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Label-free potentiometric biosensor based on solid-contact for determination of total phenols in honey and propolis

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Abstract

We developed a label-free potentiometric biosensor using tyrosinase extracted from *Musa acuminata* and immobilized by covalent bond on a surface of a solid-contact transducer. The transducer was manufactured containing two layers. The first layer contained a blend of poly(vinyl) chloride carboxylated (PVC-COOH), graphite and potassium permanganate. On this layer, we deposited a second layer containing just a mixture of poly(vinyl chloride) carboxylated and graphite. On the last layer of the transducer, we immobilized the tyrosinase enzyme by reaction with N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride.

The solid-contact potentiometric biosensor presented at low detection limit of 7.3×10^{-7} M and a linear range to catechol concentration between 9.3×10^{-7} M and 8.3×10^{-2} M. This biosensor was applied to determine the amount of total phenols in different samples of honey and propolis. The results agreed with the Folin-Ciocalteu method.

Keywords

Potentiometry; Biosensor; Tyrosinase; Grand Nain banana; Total phenols; Honey

1. Introduction

Honey is a viscous liquid of sweet flavor that is produced by bees from flower nectar [1]. Another food of bee origin is propolis that can be mixed with honey. These natural foods are rich in phenolic compounds that are responsible for antioxidant activity. Honey's chemical composition also contains vitamins, carotenoids, proteins, mineral salts and sugars that contribute to nutritional value of this food [1-4]. There are also volatile components in a honey that can be used as chemical markers that allow identifying its floral origin [5]. Honey is classified according to the floral source of the nectar. Generally, darker honeys have higher antioxidant activity than lighter honeys although the latter have major commercial interest. Honey may be useful to avoid chronic diseases due to its antioxidant activity [1,6].

In order to control the honey's quality is analyzed some parameters such as total phenol and total flavonoid content, since these parameters are related with its antioxidant activity [1]. The detection of phenolic compounds in foods is commonly performed using spectrophotometric and chromatographic techniques [7,8]. However, these methods are difficult to miniaturize or to adapt for continuous monitoring *in situ* and in some cases they require chromophores, which may be very costly.

Alternatively, many analysis methods using polyphenol oxidase (PPO) have been developed in order to determine total phenols in teas, juices, wines and foods [9,10]. PPO is an enzyme found in several species of plants, microorganisms and animals [11]. This enzyme is principally found in root, leaf, peel and fruit of the Grand Nain banana (*Musa acuminate*) [12]. PPO acts as a protecting mechanism, since

phenolic compounds are oxidized to a polymeric structure when in contact with oxygen, that safeguards the plant against microorganisms [13].

PPO is very much used in biosensors that are easy to miniaturize, inexpensive and selective. Biosensors are analytical devices that recognize a biological molecular substance through a transducer. A biologically sensitive material called bioreceptor is linked to transducer. These devices may be labelled or not. Label biosensor's use molecular substances connected to the biological material that helps to detect the target substance. Label-free biosensors are based on direct measurements of physical parameters that are detected by different types of transducers such as electrochemical, optical and thermometric [14].

For manufacturing biosensors is more usual bioreceptors based on enzymes [15]. The vast majority of the works described on the development of electrochemical biosensors made with polyphenol oxidase employs amperometric transducers [16-18]. However, electrochemical transducers based on potentiometric measurements present some advantages such as simple electronics and a high sensitivity. Besides, the analyte is not consumed during the measurements. Until now, only one potentiometric biosensor for determining adrenaline *in-vivo* using PPO was developed. In spite of its high sensitivity, this biosensor was elaborated using a carbon paste from mixture of graphite powder, enzyme and mineral oil (Vaseline™) [19]. The lifetime of this biosensor is limited due to leaching out of the enzyme. Besides, this biosensor is difficult to miniaturize by means of microfabrication process.

The purpose of this work is to develop an all-solid-state potentiometric biosensor for determining total phenols in honey and propolis based on solid-contact with good sensitivity and high mechanical resistance.

2. Materials and methods

2.1 Reagents and samples

L-tyrosine, catechol, tetrahydrofuran and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride were acquired from Sigma-Aldrich. Poly(vinyl chloride) carboxylated (1.8% carboxyl basis) was purchased from Acros Organics. Potassium hydroxide, sodium carbonate, potassium phosphate dihydrogen anhydrous (KH_2PO_4), polyvinylpyrrolidone K30 (PVP-K30, M_w 40,000), Triton X-100 and Folin-Ciocalteu reagent have been purchased from Synth, Merck, Henrifarma, All Chemistry, Becto and Anidrol, respectively. Others reagents such as methanol and powder graphite (50-300 µm, size) were analytical grade. Gaseous oxygen (99%, purity) was purchased from White Martins. Samples of Grand Nain bananas and honey were acquired in local market. Honey's samples were the same brand and expiry date.

2.2 Procedures

2.2.1 Obtainment of the Crude Extract of Tyrosinase

Crude extract of tyrosinase was prepared based on modified protocol of Nematpour *et al.* [20]. We weighed about 400 g of green-yellow banana peel (2 mm, thickness) and freeze at -20 ºC. These peels were powdered in a warring blender for 20 min with 100 mL of 0.02 M KH_2PO_4 buffer at pH 7.0, containing 2% of PVP-K30 and 1% of Triton X-100. The mixture was filtered using a sieve with a layer of gauze. Afterwards, the filtered liquid was centrifuged at 3400 rpm for 60 min. The supernatant was stored in a freezer and used as an enzymatic source for manufacturing the biosensor.

2.2.1.1 Enzymatic activity determination

Enzymatic activity of the crude extract of tyrosinase was determined as cresolase or mono-oxygenase (L-tyrosine) and catecholase or oxidase (catechol) using an Ultraviolet-Visible (UV-VIS) spectrophotometer at 420 nm with glass cuvette of 1.00 cm of optical path [12,21]. The assay was made in duplicate using L-tyrosine or catechol as primary substrates. Oxygen gas (*co*-substrate) was bubbled during 30 min for saturation of 100 mL of phosphate buffer at 0.1 M and pH 7.0.

The method is based on increasing the optical density at 420 nm due to the transformation of the primary substrate to *o*-quinones with posterior polymerization to melanin derivatives [12, 22]. The enzymatic activities of the crude extract were calculated from the initial straight region of the kinetic curve, which it is equal to the slope corrected by the dilution factor. One enzyme unit was defined as a change in absorbance at 420 nm of 0.001 per minute at 25 °C, pH 7.0 under conditions of the assay described previously [12]. Table 1 shows the assays employed for determining enzymatic activity of the crude extract of tyrosinase acting as cresolase and catecholase. The assay consisted in adding the volume presented in Table 1 to glass cuvettes.

2.2.2 Manufacturing of solid-contact potentiometric biosensor

The biosensor was elaborated in three layers. The first layer consisted of a composite containing 45% of graphite powder mixed with 5% of potassium permanganate and 50% of PVC-COOH. We added a certain amount of THF for these materials in order to dissolve the polymer up to form a paste. The paste was homogenized in a glass mortar using a glass pestle. Afterwards, THF was left to evaporate up to mixture to reach the proper viscosity to be transferred into a nylon tube

with 1 cm of inner diameter and 15 cm of length. Approximately 10 g of paste was necessary to fill the superior part of the electrode. A copper wire was inserted into the paste to obtain the electrical contact of the transducer. At the other end, we put a female BNC connector. This first layer of the transducer was left to dry for 12 hours at ambient temperature. Then, the surface of this layer was polished using abrasive papers from 150 to 3,000 meshes and oxidized with the flame of a burner. We deposited a paste of 1 mm of thickness containing graphite/PVC-COOH (1:1) without potassium permanganate on the first layer. The second layer was left to dry for 6 hours and gently polished using abrasive paper of 3,000 meshes until it presented a specular surface. Hence, the solid-contact transducer was prepared and used to fabricate the potentiometric biosensor.

The potentiometric biosensor was manufactured using the covalent bond method [23]. We added about 40 mg of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride to 100 µl of the crude extract of tyrosinase. The mixture was homogenized and dropped on the surface of the solid-contact transducer. The biosensor was stored in a refrigerator for 12 hours until the solvent evaporated and the covalent bond between the enzyme and the carboxylate groups of the transducer surface was formed.

2.2.3 Total phenols determination in honey and propolis

We analyzed five samples of honey and derivatives. Three honey samples were of floral source from wild flower, eucalypt flower and orange flower. One sample was an extract of propolis and another was a mixture of honey and propolis with guaco (*Mikania glomerata*) and watercress flavor. We poured about 4 g of each sample

weighed on analytical balance into 10.00 mL volumetric flask. These samples were dissolved with methanol.

The total phenol content in the samples was expressed as milligram of gallic acid equivalents by 100 grams of honey. All assays were made in duplicate. We employed Origin Pro software version 8.0 to elaborate the graphs of kinetic and calibration curves.

2.2.3.1 Folin-Ciocalteu method

The Folin-Ciocalteu method was adapted from Bonoli *et al.* [24]. This method is based on spectrophotometric measurements at 750 nm using a glass cuvette of 1.00 cm of optical path. A reference solution of catechol at 10,000 ppm was prepared in distilled water. We prepared six standard solutions ranging from 1 to 10 ppm of catechol in 100.0 mL volumetric flasks by diluting of the reference solution. For each one these volumetric flasks, we added an adequate volume of the reference solution (10, 20, 40, 60, 80 e 100 µl), 5 mL of methanol, 5 mL of Folin-Ciocalteu reagent and 50 mL of distilled water. The solutions were shaken for 5 min until a light green color appeared. Then, 20 mL of sodium carbonate solution at 15% was poured into each volumetric flask. Afterwards, we added the quantity of distilled water to reach 100.0 mL.

The honey samples were analyzed in a similar manner. We took 250 µl of two propolis solutions and 500 µl of the three honey solutions and we inserted these quantities into five different 10.00 mL volumetric flask. Then, we added 500 µl of Folin-Ciocalteu reagent and 6 mL of distilled water to each one of the five volumetric flasks of 10.00 mL. We shook the flasks for 5 min and added 2 mL of sodium carbonate solution at 15%. The same procedure was used to prepare the blank solution, except that sample solution was replaced by 500 µl of methanol.

2.2.3.2 Potentiometric biosensor method

The potential difference on the solid-contact potentiometric biosensor due to monocatecholate ion was measured with an ion analyzer (HI 223 model from Hanna instruments) using Ag/AgCl, 3M KCl as reference electrode (R684-105 model from Analion). All measurements of the electrochemical cell were carried out at room temperature.

Reference solutions of catechol were freshly prepared in distilled water in the concentrations between 1×10^{-5} M and 1×10^{0} M. We added an adequate quantity of each standard solution to 10.00 mL of phosphate buffer (0.1 M at pH 7.0). This method is named Spike method and we described it in previous work [25]. The potential was measured after reaching equilibrium state, which it occurred about 150 s after each addition of the standard solutions. Gaseous oxygen was continuously bubbled through the system during the measurements since this substance is the *co*-substrate of tyrosinase. The catechol concentration, corrected by the dilution factor, was plotted against relative potential (difference between initial and final potential) to produce the calibration curve.

We analyzed the phenolic compounds in the samples using the potentiometric biosensor from solutions of honey and propolis in methanol. We took between 100 and 1100 µl of each one these samples and we added this quantity to 10.00 mL phosphate buffer (0.1 M at pH 7.0). The potential was measured with the electrochemical cell described previously. The surface of the label-free biosensor was restored for new measurements by immersing the electrode during 1 to 3 min in 0.1 M of hydrogen peroxide solution as described in previous work [26].

3. Results and discussion

Polyphenol oxidase is bound to thylakoid membranes and can be found in chloroplast [22,27]. Chloroplasts are organelles that are present in the interior of plant cells. Therefore, it is necessary to destroy the cell membrane to liberate the enzyme. We used a low concentration of phosphate buffer (0.02 M) to increase the osmose effect due to phenomenon of swelling which, associated with pre-freezing of the banana peel helped to destroy the cell membrane. On the one hand, non-ionic surfactants, such as Triton X-100, help to displace PPO from thylakoids [27], in order to dissolve the cell membrane and activate the latent PPO [12]. On the other hand, it is important to add PVP-K30 to the extraction buffer, since this avoids the effect of the dopamine, the substrate that is contained into banana peel. PVP-K30 prevents inactivation of enzymes during extraction and polymerization of the phenolic substrates since it removes these from the extraction medium containing PPO [12].

We determined the enzymatic activity of crude extract of tyrosinase from banana peel by spectrophotometry in the visible region. Fig. 1 shows the enzymatic kinetic of the crude extract of tyrosinase acting as cresolase and catecholase. Table 2 lists the values of enzymatic activity for these substrates. Fig. 1A reveals that there is a lag time before of the tyrosinase to present activity as cresolase. This effect was described by Sojo *et al.* [28]. The enzymatic activity of the crude extract as catecholase was similar to the one reported by Nematpour *et al.*[20], but differs from the value found by Mataveli *et al.* [19]. Possibly, this difference in the enzymatic activity is due to soil type, seasonality, climate during growth and harvest of the fruit.

Tyrosinase presents a binuclear copper center as active site, in which copper is bound to six or seven histidine residues and to a residue of cysteine [11]. This enzyme catalyzes the oxidation of monophenols and *o*-diphenols in presence of oxygen to *o*-quinones followed of the formation of brown melanin pigments. During this process, oxygen is reduced to water and the active site of the enzyme in *oxy* state (Cu^{2+}) pass to *met* state or *deoxy* state (Cu^{1+}) when the same catalyzes monophenols or *o*-diphenols, respectively [22]. Therefore, if tyrosinase is bound to surface of potentiometric transducer, the change of *oxy* state to *deoxy* state modifies the capacitance of the sensor and a potential is developed.

We chose to develop a potentiometric biosensor based on solid-contact for improving the detection limit of phenolic compounds. Solid-contact potentiometric sensors with trace levels of sensitivity were developed in the last two decades. In the manufacturing process of these sensors, it is essential to overcome the following problems: the formation of a thin water film between the membrane interface and solid contact; flow of ions within the membrane affecting the composition of this water film and altering the boundary potential between one layer and another; spontaneous reactions of charge and discharge between the membrane and the inner electrode; leaching out of the active component from polymeric membrane to solution. The water film and spontaneous reactions of charge and discharge between the membrane and the inner electrode may be avoided using lipophilic, redox-active self-assembled monolayers such as conducting polymers [29]. However, conducting polymers are expensive and we prefer to use a blend of PVC-COOH, graphite and potassium permanganate as conductive layer. The surface of this conductive layer was oxidized by a burner to improve the adhesive properties between this layer and the second layer containing only graphite/PVC-COOH. This way, we can maybe avoid the formation of

a water film between the layers. The PVC-COOH was used due to its ability to produce hydrogen bonds between carboxyl groups in both layers. Besides, PVC-COOH allows the covalent bond with tyrosinase from crude extract in the last layer and thus reduces the leaching out of the active component from biosensor to solution. The potential instability can be overcome using a redox couple between the membrane and the conductive layer [29]. We employed potassium permanganate in first layer to improve the potential stability of the biosensor. This strategy presented good results as can be seen in Fig. 2. Fig 2A shows the response of the biosensor to monocatecholate ion, while Fig. 2B presents the responses to samples. We added different volumes of the samples and the biosensor showed good reproducibility. The biosensor was stable and re-usable during three months. The detection limit of phenolic compounds with biosensor was 7.3×10^{-7} M and the linear range response was sub-nernstian (39.7±0.3 mV decade^{-1}) from 9.3×10^{-7} M to 8.3×10^{-2} M with Pearson correlation coefficient of 0.9993 (Fig. 2C). This limit of detection was comparable to other solid-contact sensors based on the redox mechanism [30]. Moreover, this detection limit improved in one concentration decade compared the one obtained with a potentiometric biosensor where the enzyme was immobilized by occlusion [25].

The solid-contact potentiometric biosensor based on tyrosinase from crude extract of *Musa acuminate* was employed for determining phenolic compounds in honey and propolis (Fig. 2B). The results obtained with biosensor were compared with the Folin-Ciocalteu method (Table 3). We applied the student t-test to the intercept of the linear regression between the obtained results using the Folin-Ciocalteu method and solid-contact biosensor [31]. In this manner, we verified the correlation between the methods. Table 4 shows the statistical data.

According to Table 4, the biosensor was not influenced by an additive error because the straight line passes through origin since the value to calculated Student-t test is lower than critical-t value. We can estimate that 96% of the total phenols determined by Folin-Ciocalteu method were predicted by the solid-contact biosensor (Slope in Table 4). Applying paired Student-t test for comparing individual differences between the mean of both methods (Table 3) [32], we find that calculated value to Student-t test is lower than critical-t value (Table 4), indicating that there is less than 99% chance that the results of both methods are different.

Therefore, the results using the biosensor agreed with those determined by the Folin-Ciocalteu method within an acceptable value of deviation.

The Folin-Ciocalteu method takes a long time for developing color before spectrophotometric measurements. Thus, the method based on potentiometric biosensor was faster than the Folin-Ciocalteu method for determining phenolic compounds.

Finally, it is important to emphasize that common substances did not interfere with the label-free biosensor since the honey and propolis samples are very complex. We described in previous work that sugars (fructose, glucose and sucrose), salts (potassium iodide, potassium chloride, potassium sulfate, sodium benzoate, and others) and aminoacids (glycine and cysteine) presented small pK^{POT} values, therefore, low interference, although these species can form complex with the copper (II) ions from enzyme [25]. Moreover, Mataveli *et al.* studied with its biosensor for adrenaline some compounds that are present in blood samples and can interfere to PPO such as uric acid, ascorbic acid, urea, dopamine, and noradrenaline. All compounds interfered less than 10% in the biosensor response based on PPO, except ascorbic acid (13%) [19]. Nevertheless, we desired to determine total phenols, hence any catecholamines or phenolic compounds are primary substrates for the biosensor.

4. Conclusion

The label-free potentiometric biosensor using tyrosinase from crude extract of banana peel immobilized by covalent bond on surface of a solid-contact transducer presented a large range of responses to catechol concentration, good selectivity and low detection limit.

The determination of phenolic compounds in honey and propolis using the proposed biosensor presented good concordance with the results obtained with the Folin-Ciocalteu method but the biosensor method was faster than the usual procedure.

Finally, the materials used to manufacture the solid-contact potentiometric biosensor can be easily transformed into ink and a miniaturized planar biosensor for determining total phenols can be developed based on thick films techniques such as a screen-printer. A disposable potentiometric biosensor could be applied to verify the adulteration in honey samples in real time.

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Tables

Table 1 Assay for determining enzymatic activity of the crude extract of tyrosinase acting as cresolase (monooxygenase) or catecholase (oxidase).

	Amount (mL)			
Assay	Distilled water	Buffer[†]	Substrate	Crude extract
Blank	1.0	2.0	-	0.1
Cresolase	-	2.0	1.0 (L-tyrosine - 1 mM)	0.1
Catecholase	-	2.0	1.0 (Catechol - 10 mM)	0.1

[†] 0.1 M of phosphate buffer at pH 7.0, saturated with gaseous oxygen.

Table 2 Results for enzymatic activity of the crude extract of tyrosinase acting as cresolase or catecholase using gaseous oxygen as *co*-substrate.

Substrates	Tyrosinase performance	Enzymatic activity (U mL^{-1})
L-Tyrosine	Cresolase or mono-oxygenase	3±1[*]
Catechol	Catecholase or oxidase	4.9±0.1

[*]Average of two determinations and standard deviation estimate.

Table 3 Comparison between the results using label-free potentiometric biosensor and Folin-Ciocalteu method for determination of phenolic compounds in samples of honey and propolis.

	mg of gallic acid equivalents by 100 g of sample		
	Methods		
Samples	Folin-Ciocalteu	Potentiometric biosensor	Difference
Honey of wild flowers	43±2[*]	44±4	-1
Honey of eucalypt flowers	38±2	40±6	-2
Honey of orange flowers	18±4	16±1	2
Propolis Extract	81±1	77±3	4
Mixture of honey and propolis with guaco and watercress flavor	66±5	52±7	14

[*]Average of two determinations and standard deviation estimate.

Table 4 Statistical data for verifying correlation between the Folin-Ciocalteu method and solid-contact biosensor.

			Student-t value	
	Value	Standard error	Calculated	Critical
Intercept	-1.1	1.7	0.666[1]	5.841[2]
Slope	0.96	0.05	-----	-----
Difference between methods	3.4[3]	6.4	1.190[4]	4.032[5]

[1] $t_{intercept} = \frac{Coeffcient\ value}{standard\ error}$
[2] Value for 3 degrees of freedom and confidence level of 99 % (n – 2).
[3] Mean of the values difference between the methods (d) presented in the Table 3.
[4] $t_{difference\ between\ methods} = \frac{|\bar{d}|}{standard\ error}\sqrt{n}$
[5] Value for 4 degrees of freedom and confidence level of 99 % (n – 1).
n is the number of honey samples (n=5).

Figure Captions

Fig. 1 Plot of enzymatic kinetic for determination of the tyrosinase activity in the crude extract from banana peel. Substrate concentration: L-tyrosine, 0.32 mM and catechol, 3.20 mM. **A.** Cresolase; **B.** Catecholase.

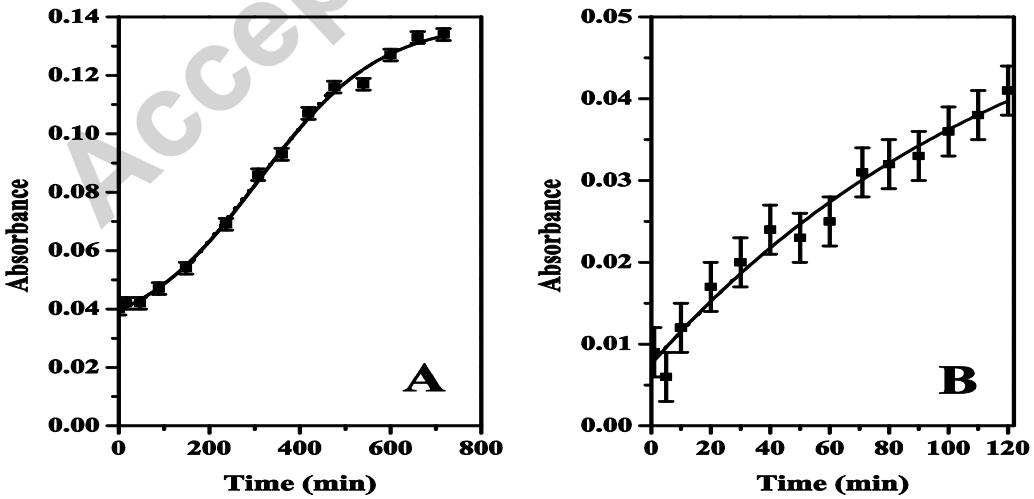
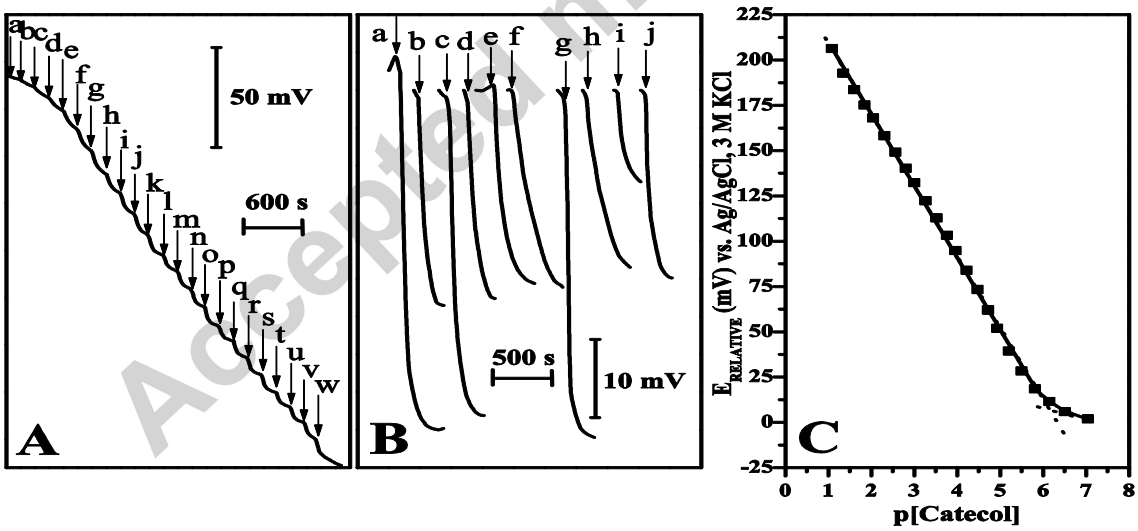
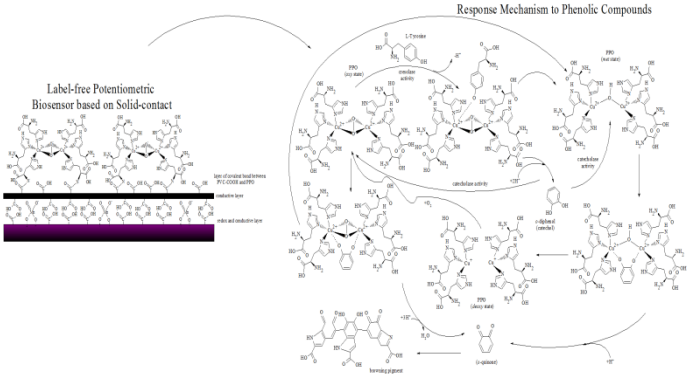


Fig. 2 Response of the label-free potentiometric biosensor based on tyrosinase from crude extract of banana peel. **A.** Potential-time curve to successive addition of catechol. Increment volume: (a, d, h, l, p and t: 100 µl); (e, i, m, q and u: 200 µl); (b: 250 µl); (f, j, n, r and v: 400 µl); (b: 500 µl); (g, k, o, s and w: 800 µl). Catechol concentration: (a to c, 1×10^{-5} M); (d to g, 1×10^{-4} M); (h to k, 1×10^{-3} M); (l to o, 1×10^{-2} M); (p to s, 1×10^{-1} M) and (t to w, 1×10^{0} M). **B.** Potential signal to honey and propolis samples. Between parentheses is showed the added volume of the sample. Honey from: a. (1100 µl) and b. (500 µl) - wild flower; c. (1100 µl) and d. (500 µl) - eucalypt flower; e. (1100 µl) and f. (1000 µl) - orange flower; g. (700 µl) and h. (200 µl) - extract of propolis; i. (100 µl) and j. (200 µl) - mixture of honey and propolis with guaco and watercress flavor. **C.** Calibration curve. Conditions: Initial volume of 0.1 M phosphate buffer at pH 7.0: 10.00 mL. Gaseous oxygen was continuously bubbled through the system during the measurements at ambient temperature.



Graphical abstract



Highlights

- A potentiometric biosensor based on polyphenol oxidase extracted from *Musa acuminata*;
- A potentiometric biosensor without molecular species bonded to enzyme (label-free);
- A potentiometric biosensor based on solid-contact with low-detection limit and large range of response in concentration to phenolic compounds;
- A potentiometric biosensor applied to determination of total polyphenols in honey and propolis.