Research Article

pISSN: 2345-5357
eISSN: 2423-4214

OPEN ACCESS

APPLIED FOOD BIOTECHNOLOGY, 2025, 12 (1): e5
Journal homepage: www.journals.sbmu.ac.ir/afb

Comparison of Two Laccase Enzymes from *Trametes versicolor* and *Trametes pubescens* for the Assessment of Phenolic Acids Content Using Laccase-Based Biosensor

Merin Shukri[1], Tsvetina Cherneva[1, 2], Angel Peshkov[1, 3], Mariana Nikolova[1, 3], Iliya Iliev[1, 3] Nina Dimcheva[1, 2,*]

1- Plovdiv University "Paisii Hilendarski", Centre of Technology, 21, Kostaki Peev Str, Plovdiv, Bulgaria
2- Plovdiv University "Paisii Hilendarski", Department of Physical Chemistry, 24, Tzar Assen Str, Plovdiv, Bulgaria
3- Plovdiv University "Paisii Hilendarski", Department of Biochemistry and Microbiology, 21, Kostaki Peev Str, Plovdiv, Bulgaria

Abstract

Background and Objective: Although biochemically similar, two laccase enzymes isolated from basidiomycetes (*Trametes* sp.) showed differences in their affinity to two types of phenolic compounds, interacting stronger with diphenols (catechol and caffeic acid), compared to interactions with benzenetriols (pyrogallol and gallic acid). Catalytic efficiency of *Trametes pubescens* laccase was detected 4-5 times higher than determined for commercial laccase (*Trametes versicolor*). In this study, the interactions of the two immobilized enzymes with di and triphenols were examined by various electrochemical techniques.

Material and Methods: Following electrochemical techniques: cyclic voltammetry, chronoamperometry and differential pulse voltammetry were used in this study. Experiments were carried out in varying substrate concentrations. Activity and sensitivity of the two alternative laccase – based biosensors were compared using DPV and chronoamperometry.

Results and Conclusion: Constant potential amperometric measurements indicated that the biosensor produced with *Trametes pubescens* laccase was much more active than biosensor based on laccase from *Trametes versicolor* when interacting with caffeic and gallic acids. The phenolic content of three different herbal extracts was evaluated with the developed laccase biosensors and results were found to be similar to those from chromatographic analysis used as a reference method. Therefore, biosensors can be used for rapid testing of phenolic content in real samples.

Conflict of interest: The authors declare no conflict of interest.

Article Information

Article history:

- Received 9 Dec 2024
- Revised 22 Jan 2024
- Accepted 3 Feb 2024

Keywords:

- Electrochemical Biosensor
- Dihydroxy and Trihydroxy Aromatic Compounds
- Herbal Extracts
- Laccase Enzyme

*** Corresponding author:**

Nina Dimcheva*

Tell: +359 32 261 309 (office)
+359 895372975 (mobile)
E-mail:
ninadd@uni-plovdiv.bg

How to cite this article

Shukri M, Cherneva T, Peshkov A, Nikolova M, Iliev I, Dimcheva N. Comparison of Two Laccase Enzymes from Trametes versicolor and Trametes pubescens for the Assessment of Phenolic Acids Content Using Laccase-Based Biosensor. Appl Food Biotechnol. 2025; 12 (1): e5.
http://dx.doi.org/10.22037/afb.v12i1. 46955

1. Introduction

Daily intake of antioxidant-rich foods and drinks is considered an important factor of a healthy regimen [1]. Antioxidants, the physiological role of which is to scavenge reactive oxygen species thus preventing oxidative damage of living cells [2] are micro-ingredients of plant cells such as those of fruits, vegetables, cereals and herbs. From natural antioxidants, phenolic acids are especially important not only because their antioxidant activity is within the highest ones, but also because they can rapidly be digested and adsorbed by gastrointestinal tract [3]. Due to their exceptional pharmacological, nutritional and wellbeing effects on humans, a wide spectrum of analytical methods for the assay of phenolic acids has been developed. Chromatographic analytical techniques receive extensive application for the quantification of these antioxidants in a variety of food samples [4-6]; however the required time-consuming sample preparation procedures stimulated the advance of various optical methods such as visible spectroscopy and fluorometry [7], or even paper-based colorimetric sensors [8]. Because of the susceptibility of phenolic acids to participate in redox processes, a range of

electroanalytical methods such as voltammetry [9,10], pulse voltammetry [11-15] and amperometric detection [9,10,16,17] have been developed.

Modern electrochemical approaches for the quantification of phenolic acids include phenolic acid detection with electrodes modified with advanced materials such as polymers [4], nitrogen doped carbon [12], carbon nanotubes [18,19] and gold nanostructures [10]. The use of nanostructured materials or composites for electrode modification offers enhanced selectivity of the determination due to either pronounced electrocatalytic effect or greatly enhanced electrode surface area [10].

Assessment of antioxidant capacity through biosensing is a novel trend in contemporary studies [9,11,20-22]. Numerous authors report an improved selectivity of the analysis when using biosensing systems for the assessment of antioxidant content [23]. Copper –containing enzymes laccase or tyrosinase were the primary choice for developing biosensing systems for the analysis of phenols [24-26]; however, whole-cells [27] and DNA-based [28] elements for molecular recognition have also been used for this purpose. Due to the formation of colored products of biocatalyzed transformations of phenolic compounds, most of the highlighted biosensing platforms rely on optical detection principle.

Biosensors with electrochemical detection for phenolic antioxidants analysis, reported in current literature [24], require nanostructured electrode surfaces and sophisticated bioreceptor immobilization protocol. Unlike these, a simple enzyme attachment to an unmodified glassy carbon electrode is discussed, which ensures electrochemical response sensitive enough to guarantee phenolic acid assay at micromolar concentrations. Therefore, the focus of the present study was to develop and optimize an electrochemical method based on two identically prepared laccase biosensors for the determination of di- and trihydroxy aromatic compounds – one bearing commercial laccase from *Trametes versicolor*, and another laccase – isolated from *T. pubescens*, which is used seldom in biosensor development. Laccases are widely used in a variety of industrial cycles such as pulp and paper production, wastewater treatment (e.g. from olive-oil mills and textile industry), brewing and food industries, pharmacy or in the construction of fuel cells [25] and biosensors for the quantification of di-substituted aromatic compounds [29].

Laccases are complex enzymes with more than one active site, which embeds three copper clusters (type T1, T2 and T3) differing in both function, and spectroscopic characteristics [25,26]. The reaction mechanism of laccases involves uptake of one electron from the substrate- a hydrogen donor, which is oxidizing to form radical with concomitant 4-electron reduction of molecular oxygen to form two water molecules [26]. The T1 copper site is responsible for binding the aromatic substrate to be oxidized via 1-electron pathway, while the T2-T3 copper cluster binds molecular oxygen and catalyzes its 4-electron reduction to water [26]. Electrons are transferred from T1 site to T2-T3 trinuclear cluster through internal molecular electron transfer. The difference between laccases from *T. versicolor* and *T pubescens* is linked to their amino acid sequences. Despite these belong to the family of fungal laccases, variations are seen in their primary structures that may affect their catalytic efficiency, substrate binding specificity, and thermal stability. Thus, amino acids surrounding the less conservative T1 copper-binding site and the overall folding of the enzymes might differ partially, which may result in differences in catalytic efficiency of the laccase manifested as substantially different catalytic constants.

Here reported biosensors function on the following principle: laccases electrocatalytically reduce the dissolved in the working medium oxygen, thus generating reductive current, which is enhanced in the presence of phenol derivatives that act as electron shuttles (mediators) between the enzyme active site and electrode surface. Gallic and caffeic acids are two representatives of phenolic acids, the positive effects of which are commented not only in terms of their antioxidant abilities, but also with respect to their potential anti-inflammatory [30] and anticancer [31] pharmacological activity that was the major reason for their use as laccase substrates in this study. As a demonstration of the applicability of biosensing method, a series of three different types rich in phenolic compounds herbal extracts were analyzed for their phenolic content and results were compared with those from high-performance liquid chromatography (HPLC) analysis.

2. Materials and Methods

2.1. Reagents

Laccase (E.C. 1.10.3.2, polyphenol oxidoreductase) enzymes from *T. versicolor* (Fluka, USA) and *T. pubescens* (a generous gift from Prof. Roland Ludwig, Department of Food Science and Technology, BOKU University of Natural Resources and Life Science, Vienna, Austria) were with homogeneous specific enzyme activities of 21 and 46 U mg^{-1}, respectively. One unit is the amount of enzyme necessary for the oxidation of 1.0 μmol of ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) per min at pH 4 and 30 °C. Laccases were dissolved in 0.05 M sodium-citrate buffer, pH 4, in such quantity to form enzyme solutions with concentrations of 950 U.ml^{-1}. The two enzymes were used without further purification.

Catechol, resorcinol, pyrogallol, caffeic acid, gallic acid, ABTS and reagents for the preparation of buffer solutions (sodium citrate, citric acid monohydrate and $NaClO_4$) were

of analytical grade (Acros, Belgium) and used without further purification. All stock solutions of the enzyme substrates were prepared with a concentration of 10 mM.

2.2. Enzyme immobilization

Enzyme electrodes were prepared based on commercial glassy carbon electrodes (2-mm diameter; Metrohm, Utrecht, The Netherlands). Prior to modification, electrodes were polished with 0.05 μm alumina slurry on a polishing cloth (Kulzer, Hanau, Germany), water-rinsed and cleaned using ultrasonication in ultrapure water for 1 min for at least two consecutive times.

Enzyme immobilization was carried out as follows [29]: 2 μl of enzyme solution were drop-cast on the electrode surface. Then, a 4-μl drop of the binder (Nafion 117 diluted with ultrapure water to 0.2%) was applied. Surface was dried at room temperature (RT). The two types of laccases were immobilized on the electrode surfaces identically and the amount of the immobilized enzyme in terms of enzyme units was equal.

After electrochemical measurements, enzyme electrodes were rinsed with ultrapure water and refrigerated at 4°C, when not in use. Regeneration of the working enzyme electrodes could be carried out after the mechanical removal of the enzyme-polymer layer via polishing procedure and following the above steps.

2.3. Electrochemical measurements

All electrochemical experiments were performed in a conventional single compartment three-electrode cell with working volume of 10 ml, connected to a computer-controlled electrochemical workstation Autolab PGSTAT 302 N (Metrohm-Autolab, Utrecht, The Netherlands) controlled by NOVA 2.1.6 software. Either a modified with enzyme glassy carbon electrode, or an enzyme– free electrode (for control experiments) was used as working electrode. A Ag|AgCl, sat. KCl (Metrohm, Utrecht, The Netherlands) was the reference and a platinum foil was the auxiliary electrode. If not otherwise specified, all reported potentials were stated against this reference electrode (Ag|AgCl, sat. KCl electrode) [29]. Cyclic voltammetry was run at scan rates of 5–20 $mV.s^{-1}$. Volt-ampere curves (voltammograms) were obtained in both background electrolyte - 10 ml of citrate buffer (pH 4, containing 0.1 M $NaClO_4$) and in the presence of enzyme substrates with a stock concentration of 10 mM until a 30 μM concentration was achieved in the cell.

Amperometric detection has been carried out at a constant potential of -0.2 V through successive additions of aliquots of 10 mM substrate stock solutions (typically from 20 to 500 μl) to 10 ml of the electrolyte in the cell. Chronoamperometric detection was carried out under constant stirring at 500 rpm.

Differential pulse voltammograms were recorded both in the absence and presence of studied compounds over the potential range from +0.6 to –0.6 V at a scan rate of 10 $mV\ s^{-1}$, pulse duration of 50 ms and an amplitude of 0.025 V, as optimized in the authors' previous studies [29,34].

Data analysis was implemented with Origin Pro 8.5 software. Non-linear regressions and statistics were carried out using embedded software module for enzyme kinetics.

2.4. Herbal extracts preparation

Herbal extracts were prepared as follows: 20 g of dry herbal mixtures were added to 1 l of pure water and boiled for 20 min at atmospheric pressure (~101 kPa). Then, the herbal extract was set to cool down to RT, filtered through nylon cloth 6.6, packed in 50 ml sealed containers and refrigerated at 4˚C until analyses. Three types of herbal extracts were subjected to electrochemical and HPLC analyses, further referred as PM1, PM3 and PM7. The three types of herbal extracts consisted of following herbs:

PM1: *Geranium sanguineous, Arctostaphylos uva-ursi, Betula alba, Polygonum hydropiper, Achillea millefolium*;

PM3: *Crataegus monogyna, Equisetum arvense, Geranium sanguineum, Urtica dioica*; and

PM7: *Fragaria vesca, Hypericum perforatum, Calendula arvensis, Frangula alnus, Polygonum hydropiper*.

These herbal combinations were selected due to their use as healing teas (pharmaceutical products) with high antioxidant content.

2.5. Chromatographic analysis

The HPLC analysis has been carried out as a referent method for phenolic acid quantification, as follows: The phenolic acid composition of the extracts was assessed using chromatographic system (Shimadzu, Japan), which consist of auto sampler (Nexera X2, SIL- 30AC); CTO-20AC column and SPD-20A UV detector (Shimadzu, Japan). Analysis was carried out using column Metitaranea Sea RP-18e (150 mm × 4.6 mm × 2 μm) (Teknokrom, Spain), mobile phase of 4% acetic acid and 100% AcCN (80:20), flow rate of 0.65 $ml.min^{-1}$, λ = 280 nm and temperature of 35 ˚C. Results were analyzed using Lab-Solution Nexera-XR-RF software and the standard phenolic acids (gallic and caffeic acids) [32,33].

The content of the phenolic compounds was calculated against a standard line constructed with its solutions at concentrations ranging from 500 $\mu g.ml^{-1}$ to 2.5 $\mu g.ml^{-1}$ of the corresponding phenolic acid with a correlation coefficient of $R^2 > 0.9991$. Total phenolic content was calculated by summing the content of each determined phenolic compound and re-calculating the total phenolic content in equivalents of gallic acid.

3. Results and Discussion

3.1. Studies of two different laccases in di – and trihydroxy aromatic compounds present by cyclic voltammetry (CV)

Electrochemical behavior of the two types of laccase-based bioelectrodes was probed using cyclic voltammetry (CV) in the absence and presence of both laccase substrates – oxygen and phenolic compounds. Voltammetric studies have shown that for the two types of laccase biosensors, reductive wave starts at potentials more negative than -0.25 V in aerated buffer solutions (i.e. in the presence of oxygen), which was not seen in deaerated solutions. It is well known that laccase is a metalloprotein capable of exchanging electrons with underlying electrode surfaces directly [34] without the need for additional electron shuttles (mediators). The efficiency of the electrical communication between the electrode and laccase depends on the enzyme orientation and distance between its active site and electrode surface [34]. Most probably, the negatively charged Nafion membrane electrostatically repulsed the negatively charged laccase active site, this way orienting the enzyme to electrode surface. The latter conformation is favorable for the electron exchange with the underlying electrode, which was manifested by a reductive wave appearing on the CV in the presence of molecular oxygen. Therefore, voltammetric studies verified the ability of immobilized laccase to carry out bioelectrocatalytic O_2 reduction to water molecules, thus proving that enzymes were electrochemically active.

Comparison of the CVs of the enzyme electrode recorded in aerated solutions in the absence and presence of pyrogallol and catechol as substrates (Fig. 1) revealed laccase-catalyzed oxidation of the two phenols to semi-quinones, followed by electrochemical regeneration of the oxidized products. When resorcinol was tested as laccase substrate, the resulting voltammograms did not show interactions between either of the immobilized enzymes, as no reduction of the product of its enzyme-catalyzed oxidation was noticed (Fig. S1, Supplementary information). These phenolic compound-depending differences in the performance of the two types of laccase biosensors were due to the difference between catechol and resorcinol in their spatial structure. The first benzenediol is with two vicinal hydroxy-groups, while resorcinol is its meta-isomer.

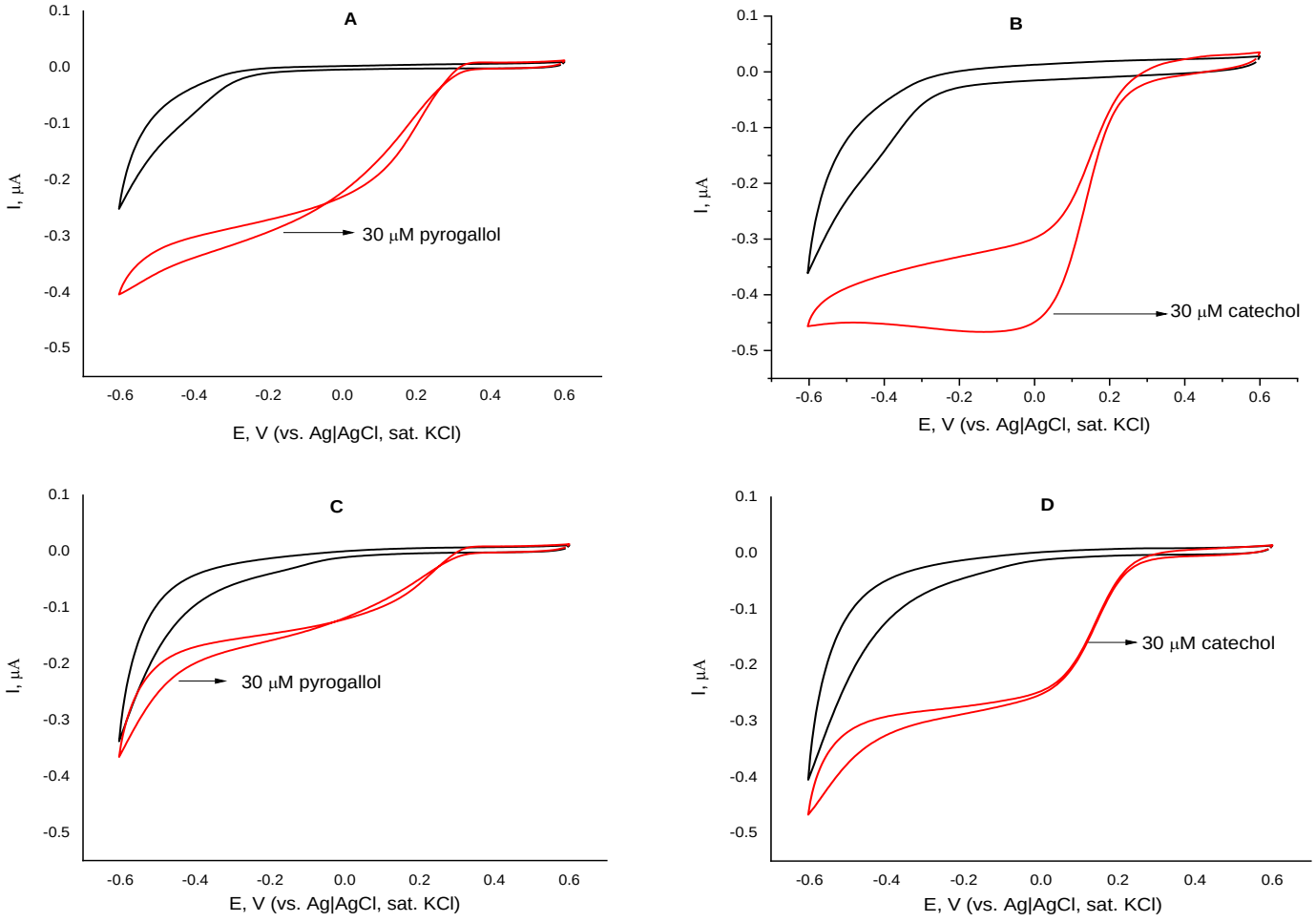

Figure 1. Cyclic voltammograms of enzyme electrodes modified with laccases from *Trametes pubescens* (A, B) and *Trametes versicolor* (C, D) in background electrolyte (black solid lines) and the presence of 30 μM laccase substrates (red solid lines): pyrogallol (A, C) and catechol (B, D).

Caffeic and gallic acids could be considered as phenolic compounds derived from catechol and pyrogallol, respectively. Their structural similarities with dihydroxyl and trihydroxyl aromatic compounds, as well as the fact that they are the usual constituents of polyphenolic complex in various natural products, motivated further interest in probing the voltammetric behavior of the produced biosensors in the presence of the two phenolic acids. On the CVs recorded in the absence of either phenolic compound (Fig. 2, black lines), a reductive wave was recorded with potentials more negative than -0.3 V that resulted from the electrochemical reduction of the dissolved molecular oxygen catalyzed by the immobilized enzyme, undoubtedly verifying that the two laccases were not only electrochemically, but also catalytically active.

The CVs recorded in the presence of either gallic or caffeic acid (Fig. 2, red lines), showed a clearly expressed reductive wave starting much earlier below +0.1 V, which was due to the fact that the two phenolic acids mediated the electrochemical reduction of dissolved oxygen and therefore significantly decreased the overpotential of the oxygen reduction on laccase-bearing electrodes. As seen from the presented plots, the interactions of the two laccases with the two phenolic acids resembled the shapes of the voltammograms recorded in the presence of catechol and pyrogallol. It is noteworthy that the efficiency of the enzyme interaction with the two phenolic acids is different being much higher in the presence of caffeic acid as it could be deduced from the pronounced reductive waves (Fig. 2C, D). Reaction with lower intensity between either of the two laccases with gallic acid was possibly resulting from electrostatic repulsion of its anionic form generated at the operating pH 4.0 [35] and the electrode surface, which also bore negative charges due to the coverage with a Nafion film.

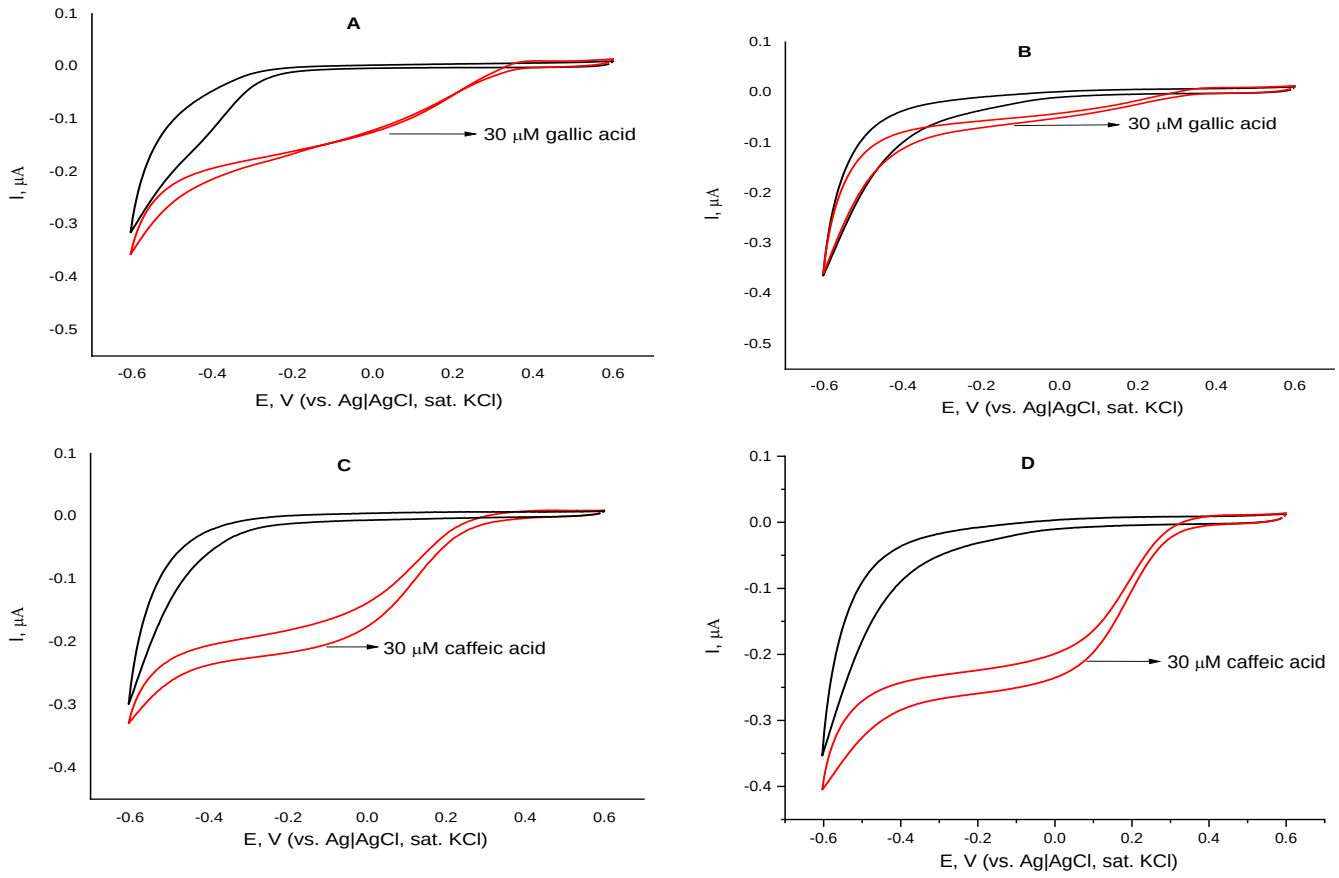

Figure 2. Cyclic voltammograms of the enzyme electrodes modified with laccases from *Trametes pubescens* (A, C) and *Trametes versicolor* (B, D) in background electrolyte (black solid lines) and in the presence of 30 μM (red solid lines) gallic acid (A, B) and caffeic acid (C, D).

3.2. Differential pulse voltamperometric response of laccase-based bioelectrodes to gallic and caffeic acids using laccases from *Trametes versicolor* and *Trametes pubescens*

Differential pulse voltammograms (DPV) of same laccase electrodes are depicted in Figure 3 (Fig. 3A, B; dashed curves). No peaks were identified on the DPVs of laccase-bearing electrodes in background electrolyte and the sharp current decay at potentials more negative than -0.4 V confirmed that oxygen reduction reaction occurred on the bioelectrode's surface. To investigate further the voltammetric behavior of immobilized laccase in the presence of the two phenolic acids – gallic and caffeic acids, the differential pulse voltammograms were recorded at varied substrates' concentrations. The addition of gallic acid aliquots to the buffer followed by the record of resulting DPV (Fig. 3A) caused a significant increase in the current with a peak at -0.2 V on the voltammograms, the height of which increased with increasing gallic acid concentration.

No shift of the peak position was reported upon raising its concentration. Similarly, in the presence of caffeic acid (Fig. 3B), a clearly expressed peak at a more positive potential than the one for gallic acid was recorded, the height of which increased proportionally to substrate concentration. The difference in the behavior of the laccase biosensor in the presence of caffeic acid as an enzyme substrate was that the reductive peak occurred at a potential of +0.2 V and the peak potential slightly shifted positively with increasing substrate concentration. It is plausible that the penetration of gallic acid was hampered by the deprotonation of its carboxylic group at the working pH due to the electrostatic repulsion between the negatively charged Nafion membrane and the gallic acid anionic form, resulting in a significant shift of the reduction potential to more negative values than those of caffeic acid. The latter was not deprotonated at the operating pH of the media [36] and hence its molecules penetrated the membrane easier. A similar behavior of the second laccase from *T. pubescens* was seen under equivalent experimental conditions.

A Michaelis type dependence between the DPV peak height and caffeic acid concentration was observed over a range from 0.01 up to 1 mM (Fig. 4A). Enzyme inhibition by the substrate of *T. pubescens* laccase became obvious at concentrations exceeding 0.5 mM, while enzyme isolated from *T. versicolor* seemed unaffected by substrate inhibition even at 1 mM concentration. Under equivalent experimental conditions, the dependence of the DPV peak current on gallic acid concentration (Fig. 4B) was based on Michaelis type kinetics only for *T. versicolor* laccase, while differential response of the biosensor based on the enzyme from *T. pubescens* decayed sharply in the presence of trihydroxy aromatic compound (Fig. 4B, red).

Despite the substantial differences between the DP voltammograms and the large peak separations recorded in the presence of either gallic or caffeic acid, DPV studies performed in combinations of the two phenolic acids did not allow discrimination between di- and triphenols. On DPVs recorded in 1:1 mixture of gallic and caffeic acids (Fig. S2, Supplementary information), the peak at -0.2 V appearing in the presence of gallic acid alone merged with the one typical for caffeic acid and the peak potential shifted negatively. With increasing the gallic acid quota up to 10 times, the two peaks broadened and turned into humps, the position and height of which varied irregularly with increasing the concentration of the mixture.

Information from DPV studies clearly demonstrated that this electrochemical technique could hardly be used for analysis of mixtures of the studied di- and triphenolic compounds, which motivated further investigating an alternative electrochemical approach that could potentially be further useful for the analysis of complex mixtures as amperometric detection.

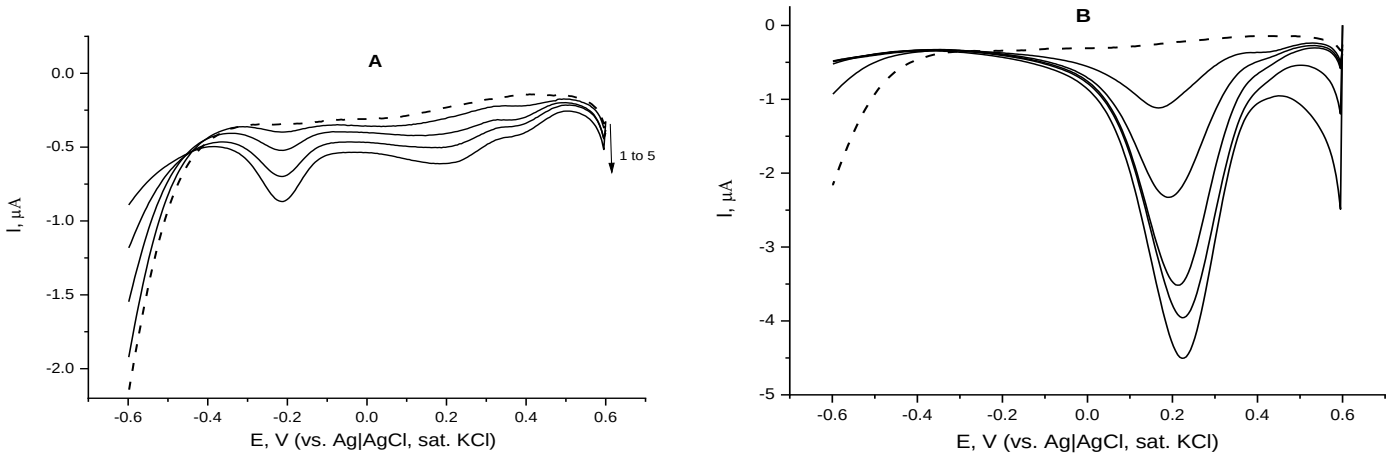

Figure 3. Differential pulse voltammograms of enzyme electrodes modified with laccase from *Trametes versicolor* in background electrolyte (black dashed lines) and in the presence of different concentrations (solid lines) of gallic acid (A) and caffeic acid (B).

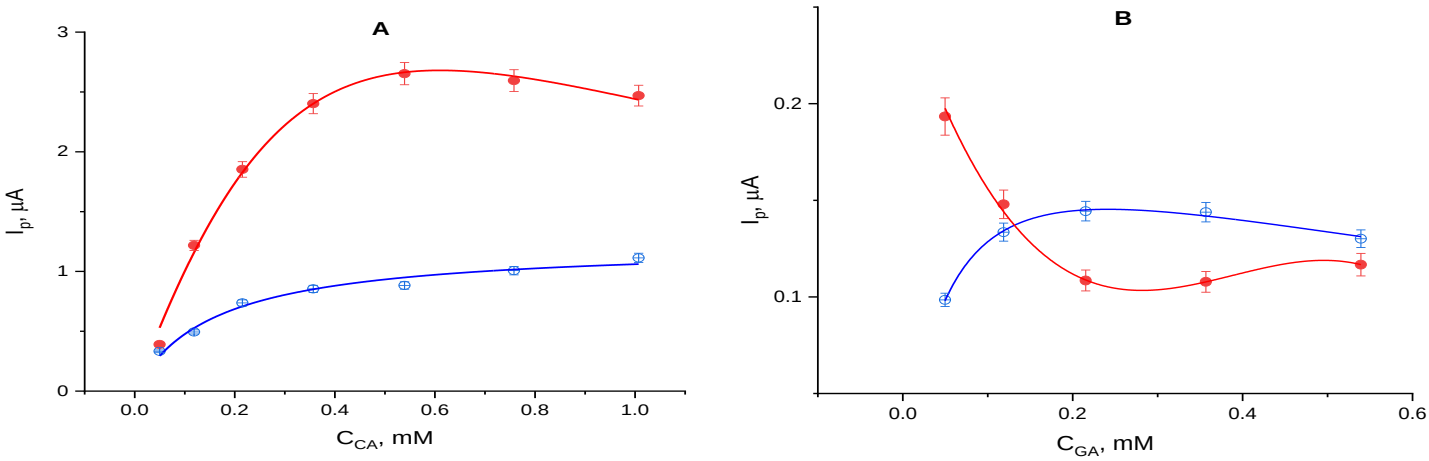

Figure 4. Dependencies of the DPV peak current on caffeic acid (A) and gallic acid (B) concentration for enzyme electrodes modified with laccases from *Trametes pubescens* (red) and *Trametes versicolor* (blue).

3.3. Amperometric detection of gallic and caffeic acids with laccase-based biosensors using laccases from *Trametes versicolor* and *Trametes pubescens*

In Fig. 5 are depicted the dependencies of the electrode response on the concentration of gallic and caffeic acids assessed with the two types of laccase-based electrodes under the working conditions selected as optimal: working potential of -0.2 V at pH 4.0 [29]. Similarities in the shapes of the curves were substantial. The two types of electrodes showed hyperbolic trends of electrode response as a function of substrate concentration. However, the apparent kinetic constants for the two immobilized laccases, determined from non-linear regression analysis of experimental data, showed significant differences. The apparent Michaelis constants for immobilized *T. versicolor* laccase (Fig. 5, A) with respect to caffeic and gallic acids have been found to be very similar (Table 1), while the corresponding apparent maximum rates of the enzyme catalyzed reaction differed significantly with the V_{max}^{app} for the caffeic acid being almost 5 times higher than that for gallic acid.

Unlike these, K_M^{app} for the immobilized laccase from *T. pubescens* (Fig. 5B) with respect to caffeic and gallic acids differed more than 3 times (0.19 mM ±0.03 against 0.06 mM ±0.04, respectively, Table 1) whereas the apparent maximum reaction rates diverged more than those estimated for *T. versicolor* laccase. These differences in the apparent kinetic constants of the two identically immobilized enzymes suggested that despite biochemical similarities of the two laccases, significant differences were reported in enzyme affinity to dihydroxy and trihydroxy aromatic compounds. Since the reaction rate was equal to the electrode response (current, in amperes [A]), significant differences in V_{max}^{app} for the two laccases (Table 1) indicated that *T. pubescens* laccase respectively oxidized caffeic and gallic acids 5 and 4 times as fast as that from *T. versicolor*. Moreover, these differences in the heterogeneous enzymatic activities were observed regardless of equivalent units of the immobilized laccases.

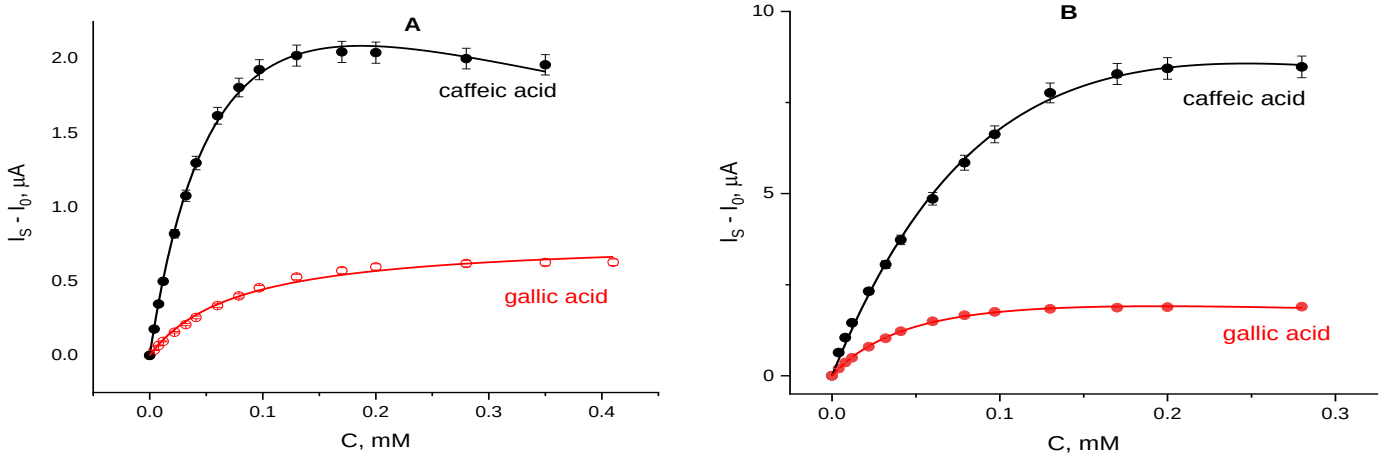

Figure 5. Dependence of the biosensor response on substrate concentration for electrodes based on laccases from A) *T. versicolor* and B) *T. pubescens* in the presence of gallic acid (red) and caffeic acid (black). Graphs were plotted based on chronoamperometric records at -0.2 V vs. Ag|AgCl (KCl sat.) in citrate buffer containing 0.1 M $NaClO_4$ at pH 4.0 and RT.

Table 1. Apparent kinetic constants of the two types of laccase biosensors based on the interaction with gallic and caffeic acids, and the regression coefficient (R^2). The kinetic constants were determined by non-linear regression analysis of the experimental data.

	Trametes pubescens laccase		*Trametes versicolor* laccase	
Kinetic constants	Caffeic acid	Gallic acid	Caffeic acid	Gallic acid
V_{max}^{app}, μA	21.90± 2.70	3.11 ± 0.12	3.77 ± 0.17	0.79 ± 0.02
K_M^{app}, mM	0.19 ± 0.03	0.06 ± 0.00	0.08 ± 0.06	0.08 ± 0.01
K_I^{app}, mM	0.32 ± 0.08	0.62 ± 0.09	0.46 ± 0.06	–
R^2	0.99_9	0.99_9	0.99_9	0.99_3

In all cases except one, significant inhibition effects by the substrate were seen at concentrations exceeding 0.1 mM with inhibition constants calculated by non-linear regression (Table 1). Only *T. versicolor* laccase showed no inhibition upon addition of gallic acid at concentrations exceeding 0.15 mM.

From the non-linear regression analysis of the kinetic curves, it was found that the electrode response obeyed the Eq.1:

$$I = \frac{V_{max}^{app} C}{\left(K_M^{app} + C\left((1 + {}^{C}/_{K_I^{app}})\right)\right)} \quad \text{Eq.1}$$

Where, V_{max}^{app} is the apparent maximum rate of the enzyme-catalyzed reaction, A;

K_M^{app} and K_I^{app} are the apparent Michaelis constant and inhibition constant, respectively, M;

C is the concentration of the respective phenolic acid, M;

I is the electrode response directly proportional to the rate of enzyme-catalyzed reaction, A.

All electrochemical measurements were done in triplicate with a RSD not exceeding 3.5 %.

3.4. Cyclic voltammetry analysis of phenolic acids in herbal extracts using laccases from *Trametes versicolor* and *Trametes pubescens*

The available three types of herbal extracts were tested for their redox behavior by performing cyclic voltammetry with the produced two types of laccase-based enzyme electrodes in the operating buffer; to which, equal volumes of each extract were added. Shapes of the resulting CVs (Figs. S3 and S4, Supplementary information) suggested that in the extract of type PM1, triphenols predominated while the other two types of herbal extracts contained a combination of di- and trihydroxy aromatic compounds. It is noticeable that the biosensor based on laccase from *T. pubescens* was responding ca. three times more intensely than the analogous one based on laccase from *T. versicolor*.

Quantitative analysis of herbal extracts has been carried out by means of constant potential amperometry (amperometric detection) using standard addition method. Method of standard additions (MSA) is typically carried out by adding small volumes of concentrated solution of the analyte to the sample [37]. The major advantage of the standard addition method is the opportunity to practically eliminate effects of the complex matrix in the real samples. As an external standard, 10 mM solutions of gallic acid were chosen and the MSA was implemented with the two discussed types of laccase biosensors. The one produced based on the laccase from *T. pubescens* (Fig. 6) guaranteed at least ten times more intense biosensor response to the same amount of PM1 herbal extract, compared to the signal of the *T. versicolor* laccase-based biosensor (Fig. 7). The latter finding led to greater RSD and significantly overestimated levels of the analyzed compounds; from which, it could be concluded that the second type of biosensor was not appropriate for further analyses. Based on the latter finding, the content of phenolic acids of the other two types of herbal extracts was analyzed using *T. pubescens* laccase-based biosensor, method of standard addition and gallic acid as an external standard.

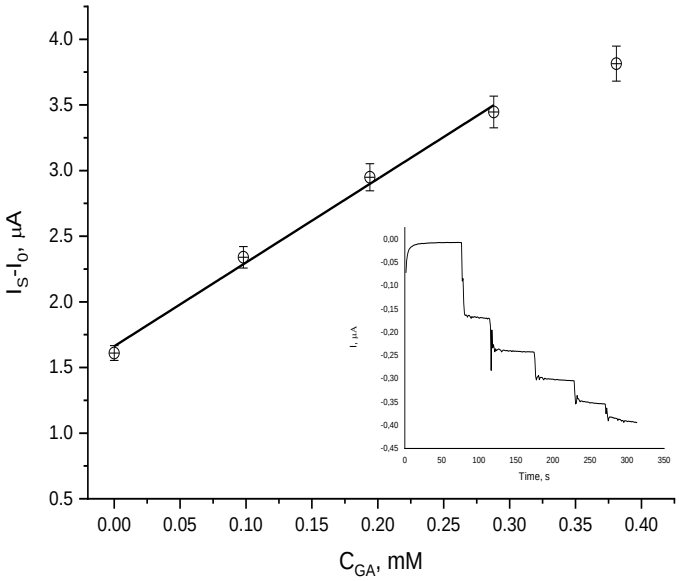

Figure 6. Standard addition method carried out with *Trametes pubescens* laccase-based biosensor: 100 μl of herbal extract type PM1 were added to the background electrolyte followed by four aliquots of 100 μl of 10 mM gallic acid (GA). Inset: authentic chronoamperometric record of the assay. Applied electrode potential E = -0.2 V vs Ag|AgCl (KCl sat.) in citrate buffer containing 0.1 M $NaClO_4$ at pH 4.0 and RT.

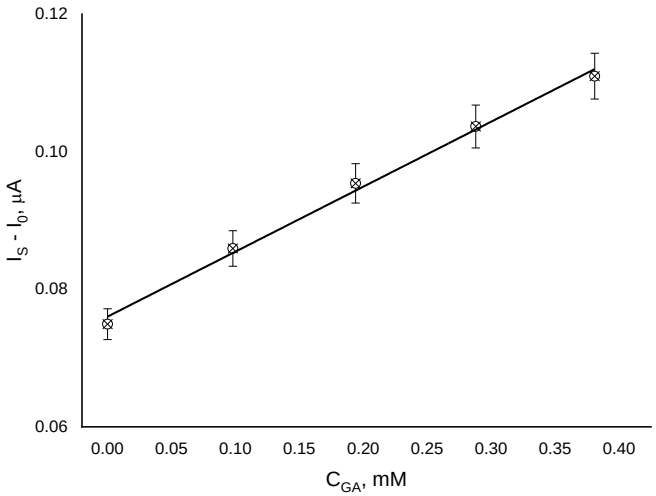

Figure 7. Linear plot of the standard addition method carried out to quantify phenolic content in herbal extract type PM1 with a laccase (*Trametes versicolor*) biosensor. All conditions as in Fig. 5.

The outcomes from biosensor analysis were compared with those from HPLC analyses of the three types of extracts (Table 2). Quantities of the phenolic compounds, estimated from the MSA, were further multiplied by dilution factor and the resulted values were recalculated into gallic acid equivalents per grams of dry weight herbs (DW). Based on HPLC analysis of the phenolic acids, the studied herbal extracts included gallic, caffeic, chlorogenic, p-coumaric and trans-ferulic acids.

Table 2. Comparison of the content of phenols expressed in milligram of gallic acid equivalents per gram of dry herbs (GAE. DW^{-1}) analyzed with laccase (*Trametes pubescens)* biosensor and high-performance liquid chromatography analysis of phenolic acid content.

Herbal extract, type	Biosensor analysis ($GAE.DW^{-1}$)	Total polyphenolic content* ($GAE.DW^{-1}$)	Recovery, %
PM1	214.2	216.0	99.0
PM3	131.0	189.0	69.3
PM7	48.3	56.5	85.4

* Determined by HPLC as a sum of all phenolic acids.

As concluded from the present results, the recovery percentage was good only for the type PM1 herbal extract while for the other two extracts biosensor method showed significant deviations from the satisfactory recovery percentages (95-105%). This might be due to the presence of significant quantities of m-benzenediols (e.g. resorcinol and its derivatives) and/or other types of polyphenols, which did not react with laccase.

The two electroanalytical techniques, DPV and constant potential amperometry, were used to find the most convenient approach for the assessment of phenolic content in model solutions to adopt it for the analysis of real samples comprising numerous interferents. However, the hypothesis that the two-model phenolic compounds, benzenediol caffeic acid and benzenetriol gallic acid, could be discriminated in their mixtures based on their DPV peaks that were not well-separated, was not verified due to the strong interferences between them when combined. The constant potential amperometry was shown as a better technique for the quantification of benzenediols and benzenetriols. Large differences in the rates of their electrochemical conversion did not provide means for their separate analysis as well.

4. Conclusion

The present results suggested that only enzymes with high homogeneous activity might be used for electroanalytical purposes. As previously discussed, low activity might lead to overestimated analyte levels (Figure 7). The decreased heterogeneous activity of *T. versicolor* laccase caused a stronger interference of a complex matrix that could not be eliminated even by the method of standard addition.

Indeed, HPLC analyses could provide not only quantitative, but also qualitative information regarding types of phenolic compounds such as benzenediols and benzenetriols. However, biosensing method provides two important advantages over the chromatographic analysis – it can be carried out rapidly with minimum sample pretreatment and equipment, which is much susceptible to miniaturization allows in-field analysis mostly.

5. Acknowledgements

This study was funded by the European Union-NextGenerationEU through the National Recovery and Resilience Plan of the Republic of Bulgaria; grant no. BG-RRP-2.004-0001-C01, DUEcoS. Authors gratefully acknowledge the generous gift of purified *T. pubescens* laccase from Prof. Roland Ludwig, BOKU, Vienna, Austria.

6. Conflict of Interest

The authors report no conflict of interest.

7. Authors Contributions

All authors designed and contributed to this study. Conceptualization, N.D. and I.I, methodology, M.S, software, T.C, validation, A.P, M.S. and T.C, formal analysis, M.S. and M.N, investigation, M.S, A.P. and M.N; resources, N.D, data curation, M.S. and M.N, writing—original draft preparation, M.S. and T.C, writing—review and editing, N.D, visualization, M.S, supervision, N.D. and I.I, project administration, I.I, funding acquisition, I.I. All authors have read and agreed to publish the final version of the manuscript.

References

1. Shahidi F. Antioxidants: Sources, efficacy and health effects. J Func Foods 2015; 18: iii. https://doi.org/10.1016/S1756-4646(15)00484-3
2. Shahidi F, Zhong Y. Measurement of antioxidant activity. J Func Foods 2015; 18: 757-781. https://doi.org/10.1016/j.jff.2015.01.047
3. Thuengtung S, Ogawa Y. Effects of Interactions Between Antioxidant Phytochemicals and Coexisting Food Components on Their Digestibility. In Encyclopedia of Food Chemistry, Melton L, Shahidi, F, Varelis, P, Eds, Academic Press: Oxford, 2019; pp. 656-660.
4. Karaosmanoglu H, Suthanthangjai W, Travas-Sejdic J, Kilmartin PA. Electrochemical analysis of beverage phenolics using an electrode modified with poly(3,4-ethylenedioxithiophene). Electro Chim Acta 2016; 201: 366-373. https://doi.org/10.1016/j.electacta.2016.02.022
5. Veeren B, Ghaddar B, Bringart M, Khazaal S, Gonthier MP, Meilhac O, Diotel N, Bascands JL. Phenolic profile of herbal infusion and polyphenol-rich extract from leaves of the medicinal plant *Antirhea borbonica*: Toxicity assay determination in zebrafish embryos and larvae. Molecules 2020; 25: 4482. https://doi.org/10.3390/molecules25194482
6. Gulcin İ. Antioxidants and antioxidant methods: an updated overview. Arch Toxicol. 2020; 94: 651-715. https://doi.org/10.1007/s00204-020-02689-3
7. Nejadmansouri M, Majdinasab M, Nunes GS, Marty JL. An overview of optical and electrochemical sensors and biosensors for analysis of antioxidants in food during the last 5 years. Sensor, 2021; 21: 1176. https://doi.org/10.3390/s21041176
8. Choleva TG, Kappi FA, Giokas DL, Vlessidis AG. Paper-based assay of antioxidant activity using analyte-mediated on-paper nucleation of gold nanoparticles as colorimetric probes. Analyt Chim Acta, 2015; 860: 61-69. https://doi.org/10.1016/j.aca.2014.12.025
9. Ye Y, Ji J, Sun Z, Shen P, Sun X. Recent advances in electrochemical biosensors for antioxidant analysis in foodstuff. Trend Anal Chem. 2020; 122: 115718. https://doi.org/10.1016/j.trac.2019.115718
10. Petrucci R, Bortolami M, Di Matteo P, Curulli A. Gold nanomaterials-based electrochemical sensors and biosensors for phenolic antioxidants detection: Recent advances. Nanomat. 2022; 12: 959. https://doi.org/10.3390/nano12060959
11. García-Guzmán JJ, López-Iglesias D, Marin M, Lete C, Lupu S, Palacios-Santander JM, Cubillana-Aguilera L. Chapter 4 - Electrochemical Biosensors for Antioxidants. In Advanced Biosensors for Health Care Applications, Inamuddin, Khan, R, Mohammad, A, Asiri, A.M, Eds, Elsevier: 2019; pp. 105-146.
12. Karikalan N, Karthik R, Chen SM, Chen HA. A voltammetric determination of caffeic acid in red wines based on the nitrogen doped carbon modified glassy carbon electrode. Sci Rep 2017, 7, 45924. https://doi.org/10.1038/srep45924
13. Munteanu IG, Apetrei C. A review on electrochemical sensors and biosensors used in assessing antioxidant activity. Antioxidant. 2022; 11: 584. https://doi.org/10.3390/antiox11030584
14. Oliveira-Neto JR, Rezende SG, De Fátima Reis C, Benjamin SR, Rocha ML, De Souza Gil E. Electrochemical behavior and determination of major phenolic antioxidants in selected coffee samples. Food Chem. 2016; 190: 506-512. https://doi.org/10.1016/j.foodchem.2015.05.104
15. Tomac I, Šeruga M. Electrochemical properties of chlorogenic acids and determination of their content in coffee using differential pulse voltammetry. Int J Electrochem Sci. 2016; 11: 2854-2876. https://doi.org/10.20964/110402854
16. Cerrato-Alvarez M, Bernalte E, Bernalte-García MJ, Pinilla-Gil E. Fast and direct amperometric analysis of polyphenols in beers using tyrosinase-modified screen-printed gold nanoparticles biosensors. Talanta 2019; 193: 93-99. https://doi.org/10.1016/j.talanta.2018.09.093
17. Tulli F, Gulotta FA, Martino DM, Paz Zanini VI, Borsarelli CD. Ultrasensitive amperometric biosensing of polyphenols using horseradish peroxidase immobilized in a laponite/Au/DNA-bioinspired polycation nanocomposite. J Electrochem Soc. 2018; 165: B452-B457. https://doi.org/10.1149/2.1191810jes
18. Arab Z, Jafarian S, Karimi-Maleh H, Roozbeh Nasiraie L, Ahmadi M. Electrochemical sensing of caffeic acid antioxidant in wine samples using carbon paste electrode amplified with CdO/SWCNTs. J Electr Sci Eng. 2023; https://doi.org/10.5599/jese.1701
19. Ma X, Yang H, Xiong H, Li X, Gao J, Gao, Y. Electrochemical behavior and determination of chlorogenic acid based on multi-walled carbon nanotubes modified screen-printed electrode. Sensors (Switzerland) 2016; 16. https://doi.org/10.3390/s16111797
20. David M, Florescu M, Bala C. Biosensors for antioxidants detection: Trends and perspectives. Biosensors, 2020; 10: 112. https://doi.org/10.3390/bios10090112
21. Dornelles Mello L, Kisner A, Oliveira Fonseca Goulart M, Tatsuo Kubota L. Biosensors for Antioxidant Evaluation in Biological Systems. Combinatorial Chemistry & High Throughput Screening, 2013; 16: 109-120. https://doi.org/10.2174/1386207311316020005
22. Forzato C, Vida V, Berti F. Biosensors and sensing systems for rapid analysis of phenolic compounds from plants: A comprehensive review. Biosensors, 2020; 10: 105. https://doi.org/10.3390/bios10090105
23. Litescu SC, Eremia S, Radu GL. Biosensors for the Determination of Phenolic Metabolites. In Bio-Farms for Nutraceuticals: Functional Food and Safety Control by Biosensors, Giardi, M.T, Rea, G, Berra, B, Eds, Springer US: Boston, MA, 2010; 234-240
24. Lzaod S, Dutta T. Recent Advances in the development of oxidoreductase-based biosensors for detection of phenolic antioxidants in food and beverages. ACS Omega 2022; 7: 47434-47448. https://doi.org/10.1021/acsomega.2c05604
25. Rodríguez-Delgado MM, Alemán-Nava GS, Rodríguez-Delgado JM, Dieck-Assad G, Martínez-Chapa SO, Barceló D, Parra R. Laccase-based biosensors for detection of phenolic compounds. TrAC Trend Anal Chem. 2015; 74: 21-45. https://doi.org/10.1016/j.trac.2015.05.008
26. Tarasov A, Stozhko N, Bukharinova M, Khamzina E. Biosensors based on phenol oxidases (laccase, tyrosinase and their mixture) for estimating the total phenolic index in food-

related samples. Life, 2023; 13: 291. https://doi.org/10.3390/life13020291

27. Kutraite I, Malys N. Development and application of whole-cell biosensors for the detection of gallic acid. ACS Synthetic Biology, 2023; 12: 533-543. https://doi.org/10.1021/acssynbio.2c00537
28. Sun B, Yang Y, Yu S, Bao L, Shi H, Dang Q, Liu Y, Yang L, Ma Q, Shi X. Electrochemical biosensor based on ds-DNA/N-G@CS/GCE for highly sensitive and rapid measurement of antioxidant activity. ECS Adv. 2023; 2: 026501. https://doi.org/10.1149/2754-2734/acd017
29. Shukri ME, Dimcheva N. Biosensing L-DOPA with laccase-based enzyme electrodes: a comparative study. Bul Chem Comm. 2024; 56: 95-99. https://doi.org/10.34049/bcc.56.D.S1P36
30. Nussinovitch U. Emerging Technologies for Heart Diseases: Volume 1: Treatments for Heart Failure and Valvular Disorders; Elsevier: 2020; pp. 1-524.
31. Espíndola KMM, Ferreira RG, Narvaez LEM, Silva Rosario ACR, Da Silva AHM, Silva AGB, Vieira APO, Monteiro MC. Chemical and pharmacological aspects of caffeic acid and its activity in hepatocarcinoma. Front Oncol. 2019; 9. https://doi.org/10.3389/fonc.2019.00541
32. Karcheva-Bahchevanska D, Lukova P, Nikolova M, Mladenov R, Iliev I. Inhibition effect of Bulgarian lingonberry (*Vaccinium vitis-idaea L.*) extracts on α-amylase activity. Comptes rendus de l'Académie bulgare des Sci. 2019; 72(2): 212-219. https://doi.org/10.7546/crabs.2019.02.10
33. Karcheva-Bahchevanska DP, Lukova PK, Nikolova MM, Mladenov RD, Iliev IN. Effect of extracts of bilberries (*Vaccinium myrtillus L.*) on amyloglucosidase and α-glucosidase activity. Folia Med. 2017; 59(2): 197-202. https://doi.org/10.1515/folmed-2017-0028
34. Pimpilova M, Kamarska K, Dimcheva N. Biosensing dopamine and L-epinephrine with Laccase (*Trametes pubescens*) immobilized on a gold modified electrode. Biosensors, 2022; 12: 719. https://doi.org/10.3390/bios12090719
35. Ertan K, Sahin S, Sumnu G. Effects of alkaline pH and gallic acid enrichment on the physicochemical properties of sesame protein and common vetch starch-based composite films. Int J Biol Macromol. 2024; 257: 128743. https://doi.org/10.1016/j.ijbiomac.2023.128743
36. Genaro-Mattos TC, Maurício AQ, Rettori D, Alonso A, Hermes-Lima M. Antioxidant activity of caffeic acid against iron-induced free radical generation-a chemical approach. Plos One 2015; 10. https://doi.org/10.1371/journal.pone.0129963
37. Andersen JET. The standard addition method revisited. TrAC Trends Anal Chem. 2017; 89: 21-33. https://doi.org/10.1016/j.trac.2016.12.013

Research Article

pISSN: 2345-5357
eISSN: 2423-4214

OPEN ACCESS

APPLIED FOOD BIOTECHNOLOGY, 2025, 12 (1): e5
Journal homepage: www.journals.sbmu.ac.ir/afb

مقایسه دو آنزیم لاکاز به‌دست آمده از *Trametes versicolor* و Trametes pubescens برای ارزیابی میزان فنولیک اسیدها با استفاده از حسگر زیستی مبتنی بر لاکاز

مرین شکری[۱]، تسوتینا چرنووا[۱و۲]، آنجل پشکوف[۱و۳]، ماریانا نیکولوا[۱و۳]، ایلیا ایلیف[۱و۳]، نینا دیمچوا[۱و۲*]

۱- دانشگاه پلوودیو "پایسی هیلندارسکی"، مرکز فناوری، ۲۱، خیابان کوستاکی پیف ، پلوودیو ، بلغارستان
۲- پلوودیو گروه شیمی فیزیک،۲۴، خیابان کوستاکی پیف ، پلوودیو ، بلغارستان
۳- پلوودیو گروه بیوشیمی و میکروبیولوژی،۲۱، خیابان کوستاکی پیف ، پلوودیو ، بلغارستان

تاریخچه مقاله

دریافت ۹ دسامبر ۲۰۲۵
داوری ۲۲ ژانویه ۲۰۲۵
پذیرش ۳ فوریه ۲۰۲۵

واژگان کلیدی

- آنزیم لاکاز
- حسگر زیستی الکتروشیمیایی
- ترکیبات معطر دی‌هیدروکسی و تری‌هیدروکسی
- عصاره‌های گیاهی

نویسنده مسئول

نینا دیمچوا

تلفن: ۳۰۹ ۲۶۱ ۳۲ ۳۵۹+
همراه، ۳۵۹ ۸۹۵۳۷۲۹۷۵+
پست الکترونیک:
ninadd@uni-plovdiv.bg

چکیده

سابقه و هدف: دو آنزیم لاکاز جدا شده از بازیدیومیست‌ها (*Trametes sp.*)، علی‌رغم تشابه بیوشیمیایی، از نظر میل ترکیبی با دو نوع ترکیب فنولی تفاوت دارند، برهمکنش‌ها با دی‌فنول‌ها (کاتکول و کافئیک اسید) قوی‌تر از برهمکنش با بنزنتریول‌ها (پیروگالیک اسید و پیروگالول) می‌باشد. کارایی کاتالیزوری لاکاز *Trametes pubescens* ۴-۵ برابر بیشتر از لاکاز تجاری (*Trametes versicolor*) تشخیص داده شد. در این مطالعه، برهمکنش دو آنزیم تثبیت شده با دی و تری فنول‌ها با روش‌های گوناگون الکتروشیمیایی مورد بررسی قرار گرفت.

مواد و روش ها: در این مطالعه از روش‌های الکتروشیمیایی: ولتامتری سیکلی، کرونوآمپرومتری و ولتامتری پالس تفاضلی استفاده شد. آزمایش‌ها در غلظت‌های گوناگون رشدمایه[۱] انجام شد. فعالیت و حساسیت دو حسگر زیستی جایگزین مبتنی بر لاکاز با استفاده از DPV (کرونوآمپرومتری) و کرونوآمپرومتری مقایسه شد.

یافته‌ها و نتیجه‌گیری: اندازه‌گیری‌های ثابت پتانسیل آمپرومتریک نشان داد که هنگام برهمکنش با اسیدهای کافئیک و گالیک، حسگر زیستی تولید شده با لاکاز *Trametes pubescens* بسیار فعال‌تر از حسگر زیستی مبتنی بر لاکاز *Trametes versicolor* است. میزان ترکیبات فنولی سه عصاره گیاهی گوناگون با حسگرهای زیستی لاکاز توسعه‌یافته ارزیابی شد و نتایج مشابه آنالیز کروماتوگرافی مورد استفاده در روش مرجع بود. بنابراین، از حسگرهای زیستی می‌توان برای آزمایش سریع میزان ترکیبات فنولی در نمونه های واقعی استفاده کرد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[۱] Substrate هر ماده‌ای که نیاز غذایی و بستر زیستِ ریزاندامگان‌ها را فراهم کند