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CATALYTIC ACTIVITY OF *AGARICUS BISPORUS* MUSHROOM TYROSINASE TO ACETAMINOPHENON IN THE PRESENCE OF L-PROLINE

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Secondary reaction of amino acid L-proline with 4-acetamido-o-benzoquinone as a product of the enzymatic oxidation of acetaminophen mediated by mushroom tyrosinase isolated from Agaricus bisporus has been studied by amperometric detection in the batch injection analysis configuration and by ultraviolet-visible spectrophotometry. Actually, this scientific work is focused on finding out whether it would be possible to develop an indirect electrochemical method based on the amperometric biosensing suitable for monitoring of the L-proline in various honey samples. For optimum content of acetaminophen (100 μmol dm^{-3}) and tyrosinase (3.33 μg cm^{-3}), a satisfactory linear range from 0.1 to 0.5 mmol dm^{-3} L-proline was obtained for absorption maximum at 332 nm. However, it is necessary to mention that the indirect determination of L-proline by the amperometric tyrosinase biosensor still presents a challenge in the field of developing biological sensors for monitoring of this amino acid.

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Introduction

L-proline known as a non-essential hydrophilic low-molecular nitrogenous substance belongs to a twenty of basic coded α-amino acids participating in the biosynthesis of proteins. Only the L-proline does not contain the primary amino group ($-NH_2$), but this structure is a part of the alkyl side chain forming a cycle of the R-NH-R type being called imino acid [1].

Generally, the total content of amino acids (TCA) in honey and products made of is usually attributed to L-proline due to its dominant content. In the E.U. legislative for foodstuffs [2,3], the values of TCA are also used as important criterion for estimating the matureness of honey or indicia counterfeiting in some cases [4,5].

A relatively simple spectrophotometric method was developed for determination of L-proline based on the specific reaction of compounds containing the amino group with 2,2-dihydroxyindan-1,3-dion, known as ninhydrin. This method is also included in the harmonized methods of the European Commission [6,7]. Unfortunately, it is important to note that the determination of L-proline by this official procedure requires a high consumption of reagents with time-consuming sample preparation, except the analysis alone [2,8].

For that reason, the aim of our study was to develop a simple screening analytical method based on electrochemical biosensing [9] that could offer a possible way how to selectively and fast determine the TCA in honey and mead. This contribution represents an initial research, where a secondary reaction of L-proline with 4-acetamido-*o*-benzoquinone (4-AOBQ) as a product of tyrosinase oxidation catalysis of acetaminophen [10,11] has been studied by amperometry in the batch configuration and with ultraviolet-visible (UV-VIS) spectrophotometry.

Basically, this study could also help to clarify if this secondary chemical reaction can be used for development of an indirect electrochemical method. So far, the reactions of aliphatic or aromatic amines with aldehydes or ketones by nucleophilic addition forming a carbinolamines [12], and followed by a dehydration step for generating an imine were described [13]. Of all encoding α-amino acids, only L-proline does not have the free amino group in its molecular structure due to the cyclic structure of the side chain [1,14]. Each α-amino group is protonated and the present α-carboxyl group is deprotonated under biological conditions (pH ~ 7). L-proline can react with the already mentioned carbonyl compounds to give carbinolamines which then dehydrate to give rise the enamines. Additionally, in 1955, it has been found that L-proline also spontaneously reacts with several quinones that can be theoretically classified as "week ketones" [15]. Especially, native quinones serve as the electron acceptors and many of such compounds are also products of the tyrosinase biocatalytic activity [16].

Tyrosinase (EC 1.14.18.1) from edible and cultivated mushroom *Agaricus bisporus* [17] belongs to the group of cooper metalloenzymes [18]. Unlike other

polyphenol oxidases, it is able to catalyze a hydroxylation of monophenols in *ortho* position due to cresolase activity [19]. Subsequently, due to catecholase activity, the oxidation of the *o*-diphenols by molecular dioxygen continues to form the corresponding quinones and water [20].

According to the previous characterisation of tyrosinase catalytic activity, it should be evident that an acetaminophen is oxidized analogically. However, it is necessary to note that each enzymatic reaction is usually affected by other accompanying chemical substances. Only a few scientific works were focused on studying of L-proline effect on mushroom tyrosinase [21]. In 2003, Valero et al. described a kinetic study of catalytic oxidation of acetaminophen by the tyrosinase in the presence of L-proline in details [22]. In the first step, the acetaminophen was hydroxylated to 4-acetamido-1,2-catechol with its subsequent oxidation to 4-AOBQ. Proposed reaction mechanism is shown in Fig. 1.

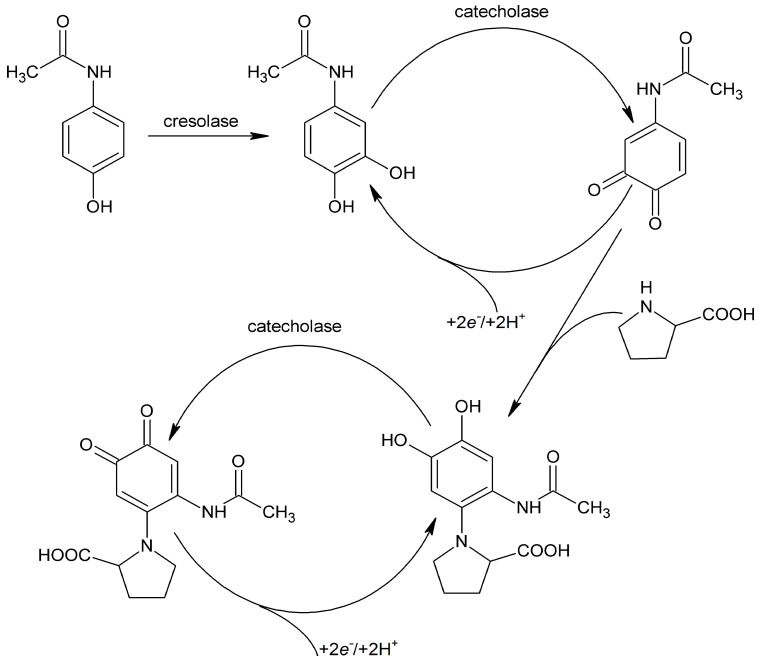


Fig. 1 Reaction pathway proposed for oxidation of acetaminophen by tyrosinase in the presence of L-proline

Additionally, this study had also shown that the resulting 4-AOBQ was able to be electrochemically reduced with the involvement of $2e^-$ and $2H^+$ at a constant voltage (i.e., principle of amperometric biosensor for tyrosinase). In the presence of L-proline, the 4-AOBQ may be a subject of the respective side reaction producing a 4′,5′-dihydroxy-2′-*N*-prolylacetanilide which cannot be reduced under the same voltage. Unfortunately, one has to admit the catalysis by tyrosinase enzyme proceeding *via* oxidation of 4′,5′-dihydroxy-2′-*N*-prolylacetanilide and giving rise to 4-acetamido-5-*N*-prolyl-*o*-benzoquinone, which is an electrochemically reducible compound [22].

Experimental

Chemicals and Reagents

Acetaminophen, *N,N*-dimethylformamide (DMF), the reference standard of L-proline, glutaraldehyde solution (25% in water), and lyophilized powder of mushroom tyrosinase (EC 1.14.18.1) were purchased from Sigma-Aldrich. Multi-walled carbon nanotubes (MWCNTs) characterized by diameter: 10-30 nm, length: 5-15 μm, and specific surface area: 40-300 m^2 g^{-1} were taken from Shenzhen Nanotech (Shenzhen, China). Strong mineral acids (65% HNO_3 and 96% H_2SO_4) from Lach-Ner (Neratovice, Czech Republic) were needed for functionalization of multi-walled carbon nanotubes (MWCNTs). Deionized water (ρ = 18.3 MΩ cm; Milli-Q system, Millipore) and salts (KH_2PO_4 and $Na_2HPO_4.2H_2O$) from Penta (Prague, the Czech Repubic) was used for preparation of 0.01 M phosphate buffer (pH 7.0).

Apparatus and Instrumentation

Amperometric Detection in Batch Configuration. Each amperometric measurement was performed in the three-electrode configuration consisting of the tyrosinase biosensor (working), Ag/AgCl/3M KCl (reference), and Pt-wire (auxiliary) electrodes. Amperometric detection (AD) was carried out by using an AUTOLAB electrochemical system (model "PGSTAT 101", operated *via* the Nova 1.11 software; Metrohm, Prague, the Czech Republic).
Spectrophotometric Measurements. All spectrophotometric measurements in the ultraviolet and visible region were carried out with quartz cuvettes (l_c = 1.0 cm) using a UV-VIS spectrophometer (Shimadzu; Kyoto, Japan).

Preparation of Amperometric Tyrosinase Biosensor

Functionalization of MWCNTs was realized by an acidic oxidation in the mixture of HNO_3 and H_2SO_4 at a ratio of 3:1 (v/v). In this case, a portion of 100 mg untreated MWCNTs was added to 50 cm^3 mixture of acids and left for 60 min at laboratory temperature. The resulting suspension was heated at 100 °C in water bath for 4 hours, subsequently washed with deionized water, and centrifuged at 4 000 min^{-1} for 10 min to the neutrality controlled by litmus pH-test strip. Drying of acid-oxidized MWCNTs was then performed with the aid of infrared lamp for 4 hours [22]. An amount of 2.0 mg cm^{-3} of treated MWCNTs in DMF was homogenized using an ultrasonic bath at laboratory temperature for 1 hour. For firm immobilization of the MWCNTs, 10 mm^3 of fresh suspension was allowed to

slowly evaporate on the surface of the glassy carbon electrode (GCE; with diameter of 3 mm) at ambient temperature for one day [24].

The tyrosinase immobilization onto the transducer surface (GCE/MWCNTs) included three separate steps. At first, 5 mm^3 of enzyme solution (2.0 mg cm^{-3}) in 0.01 M phosphate buffer (pH 7.0) was applied and left to dry out under laboratory conditions for 30 min. Using this approach, the enzyme had been adsorbed onto the MWCNTs and then linked by adding 3 mm^3 1% glutaraldehyde for 20 min to avoid an excessive leaching of the enzyme from the polymer used. Finally, 5 mm^{-3} neutralized 1% Nafion in 55% ethanol was applied and left to dry under previously mentioned conditions for 30 min. If not in use, the biosensor was stored in a refrigerator at 5 °C.

Procedure

All experiments were performed in the 0.01 M phosphate buffer (pH 7.0) due to the optimum catalytic activity of tyrosinase used as the enzyme of choice [25]. In spectrophotometric measurements, vials of total volume 6 cm^3 usually contained a 100 μmol dm^{-3} acetaminophen with dissolved 10 μg tyrosinase and different amount of L-proline. This mixture had to be left for minimally 2 hours and, after that, the respective spectra were recorded from 700 to 200 nm.

Amperometry in the batch configuration was performed usually in 10 cm^3 of buffer at a constant voltage of –0.1 V vs. Ag/AgCl and a stirring rate of 400 min^{-1}. Otherwise, each other change in the experimental conditions or instrumental parameters is specified below, in the legends of the corresponding figures.

Results and Discussion

Construction of Amperometric Tyrosinase Biosensor

The overall optimization of amperometric biosensing involved the search of the best possible working conditions, such as optimal amount of all the components necessary for preparation of the tyrosinase biosensor itself, appropriate setting of the working potential and stirring rate. Similar tyrosinase biosensor based on the GCE covered by thin film of MWCNTs / Nafion / tyrosinase nano-biocomposite for monitoring the native phenolic compounds was already reported in 2007 [26]. In our case, the tyrosinase biosensor was not made by applying this mixture on the GCE surface, but using of multi-step immobilization of the individual layers according to the following sequences: 1) immobilization of MWCNTs [27], 2) direct absorption of the enzyme, 3) crosslinking of biomolecules by glutaraldehyde, and 4) fixation of the resulting enzyme aggregate by its covering with Nafion

layer [28].

This multi-step immobilization usually guarantees maximum availability of the substrate to the active center of the enzyme. It was found that 10 μg tyrosinase is an optimal content in order to achieve the sufficient sensitivity, when a higher content of glutaraldehyde (3 mm^3 of 1% aqueous solution) causes a dramatic increase of background current.

Effect of the Stirring Rate

In the batch configuration, the intensity of stirring has a significant effect on the rate of analyte transport toward the biosensor active layer, which can cause a principal change in the biosensor sensitivity. From the stirring rates of 100, 200, 300, 400, 500, and 600 min^{-1}, a value of 400 min^{-1} was chosen because higher values already did not lead to further increase of the response.

Effect of the Working Potential

For amperometric biosensors, the working potential always plays a key role in almost each application. Generally, electrochemical detection of the electroactive species (products of biological catalysis) is being performed at a constant voltage. The highest reduction response of 4-AOBQ was obtained at –0.1 V; therefore, this value could be selected as optimum, corresponding also to the previously reported data [29]. Moreover, it should be mentioned that the biosensor provides a satisfactory current response also at +0.05 V.

This voltage contributed to a very high selectivity of acetaminophen determination because any electrochemical reduction of the accompanying substances were not expected. However, it was not suitable in our case of the indirect determination of L-proline.

Effect of L-proline on the Functioning of Mushroom Tyrosinase

Figure 2A demonstrates a typical amperometric response of the tyrosinase biosensor for a number of successive additions of acetaminophen with the corresponding calibration curve. Moreover, the amperometric responses of this biosensor in 20, 60 and 100 μmol dm^{-3} acetaminophen in dependence on the successive additions of 500 μmol dm^{-3} L-proline are shown in the neighboring Fig. 2B.

Figure 2A shows that the tested amperometric biosensor for tyrosinase is a suitable analytical tool for the determination of acetaminophen, which is also cha-

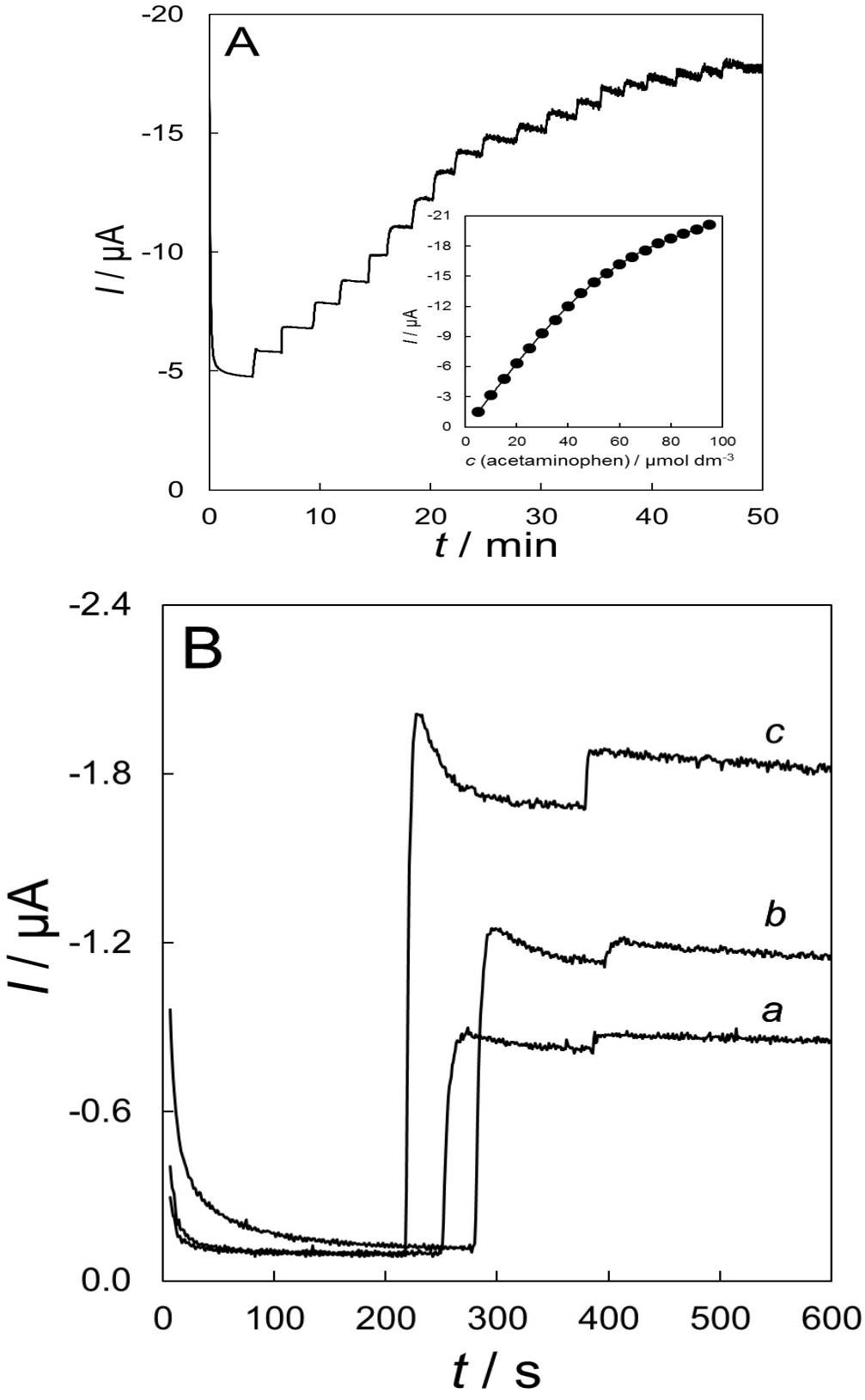


Fig. 2 Typical amperometric record during the model calibration obtained at the tyrosinase biosensor for successive additions of 5 µmol dm^{-3} acetaminophen (A). The amperometric response with the corresponding curve of this biosensor in 20 (a), 60 (b) and 100 µmol dm^{-3} acetaminophen (c) on successive additions of 500 µmol dm^{-3} L-proline always at 380 s; measured at –0.1 V (B).

racterized by linear calibration from 5×10^{-6} to 4.5×10^{-5} mol dm^{-3} and the response time less than 2 s. However, the secondary chemical reaction of 4-AOBQ with L-proline is many times slower than that proceeding *via* direct electrochemical reduction at –0.1 V. For this reason, only a minimal amount of 4-AOBQ participates in the chemical reaction with L-proline. Thus, one can assume that the use of more positive potentials could be reflected in a slower electrochemical reduction of 4-AOBQ, thus causing the sensitivity deterioration.

Our prediction that addition of L-proline would lead to a decrease of the reduction current signal has not, however, been confirmed experimentally. A significant decrease was observed only after adding of 500 mm^3 of pure water into 10 cm^3 of phosphate buffer. This phenomenon could probably be attributed to the decrease of conductivity of the supporting electrolyte used as a consequence of dilution. Contrarily, it could be observed that the successive addition of L-proline had increased the reduction current signal (see Fig. 2B); probably, due to the electrochemical reduction of 4-acetamido-5-*N*-prolyl-*o*-benzoquinone (see Fig. 1; [22]). Unfortunately, a notable increase of absorbance at 332 nm could be detected after two hours, which indicated a very slow pathway of the respective reaction (see Fig. 3C). According to these findings, it can be concluded that the indirect determination of L-proline in various honey products by amperometric tyrosinase biosensor remains a challenge for further research.

UV-VIS Spectrophotometry

Additionally, UV-VIS spectrophotometry had to be used to confirm the fact that L-proline reacts with the 4-AOBQ at optimum working conditions. In this case, it has been necessary to find out whether the increasing content of L-proline may cause a significant change in the shape of the final spectra.

During spectrophotometric measurements, it has been found that acetaminophen provides one intensive absorption maximum at wavelength at 240 nm. In the presence of tyrosinase, the absolute height of this maximum decreases with the increasing duration of catalysis. Moreover, other absorption signals corresponding to the formation 3´-hydroxy-acetaminophen and the already mentioned 4-AOBQ could also be possibly recognized at 280 nm and 370 nm, respectively. It is also very important to quote that the sensitivity of these two maxima has been many times lower than that at 240 nm, even after three hours (not shown). Furthermore, it has been ascertained that presence of L-proline significantly increases the intensity of absorbance at 332 nm. Thus, it was necessary to find an optimal amount of tyrosinase, time of catalysis, and a concentration ratio between acetaminophen and L-proline.

As found out, a content of 3.3 μg cm^{-3} tyrosinase enzyme was sufficient to achieve the highest value of absorbance at 332 nm; see Fig. 3A. The parent Fig. 3B

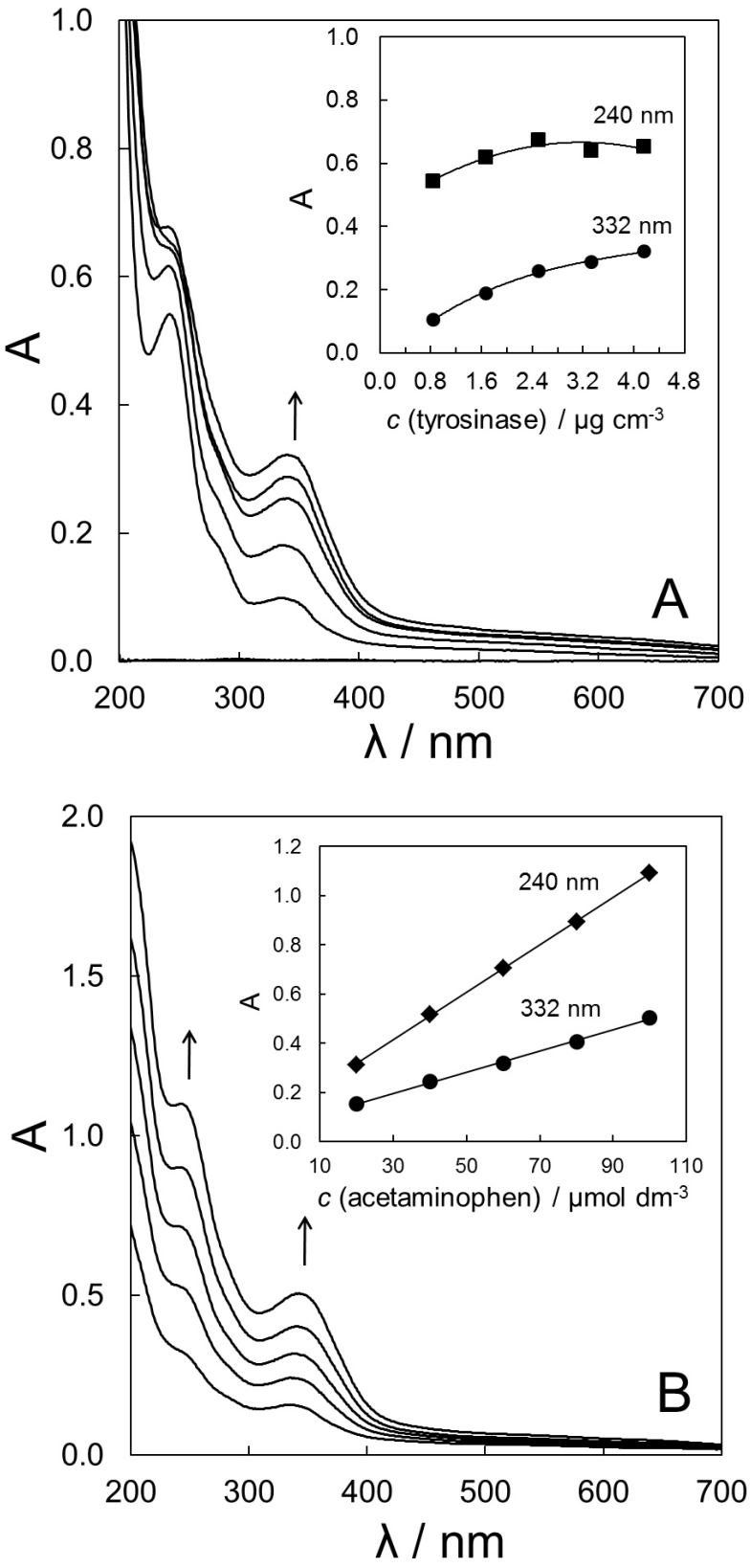
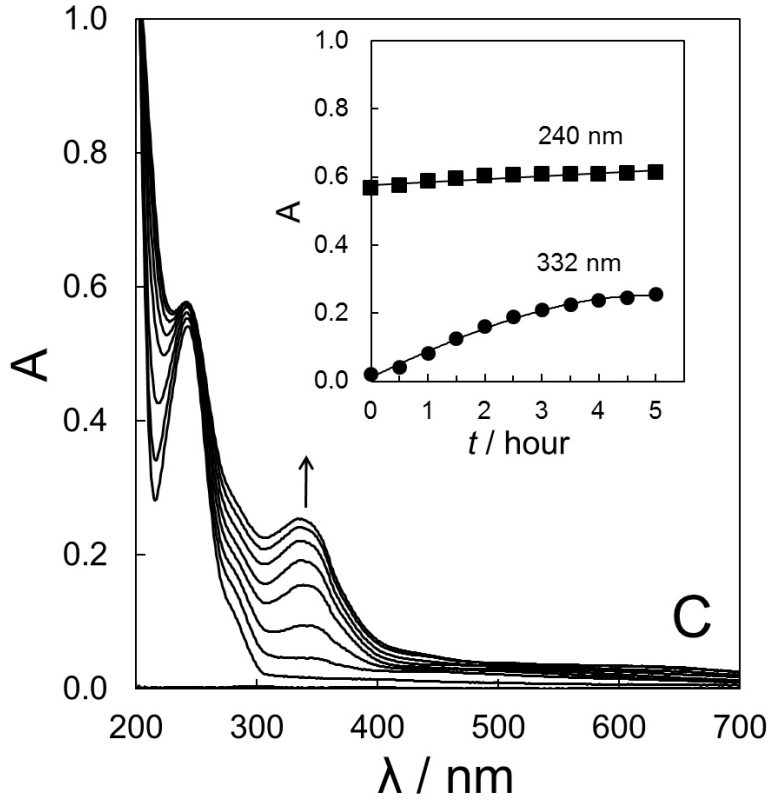


Fig. 3 UV-VIS spectrophotometry of the mixture containing 50 μmol dm^{-3} acetaminophen, 500 μmol dm^{-3} L-proline and different amounts of tyrosinase measured after 2 hours (A). UV-VIS spectrophotometry of the mixture containing 500 μmol dm^{-3} L-proline, 3.3 μg cm^{-3} tyrosinase and different amounts of acetaminophen measured after 2 hours (B)

shows that the absorbance obtained at 240 and also at 332 nm linearly increase with the increasing content of acetaminophen. Therefore, the sensitivity of an indirect tyrosinase biosensor could be controlled *via* the content of acetaminophen, but it should be mentioned that an enzymatic activity is generally limited by the amount of substrate [30]. Consequently, the value of 100 μmol dm^{-3} was considered as the maximal permissible quantity.

From kinetic study of the enzymatic oxidation of acetaminophen in the presence of L-proline (see Fig. 3C), it has been found that a time longer than two hours is needed to achieve the constant absorbance, which indicates that this time period is too long for continuous amperometric detection. Fortunately, it can be noted that the values of absorbance at 332 nm increased linearly in dependence on L-proline content (see Fig. 3D); the lowest value of calibration curve corresponding to 11.51 mg dm^{-3} L-proline.

Thus, it should be possible to determine its content in honey, where critical value is 180 mg per 1.0 kg; the smaller amount then indicating the counterfeiting of honey.



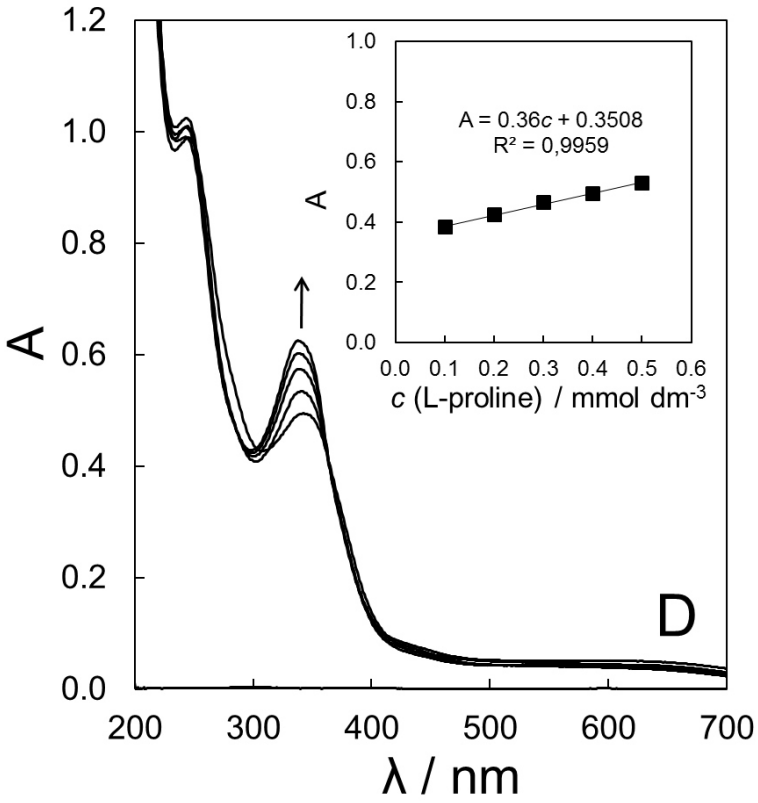


Fig. 4 UV-VIS spectrophotometry of the mixture containing 50 μmol dm^{-3} acetaminophen, 500 μmol dm^{-3} L-proline and 1.7 μg cm^{-3} tyrosinase measured every 30 minutes (C). UV-VIS spectrophotometry of the mixture containing 100 μmol dm^{-3} acetaminophen, from 100 to 500 μmol dm^{-3} L-proline and 3.3 μg cm^{-3} tyrosinase measured after 2 hours (D)

Conclusion

This study is definitely an initial step in developing and characterizing a new analytical method which could be used for monitoring the content of L-proline in the honey products. It may well be that these types of biosensors will eventually be found as practically inapplicable due to a very slow secondary reaction of L-proline with quinones incurred by enzymatic oxidation of naturally occurring phenolic compounds in honey [31].

A detailed spectrophotometric study has confirmed that L-proline reacts with the 4-AOBQ at laboratory conditions. Due to the above-stated slow reaction between L-proline and this *ortho*-quinone, it would be a good idea to try also other *ortho*-phenols, when bearing in mind that the determining factor is not the rate of enzymatic oxidation of phenols, but the rate of the secondary reaction with the L-proline.

Acknowledgement

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