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A New Voltammetric Approach for the Determination of Biomimetic Catalyst Kinetic Constants Based on Substrate Consumption

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Abstract: In the present study, a new voltammetric method is introduced for the determination of kinetic parameters of artificial metalloenzymes (biomimetic complexes) mimicking the catecholase activity towards 3,5-di-*tert*-butylcatechol (3,5-DTBC) or 2,5-di-*tert*-butylhydroquinone, and representing a promising alternative to common spectrophotometric method. To demonstrate the effectiveness of the electrochemical approach, two binuclear copper(II) complexes bearing the same pentadentate ligand and different ionic ligands were selected. Apparent kinetic parameters, such as maximum velocity (V_{max}), Michaelis-Menten constant (K_m), and turnover number (k_{cat}), obtained by the described method employing differential-pulse voltammetry were in good agreement with the data evaluated from UV-Vis spectrophotometric measurements.

Keywords: Artificial enzyme · Binuclear copper(II) complexes · Voltammetry · Michaelis-Menten kinetics · Kinetic spectrophotometric assay

Generally, biomimetic catalysis refers to accelerated chemical reaction that mimics certain key features of natural enzymatic processes [1]. Nowadays, among scientists, synthesis and characterization of biomimetic catalysts gains a special interest thanks to their wide use in various areas, such as chemical engineering [2], battery technologies [3], analytical devices for medicinal diagnosis and food quality evaluation [4,5], environmental decontamination systems [6,7], or photoelectrochemical solar cells [8,9]. Biomimetic catalysts can be prepared from some metal complexes, replacing unstable and expensive natural enzymes. For instance, various types of copper complexes represent potential anticancer agents applicable in cancer chemotherapy [10,11], water oxidation catalysts in artificial photosynthesis [12], catalysts to remove organic pollutants from the environment [13]. The same complexes can also act as artificial metalloenzymes (ArMs), mimicking the polyphenol oxidase (cresolase and catecholase) activity, and thus serving as stable biorecognition elements in the construction of biomimetic sensors for diagnosis of neurodegenerative disorders [14].

Investigation of the catalytic activity of newly synthesized ArMs cannot be performed without kinetic assays which include measurements of the analytical signal under dynamic conditions in which the substrate consumption and/or product formation are monitored for a certain time up to saturation [15]. Until now, a measurement of catecholase activity of biomimetic tyrosinase catalysts by UV-Vis spectrophotometry is usually recommended as a standard analytical procedure. However, electrochemical approaches describing the kinetics of catalytic processes come to the fore, namely amperometric detection of the biocatalytically generated products [16,17], and/or principles of cyclic voltammetry enabling to electrochemically mediate the enzymatic reaction that involves a substrate and a mediator [18,19].

This contribution offers more-or-less universal approach based on the voltammetric detection of phenolic substrate consumption during the catalytic oxidation mediated by binuclear copper(II) complexes (see Figure 1). To demonstrate this new method in full extent, a comparison of the developed voltametric method with commonly used spectrophotometric assay is included and discussed.

For the developed method, two phenolic isomers, differing in the position of the hydroxyl group on benzene ring were selected, namely 3,5-di-*tert*-butylcatechol (3,5-DTBC) and 2,5-di-*tert*-butylhydroquinone (2,5-DTBHQ). They gave rise to anodic peak at +0.722 V and +0.687 V *vs.* SCE at the GCE in MeOH containing 0.1 $\mathrm{mol\,L^{-1}}$ $LiClO_4$, respectively. A compromise between sensitivity and linear range of voltametric detection was necessary to complete the optimization experiments. If a potential step of 5 mV, potential amplitude of 25 mV, and a scan rate of 50 $\mathrm{mV\,s^{-1}}$ are set, linear relationships between the anodic peak

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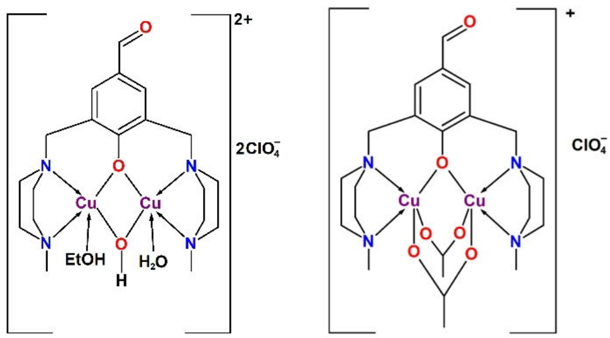

Fig. 1. Structures of the copper(II) complexes taken for studies; complex **1** $[Cu_2L(OH)(H_2O)(EtOH)](ClO_4)_2$ (left) and complex **2** $[Cu_2L(OAc)_2(H_2O)](ClO_4)$ (right).

current (I_p^a) and concentration of substrates (c) have been obtained obeying the following equations: $I_p^a = 0.0218c - 0.6704$ with $R^2 = 0.9997$ and $I_p^a = 0.0207c - 0.4650$ with $R^2 = 0.9999$ for linear range 50–1000 μmol L^{-1} 3,5-DTBC and 2,5-DTBHQ.

The satisfactory precision of the voltammetric method is presented via the relative standard deviations calculated for five repetitive measurements with 100 μmol L^{-1} 3,5-DTBC and 2,5-DTBHQ, where fairly low values of ±0.5 % and ±0.4 % are below the significance level of 5 %.

Although the tyrosinase biomimetic catalytic activity of copper(II) complexes has been mostly studied using spectrophotometric techniques, it is necessary to quote that the application of artificial enzymes in biosensing usually concerns the electrochemical approaches. However, it should be emphasised that electrochemical studies on artificial enzyme kinetics together with their comparison and validation against spectrophotometric techniques have never been the subject of interest in the area of biomimetics. Thus, herein, kinetic constants for two different dinuclear copper(II) complexes towards 3,5-DTBC and 2,5-DTBHQ are obtained from electro-chemical measurements and for the first time directly compared with spectrophotometric data.

Figure 2A shows typical adsorption bands obtained during the oxidation of 3,5-DTBC by air oxygen being catalysed by the complex **1**. At a constant content of catalyst, kinetic curves recorded for the 3,5-di-*tert*-butyl-*o*-benzoquinone (3,5-DTBQ) formation were obtained (Figure 2B), where a calibration curve for 2–100 μmol L^{-1} 3,5-DTBQ described by equation $A = 0.0023c + 0.0015$ with $R^2 = 0.9995$ was used for calculation of reaction velocity (V_0) values and construction of Lineweaver-Burk plot (see Figure 2D).

In contrast to the above-mentioned conventional method, the substrate consumption over a certain time-period was monitored via the anodic oxidation of the remaining 3,5-DTBC (Figure 3A), when a negligible amount is assumed to be converted electrochemically and not affecting the overall catalysed oxidation. As shown in Figure 3B, kinetic curves drawn as hyperbolic decline were obtained. If the V_0 values were plotted against 3,5-DTBC concentration, the analogical (hyperbolic) saturation curve could be achieved as in the previous case (Figure 3C).

Table 1 offers a comparison of catalytic rate constants determined using DPV and UV-Vis spectrophotometry in the biomimetic catecholase activity of dinuclear copper(II) complexes towards two isomers of di-*tert*-butyl-dihydroxybenzene. Comparable kinetic parameters of catalytic oxidation of 3,5-DTBC with the data presented in the literature [21] were found having demonstrated that the new voltammetric approach also provides accurate results in accordance with traditional kinetic spectrophotometric assay, with the only limitation–substrate (product) must be electroactive.

In fact, isomers of di-*tert*-butyl-dihydroxybenzene were selected by a random choice because their oxidation products (*ortho* or *para* di-*tert*-butyl-quinones) are not subject of undesired polymerization, which is typical for common phenolic substances, as well as neurotransmitters [22]. The resulting polymers often adhere onto the walls of a cuvette and may thus deteriorate the final results. Such a problem can be overcome by the presented method via the choice of the working conditions, namely selection of the electrode material, composition of the working medium, and setting the parameters of the selected voltammetric technique.

In comparison with the already introduced amperometric methods employed in enzymatic kinetics investigation [16,17], the voltammetric approach can be

Table 1. Kinetic parameters for the tyrosinase biomimetic dinuclear copper(II) complexes.

Substrate	DPV			UV-Vis		
	k_{cat}/h^{-1}	K_m/μmol L^{-1}	V_{max}/μmol L^{-1} s^{-1}	k_{cat}/h^{-1}	K_m/μmol L^{-1}	V_{max}/μmol L^{-1} s^{-1}
3,5-DTBC^a	28.3 ± 4.1	267.7 ± 25.8	1.18 ± 0.12	23.3 ± 1.0	270.4 ± 10.7	0.97 ± 0.04
2,5-DTBH^a	27.84 ± 2.7	297.2 ± 18.8	1.16 ± 0.07	27.9 ± 1.8	242.7 ± 15.7	1.16 ± 0.08
3,5-DTBC^b	32.9 ± 3.0	225.1 ± 34.4	1.37 ± 0.08	38.8 ± 4.2	223.0 ± 24.4	1.62 ± 0.18
2,5-DTBH^b	25.2 ± 1.8	572.8 ± 20.6	1.05 ± 0.05	24.2 ± 0.7	584.0 ± 16.2	1.01 ± 0.03

Values given as coverage intervals $\bar{x} \pm st_{1-\alpha}/\sqrt{n}$, where $\bar{x}$ is the arithmetic mean, s the standard deviation taken from the Lineweaver-Burk plot results obtained for $[Cu_2(L)(OH)(H_2O)(EtOH)][ClO_4]_2{}^a$ $[Cu_2(L)(OAc)_2]ClO_4{}^b$, and $t_{1-\alpha}$ the critical value (4.303) of Student's t-distribution for 3 repetition of each analysis at $\alpha = 0.05$.

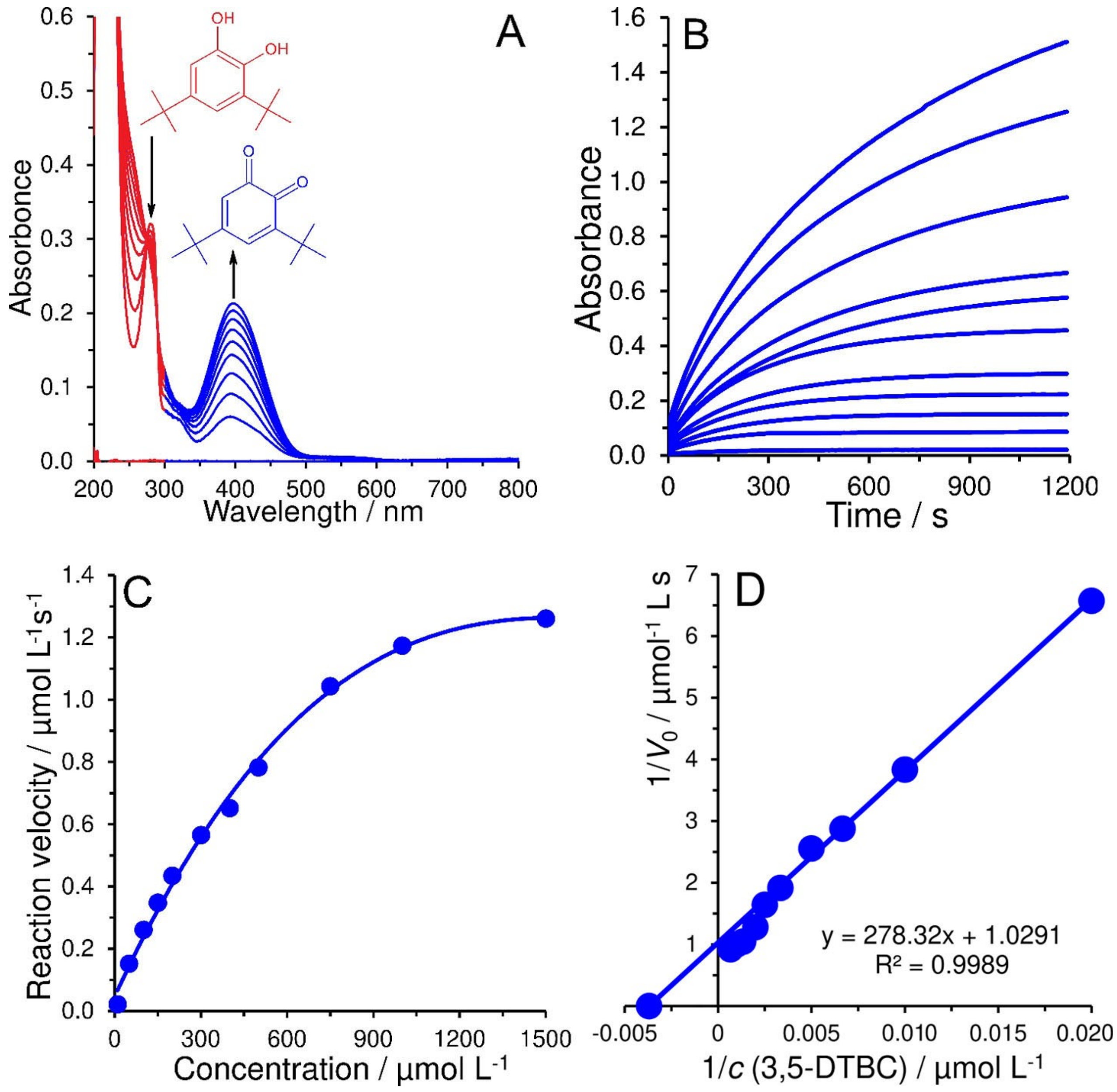

Fig. 2. UV-Vis absorption spectra obtained from mixture of 150 µmol L^{-1} complex **1** and 150 µmol L^{-1} 3,5-DTBC in pure MeOH containing 0.1 mol L^{-1} $LiClO_4$ every 5 min (A). Kinetic curves obtained for the oxidation of 10, 50, 100, 150, 200, 300, 400, 500, 750, 1000, and 1500 µmol L^{-1} 3,5-DTBC catalysed by 150 µmol L^{-1} complex **1** in pure MeOH containing 0.1 mol L^{-1} $LiClO_4$ at 394 nm (B). Michaelis-Menten saturation curve for complex **1** showing the relation between the concentration of 3,5-DTBC and reaction rate (C), with the appropriate Lineweaver Burk plot (D).

additionally utilized in studies of the catalytic activity of biomimetic complexes. In case of an amperometric method, the constant potential applied changes the oxidation state of the complex in time, which may lead to the undesired effect on the resulting catalytic activity.

Therefore, it can be concluded that newly proposed voltammetric approach is applicable to the continual investigation of the kinetics of biomimetic catalytic reactions, utilizing the monitoring of the change in the current signal related to the oxidation of substrate (consumption) or reduction of the product (formation) in the course of time. This has been successfully tested on two dinuclear copper(II) complexes towards two different di-*tert*-dihydroxybenzenes (3,5-DTBC and 2,5-DTBHQ) in non-aqueous media (MeOH), when the results of catecholase activity were comparable to those obtained by traditional spectrophotometric assay. Furthermore, the new voltametric method offers an eventuality of how to receive the kinetic parameters of a biomimetic catalyst in the catalysed reaction where the products may undergo the undesired polymerization.

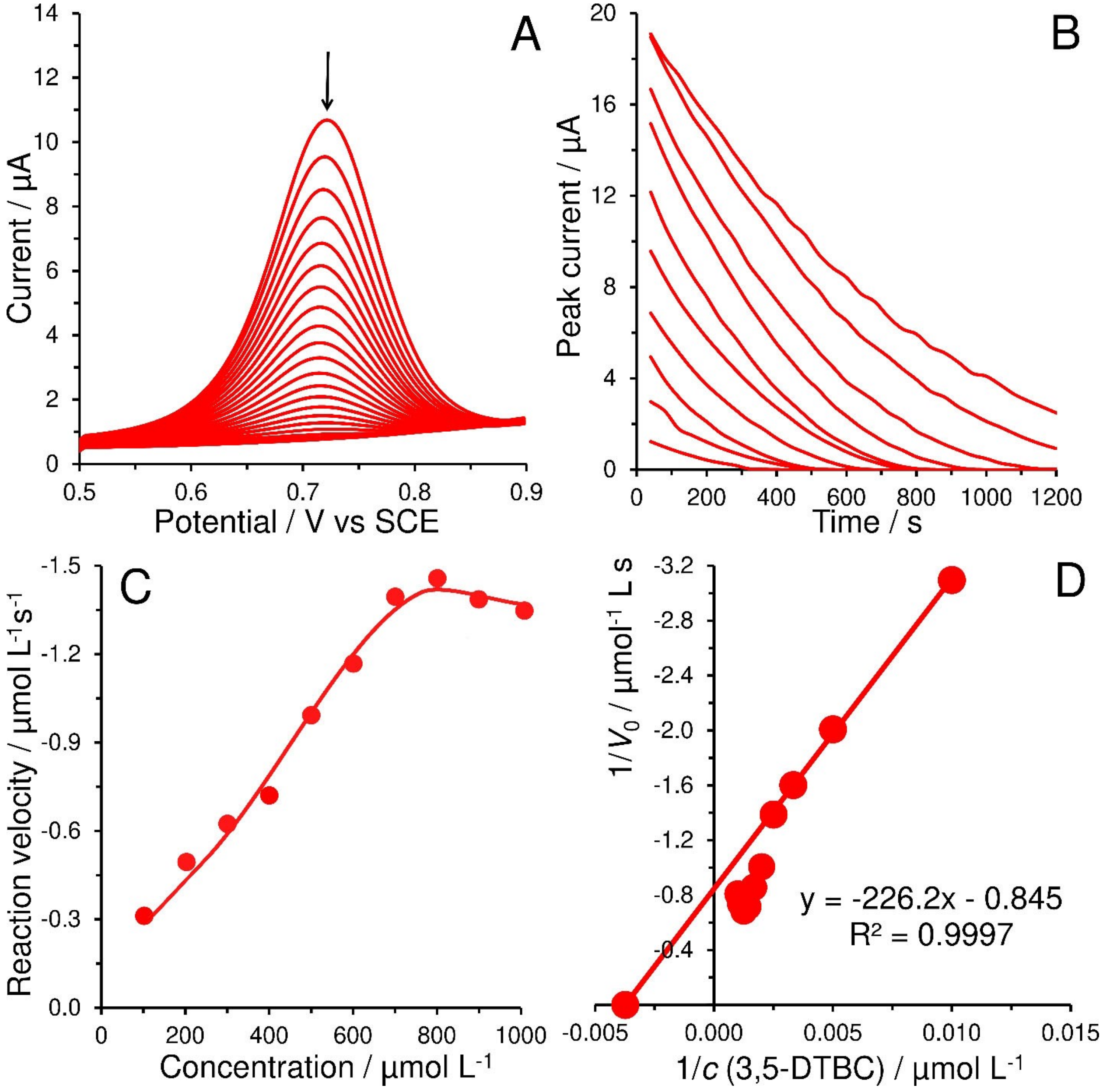

Fig. 3. Voltammetric curves of 500 μmol L−1 3,5-DTBC in the presence of 150 μmol L^{-1} complex **1** recorded every 40 s at GCE in pure MeOH containing 0.1 mol L^{-1} $LiClO_4$ at potential step of 5 mV, potential amplitude of 25 mV, and scan rate of 50 mV s^{-1} (A). Kinetic curves obtained for the oxidation of 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 μmol L^{-1} 3,5-DTBC catalysed by 150 μmol L^{-1} complex **1** in pure MeOH containing 0.1 mol L^{-1} $LiClO_4$ (B). Michaelis-Menten saturation curve for complex **1** showing the relation between the concentration of 3,5-DTBC and reaction rate (C), with the appropriate Lineweaver-Burk plot (D).

Last but not least, there is also a possibility to choose special working electrode, which would allow one to detect even slight changes in the current response, thus reducing the catalyst consumption, as well as the overall time of measurement(s). Both these aspects seem to be a distinct advantage compared to spectrophotometry.

Experimental

All the chemicals, namely 98 % 4-hydroxybenzaldehyde, 95 % paraformaldehyde, 99 % *N*-methylpiperazine, 98 % copper(II) perchlorate hexahydrate, 98 % copper(II) acetate hydrate, 98 % anhydrous sodium perchlorate, 99.99 % anhydrous lithium perchlorate, 99 % triethylamine, 98 % 3,5-di-*tert*-butylcatechol (3,5-DTBC), 98 % 3,5-di-*tert*-butyl-*o*-benzoquinone (3,5 DTBQ), 99 % 2,5-di-*tert*-butylhydroquinone (2,5 DTBHQ), 99 % 2,5-di-*tert*-butyl-*p*-benzoquinone

(2,5-DTBQ), together with 99.99 % methanol (MeOH), 99.99 % acetonitrile (MeCN), 99.99 % diethyl ether, and 99.8 % chloroform, were purchased from Merck KGaA (Darmstadt, Germany). Two different dinuclear copper(II) complexes (see Figure 1) bearing 2,6-*bis*(4-methylpiperazine-1-yl-methyl)-4-formylphenol (L) were synthetized according to the already published procedures [20,21].

The measurement of tyrosinase biomimetic catalytic activity of selected copper(II) complexes was performed at the UV-Vis spectrophotometer UV2450 from Shimadzu (Kyoto, Japan) with 1 cm quartz cuvette from Fisher Scientific (Pardubice, Czech Republic) in the region of 200–800 nm at a scanning speed of 0.5 nm s^{-1}.

All voltammetric measurements were carried out in a conventional three-electrode cell containing glassy carbon electrode (GCE) of diameter 3 mm (working), saturated calomel electrode (SCE) connected to a salt bridge with the supporting electrolyte (reference) and a platinum sheet (auxiliary electrode). These electrodes were connected to the AUTOLAB PGSTAT101 potentiostat/galvanostat from Metrohm (Prague, Czech Republic) operated by the NOVA 1.11 software.

A standard kinetic spectrophotometric assay was used for the determination of 3,5-DTBC and 2,5-DTBHQ oxidation kinetics measured in the concentration range of 10–1500 μmol L^{-1} in the presence of 150 μmol L^{-1} biomimetic copper(II) complexes forming 3,5-DTBQ and 2,5-DTBQ in MeOH with 0.1 mol L^{-1} $LiClO_4$. The formation of 3,5-DTBQ and consumption of 2,5-DTBHQ were monitored at a wavelength of 394 and 294 nm, respectively.

In a potential range from 0 to +1.0 V (*vs* SCE), anodic oxidations of 100–1000 μmol L^{-1} 3,5-DTBC and 2,5-DTBHQ in the presence of 150 μmol L^{-1} selected copper(II) complexes were investigated for every 40 s by differential pulse voltammetry at the GCE in MeOH with 0.1 mol L^{-1} $LiClO_4$ at a potential step of 5 mV, potential amplitude of 25 mV, and scan rate of 50 mV s^{-1}.

For the coefficients of determination (R^2) higher than 0.9950, kinetic curves were properly fitted by the cubic splines obeying the equations ($f(x) = a_3x^3 + a_2x^2 + a_1x + a_0$) and characterizing the change in absorbance (A) or the anodic peak current (I_p^a) in relation with time (t). The obtained a_1 values were used to determine the reaction velocities (V_0) and subsequent construction of Michaelis–Menten saturation curves, where the appropriate Lineweaver-Burk plots were preferred for calculations of maximum velocities (V_{max}) and Michaelis constants (K_m). Values of turnover number (k_{cat}) were calculated as ratio of V_{max} to total complex concentration (c_T).

All the values are presented as coverage intervals $\bar{x} \pm st_{1-\alpha}/\sqrt{n}$, where $\bar{x}$ is the arithmetic mean, s the standard deviation taken from the Lineweaver-Burk plot results obtained for complexes **1** and **2**, and $t_{1-\alpha}$ is the critical value (4.303) of Student's t-distribution for 3 replicates of each analysis for the 95 % confidence interval.

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Data Availability Statement

All data related to this manuscript will be made available upon suitable request.

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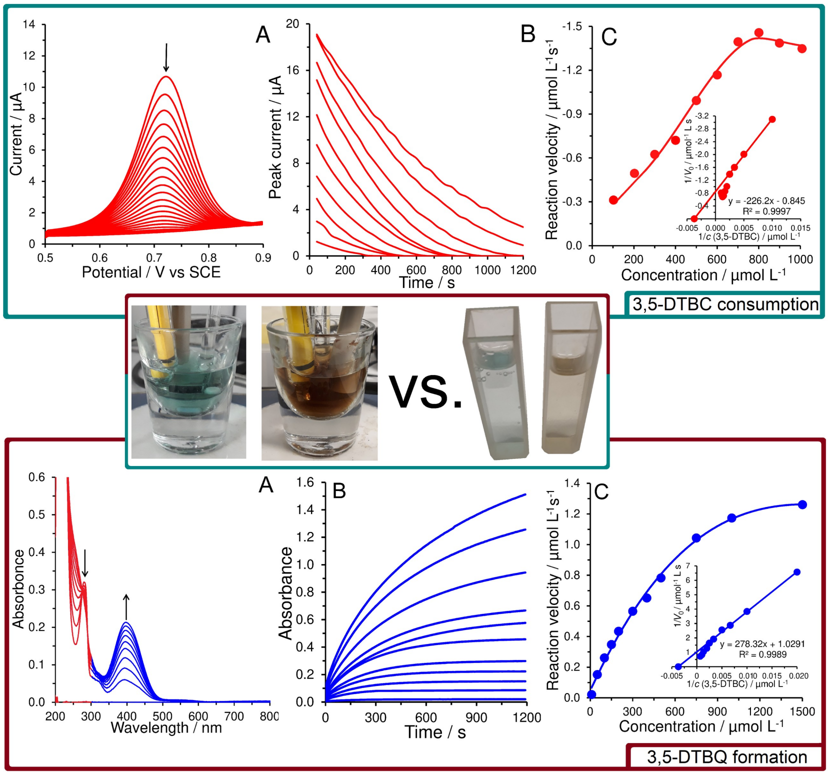
SHORT COMMUNICATION



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