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On the Performance of Amperometric Tyrosinase Carbon Paste Biosensor in the Presence of Catechol, Resorcinol, or Hydroquinone

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Abstract: This study was aimed to understand which substitution position of hydroxy groups on benzene ring is the most suitable for active site of enzyme tyrosinase, and also to determine if tyrosinase is a useful biological component for the development a biosensor based on carbon material for the determination of polyphenolic compounds in food samples. Electrochemical properties of diphenols (catechol, resorcinol, and hydroquinone) at bare carbon paste electrode (CPE) and CPE surface-modified with conductive copolymer Nafion and enzyme mushroom tyrosinase (CPE/Tyr/Nafion) were investigated using cyclic voltammetry and hydrodynamic amperometry.

Keywords: Catechol; Cyclic voltammetry; Biosensor; Carbon paste electrode; Hydrodynamic amperometry; Hydroquinone; Polyphenols; Resorcinol; Tyrosinase.

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Introduction

Diphenols represent a simple polyphenolic compounds which are significant antioxidants [1]. Antioxidants are chemical compounds with positive effect on human health located in plants and secondarily in foodstuffs [2,3]. Enzyme tyrosinase catalyzes oxidation reaction of

polyphenols to quinoid form in the presence of dissolved oxygen [4]. Products of this enzymatic reaction can be then detected electrochemically.

Three isomers of diphenols (catechol; *o*-dihydroxybenzene, resorcinol; *m*-dihydroxybenzene, and hydroquinone; *p*-dihydroxybenzene) were studied to ascertain, which substitution position of hydroxyl groups on benzene ring provides the most sensitive current response. Chemical structures of selected diphenols are shown in Fig. 1.

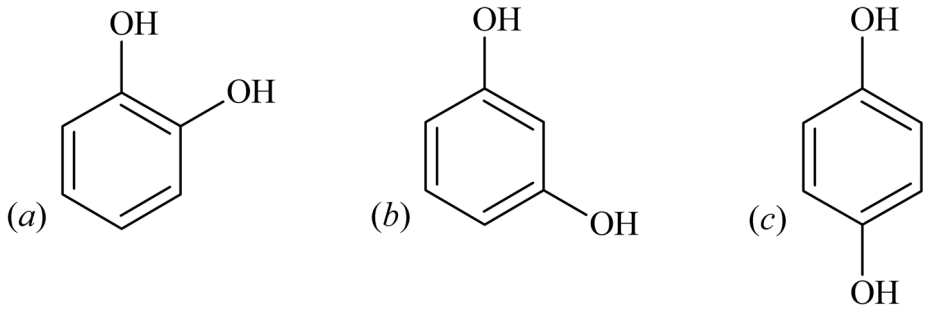

Fig. 1: *Chemical structures of a) o-dihydroxybenzene (catechol), b) m-dihydroxybenzene (resorcinol), and c) p-dihydroxybenzene (hydroquinone).*

Such variants of substituted benzene rings are possible to find in chemical structure of many significant antioxidants such as flavonoids, stilbenes, phenolic acids and tocopherols (see examples in Fig. 2) [5]. The electrochemical study of tyrosinase activity on diphenols substrates can help to understand possibilities of analytical applications of tyrosinase-based enzyme biosensors for the determination of polyphenolic compounds in real samples.

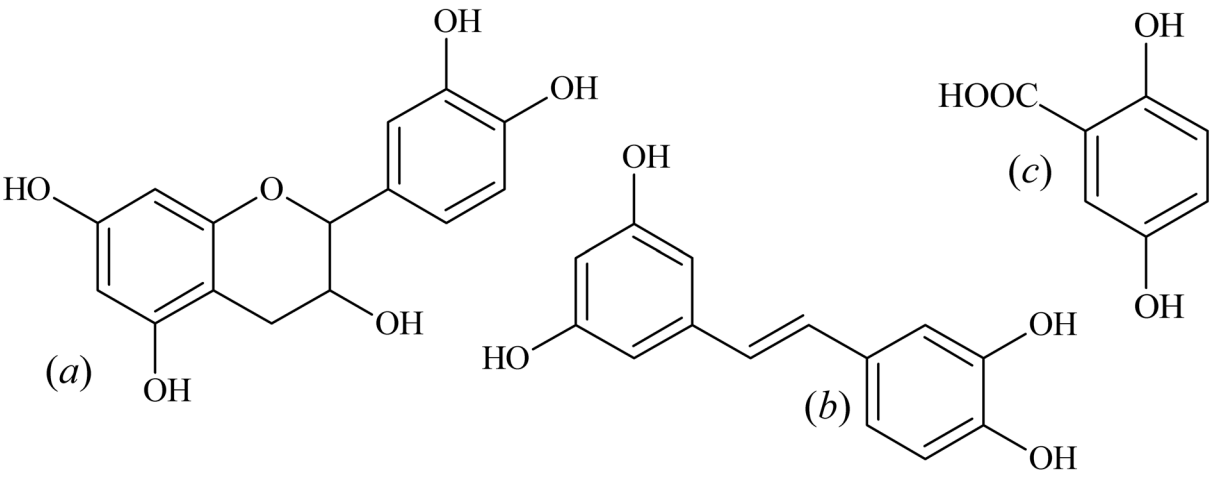

Fig. 2: *Chemical structures of commonly occurring polyphenols in food: a) epicatechin (flavonoid), b) piceatannol (stilbene), and c) gentisic acid (phenolic acid).*

The active site of the enzyme consists of a dinuclear copper centre in which the metal is bound by six or seven histidine residues and a single cysteine residue [6]. The copper is probably Cu^{I}, which may assist redox reactions via oxidation to Cu^{II} [7]. The chemical structure of the tyrosinase active site together with its cresolase and catecholase activity [8,9] is demonstrated in Fig. 3. Response time and current of amperometric signal associated with the detection of products of enzymatic reaction reflect the availability of hydroxyl groups in the active site of the used enzyme for enzyme conversion. Therefore, it is possible to find, which arrangement of hydroxyl groups on benzene ring suits enzyme active site the most.

Influence of enzyme mushroom tyrosinase (ex. *Agaricus bisporus*) on oxidation of diphenols was studied electrochemically using carbon paste electrode with the aid of cyclic voltammetry (CV) and hydrodynamic amperometry (HA). Electrochemical experiments were performed in neutral media with pH 7.0 because the active range of tyrosinase lies within pH 6-7 [10]. Carbon paste is a typical heterogeneous electrode material [11], which can be used for easy laboratory preparation of physical transducer. It is a mixture of an electrically conductive component (usually a graphite powder) embedded in a binder, basically an insulating liquid [12]. Carbon paste electrode (CPE) can be useful to develop an amperometric biosensor operating in negative constant working potential due to its material stability and wide potential window from –1.7 V to +1.2 V *vs*. Ag/AgCl for neutral electrolyte [13].

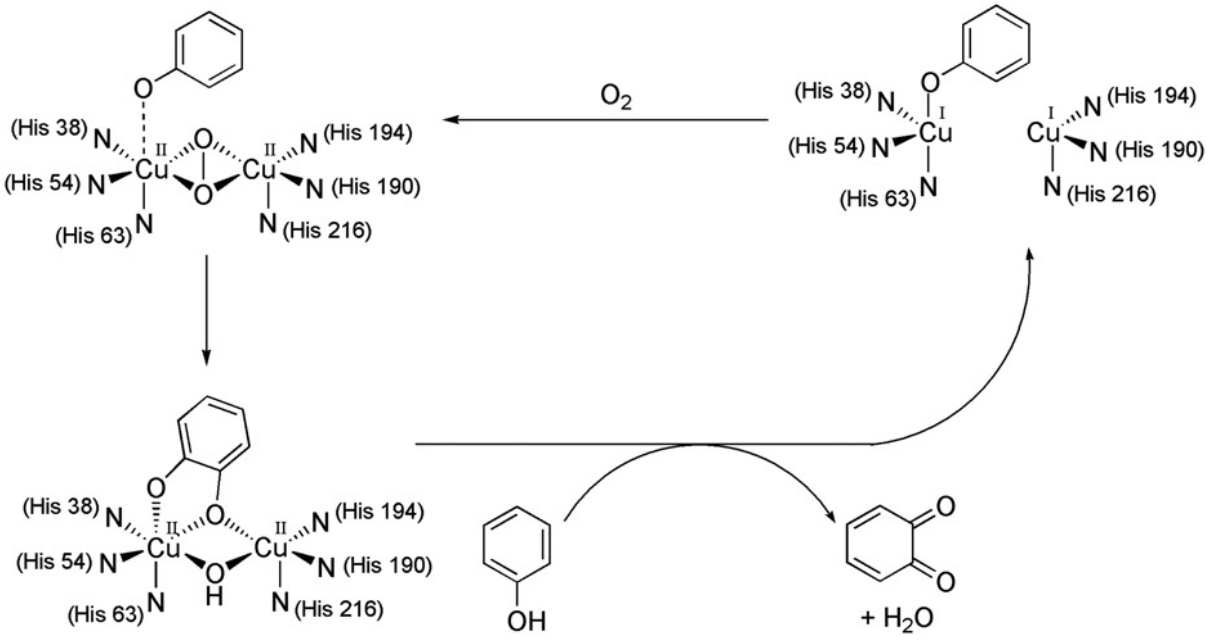

Fig. 3: *Chemical structure of tyrosinase active site and mechanism of its activity.*

Experimental

Chemicals and Reagents

Catechol (*o*-dihydroxybenzene), hydroquinone (*p*-dihydroxybenzene), resorcinol (*m*-dihydroxybenzene), Nafion (5 % m/m solution in lower alcohols), and mushroom tyrosinase (E.C. 1.14.18.1, 4276 units/mg solid) were purchased from Sigma-Aldrich (Austria). Carbon powder CR-2 (2 μm average particle diameter) was obtained from Maziva Týn nad Vltavou (Czech Republic). Paraffin oil (Uvasol) was from Merck (Germany). Other chemicals and reagents were of analytical grade (Sigma-Aldrich). Highly purified water (resistivity 18 MΩ cm) was prepared using purification system Milli-Q (Millipore) and used throughout. Phosphate buffer solution (0.1 mol L^{-1}, pH 7.0) was used as the supporting electrolyte.

Preparation of Biosensor

Carbon powder (0.5 g) and mineral oil (0.13 mL) were intensively mixed in a porcelain mortar for 30 min. The resultant carbon paste was pressed into a piston-like electrode holder with diameter 3 mm [14] and the surface of the CPE was polished with a wet filter paper. Nafion® solution was dropwise neutralized with ammonia (5 % m/m water solution) against indicator paper. Membrane solution was prepared by mixing neutralized 5% Nafion® in 55 % ethanolic solution (40 μL), pure water (60 μL) and enzyme solution in PB (150 μL; 500 μg mL^{-1}) [15]. An aliquot (10 μL) was applied to the electrode surface and left for one hour under laboratory conditions to dry out. If not in use, the biosensors were stored dry in a refrigerator at temperature of 5 °C.

Instrumentation

Conventional three-electrode arrangement was used for all experiments. The setup consisted of working carbon paste electrode (diameter 3 mm) with immobilized tyrosinase, a reference electrode (Ag|AgCl|3.0 mol L^{-1} KCl) and an auxiliary electrode (platinum metal wire). A miniature potentiostat (PalmSens, The Netherlands) operated by the corresponding software (PSTrace, version 4.2) was used for electrochemical measurements. All potentials discussed in this work are referred to the above mentioned reference electrode.

Methods

Cyclic voltammetry and hydrodynamic amperometry were used as electrochemical techniques for laboratory experiments. Measurements were performed in 10 mL of non-deareated 0.1 M PB (pH 7.0) as the supporting electrolyte at room temperature 25 ± 1 °C. Potential range for cyclic voltammetry was set from –0.5 to 1.3 V, scan rate 0.05 V s^{-1}, and potential step 5 mV. Usually 10 µL of 0.01 M of corresponding analyte was pipetted into 10 mL PB in voltammetric glass cell. Batch hydrodynamic amperometric measurements were carried out also in the same glass cell containing 10 mL PB with constant stirring of 400 rpm. If not stated otherwise, the detection potential of –0.24 V was applied to the working electrode.

Results and Discussion

Stability of Nafion Membrane

Usually a few freshly immobilized sensor based on CPE showed signs of cracked membrane. This phenomenon was never observed in the immobilization of enzyme at glassy carbon electrode (GCE) or screen printed carbon electrode (SPCE). Integrity of enzyme membrane based on Nafion is much better for solid electrode materials than for pastes. A content of Nafion in membrane (> 2.0 % w/w) leads to faster evaporating of immobilized membrane solution and rearranging of thick Nafion layers thus causing the cracks in the final membrane. Signs of membrane damage made from 0.8 % Nafion solution were not observed at SPCE after one month. Microscopic images of working electrode surfaces are shown in Fig. 4. Tyrosinase biosensor based on SPCE could possess some advantages as transducer than biosensor with CPE, because of better membrane stability and the electrochemical properties similar to CPE due to its heterogeneous carbon origin [16].

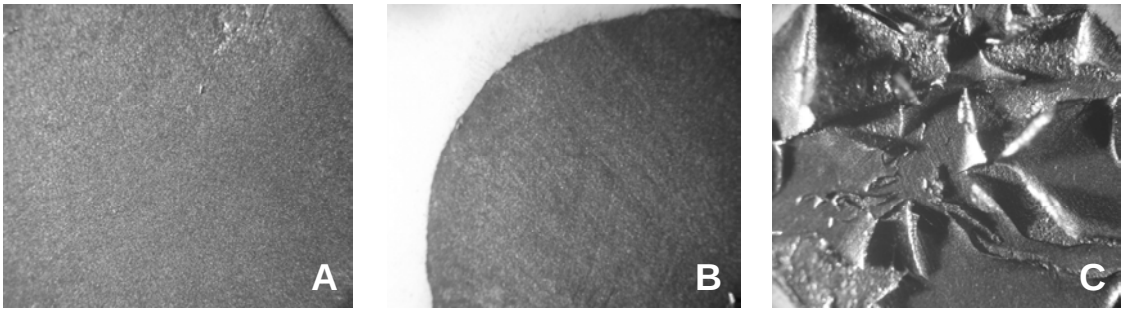

Fig. 4: *Microscopic images (total magnification 40x) of bare CPE (A), fixed CPE/Tyr/Nafion (B) and cracked CPE/Tyr/Nafion surfaces (C).*

Electrochemical Behaviour of Diphenols at CPE and CPE/Tyr/Nafion

Electrochemical behavior of diphenols was studied using cyclic voltammetry. Voltammetric measurements of diphenols were performed at both bare CPE and tyrosinase biosensor. Position of reduction peak potentials (E_p) and height of peak current responses (I_p) were investigated. Comparison between voltammetric responses of diphenols at bare CPE and CPE/Tyr/Nafion is shown in Fig. 5. Kinetics study of CPE/Tyr/Nafion, influence of scan rate (v), and effects of substrate (diphenols) concentration were not the aims of this study. Constant value of scan rate 0.05 V s^{-1} was used for CV measurements.

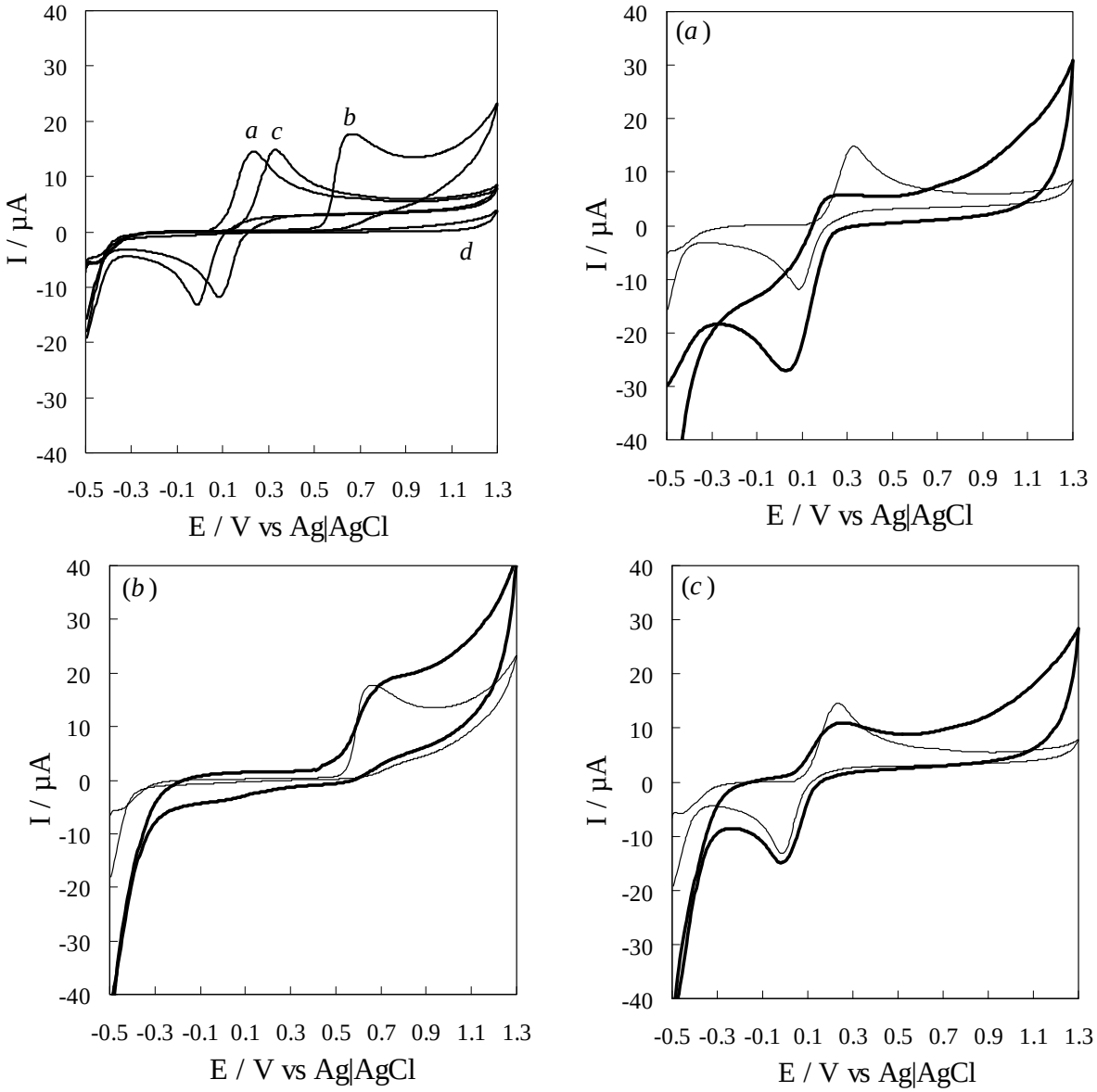

Fig. 5: *Cyclic voltammograms (first cycles) of 0.5 × 10^{-3} mol L^{-1} catechol (a), resorcinol (b), hydroquinone (c) and pure PB (d).* Bare CPE (thin line) and CPE/Tyr/Nafion (thick line) in 0.1 mol L^{-1} PB (pH 7.0) at scan rate 0.05 V s^{-1}.

Presence of Nafion used for immobilization of tyrosinase resulted in increase of background current to 1.5–2.0 µA in comparison with bare CPE (1.0 µA). Decline of oxidation peak and rise of reduction peak express the catalytic effect of tyrosinase. This effect causes shifts of redox peaks (peak potentials) because the presence of biocatalyst decreases free Gibbs function (ΔG) as energetic barrier. Significant catalytic activity of tyrosinase on the oxidation of catechol comparing to other used diphenols was observed (see Fig. 5a). Therefore, it can be presumed that tyrosinase preferentially oxidases polyphenols with hydroxyl groups in *ortho* substitution pattern rather than in *meta* or *para* position. *Ortho* diphenols probably provides suitable sterical arrangement to the active site of tyrosinase.

Influence of Stirring Speed

The speed of stirring affects the mass transport rate of the analyte to the biosensor's membrane and therefore, it may significantly influence the current response in a closed dynamic system. The experiments were carried out at 100, 200, 300, 400 and 500 rpm, from which the stirring speed of 400 rpm was found as an optimum. At higher speed, no significant increase of the current response was observed.

Response Time of CPE/Tyr/Nafion Biosensor

In amperometric techniques, response time is one of the most important parameters describing the performance and quality of analytical devices, especially the biosensors, because it reflects kinetics of electrodes reactions. From selected diphenols, catechol revealed the shortest response time (10 s). Evidently, tyrosinase catalyzes predominantly the oxidation of polyphenols having their hydroxyl groups in *ortho* position. Polyphenolic compounds with hydroxy group in *meta* or *para* positions need a longer time for their enzymatic oxidation.

Optimal Peak Potential for Amperometric Detection

Selectivity of an amperometric biosensor is controlled not only by the specificity of the biocomponent, but also by the choice of operating potential. The presence of electroactive species in the sample matrix, which could contribute to measured current, must be considered [17]. Final products of enzymatic oxidation of diphenols could be electrochemically reduced at negative potentials. Therefore, interval of working potentials from 0 to –0.3 V was measured and optimal detection potentials were selected for all diphenols. The relationship

between current response and applied working potential in amperometric detection is demonstrated in Fig. 6.

Mushroom tyrosinase (ex. *Agaricus bisporus*) is not suitable for construction of biosensor for determination of resorcinol, because very low current responses are obtained at CPE/Tyr/Nafion electrode. Minor inhibitory effect of resorcinol in determination of other phenols was observed during their electroanalysis. The solution is to use a different type of tyrosinase, which catalyzes the oxidation of resorcinol and also prevent its inactivation [18,19]. Optimal potentials for amperometric detection were found approximately around -0.15 V (catechol), -0.10 V (resorcinol), and -0.25 V (hydroquinone).

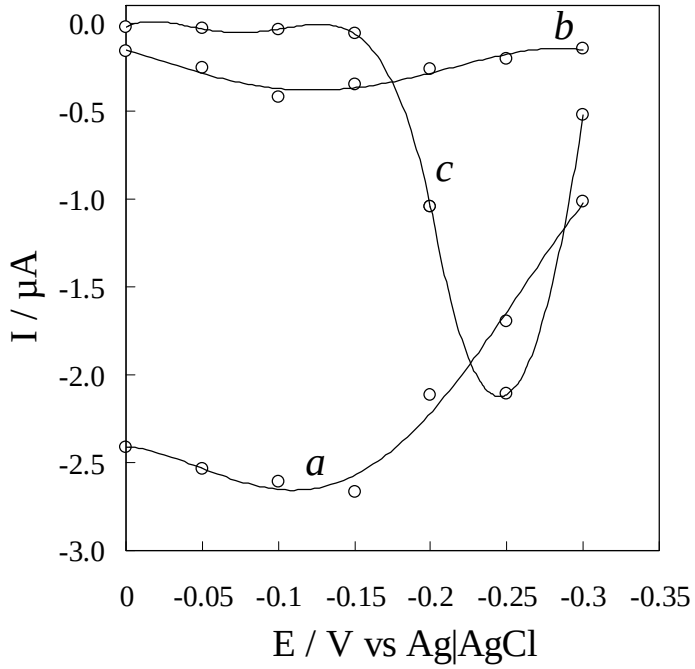

Fig. 6: *Dependence of the amperometric response on the applied electrode potential of the CPE/Tyr/Nafion biosensor:* 10 µmol L^{-1} catechol (a), 90 µmol L^{-1} resorcinol (b), and 90 µmol L^{-1} hydroquinone (c); supporting electrolyte 0.1 M PB (pH 7.0); speed of stirring 400 rpm; temperature 25 °C.

Sensitivity of diphenols at CPE/Tyr/Nafion

No amperometric response was observed at bare CPE for used freshly prepared analytes, which were clear solutions. Such observation is not true for solutions old for more than two weeks; analytes showed signs of oxidation (change to yellow or brown color) when solutions were stored under laboratory conditions. Apparently, colored solutions contained mixture of partially oxidized polyphenols and their quinoid forms.

Detection limit (LOD) and limit of quantification (LOQ) were not calculated for every tested isomer but a linear relationships between the concentration of each diphenol and measured current were evident during hydrodynamic amperometry. Typical hydrodynamic

amperograms of all diphenols are shown in Fig. 7. Attention was paid only to the difference of current responses for the same concentrations of analytes. Current response acquired for catechol was higher for more than twenty times comparing to other diphenols in PB at CPE/Tyr/Nafion biosensor when applying optimal working potential –0.24 V. Almost five times higher current response was measured for hydroquinone in comparison to resorcinol at working potential –0.2 V.

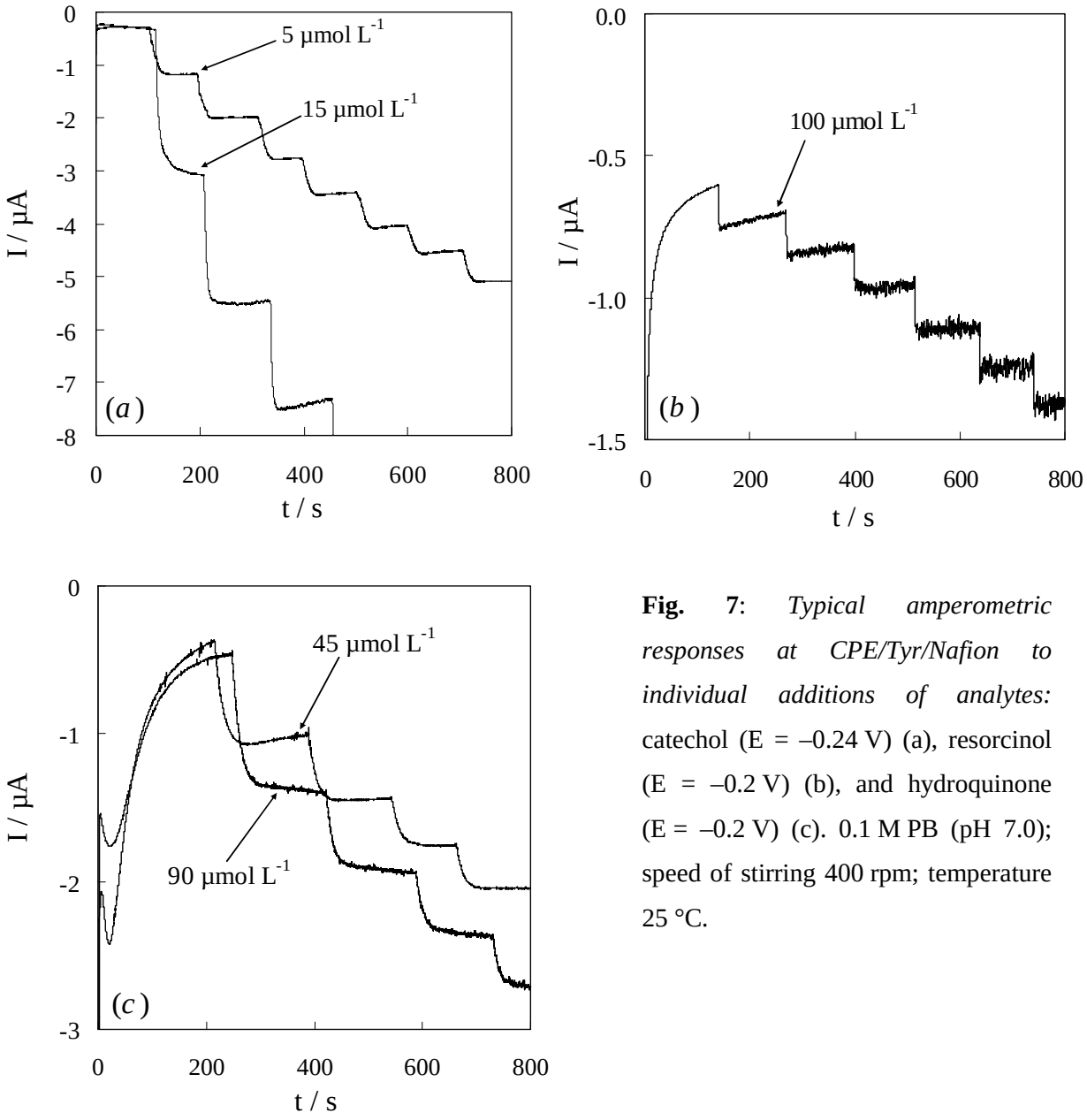

Fig. 7: *Typical amperometric responses at CPE/Tyr/Nafion to individual additions of analytes:* catechol (E = –0.24 V) (a), resorcinol (E = –0.2 V) (b), and hydroquinone (E = –0.2 V) (c). 0.1 M PB (pH 7.0); speed of stirring 400 rpm; temperature 25 °C.

The enzyme tyrosinase is capable to oxidize phenol to catechol due to its cresolase (monooxygenase) activity. Moreover, tyrosinase can catalyze an oxidation reaction of catechol to the corresponding quinone (oxidase activity) [20]. These two different catalytic properties are typical for enzyme tyrosinase. On the contrary, tyrosinase catalyzes oxidation

reaction of hydroquinone with difficulties. This inability of tyrosinase to catalyze the oxidation of hydroquinone has been often used to distinguish between tyrosinase and laccase, a phenolase, or possibly a group of phenolases, which possess a great activity towards hydroquinone [21]. If a small amount of catechol is added to an aqueous solution of hydroquinone and tyrosinase, then the hydroquinone is oxidized [22].

Conclusions

In this study, lower current responses of *m*-diphenol (resorcinol) and *p*-diphenol (hydroquinone) were measured comparing to *o*-diphenol (catechol) at CPE with immobilized mushroom tyrosinase in Nafion coating. On the basis of results of abovementioned experiments, it can be concluded that the used tyrosinase enzyme difficultly catalyzes the oxidation of polyphenols having hydroxyl group in *meta* or *para* positions. Contrary, tyrosinase easily catalyzes oxidation reaction of polyphenolic compounds which bear hydroxyl group in *ortho* position on benzene ring in presence of dissolved oxygen.

Significant antioxidants, like catechins and stilbenes, usually contain substituted benzene ring with hydroxyl groups in *ortho* and *meta* position in its molecule. Tyrosinase biosensor could be useful for determination of total content of polyphenols in food of plant origin. Biosensor based on carbon materials with immobilized tyrosinase and laccase together could then cover the whole range of polyphenols presented in real samples.

Acknowledgement

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