper species $(CuSH)^+$, $Cu(SH)_2$ together with both simulated and experimental kinetic curves for APX are displayed in fig. II.2.6.

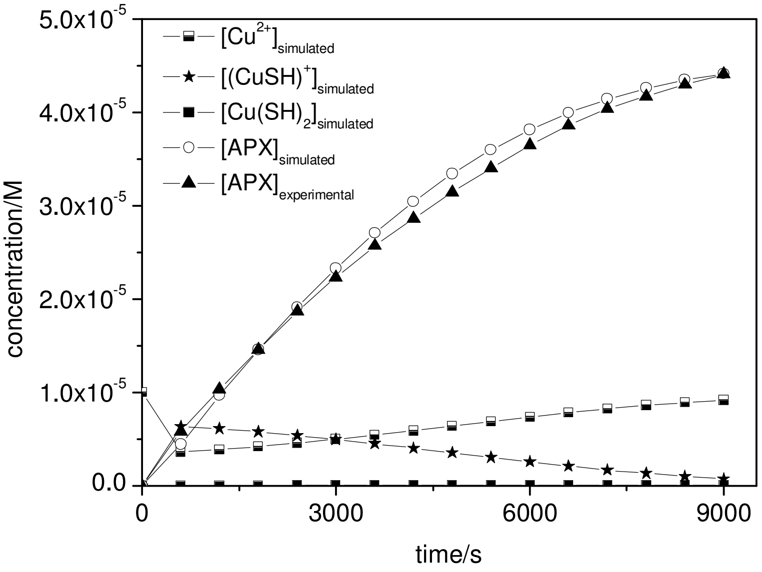


Fig. II.2. 6 Simulated and experimental extended kinetic curves of main reaction intermediates and product $[OAP]_0 = 1 \cdot 10^{-4}$M, $[O_2]_{dissolved} = 1.17 \cdot 10^{-3}$M, $[CuCl_2]_0 = 1 \cdot 10^{-5}$M, T = 30°C

The optimized parameters k_1, k_2, k_3, k_{11} and concentrations $[O_2]$ and $[SH_2]$ can be used to decide which terms in the denominator of equation (II.2.22) can be neglected. With present data equation (II.2.22) becomes simpler but still difficult to integrate:

$$-\frac{d[Cu(II)]_0^*}{dt} = \frac{k_1 k_{11}[SH_2]^2[Cu(II)]_0^*}{k_2[O_2]+k_1[SH_2]} \qquad (II.2.32)$$

For air saturated solutions $k_2[O_2] \approx 4.9\ 10^{-4}\ s^{-1}$ and $k_1[SH_2] \approx 5.9\ 10^{-3}\ s^{-1}$. If the first term in the denominator is neglected as a rough approximation, one obtains $k_{in1} \approx k_{11}$, a result obtained also from equation (26) since $k_2[O_2]/k_3 = 1.7\ 10^{-6} << 1$. This explains the apparent independence of the inhibition constant on the dioxygen concentration. For dioxygen saturated solution the approximation is even rougher but still in accord with the experimental behaviour. It can be concluded that only the first limiting approximation $k_2k_3[O_2] << (k_1k_3 + k_1k_2[O_2])[SH_2]$, although rough, can explain the results, while the second one, predicting an inverse proportionality of the inhibition constant with the oxygen concentration is in disagreement with the results.

II.3. Kinetics of substituted 2-aminophenols oxidation catalyzed by transition metals ions

In this work the kinetics behavior of substituted 2-aminophenol: 2-amino-*p*-chlorophenol (4ClOAP) and 2-amino-*p*-cresol ($4CH_3OAP$) oxidation with O_2 in oxygenated aqueous solutions catalyzed by transition metal salts is presented and discussed.

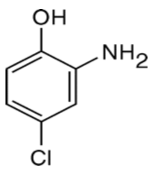


Fig. II.3.1. 2-amino-*p*-chlorophenol

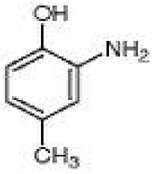


Fig. II.3.2. 2-amino-*p*-cresol

The presence of the substituents in 4th position can influence the final reaction products formation by steric and electronic effects.

The reaction rates in initial conditions (1% conversion) were calculated as the slopes of the linear parts of the extended kinetic curves substrate concentration towards time and the turnover frequencies were obtained by dividing the slopes values to salt concentrations. To illustrate the differences which appear on substituted 2-aminophenol oxidation in the presence of Cu (II) salts, the turnover frequencies were represented *vs.* the initial concentrations of substrate (fig. II.3.17.and II.3.18.).

In figure II.3.19. the turnover frequencies of OAP, 4ClOAP, $4CH_3OAP$ oxidation catalyzed by copper (II) salt were compared. The higher values were for OAP oxidation.

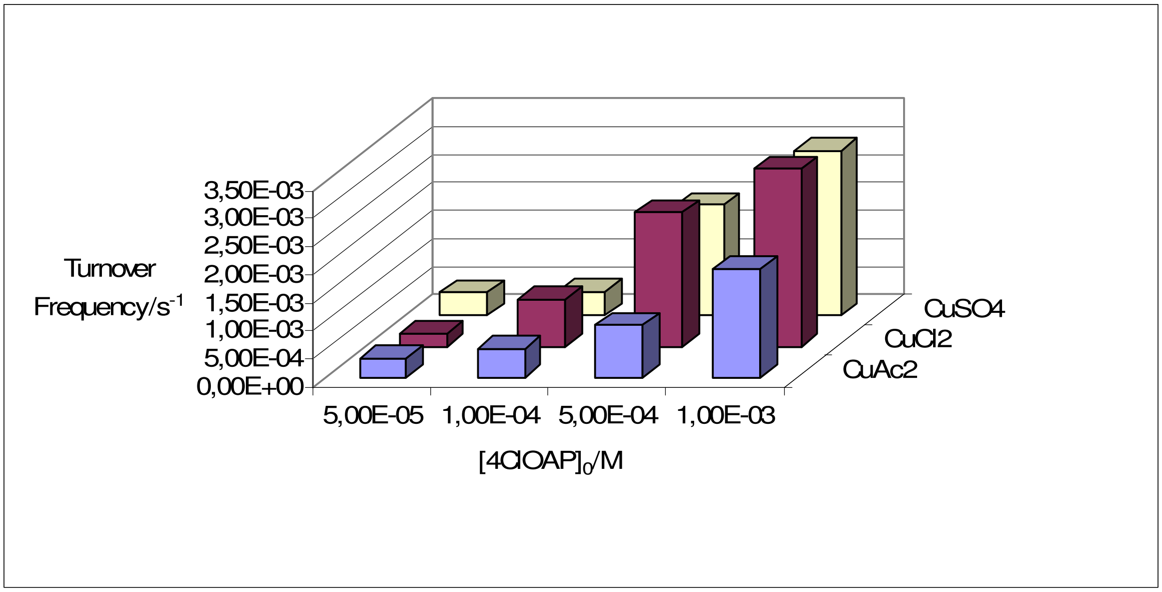


Fig. II.3.17. Turnover frequency for three copper (II) salts: $[CuCl_2]$, $[CuSO_4]_0$, $[Cu(CH_3COO)_2]_0$ $= 1\cdot10^{-5}$M, $[4ClOAP]_0 = 1\cdot10^{-3}$M, T=30°C

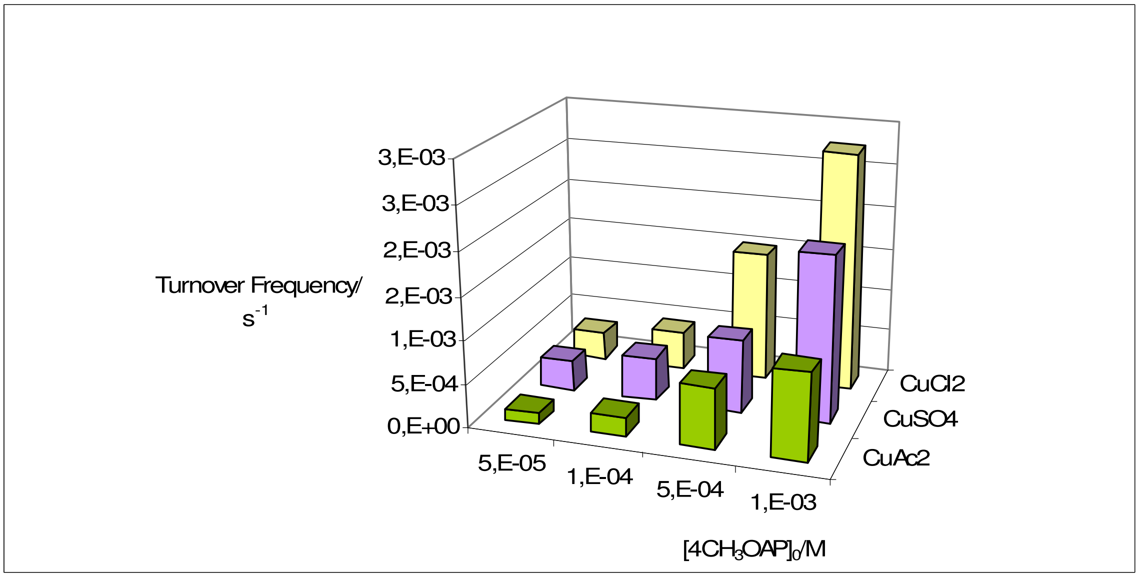


Fig. II.3.18. Turnover frequency for three copper (II) salts: $[CuCl_2]$, $[CuSO_4]_0$, $[Cu(CH_3COO)_2]_0$ $= 1\cdot10^{-5}$M, $[4CH_3OAP]_0 = 1\cdot10^{-3}$M, T = 30°C

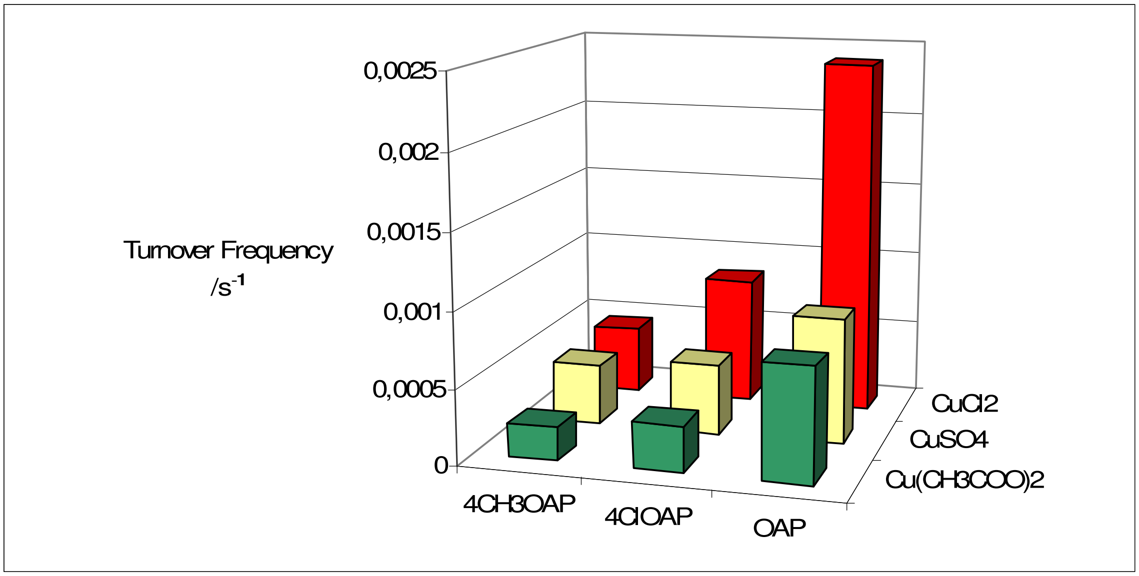


Fig. II.3.19. Turnover frequency for three copper (II) salts: [$CuCl_2$], [$CuSO_4$]$_0$, [$Cu(CH_3COO)_2$]$_0$ = $1 \cdot 10^{-5}$M, [OAP]$_0$, [4ClOAP]$_0$,[$4CH_3OAP$]$_0$ = $1 \cdot 10^{-3}$M, T = 30°C

II.4. Kinetics of 2-aminophenols oxidation in the presence of mushroom tyrosinase

In this chapter, three *o*-aminophenols: OAP, 4ClOAP and $4CH_3OAP$ were oxidized in oxygenated aqueous solutions in the presence of mushroom tyrosinase. Tyrosinase (TYR) is a metalloenzyme which catalyzes the oxidation of phenols, and its catalytic active site contains two copper atoms, each coordinated to three histidine residues.

Catalytic efficiency of tyrosinase in the reaction of 2-aminophenols oxidation [105], was calculated using the apparent first-order constants obtained from kinetic curves of product absorbance *vs.* time.

At low substrate conversions, each reaction follows a first order kinetics, therefore on the initial part, the product absorbance *vs.* time, a kinetic equation $A=A_0\ (1-e^{-kt})$ characteristic for a first order process was fitted.

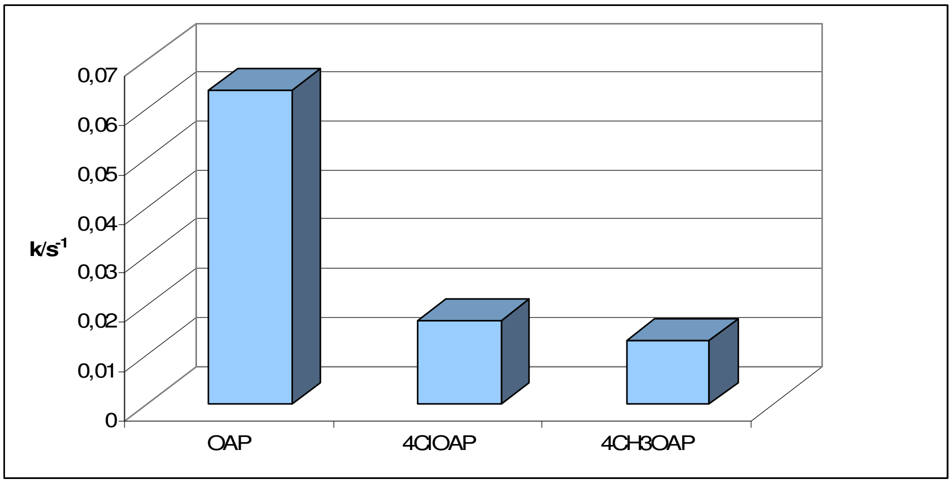


Fig.II.4.4. Catalytic efficiency of tyrosinase on several 2-aminophenols oxidation

The turnover frequencies of OAP, 4ClOAP and 4CH$_3$OAP oxidation with O$_2$ catalyzed by tyrosinase were calculated-by dividing the slopes values (1% conversion) to enzyme concentrations.

Table II.4.1. Turnover Frequencies of OAP, 4ClOAP and 4CH$_3$OAP oxidation with O$_2$, catalyzed by mushroom tyrosinase

	OAP+O$_2$+TYR	4ClOAP+O$_2$+TYR	4CH$_3$OAP+O$_2$+TYR
Turnover Frequencies	$3,14 \cdot 10^4$ s^{-1}	$1,78 \cdot 10^4$ s^{-1}	$0,81 \cdot 10^4$ s^{-1}

II.5. Kinetic behavior of phenolic pollutants oxidation in the presence of horseradish peroxidase

In this chapter, three *o*-aminophenols: OAP, 4ClOAP and 4CH$_3$OAP were oxidized in oxygenated aqueous solutions in the presence of horseradish peroxidase (HRP). HRP is a metalloenzyme and its cofactor consists of an iron (III) ion and protoporphyrin IX.

Catalytic efficiency of peroxidase in the reaction of 2-aminophenols oxidation [106], was calculated (as for tyrosinase) using the apparent first-order constants obtained from kinetic curves of product absorbance *vs.* time. At low substrate conversions, each reaction follows a first order kinetics, therefore on the initial part the product absorbance *vs.* time was fitted a kinetic equation $A=A_0\,(1-e^{-kt})$ characteristic for a first order process.

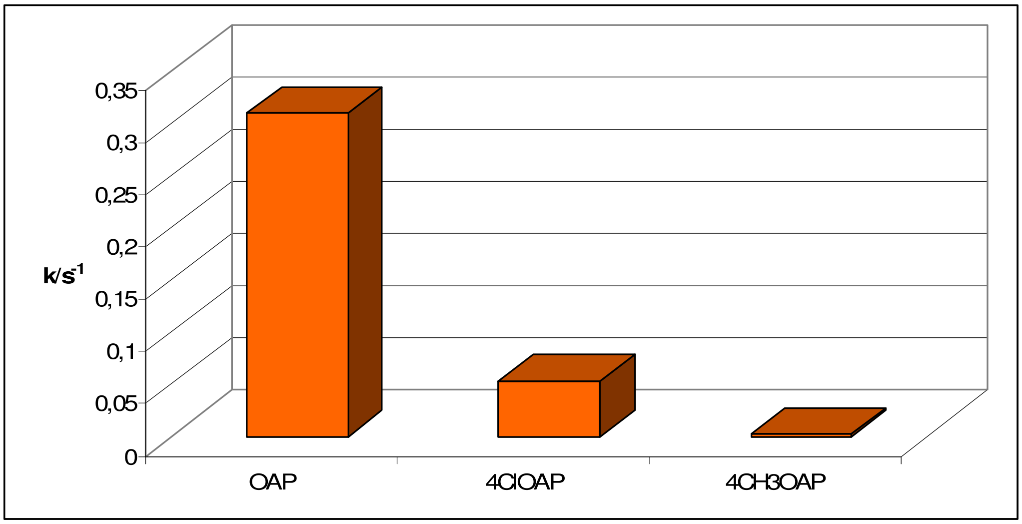


Fig.II.4.5. Catalytic efficiency of peroxidase on several 2-aminophenols oxidation

The turnover frequencies of OAP, 4ClOAP and $4CH_3OAP$ oxidation with O_2 catalyzed by tyrosinase were calculated and given in Fig. II 4.5 and Table II. 4.1

Table II.4.1. Turnover Frequencies of OAP, 4ClOAP and $4CH_3OAP$ oxidation with O_2, catalyzed by horseradish peroxidase

	$OAP+O_2+HRP$	$4ClOAP+O_2+HRP$	$4CH_3OAP+O_2+HRP$
Turnover Frequencies	$5{,}93\cdot10^4\ s^{-1}$	$2{,}18\cdot10^4\ s^{-1}$	$1{,}41\cdot10^4\ s^{-1}$

Comparing turnover frequencies obtained from OAP, 4ClOAP, 4CH3OAP oxidation with molecular oxygen both in the presence of HRP, TYR (Chapter II.4.) and in the presence of copper salts (Chapter II.3.) the same trend was observed: the highest value was obtained for OAP oxidation, followed by 4ClOAP and the lowest value was obtained for $4CH_3OAP$ oxidation.

Inactivation pathways of several 2-aminophenols derivatives oxidation catalyzed by horseradish peroxidase

The generation of reactive oxygen species (ROS) during the oxidative process, which finally inactivate the horseradish peroxidase, raises some difficulties in obtaining significant conversions. ROS such as $O_2^{\cdot}$ or $HOO^{\cdot}$ react with native form of enzyme or with enzyme intermediates, resulting inactive complexes [46, 107].

The operational inactivation of HRP was detected using the Selwyn test. Accordingly, the extended kinetic curves (substrate concentration/product concentration *vs.* ($[HRP]_0$·time) should be superposed in the absence of inactivation. It was observed that the progress kinetic curves at different initial concentrations of HRP are not superposed, evidencing the inactivation of HRP during the reaction.

To elucidate the possible reactions of hydrogen peroxide with phenolic substrate or peroxidase active intermediates a series of experimental studies were made: OAP was oxidized to APX with H_2O_2 in air-free solution [82].

According to literature data the following kinetic model can be proposed for the generation of aminophenoxyl radicals together with ROS species, during the oxidation of OAP with H_2O_2:

Based on the experimental results and according to literature data [1, 21, 81, 109], a kinetic model was proposed for the generation of aminophenoxyl radicals together with ROS species, during the oxidation of OAP with H_2O_2:

$$SH_2 + H_2O_2 \xrightarrow{k_1} SH^\bullet + HO^\bullet + H_2O \qquad (II.5.6)$$

$$H_2O_2 + HO^\bullet \xrightarrow{k_2} HOO^\bullet + H_2O \qquad (II.5.7)$$

$$HOO^\bullet + H_2O_2 \xrightarrow{k_3} HO^\bullet + H_2O + O_2 \qquad (II.5.8)$$

$$SH_2 + O_2 \xrightarrow{k_4} SH^\bullet + HOO^\bullet \qquad (II.5.9)$$

$$2HOO^\bullet \xrightarrow{k_5} H_2O_2 + O_2 \qquad (II.5.10)$$

where SH_2 is OAP and SH• the corresponding aminophenoxyl radical. The rate constants $k_2 - k_5$ are reported in literature and their values were presented in table II.5.1.

For the first reaction (II.5.6), the apparent first-order rate constant was estimated $k' = (2.25 \pm 0.18) \cdot 10^{-4} s^{-1}$ when the excess concentration of H_2O_2 was $1 \cdot 10^{-1}$M. The partial reaction order towards H_2O_2, was estimated nearly equal to 1, and the second-order rate constant was calculated as the ratio of k' and the exceeding concentration of H_2O_2 (for the present data set $k_1 = 2.25 \cdot 10^{-3}\ M^{-1} \cdot s^{-1}$).

Table II.5.2. Rate constants of the mechanism described at OAP oxidation with H_2O_2

Rate constant	Value/ $M^{-1}s^{-1}$	Reference
k_2	$2,7 \cdot 10^{7}$	109
k_3	0,5	109
k_4	$4,7 \cdot 10^{-2}$	81
k_5	$1,6 \cdot 10^{5}$	81, 46

Detecting operational inactivation of horseradish peroxidase using an isoconversional method

a) L-DOPA oxidation

According to the isoconversional method, as long as the concentration of HRP is much lower than the both substrates concentrations L-DOPA and H_2O_2, a quasi-steady state is attained and the time evolution of the system can be described by a single reaction rate.

$$\left(\frac{d[DC]}{dt}\right)_i /[HRP]_{0,i} = f\left([DC]_{isoconv}\right) \cdot e^{-k_{in}t} \qquad (II.5.14)$$

where DC is dopacrom, the product of reaction.

The inactivation constant k_{in1} can be estimated using a nonlinear regression analysis of eq. II.5.14, with $(d[DC]/dt)_{isoconv}/[HRP]_{0,i}$ as dependent variable, t as independent variable and $f\left([DC]_{isoconv}\right)$ and k_{in1} as estimated parameters, or using a linear regression analysis of the logarithmic form of the same equation.

$$\ln\left(\left(\frac{d[DC]}{dt}\right)_i \cdot \frac{1}{[HRP]_{0,i}}\right) = \ln\left(f\left([DC]_{isoconv}\right)\right) - k_{in}t \qquad (II.5.15)$$

The evaluation of the inactivation kinetic constant k_{in1} is illustrated in fig. II.5.19.

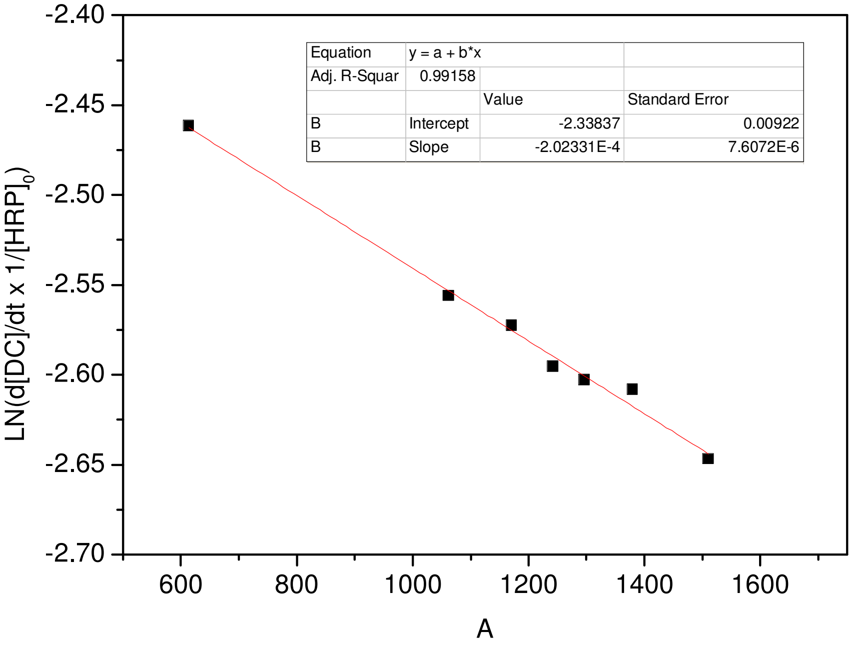


Fig. II.5.19. Estimation of inactivation kinetic constant k_{in}. Conditions: $[\text{L-DOPA}]_0 = 5\cdot10^{-5}$ M, $[H_2O_2]_0 = 8\cdot10^{-3}$ M, $[DC]_{isoconv} = 2\cdot10^{-5}$ M [110]

The apparent first-order rate constant being $k_{in} = (2.033\pm0.076)\cdot10^{-4}\ s^{-1}$, the second-order inactivation constant can be estimated as the ratio $k_{in}/[H_2O_2] = 2.52\cdot10^{-2} M^{-1}\cdot s^{-1}$.

b) OAP oxidation

Following the same reasoning as for L-DOPA an overall inactivation constant k_{in} was estimated for OAP oxidation by linear regression of the equation:

$$\ln\left(\left(\frac{d[APX]}{dt}\right)_i \times \frac{1}{[HRP]_{0,i}}\right) = \ln\left(f\left([APX]_{isoconv}\right)\right) - k_{in}t \qquad (II.5.24)$$

The results are illustrated in Fig. II.5.20.

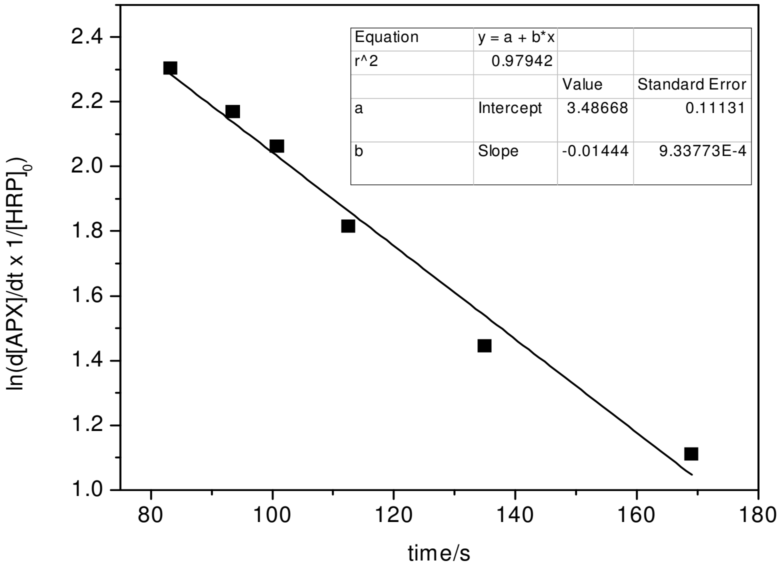


Fig. II.5.20. Estimation of inactivation kinetic constant k_{in}. Conditions: $[OAP]_0 = 1 \cdot 10^{-4}$M, $[H_2O_2]_0 = 2.5 \cdot 10^{-5}$ M, $[APX]_{isoconv} = 1 \cdot 10^{-5}$M, pH =7.00, T = 30°C,

A second-order inactivation constant can be calculated as the ratio of the apparent first-order rate constant and the initial concentration of H_2O_2. In the actual work conditions the calculated second-order rate constant is $5.76 \cdot 10^2$ $M^{-1} \cdot s^{-1}$.

II.6. Influence of Cu (II), Mn (II) and Co (II) salts on several 2-aminophenols kinetics oxidation in the presence of horseradish peroxidase

As described in the previous chapter, phenols oxidation in the presence of peroxidase generates certain reactive oxygen species, such as $O_2^{\cdot}$ or $HOO^{\cdot}$, that can react with the native form of the enzyme or with Compound II, resulting inactive complexes.

This inconvenient can be avoided by adding transition metals ions such as Mn(II), Co (II) and Cu(II) to the reacting system some of them being efficient superoxide anion or peroxy radicals scavengers.

The addition of manganese salts in the oxygenated reacting system of OAP and 4ClOAP oxidation, determined the increase of APX and ClAPX formation. Literature studies demonstrate that Mn^{2+} reacts with superoxide anion radical or with peroxy radical, resulting the strong oxidant Mn^{3+} and finally generates H_2O_2 [111]. Since H_2O_2 exists in the system, generation of final reaction products is much faster when oxidation is done in the presence of HRP.

The increase of the H_2O_2 amount in the system facilitates the reaction between Compound II and H_2O_2 and can lead to the inactivation of the native enzyme, due to the formation of Compound III. To verify if the enzyme inactivation occurs during the oxidation reaction in the presence of Mn(II) salts, Selwyn test was applied which indicates the presence of inactivation.

Cobalt sulphate added to the solution has also an activating effect on the enzyme oxidation reaction of OAP [108]. However, Co(II) is not known as peroxy radical scavenger and its activating effect might be assigned to induced conformational changes of enzyme [88].

The influence of Cu(II) salts ($CuCl_2$, $Cu(CH_3COO)_2$, $CuSO_4$) on the kinetics of several 2-aminophenols (OAP, 4ClOAP, $4CH_3OAP$) oxidation with O_2 in aqueous solution saturated with oxygen, in the presence of HRP was studied. The increase of the reaction rate is due to the generation of hydrogen peroxide in the system (cap. II.2).

II.7. General Conclusions

According to the proposed objectives, experimental studies lead to the following general conclusions:

- The original part of the thesis presents kinetics studies of several phenolic pollutants oxidation such as 2-aminophenol, 4-cloro-2-aminophenol and 2-amino-*p*-crezol with O_2 in oxygenated aqueous solutions, using simple salts of transitional metals such as $CuCl_2$, $Cu(CH_3COO)_2$, $CoSO_4$, $MnSO_4$, $MnCl_2$, $Mn(NO_3)_2$, $Mn(CH_3COO)_2$ as catalysts, and as biocatalyst mushrooms tyrosinase and horseradish peroxidase. This enzymes have transitional metal ions in their active site, such as Cu(II) for tyrosinase and Fe(III) for peroxidase;

- For 2-aminophenol oxidation with molecular oxygen catalyzed by Cu(II) salts ($CuCl_2$, $Cu(CH_3COO)_2$, $CuSO_4$), a kinetic model was proposed implying 11 steps (catalytic and non-catalytic). Kinetic process indicates a substrate inhibition due to the formation of [bis(o-iminosemiquinonate)] copper (II) complex, which was isolated and characterized by IR spectrum;

- Several rate constants of the reaction steps were taken from literature and the others were evaluated either experimentally or numerically using an optimization procedure. The inhibition constant was evaluated using an isoconversional method. A fair agreement between the measured and simulated inhibition constant was obtained.

- For substituted 2-aminophenols oxidation, 4-cloro-2-aminophenol and 2-amino-*p*-cresol catalyzed by copper salts, the reaction was slower than the oxidation of 2-aminophenol, due to the substitute effect from 4th position;

- The initial reaction rate of 4-cloro-2-aminophenol and 2-amino-*p*-cresol oxidation catalyzed by copper salts, are higher in the presence of $CuCl_2$ than in the presence of $CuSO_4$ and $Cu(CH_3COO)_2$;

- The addition of transitional metal salts, such as Co(II), Mn(II) and Cu(II), on 4ClOAP oxidation with O_2, leads to the increase of the substrate conversion to final product, Cu(II) ions having higher catalytic efficiency compared to Co(II) and Mn(II) ions;

- The value of the first-order kinetic constant on *o*-aminophenol, 4-cloro-2-aminophenol and 2-amino-*p*-cresol oxidation with O_2 in the presence of mushrooms tyrosinase was higher for OAP oxidation compared to the other two substituted amino-phenols;

- The first-order kinetic constant was higher on OAP oxidation in the presence of horseradish peroxidase, compared to 4ClOAP and $4CH_3OAP$ oxidation;

- During OAP and 4ClOAP oxidation process in the presence of HRP, the enzyme is inactivated (the inactivation was proven using Selwyn test), due to the generation of reactive oxygen species and H_2O_2. Generation of ROS species was showed in the proposed kinetic model of OAP oxidation with H_2O_2, in air-free solution;

- A kinetic model of OAP oxidation with excess H_2O_2 in the presence of HRP was proposed and an enzymatic inactivation constant was estimated by an isoconversional method. In the same way, by the same method, an enzymatic inactivation constant was estimated in the case of L-DOPA oxidation in the presence of HRP and excess of hydrogen peroxide;

- Addition of some transitional metal salts such as Co(II), Mn(II) and Cu(II), in the enzymatic oxidation process of some amino-phenols, had an activating effect;

- During OAP and 4ClOAP oxidation in the presence of HRP and of Mn(II) ions, the increase of H_2O_2 amount in the system was followed by the inactivation of the native enzyme, because of Compound III increase; the inactivation was proved by Selwyn test;

- For OAP, 4ClOAP and $4CH_3OAP$ oxidation with molecular oxygen catalyzed by copper salts and metalloenzymes, such as HRP and TYR, the higher turnover frequency was obtained for OAP oxidation as compared to 4ClOAP and $4CH_3OAP$ oxidation.

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