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Utilization of enzyme extract self-encapsulated within polypyrrole in sensitive detection of catechol

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ABSTRACT

Utilization of polyphenol oxidases (laccase, tyrosinase) in biosensor technology is an efficient approach towards phenol detection, which is significant in numerous fields such as environmental monitoring, food industry *etc.* The use of crude extract instead of pure enzyme eliminates the need for costly and laborious processes of enzyme separation and purification. This study employs polyphenol oxidase extract, biosynthesized by white-rot fungi *Trametes pubescens* (*TP*) for the development of amperometric biosensors for catechol detection. The catalytic activity of the crude extract was firstly used to induce the bio-synthesis of conducting polymer - polypyrrole (Ppy), resulting in the self-encapsulation of the enzyme extract within the conducting material. The viability and biological integrity of the enzyme extract was preserved after the synthesis and was able to efficiently detect phenolic compounds such as catechol. Comparative evaluations between the biosynthesized Ppy based biosensor (bio-Ppy) and the biosensor based on bio-PPy with additional enzyme extract (bio-Ppy-*TP*) were performed. Lastly, the performance of these two biosensors was compared with that of a third one, based on chemically synthesized Ppy with enzyme extract (chem-Ppy-*TP*). All three types of biosensors proved high efficiency for catechol detection at low concentration (1–60 μM) and were employed for real sample detection in fruit wines showing linear correlation with the spectrophotometric results obtained with the Folin-Ciocalteau standard test.

1. Introduction

Phenolic compounds are widely scattered, being encountered in a broad range of industries such as environmental and industrial fields. Some of them have been listed as hazardous substances due to their various sources (insecticides, disinfectants) along with the ability to contaminate the environment as a byproduct of industrial or agricultural processes. Their toxicity and persistence in the environment after release in ground and surface water increases the risk for the environment and for human health [1]. As such, the development of simple and reliable analytical methods for the detection of phenols is essential in fields such as chemical, food or pharmaceutical industry [2,3]. For instance, in food chemistry, wine analysis is an essential area regarding quality assessment [4,5]. Wine represents a complex sample due to several classes of compounds such as ethanol, glycerol and saccharides. A wide variety of polyphenols, commonly known as tannins are also present in wine in lower concentration. They play an important part in food quality due to their antioxidant properties [6,7], yet their detection is often costly and complex being that they are seldom determined separately, instead they are quantified collectively in a total polyphenol index [8–10]. The most common methods employed are chromatographic, yet they require costly and advanced equipment.

Biosensor technology proves highly suitable for this type of detection due to facility in preparation, reproducibility and low cost [11–13]. In particular, amperometric biosensors exhibit high specificity derived from the selectivity of the biocatalyst and the accuracy of the electrochemical transducer. The immobilization of oxidoreductases on electrodes is one of the most common concepts in biosensors and its relevance is given by the facility of monitoring the enzymatic reaction by electrochemical means [14]. Polyphenol oxidases (PPOs) such as laccase and tyrosinase are a group of enzymes highly employed for the electrochemical detection of phenols in wastewaters [15,16], food and beverages [17,18] and pharmaceuticals [19,20]. The complexity of polyphenol-containing samples led to the co-immobilization of these enzymes on the electrode [21–24] with the aim of widening the range of phenols available for detection, due to the difference between the catalytic mechanisms of these enzymes [25].

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Another aspect to be considered is the modification of electrodes with conducting polymers (CPs), which represents a valuable tool in the design of electrochemical devices due to the ability to facilitate charge transfer through their intrinsic conductivity, thus improving the contact between the biorecognition molecule and analyte [26–28]. The use of polypyrrole (Ppy) for this purpose has been the subject of extended research over the last decades since it can act both as transducer and as an immobilization matrix [29]. The established techniques to achieve this type of modification are the immobilization of enzymes prior to polymerization of desired monomer (*e.g.* pyrrole) [30–33] or the direct electrochemical entrapment during electrosynthesis [29,34,35]. Albeit the convenience of these approaches, they are prone to some limitations such as the tendency of the biocatalyst to leach out from the polymer coating, leading to diminished performance over time or the possibility of biomolecule denaturation/inactivation given by the harsh conditions encountered during polymer electrosynthesis [26]. This can result in reduced response towards analyte [36].

More recently, biocatalytic methods for Ppy synthesis and, consequently, immobilization of biomolecules, have emerged. Enzymes can replace large quantities of chemical oxidants and allow for thermodynamically efficient reactions whilst maintaining their biological function and integrity [37]. Enzymatic catalysis of CPs usually implies environmentally friendly conditions such as physiological temperature and pH [38]. The biocatalytic polymerization of pyrrole has been investigated following different approaches. It has been a result of oxidation with hydrogen peroxide (H_2O_2) generated *in situ* by glucose oxidase [39] or by lactate oxidase [40] or the result of the reaction between either peroxidase [41–43] or laccase [44,45] and the pyrrole monomer. Ultimately, this process entails a one-step *in situ* polymerization and immobilization of the enzymes employed as biocatalysts in the first place or so-called 'self-encapsulation' of the biomolecule within the conducting polymer. This leads to the development of a conducting material with intrinsic biocatalytic properties, which is ideal for biosensor development. Moreover, this type of synthesis dismisses the use of strong oxidants or harsh synthesis conditions, which, in turn, increases the chances for biomolecule viability and functional integrity. The Ppy prepared as such is more strongly linked to the enzymes, thus, the liability for biomolecule leaching is diminished. The biocatalytic synthesis of CPs using glucose oxidase was recently investigated in terms of applicability in electrochemical devices [39,46,47] and led to several interesting applications such as the visualization of oxidoreductases on electrode surface and improvement of upper detection limit in biosensorics [48,49].

The present study explores this alternative by using crude polyphenol oxidase (PPO) extract from submerse cultivation of the white-rot fungi strain, *Trametes pubescens* (*TP*), which is self-encapsulated within the Ppy biosynthesized *in situ* (bio-Ppy). Firstly, using the enzyme extract eliminates the need for costly and laborious processes of enzyme separation and purification as well as the need for co-immobilization of separate polyphenol oxidases such as laccase and tyrosinase. In addition, the Ppy incorporating the enzyme extract represents a suitable material for electrode modification for the detection of phenols. As such, the *TP* extract used fulfills a two-fold purpose: (i) catalyzes Ppy biosynthesis; (ii) remains available for performing phenol detection. In order to assess the viability and functional integrity of the enzyme extract upon being immobilized within the bio-Ppy, an additional biosensor including both bio-Ppy and additional *TP* extract was prepared. Lastly, the performance of these two biosensors was compared with a third one based on chemically synthesized Ppy (chem-Ppy) mixed with the *TP* extract. The amperometric response of these three biosensors to three common phenols (catechol, gallic acid, caffeic acid) was compared and catechol was determined as the most suitable analyte. Considering the nutritional importance of polyphenols, the amperometric detection of catechol in fruit wines was chosen as real sample study. Fruit wines represent rich sources of antioxidants and, depending on the type of fruit, different catechol contents have been detected and compared with the results obtained with the standard Folin-Ciocalteau spectrophotometric method.

2. Experimental

2.1. Microorganism cultivation and synthesis of Ppy

The white-rot fungi strain *Trametes pubescens* (*TP*) was acquired from the Culture Collection of Faculty of Biology, *Alexandru Ioan Cuza* University of Iasi and deposited in the Microbial Culture Collection of Bioaliment Research Center of Faculty of Food Science and Engineering of *Dunărea de Jos* University of Galaţi, Romania. The inoculation of agar plugs from the growing zone of fungi on Petri dishes in 100 mL Sabouraud Dextrose Agar (SDA) media led to the formation of a vegetative mycelial suspension during incubation at 27 °C for 4–5 days (Heidolph® Model 1000 Incubator Heating Unit). Submerse cultivation entailed inoculation with 1 mL of the obtained mycelial suspension using 100 mL YPD broth (5%) (pH 5.5) and incubation on an orbital shaker (Heidolph® Unimax 1010 Orbital Shaker) at 27 °C and 180 rpm.

The crude enzyme extract within 72 h of submerge cultivation was separated from the culture by centrifugation and the pyrrole monomer (30 mM/100 mL) was added at this point in the resulting supernatant. The formation of Ppy was observed within 96 h. The chemical synthesis of Ppy entailed addition of 30 mM pyrrole and 5 mM hydrogen peroxide (H_2O_2) in 100 mL 0.1 M phosphate buffer solution pH 2.0 and 48 h reaction time [50].

The first biosensor was based on the bio-synthesized Ppy as collected from the supernatant within 96 h (bio-Ppy biosensor). The second biosensor was based on the mixture (1:1 ratio) of bio-Ppy and unmodified *TP* extract (bio-Ppy-*TP* biosensor). The third biosensor was based on the mixture (1:1 ratio) of chemically produced Ppy and unmodified *TP* extract (chem-Ppy-*TP* biosensor).

2.2. Preparation of bioactive layers

For the bio-Ppy biosensor, 20 mg of gold gelatin sheet was mixed with 400 µL of Ppy suspension (synthesized in the culture supernatant) in an Eppendorf tube and then placed in a beaker, which contained previously heated (38 °C) 0.1 M PBS pH 7.0. After the dissolution of gelatin, 50 µL mixture was placed on top of the glassy carbon electrode (GCE) and let to dry. For the bio-Ppy-*TP* biosensor, 200 µL *TP* extract and 200 µL Ppy suspension were used. For the chem-Ppy-*TP* biosensor, 200 µL *TP* extract and 200 µL chem-Ppy suspension (after thorough washing with PBS 0.1 M pH 7.0) were used. The same procedure was employed for each sensor. Afterwards, the prepared electrodes were kept in refrigerator for 30 min-1 h and subsequently immersed in 2.5% glutaraldehyde solution for 5 min. The biolayer concept is described in Scheme 1. The electrode storage was done in 0.1 M PBS pH 7.0 at refrigerator conditions.

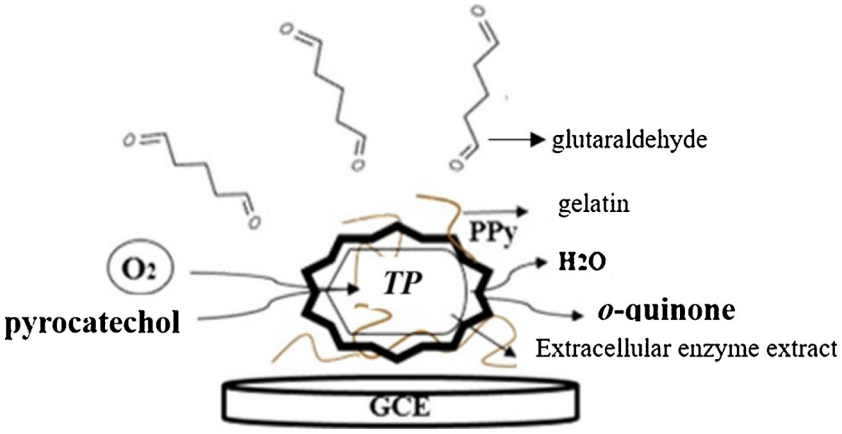
2.3. Equipment and principle of measurements

For each biosensor, the optimization of operational parameters (pH and potential) was performed. Catechol was selected as the model phenolic compound. Progressive concentrations of catechol were added in steady-state conditions under constant magnetic stirring and the oxygen consumption was monitored at negative potential.

Experiments were carried out using a Gamry potentiostat connected to a computer and Gamry Echem Analyst software was used for data analysis. A conventional 3-electrode electrochemical system ($Ag/AgCl_{(3M\ NaCl)}$ reference electrode, a Pt wire - counter electrode, GCE - working electrode) was used for amperometric measurements.

2.4. Real sample investigations and interpretation of results

For analysis in real system, three commercial fruit wines were



Scheme 1. Biolayer design for the Ppy-*TP* biosensors.

selected (blueberry, blackberry and pomegranate). To compare the biosensor performance in amperometric detection of phenols, the standard Folin-Ciocalteau spectrophotometric assay for total phenol content was used [51]. The protocol involves the use of gallic acid as standard (since it is representative for wine samples) and absorbance reading at 765 nm [52]. The assay can be performed using catechol as standard (since the biosensors developed exhibited highest preference for catechol) and, in this case absorbance was read at 650 nm [53]. Both calibration curves were linear ($y = 0.0015x + 0.0032$ ($R^2 = 0.9953$) for gallic acid, $y = 0.0016x + 0.0045$ ($R^2 = 0.9952$) for catechol) and the catechol assay was preferred for comparison in the real sample study.

Hyperbolic dependency between the amperometric signals and analyte concentration was observed and the calculation of kinetic parameters such as I_{max} and Michaelis-Menten constant (K_m) were determined from the reciprocal curve of the calibration plot *i.e.* the Lineweaver-Burk slope (1/I *vs* 1/C). The limit of detection (LOD) was calculated based on the response to minimum ten separate additions of 1 μM analyte using the formula 3x Standard Deviation of Low Concentration/ Slope of the calibration line.

3. Results and discussion

3.1. Optimization of operational parameters involved in the amperometric detection of catechol with the Ppy-TP biosensors

For each type of biosensor developed, pH and working potential optimizations were performed. The pH dependence within the range 4.5–7.5 and the potential dependence within -0.6 - -0.8 V *vs* Ag/AgCl are illustrated in Fig. 1.

The optimum pH for the bio-Ppy and bio-Ppy-*TP* biosensors is 5.0 and for the chem-Ppy-*TP* biosensor, the optimum pH value was 5.5. This analysis provides insight into the behaviour of the immobilized enzyme. Our previous research on the mechanism of biocatalysis responsible for the polymerization of pyrrole involving white-rot fungi has shown that the *TP* extract is a multi-enzymatic system in which laccase enzyme is predominant [54]. This is confirmed herein since pH values in the range 5.0–5.5 are commonly optimum for laccase catalysis [55,56]. The optimum range for biosensor activity is very similar to the one observed for soluble and purified laccase [57], indicating that the presence of Ppy did not affect the pH profile of the enzyme. The presence of other polyphenol oxidases such as tyrosinase in the *TP* extract is observed as well by the shoulder in the 6.5–7.5 pH range, which is reported as optimum for the activity of immobilized tyrosinase in phenol biosensors [29,58,59].

Optimum operational potential value was −0.7 V *vs* Ag/AgCl for the bio-Ppy biosensors and -0.65 V *vs* Ag/AgCl for the chem-Ppy-*TP* one. The optimization of operational parameters reveals that the type of synthesis employed in preparation of Ppy has a minor effect on the optimum conditions for amperometric detection of catechol.

3.2. Analytical characteristics and kinetic constants

Catechol detection mechanism was based on oxygen consumption during its bioconversion into quinone. The amperometric response was examined in optimized conditions of potential and pH after a steady state current value was reached. Progressive catechol additions started at concentrations of 1 μM and each addition led to amperometric increase, which was considered proportional with the catechol concentration added. Thus, standard graphs of current *vs* catechol

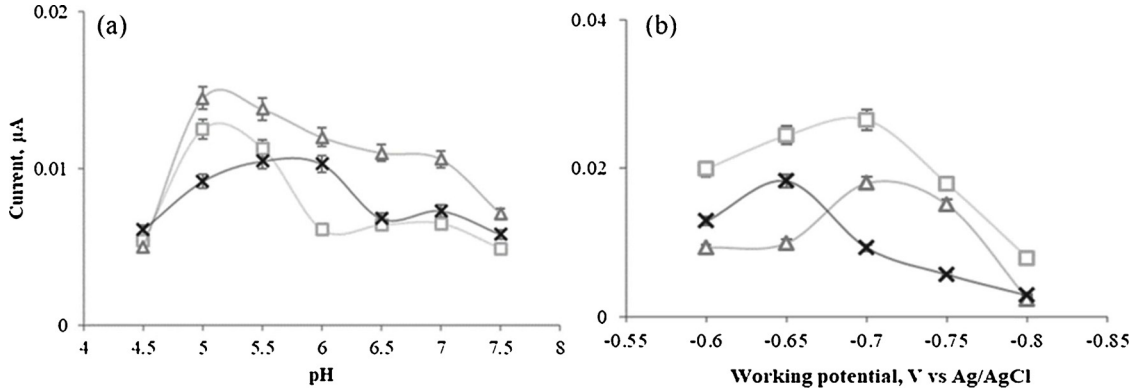


Fig. 1. (a) the effect of pH and (b) the effect of working potential on the response of the: bio-Ppy biosensor (□), bio-Ppy-*TP* biosensor (△) and chem-Ppy-*TP* biosensor (×); additions of 1 μM catechol.

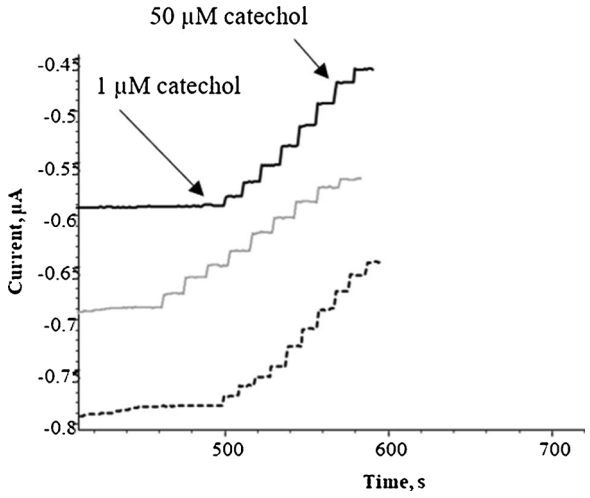


Fig. 2. The amperometric response of the developed biosensors towards catechol: bio-Ppy biosensor (▬), bio-Ppy-*TP* biosensor (—) (0.1 M PBS pH 5.0, V = −0.7 V *vs* Ag/AgCl), chem-Ppy-*TP* biosensor (▬) (0.1 M PBS pH 5.5; V = −0.65 V *vs* Ag/AgCl).

Table 1
Substrate selectivity of the Ppy-*TP* biosensors.

Substrate*	Current response (%)		
	bio-Ppy	bio-Ppy-*TP*	chem-Ppy-*TP*
Catechol	100	100	100
Caffeic acid	80	44.9	44.9
Gallic acid	86.6	48.9	57.2

* Substrate concentration: 10 µM.

Table 2
Analytical performance of Ppy-*TP* biosensors.

Type of biosensor	Linear range, µM	Sensitivity*, µA/mMcm^{-1}	LOD, µM	I_{max}, µA	$K_{m,}$ mM
bio-Ppy	1-60	30.40 ± 0.45	5.0	0.147	0.028
bio-Ppy-*TP*	1-70	34.61 ± 1.28	2.1	0.260	0.049
chem-Ppy-*TP*	1-60	37.45 ± 0.48	1.8	0.223	0.070

* Results of triplicate measurements.

Table 3
Comparison of analytical performances of various Ppy-PPO biosensors reported in literature.

Enzyme-analyte	Linear range (µM)	Sensitivity (µA/mMcm^{-1})	Reference
Tyr/Ppy – catechol	1 – 30	4.75 × 10^3	[60]
Tyr/nanoAu/Ppy – phenol	0.05 – 70	7.0 × 10^3	[28]
Tyr/Ppy – phenol	3.3 – 220.3	17	[61]
Tyr/Ppy – catechol	5.6 – 74.3	70	
Tyr/Ppy – phenol	1.35–222.3	1.75	[62]
Tyr/Ppy – catechol	1.6 – 118.8	3.46	
Tyr/Ppy – catechol	3 – 50	0.9 × 10^{-3}	[63]
Tyr/MWCNTs/Ppy – catechol	3 – 50	8 × 10^{-3}	
PPO extract* /bioPpy – catechol	1-60/70	30 – 40	This work

* PPO extract refers to polyphenol oxidase extract from *Trametes pubescens*.

concentration were plotted. Substrate addition was stopped when the calibration curve started to deviate from linearity. The comparison between the amperometric responses of the Ppy-*TP* biosensors, given in

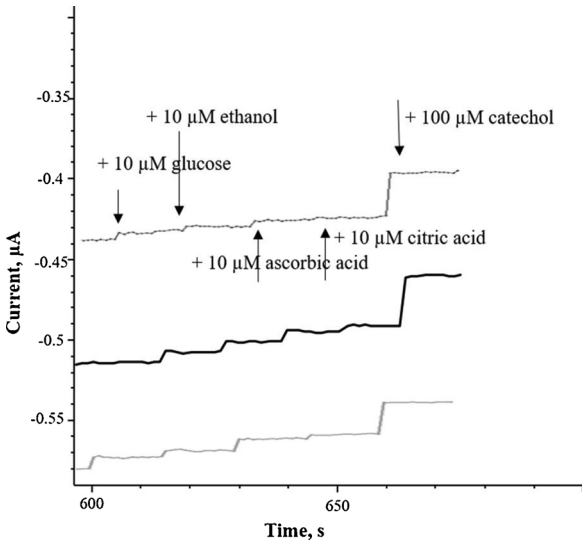


Fig. 3. Interference effect in the amperometric detection of catechol with the: bio-Ppy biosensor (▬), bio-Ppy-*TP* biosensor (—) (0.1 M PBS pH 5.0, V = −0.7 V *vs* Ag/AgCl), chem-Ppy-*TP* biosensor (▬) (0.1 M PBS pH 5.5; V = −0.65 V *vs* Ag/AgCl); the interfering compounds were added in the shown order and concentration for each biosensor experiment.

Fig. 2, reveals similarity for all the developed sensors and very good sensitivity for low concentrations of catechol.

The amperometric response towards other phenolic compounds (Fig. S1) was tested to determine the most suitable analyte for the developed biosensors, which proved to be catechol. The bio-Ppy biosensor exhibited the highest ability to detect other similar phenols (gallic acid, caffeic acid). Table 1 illustrates the substrate selectivity by percentage considering the current response to catechol as 100%.

The linear range and sensitivity were very similar for all the Ppy-*TP* biosensors proving efficiency for the detection of catechol in minimal concentrations. The calibration curves are depicted in Fig. S2 and the analytical parameters are given in Table 2.

Table 2. Analytical performance of Ppy-*TP* biosensors.

The linear ranges of the developed biosensors are between 1.0–60/70 µM with sensitivities ranging from 30.4 µA/mMcm^{-1} for the bio-Ppy biosensor to 37.45 µA/mMcm^{-1} for the chem-Ppy-*TP* biosensor. The detection limit is in the range of 5 to 1.8 µM, slightly improving when additional enzyme extract is added to the bio-Ppy electrode or when the chem-Ppy is used. On the contrary, the Michaelis-Menten constant (K_m), which illustrates the affinity of the enzyme towards substrate, has the lowest value for the simple bio-Ppy biosensor and slightly increases when additional enzyme extract is added. This is presumably due to the structural and/or conformational modification of the enzyme during self-encapsulation within bio-Ppy.

Since all the biosensors exhibit very similar behaviour, it can be confirmed that: (i) the enzymatic extract was incorporated within the *in situ* biosynthesized Ppy structure; it preserved its viability and ability for catechol detection, which was observed from the bio-Ppy *vs* bio-Ppy-*TP* comparison; (ii) the biosynthesis of Ppy is a valid and suitable method for biomolecule immobilization, comparable, in terms of efficiency, with the chemical synthesis using H_2O_2, as observed from the comparison between the bio-Ppy-*TP* and chem-Ppy-*TP* biosensors.

Table 3. Comparison of analytical performances of various Ppy-PPOs biosensors reported in literature.

The comparison with literature data (Table 3) shows that the biosensors developed have adequate linear ranges, very similar with several biosensors based on polyphenol oxidases and Ppy. The sensitivity is in normal ranges especially considering that the enzyme extract was used as such and not concentrated or purified.

Table 4
Real sample analysis of catechol content in fruit wines.

Analysis	Blueberry wine(mg/L)	Recovery* (%)	Blackberry wine(mg/L)	Recovery* (%)	Pomegranate wine(mg/L)	Recovery* (%)
Folin-Ciocalteau (catechol equiv.)	900.7		1519.5		2138.3	
bio-Ppy biosensor	1011 ± 179	112.34	1478 ± 147	97.20	2086 ± 231	97.53
bio-Ppy-*TP* biosensor	985 ± 22	109.40	1539 ± 51	101.28	2217 ± 45	103.70
chem-Ppy-*TP* biosensor	926 ± 85	102.85	1545 ± 43	101.71	2092 ± 24	97.83

* Results of triplicate measurements.

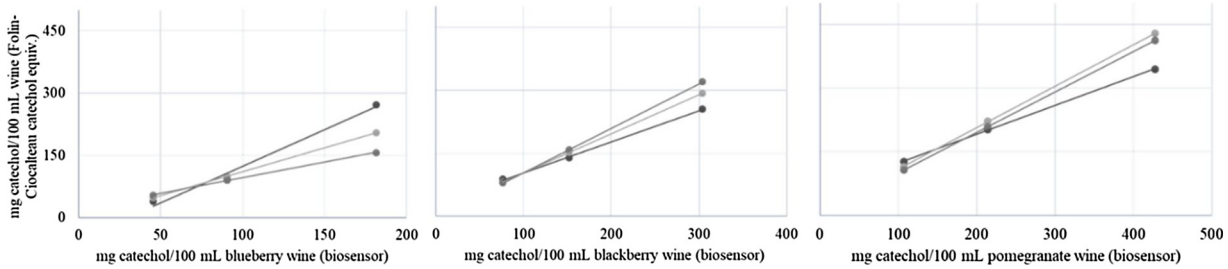


Fig. 4. Correlation between the detection of catechol by Folin-Ciocalteau spectrophotometric method and with the Ppy-*TP* biosensors (bio-Ppy ●, bio-Ppy-*TP* ●, chem-Ppy-*TP* ●).

3.3. Study of interference effect in the amperometric detection of catechol with the Ppy-TP biosensors

The amperometric determination of catechol can be dependent on the ability of other substances to be detected at the operational potential, also known as interference effect. Wines are very complex samples and it has been considered adequate to study the interference of the major compounds such as glucose, ethanol and organic acids (ascorbic and citric). Fig. 3 illustrates the current response registered for the Ppy-*TP* biosensors to the possible interfering compounds found in wine samples.

The interference study showed that the response of the biosensors to common compounds in wine is moderate to minimal with least interference on the bio-Ppy-*TP* system. These results are strictly related with the recovery rate in real system.

3.4. Amperometric detection of catechol in fruit wines

All the biosensors prepared were tested for catechol detection in fruit wines (blueberry, blackberry and pomegranate). As standard method for determination of phenol content, Folin-Ciocalteau spectrophotometric method was employed. Table 4 depicts the recovery rates for each biosensor and each type of wine and Fig. 4 illustrates the correlation between catechol determination by spectrophotometry and by amperometry.

The best recovery rates are obtained for the blackberry wine by all three biosensors. This is presumably due to the moderate content of phenols found in this wine. When the wine sample has a higher phenol content, such as the pomegranate sample, the recovery is slightly lower because the biosensors are better suited for the detection of trace amounts of phenolic compounds. This was proved by the highest recovery rates obtained for the blueberry wine, which has the lowest phenol content. In this case, the recovery rate was slightly over 100% due to the interference effect, which was mostly noticed for the bio-Ppy sensor. Overall, the correlation between spectrophotometric and amperometric detection of catechol is linear, proving suitable performance of the developed biosensors in real samples.

4. Conclusions

The present study displays a promising approach in biosensor technology, based on self-encapsulation of polyphenol oxidase enzyme extract (biosynthesized by *Trametes pubescens*) within conducting material - Ppy through biosynthesis resulting in a suitable material for electrode modification. The biological integrity of the *TP* extract was maintained through the self-encapsulation process. As such, the enzyme extract fulfills a two-fold purpose: (i) it catalyzes the Ppy biosynthesis; (ii) it remains available for catechol detection. Additionally, it was confirmed that bio-Ppy biosensor exhibits very similar characteristics to the one based on chemically synthesized Ppy, which makes the biocatalytic synthesis of Ppy, a promising approach.

The analytical parameters of the developed biosensors were adequate and comparable with literature data on the subject. The linear range albeit short, was within minimal range of concentration, thus being adequate for samples with moderate to low phenol content. The correlation between Folin-Ciocalteau spectrophotometric method and the amperometric analysis was linear, proving that the developed biosensors are effective in the detection of catechol in wine samples.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.enzmictec.2019.04.015.

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