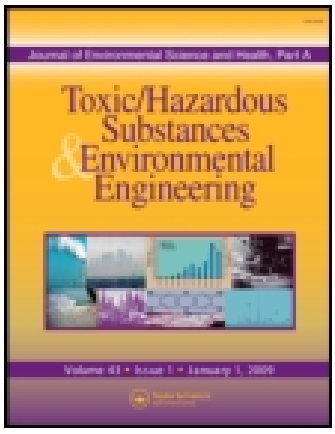

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Biocatalytic spectrophotometric method to detect paracetamol in water samples

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Biocatalytic spectrophotometric method to detect paracetamol in water samples

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A biocatalytic methodology based on the quantification of the laccase inhibition during the oxidation of a standard substrate ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) for the indirect determination of paracetamol in drinking water has been developed. The method displayed a fast response time (20 s), and high selectivity to paracetamol in presence of interfering substances such as naproxen, estradiol, ketoprofen, sulfamethoxazole, and diclofenac. The limit of detection (LOD) and limit of quantification (LOQ) were noticed to be 0.55 μM and 8.3 μM, respectively. By comparing the catalytic constants value K_M and k_{cat} for ABTS oxidation in the absence and presence of various concentrations of paracetamol, a competitive-type inhibition was disclosed. On the other hand, the close value between K_i and K_M indicates similar binding affinity of the enzyme to ABTS and paracetamol corroborated by docking studies. The methodology was successfully applied to real water samples, presenting an interesting potential for further development of a biosensor to paracetamol detection.

Keywords: Emerging pollutants, laccase, paracetamol, pharmaceuticals, pollutant detection.

Introduction

In recent years, discharge of pharmaceutical compounds (PhCs) to water bodies has gained attention in several research fields because, on one hand, the effects of these compounds on human health and the environment have not been fully determined.[1] On the other hand, this issue is of environmental concern due to the lack of legal requirements for discharge these ubiquitous, persistent and biologically active substances.[2] Pharmaceutical substances are used in human and veterinary medicine and can enter the aquatic environment following manufacture, use or ingestion/excretion.[2] Although PhCs are usually present at trace concentrations in the environment, mainly water bodies, their presence have raised questions regarding their chemical persistence, microbial resistance, and synergistic effects.[1, 3]

Paracetamol (4-hydroxyacetanilide) is one of the most widely used drug substances in the world as a pain reliever. Paracetamol has been identified as one of the most frequently detected anthropogenic compounds in water bodies in the United States.[4] At the water treatment facilities, paracetamol is not completely biodegraded and is usually oxidized during the chlorination step, producing several side toxic products.[5, 6] Since a large number of cases of paracetamol contamination have been reported,[2, 3, 7] research efforts have been made to develop new specific methods to determine the presence of paracetamol in environmental samples. Current analytical methods for the quantification of paracetamol include optical, electrochemical and chromatographic.[8] Some of these methods are very accurate and highly sensitive.

The increased analytical sensitivity has allowed paracetamol occurrence studies to be undertaken at μg L^{-1} or ng L^{-1}.[4, 7] However, these methods present several disadvantages such as the requirement of expensive and sophisticated instruments, long analysis times and sample pretreatment rendering them unsuitable for routine analyses. Methods based on enzymes are an attractive alternative for pollutants detection and quantification because enzymes usually exhibit high activity, specificity, precision, and are fast and easy to use.[9, 10]

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Oxidative enzymes as laccases and peroxidases are able to recognize and catalyze the transformation of a variety of pharmaceutical compounds to less toxic or more biodegradable products.[11–15] The high affinity and reactivity towards these compounds have been applied for the development of multiple biosensors for emerging pollutants.[16–21]

In this work, a biocatalytic approach using laccase is proposed to detect and quantify paracetamol from different water samples. The methodology is based on the quantification of the degree of laccase inhibition during the oxidation of a standard substrate (ABTS). The method is selective to determination of paracetamol in presence of other pharmaceuticals such as naproxen, estradiol, ketoprofen, sulfamethoxazole, and diclofenac. The method was applied to determine the paracetamol concentration in bottled and tap water, and also in water samples from the secondary unit of a water treatment process.

Material and methods

Chemicals

Paracetamol, estradiol, sulfamethoxazole, diclofenac, naproxen, ketoprofen, ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) were purchased from Sigma (St. Louis, MO, USA). Laccase solution (40.9 μM) from *Coriolopsis gallica* UAMH 8160 (MW 66 kDa *via* SDS page, pI 3.4, 21% carbohydrate content, purity index $A_{280}/A_{606} = 5$) was obtained as described elsewhere[22] and provided by Altaenzymes. Buffer salts and isopropanol were procured from J.T. Baker (Phillipsburg, NJ, USA).

Enzymatic activity

The enzymatic activity was spectrophotometrically measured by monitoring the oxidation of ABTS to its cation radical with the subsequent color formation, which is proportional to laccase activity. The assay was performed in 600 mM sodium acetate buffer, pH 4.5 and 25°C using 4 nM of laccase and 47 μM of ABTS. The progress of the reaction was monitored for 2 min based on the increase in product absorbance at 436 nm (μ_{436} = 29,300 M^{-1} cm^{-1})[23] using a Cary 50 spectrophotometer. The initial reaction rate was measured during the first 20 seconds.

Inhibition assays

The effect of paracetamol, naproxen, estradiol, sulfamethoxazole, diclofenac, and ketoprofen on laccase activity was determined by measuring the changes in the reaction rate for ABTS oxidation due to increasing concentrations of each pharmaceutical, ranged from 0.02 to 0.2 mM. The percent of inhibition was calculated according to Eq. (1):

$$\%\ \text{Inhibition} = \left(\frac{V_0 - V_1}{V_0}\right) * 100 \qquad (1)$$

where V_0 and V_1 are the initial reaction rates, measured during the first 20 sec, in the absence and in the presence of different pharmaceutical concentrations, respectively. The assays were also carried out at different enzyme and ABTS concentrations.

Kinetic constants

To determine the kinetic constants V_{max} and K_M, the oxidation of different ABTS concentrations from 10 to 450 μM was assayed and followed as described in the section describing the enzymatic activity. The kinetic data were adjusted to the Michaelis–Menten equation using an iteration procedure following the Marquardt–Levenberg nonlinear least-squares algorithm from Origin 7.0 software.

To determine the inhibition constant, the kinetic parameters for ABTS oxidation were evaluated in the presence of different concentrations of paracetamol, ranging from 20 to 100 μM. K_i was calculated from the plot of K_{Mapp} versus paracetamol concentration as the intercept on the paracetamol-axis.

Docking studies

Docking was carried out using Autodock Vina[24] in a Mac OS X i7 computer; all docking runs used an exhaustiveness parameter of 1000, and 20 results were collected for each ligand. The receptor employed is the structure PDBID 4A2E[25] by adding hydrogen atoms using MGLtools.[26] The copper atoms required for function and structure were retained. All analysis was carried out using PyMOL,[27] in conjunction with Autodock Vina[28] plugin. Ligands were obtained from the UCSF Zinc database and, when required used in the dominant protonated/deprotonated form (at pH 4.5) as a better representation of the experimental results. All ligand bonds (i.e., single bonds) are allowed to rotate during docking operation to locate their minima during the bond to the protein.

Analysis of spiked water samples

To evaluate the potential environmental interferences of the sample matrix in paracetamol detection, three different spiked water samples were analyzed. First, the presence of paracetamol was evaluated in spiked commercial potable bottle and tap water samples at three different concentrations, i.e., 4, 8 and 10 μM. Second, a real wastewater effluent from the secondary treatment of the municipal wastewater treatment plant of Puebla City, containing 4, 8 and 10 μM of paracetamol, was also assayed. This effluent

was filtered to remove particulate matter and suspended solids, and then stored at 4°C until its use. The wastewater sample was analyzed according to standard methods and the main characteristics are summarized in Table A1 of the Appendix. Three replicate experiments were performed for all samples. Inhibition of the enzyme catalytic activity was carried out as described in a previous section.

Results and discussion

The development of methodologies and devices for the detection and quantification of pollutants by inhibition of enzyme catalytic activity has had an exponential growth in the last few years. The capacity of laccase to oxidize several organic aromatic compounds allows the development of methodologies to detect emerging aromatic pollutants by inhibition mechanism. The oxidation of ABTS by laccase follows Michaelis–Menten kinetics (Fig. A1) with a k_{cat} and a K_M of 14, 605 min^{-1} and 45 μM, respectively. The oxidation of ABTS by laccase may be inhibited by the presence of other aromatic compounds with the capacity to be recognized by the catalytic site of the enzyme.

Indeed, as it can be seen from Figure 1, the initial reaction rate of laccase for ABTS oxidation is decreased by the presence of paracetamol in a concentration-dependent manner; however, other aromatic pharmaceuticals such as naproxen, diclofenac, estradiol, ketoprofen, and sulfamethoxazole did not show any effect on the initial reaction rate (Fig. 1). On the other side, the final absorbance for

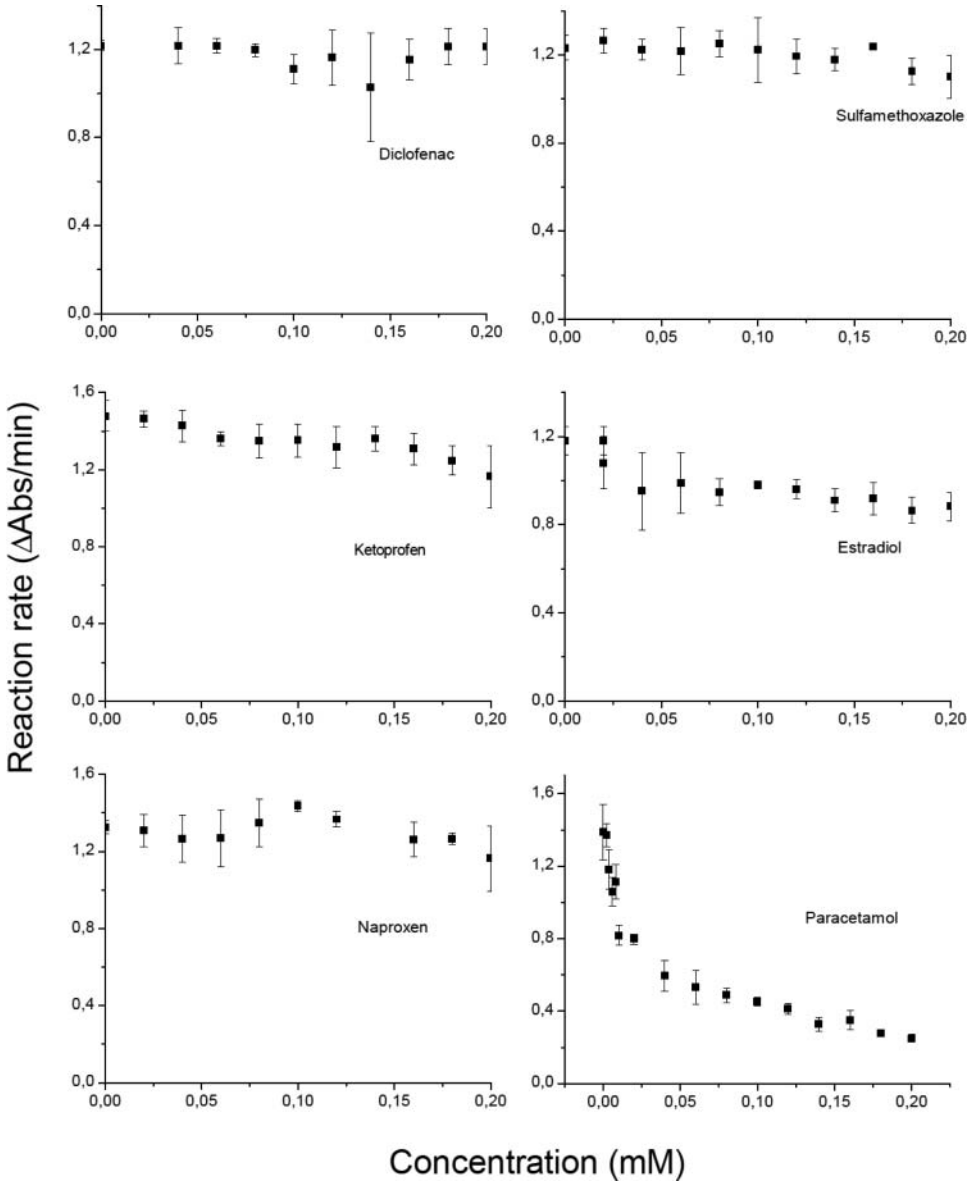


Fig. 1. Laccase inhibition induced by the different pharmaceutical compounds during ABTS oxidation. Reaction conditions: Acetate buffer 60 mM pH 4.5, ABTS 47 μM, Laccase 4 nM.

100% ABTS conversion must be around 1.4, which is reached when all the ABTS is oxidized after 2 min of reaction time, in the absence of the pharmaceuticals and in presence of ketoprofen, naproxen and sulfamethoxazole (Figs. 2 and A2).

However, for diclofenac, estradiol and paracetamol, the final absorbance decreased, which suggests the inhibition of laccase or the reaction of the oxidized product ABTS* with the pharmaceuticals, as previously reported with these substrates in the presence of other redox mediators[11, 14] (Figs. 2 and A1). In the case of paracetamol, the initial reaction rate, as well as the final absorbance, changed. To improve the sensitivity of the method, the inhibition at 10 μM of ABTS was assayed. Figure 3 shows the linear range between reaction rate and paracetamol concentration. The dynamic range spans from 2 to 14 μM (Fig. 3). Equation (2) describes the relationship between the variables:

$$V = -146.4[I] + 2738.1 \quad (2)$$

where V and I are the initial velocity (min^{-1}) and the paracetamol concentration (μM), respectively. The linear correlation was high, with a correlation coefficient of 0.94. As the rest of the pharmaceuticals did not affect the initial reaction rate this

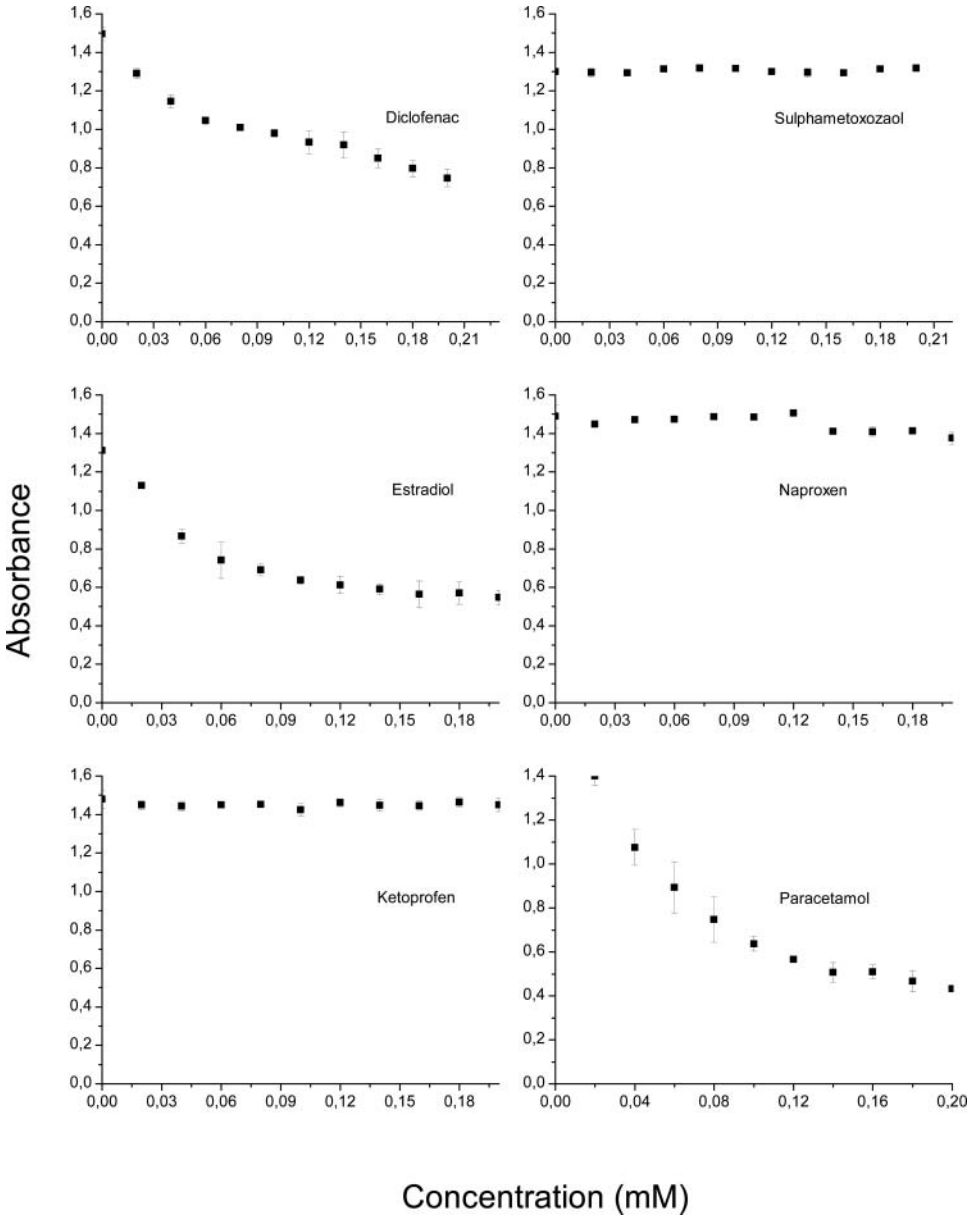


Fig. 2. Changes in the final absorbance of ABTS radical in the presence of the different pharmaceutical compounds. Reaction conditions: Acetate buffer 60 mM pH 4.5, ABTS 47 μM, Laccase 4 nM.

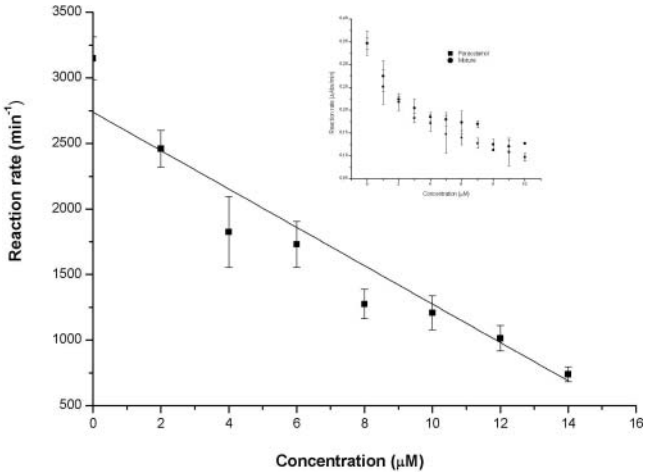


Fig. 3. Calibration curve for paracetamol detection based on the laccase inhibition for ABTS oxidation. Inset. Comparison of laccase inhibiton by paracetamol alone and in the mixture with naproxen, sulfamethoxazole, ketoconazol, diclofenac, estradiol, ketoprofen. Reaction conditions: Acetate buffer 60 mM pH 4.5, ABTS 10 μM, Laccase 4 nM.

Table 1. Detection test of paracetamol in spiked water samples.

Water sample	*Paracetamol added (μM)*	*Paracetamol found (μM)*	*CV (%)*	*Recovery (%)*
Potable	4	4.5	3.1	112
	8	8.3	5.3	104
	10	9.6	2.0	96
Tap	4	4.5	0.94	112
	8	7.2	0.78	88
	10	10.2	2.25	102
Treated wastewater	4	4.2	2	105
	8	8.5	6.8	106
	10	9.9	5.5	99

parameter may be used to selectively detect and quantify paracetamol. Indeed, the inset of Figure 3 shows that the presence of the other compounds in the reaction mixture did not affect the degree of inhibition caused by paracetamol.

Two important criteria for the development of analytical methods are the limit of detection (LOD) and the limit of quantification (LOQ). These parameters are defined as the lowest content of the analyte that can be detected (LOD) or measured (LOQ) with reasonable statistical certainty [Commission Regulation (EC) number 333/2007], and these are numerically equal to 3 and 10 times the standard deviation of the blank mean determinations, respectively. Using these values and Eq. (2), the LOD and LOQ are 0.55 μM and 8.3 μM.

Analysis of spiked water samples

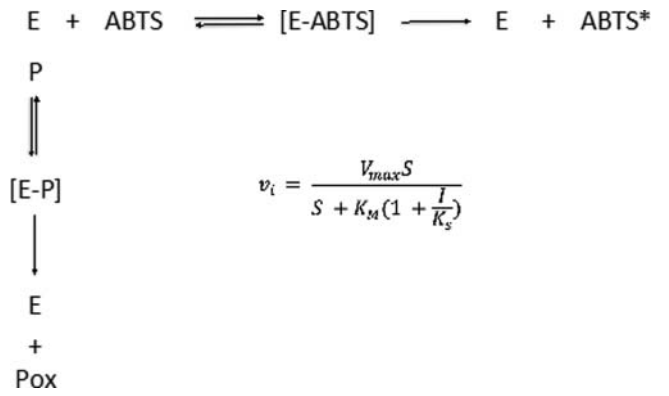
To validate the applicability, the proposed method was applied to detect and quantify paracetamol in three different water samples. The paracetamol concentration in the samples was calculated using Eq. (2). The results are shown in Table 1. As it can be seen, the coefficients of variation are low, revealing a good precision; in addition, the recovering percentage is high, within the range 98–112%. Better predictions are obtained at paracetamol values above the detection limit, as expected.

Insight into the kinetic mechanism of inhibition

The study of the mechanism of enzyme inhibition is important towards the development of a biosensor.[10] The catalytic constants for ABTS oxidation in the presence of paracetamol are listed in Table 2. The ANOVA analysis (Table A2) indicates that at 0.05 level k_{cat} values are not significantly different; while the K_M values are significantly different (Table A3). The presence of increasing amounts of paracetamol in the reaction mixture increases the K_M values of laccase for ABTS oxidation (Table 2), which suggests a competitive-type inhibition. In this case, paracetamol would bind the free enzyme to form a complex, enzyme-paracetamol (Scheme 1). The K_i value is 39 μM (Fig. A3), close to the K_M value for ABTS, which indicates that the enzyme has a similar affinity towards paracetamol. Changes in concentrations of both enzyme and ABTS prove that the inhibition is competitive because a higher ABTS concentration (100 μM) lessens the

Table 2. Kinetic constants, V_{max} y K_M and K_i, for ABTS oxidation by laccase in the presence on paracetamol as inhibitor.

ParacetamolmM	$V_{Max}(min^{-1})$	$K_M(mM)$	$K_i(mM)$
0	14,590 ± 1535	0.048 ± 0.012	0.039 ± 0.0041
0.02	14,163 ± 1791	0.121 ± 0.021	
0.04	13,481 ± 255	0.110 ± 0.003	
0.06	13,651 ± 1194	0.168 ± 0.021	
0.08	14,163 ± 1450	0.226 ± 0.034	
0.10	14,163 ± 1023	0.223 ± 0.026	



Scheme 1. Schematic representation of competitive inhibition kinetics; E, enzyme, P, paracetamol, P_{ox}, oxidized paracetamol.

inhibition; meanwhile, lower enzyme concentration (2 nM) did not affect the inhibition significantly (Fig. A4).

According to a competitive enzyme inhibition, the I_{50} value, the concentration of inhibitor that produces a decrease in laccase catalytic activity by 50% can be calculated using Eq. (3):

$$I_{50} = \frac{K_M + S}{K_M\left(1 + \frac{I}{K_i}\right) + S} \quad (3)$$

At 10 μM ABTS concentration the I_{50} value is 47 μM, which is not in accordance with the value obtained from Eq. (2) (9.3 μM). This discrepancy may be partially explained by the fact that paracetamol could reduce the ABTS radicals produced. Indeed, the ABTS radical reduction by paracetamol presents a hyperbolic kinetic behavior (Fig. A5), with a maximum reaction rate of 200 μM/min. This chemical oxidation of paracetamol allows for an inhibition of laccase higher than expected just for a competitive inhibitor. It is important to note that paracetamol is not oxidized by the free laccase under the reaction conditions assayed (data not shown). The oxidation of paracetamol by immobilized tyrosinase has been reported but longer reaction times are needed.[29]

Docking studies

To gain structural insight into the mechanism on the proposed inhibition, we docked all of the ligands employed in the kinetic experiments against a high resolution laccase structure.[25] Laccases have three copper binding sites (T1-T3) with four Cu^{2+} ions. T1site, being a mononuclear center, possesses one copper atom and T2 and T3, being trinuclear centers, possess three copper atoms. Laccases have two conserved channels that allow the passage of di-oxygen and release of water molecule from the T2/T3 center. The T1 Cu atom constitutes the substrate binding pocket.

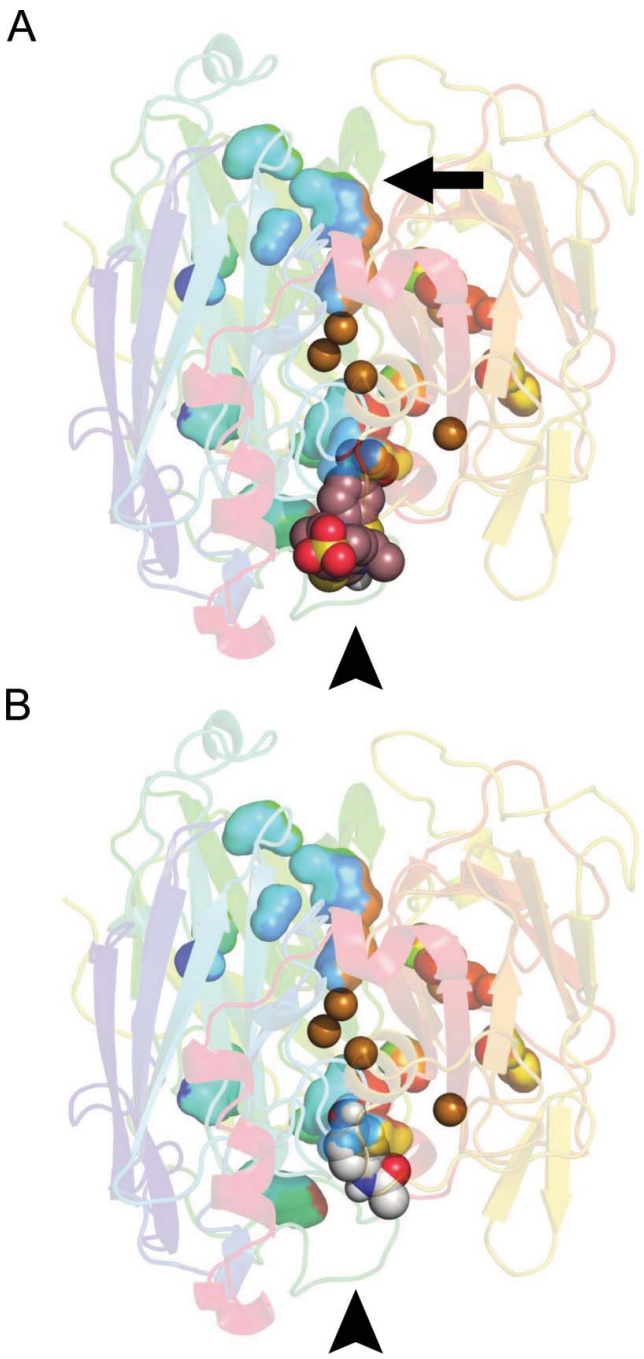


Fig. 4. Three-dimensional representation of docking results. Lacasse is shown its copper ions (yellow spheres). The two susbtrate channels are represented with their accessible surfaces while the ligands are shown as CPK; the upper channel is indicated by an arrow and the lower one with an arrowhead. In (A) is ABTS while in (B) paracetamol is shown. The lower channel has been suggested to be the entrance to the catalytic site.

The geometry of this pocket defines the substrate specificity of the enzyme.[30]

Previous docking studies on laccases have emphasized steric effects as a key determinant for substrate binding.[31]

Our results show that paracetamol and ABTS are small enough to fit and rotate in the active site to bind in close proximity to the Cu^{1+} atom (Fig. 4) with predicted interaction energies of −6.7 and −7.8 kcal/mol. The solvent accessible region at active site cavity comprises residues H85, P100, A101, F102, H130, S131, H132, L133, S134, T135, Y137, F364, P366, H418, F468, H470, H472, D474, L477, E478, A479, G480, F481, and copper 1518. For ABTS at the entrance channel, the deeply buried sulfonate group interacts with the backbone of residues A101, H132 and L477 as well as the sidechain of H132. The latter is a residue that coordinates one of the Cu atoms at the trinuclear center. The other sulfonate is interacting to sidechain D510 and the backbone of T638. In the case of paracetamol, main interactions with laccase residues are the same as for the deeply buried sulfonate, namely, the backbone of residues A101, H132 and L477 as well as the sidechain of H132.

The consequence of all these interactions is as expected from the kinetics experiments; that is, ABTS and paracetamol compete for the same binding site. It is worth noting that while the flexible docking used in these experiments allows for the rotation of the single bonds in the ligand, it does not explore the flexibility of the protein. Thus, it is possible that the omission of these properties result in almost the same binding affinity of ABTS and paracetamol to laccase.

Conclusions

A simple biocatalytic methodology for the detection of paracetamol was proposed. This methodology is based on monitoring the change of laccase initial rate for ABTS oxidation in the presence of paracetamol, occurring in approximately 20 s. Due to other aromatic pharmaceuticals such as naproxen, diclofenac, estradiol, ketoprofen, and sulfamethoxazole did not show any effect on the initial reaction rate, the methodology is useful for measuring paracetamol in presence of other interferents. The method was tested to determine the paracetamol concentration in bottled and tap water, and also in water samples from the secondary unit of a water treatment process. The recovering percentage was in the range 98–112%. The limit of detection (LOD) and limit of quantification (LOQ) were 0.55 μM and 8.3 μM, respectively.

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Appendix

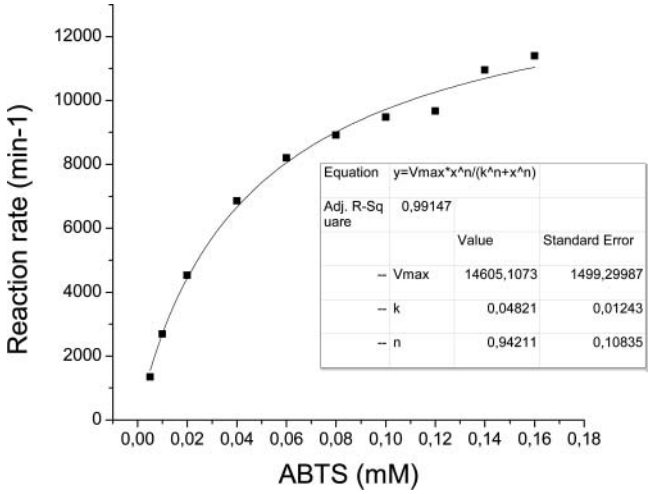

Fig. A1. Kinetic behaviour of laccase for ABTS oxidation.

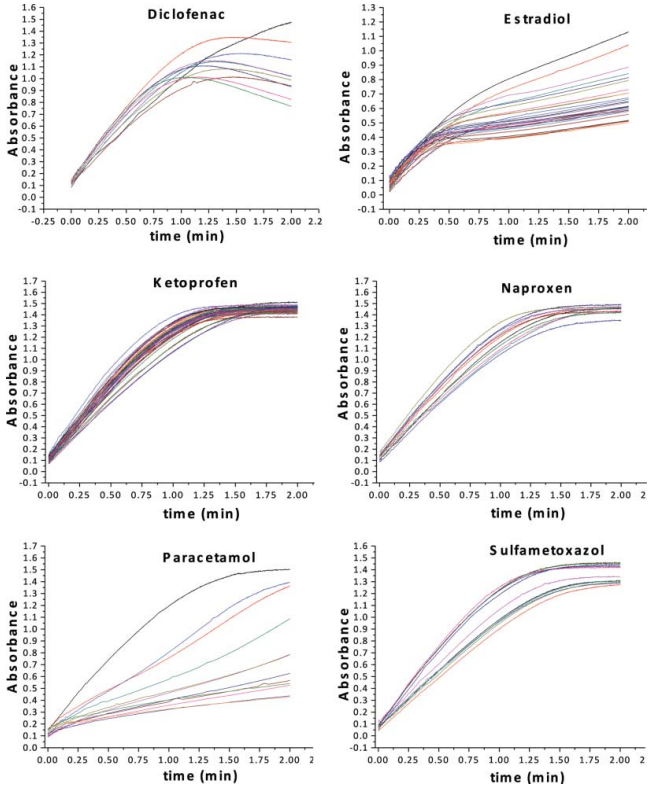

Fig. A2. Effect of the pharmaceuticals on the reaction progress of ABTS oxidation by laccase.

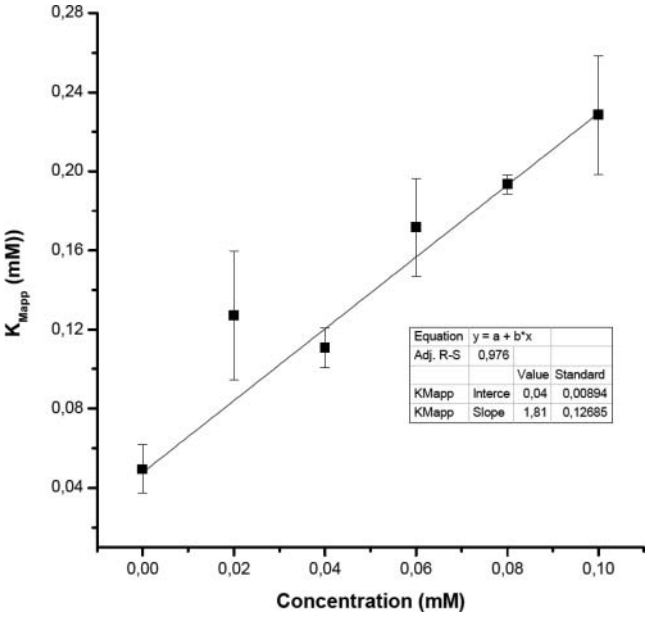

Fig. A3. Plot of K_{Mapp} versus paracetamol concentration for K_i determination.

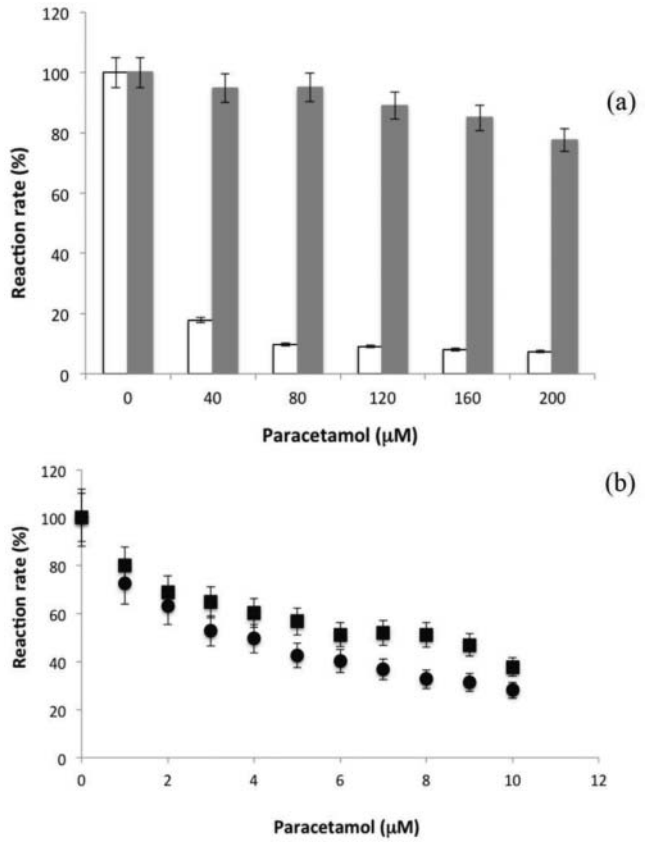

Fig. A4. Effect of ABTS (a) concentration on the inhibition of laccase by paracetamol. Grey bars 100 μM ABTS; white bars 10 μM ABTS. (b) Effect of laccase concentration on the enzyme inhibition by paracetamol (ν) 2 nM, (•) 4 nM laccase.

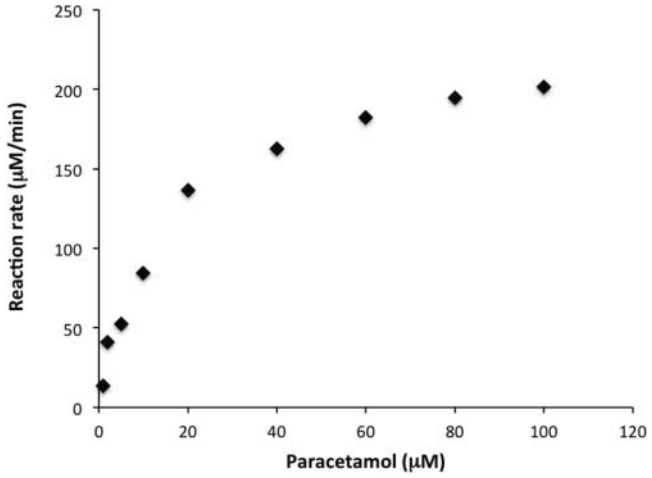

Fig. A5. Reaction rate reduction of ABTS radical by paracetamol in the absence of the enzyme. The radical cation ABTS·+ was generated by persulfate oxidation of ABTS by mixing equal volumes of ABTS (7.0 mM) and potassium persulfate (4.95 mM). The resulting solution was allowed to stand overnight at room temperature in the dark. Acetate buffer (0.06 M, pH 4.5) was used to dilute the ABTS·+ solution to obtain an absorbance of 1 ± 0.015 at 436 nm. Then, increasing amounts of paracetamol were added, and the decrease in the absorbance was registered versus time.

Table A1. Physicochemical characterization of the wastewater applied as matrix for paracetamol detection.

Parameter	*Value*
pH	7.80
Colour	50 UPC
Turbidity	19 UNF
Conductivity	1.41 $mScm^{-1}$
Total Solids	1.25 mgL^{-1}
Total Suspended Solids	0.02 mgL^{-1}
Total Dissolved Solids	1.23 mgL^{-1}
Chemical Oxygen Demand	141 mgL^{-1}
Biochemical Oxygen Demand (DBO_5)	560 mgL^{-1}

Table A2a. ANOVA analysis of V_{max} values for ABTS oxidation at different paracetamol concentrations.

Descriptive Statistics					
	Sample size	*Paracetamol mM*	*Vmax (DAbs/min)*	*Standard deviation*	*SE of mean*
B	3	0	1.7224	0.1808	0.10439
C	3	2	1.7035	0.3088	0.17828
D	3	4	1.5858	0.0814	0.04698
E	3	6	1.618	0.1546	0.08926
F	3	8	1.5216	0.1762	0.10172
G	3	10	1.6773	0.1409	0.08136

Table A2b. One way ANOVA.

	DF	*Sum of squares*	*Mean squares*	F *value*	*Prob* > F
Model	5	0.08891	0.0178	0.5093	0.76425
Error	12	0.41895	0.0349		
Total	17	0.50785			

Null hypothesis: the means of all values are equal.
Alterntive hypotesis: The means of one or more levels are different.
At the 0.05 level, the population means are NOT significantly different.

Table A2c. Fit Statistics.

R-square	*Coff var*	*Root MSE*	*Data mean*
0.17507	0.11406	0.18685	1.6381

Table A2d. Means comparisons; Tukey test.

	Mean diff.	*SEM*	q *value*	*Prob*	*Alpha*	*Sig*	*LCL*	*UCL*
Level 2-1	−0.019	0.153	0.175	1.000	0.05	0	−0.531	0.494
Level 3-1	−0.137	0.153	1.266	0.940	0.05	0	−0.649	0.376
Level 3-2	−0.118	0.153	1.091	0.967	0.05	0	−0.63	0.395
Level 4-1	−0.104	0.153	0.968	0.980	0.05	0	−0.617	0.408
Level 4-2	−0.085	0.153	0.793	0.992	0.05	0	−0.598	0.427
Level 4-3	0.032	0.153	0.298	1.000	0.05	0	−0.48	0.545
Level 5-1	−0.201	0.153	1.862	0.771	0.05	0	−0.713	0.312
Level 5-2	−0.182	0.153	1.686	0.832	0.05	0	−0.694	0.331
Level 5-3	−0.064	0.153	0.595	0.998	0.05	0	−0.577	0.448
Level 5-4	−0.096	0.153	0.894	0.986	0.05	0	−0.609	0.416
Level 6-1	−0.045	0.153	0.418	1.000	0.05	0	−0.558	0.467
Level 6-2	−0.026	0.153	0.243	1.000	0.05	0	−0.539	0.486
Level 6-3	0.091	0.153	0.848	0.989	0.05	0	−0.421	0.604
Level 6-4	0.059	0.153	0.549	0.999	0.05	0	−0.453	0.572
Level 6-5	0.156	0.153	1.443	0.902	0.05	0	−0.357	0.668

Sig equals 1 indicates that the means difference is significant at the 0.05 level.
Sig equals o indicates that the means difference is NOT significant at the 0.05 level.

Table A3a. ANOVA analysis of K_M values for ABTS oxidation at different paracetamol concentrations.

Descriptive Statistics					
	Sample size	*Paracetamol μM*	*KM (mM)*	*Standard deviation*	*SE of mean*
B	3	0	0.049	0.012	0.007
C	3	2	0.127	0.032	0.019
D	3	4	0.111	0.010	0.006
E	3	6	0.172	0.025	0.014
F	3	8	0.193	0.005	0.003
G	3	10	0.228	0.030	0.017

Table A3b. One way ANOVA.

	DF	*Sum of squares*	*Mean squares*	F *value*	*Prob* > F
Model	5	0.0619	0.01237	26.2173	4.56E-06
Error	12	0.0057	4.72E-04		
Total	17	0.0675			

Null hypothesis: the means of all values are equal.
Alterntive hypotesis: The means of one or more levels are different.
At the 0.05 level, the population means are significantly different.

Table A3c. Fit Statistics.

R-square	*Coff var*	*Root MSE*	*Data mean*
0.91613	0.148	0.0217	0.1468

Table A3d. Means comparisons; Tukey test.

	Mean diff.	*SEM*	q *value*	*Prob*	*Alpha*	*Sig*	*LCL*	*UCL*
Level 2-1	0.078	0.018	6.190	0.009	0.05	1	0.018	0.137
Level 3-1	0.061	0.018	4.888	0.042	0.05	1	0.002	0.121
Level 3-2	−0.016	0.018	1.302	0.934	0.05	0	−0.076	0.043
Level 4-1	0.122	0.018	9.748	0.000	0.05	1	0.063	0.182
Level 4-2	0.045	0.018	3.559	0.194	0.05	0	−0.015	0.104
Level 4-3	0.061	0.018	4.861	0.044	0.05	1	0.001	0.121
Level 5-1	0.144	0.018	11.477	0.000	0.05	1	0.084	0.204
Level 5-2	0.066	0.018	5.287	0.026	0.05	1	0.007	0.126
Level 5-3	0.083	0.018	6.589	0.006	0.05	1	0.023	0.142
Level 5-4	0.022	0.018	1.729	0.818	0.05	0	−0.038	0.081
Level 6-1	0.179	0.018	14.270	0.000	0.05	1	0.119	0.239
Level 6-2	0.101	0.018	8.080	0.001	0.05	1	0.042	0.161
Level 6-3	0.118	0.018	9.382	0.000	0.05	1	0.058	0.177
Level 6-4	0.057	0.018	4.522	0.065	0.05	0	−0.003	0.116
Level 6-5	0.035	0.018	2.793	0.408	0.05	0	−0.025	0.095

Sig equals 1 indicates that the means difference IS significant at the 0.05 level.
Sig equals o indicates that the means difference is NOT significant at the 0.05 level.