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Tyrosinase biosensor for benzoic acid inhibition-based determination with the use of a flow-batch monosegmented sequential injection system

Jolanta Kochana*, Joanna Kozak, Agata Skrobisz, Michał Woźniakiewicz

Department of Analytical Chemistry, Faculty of Chemistry, Jagiellonian University, Ingardena 3, 30-060 Kraków, Poland

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ABSTRACT

Amperometric tyrosinase based biosensor for benzoic acid determination with the use of a flow-batch monosegmented sequential injection system is proposed. The functioning of the biosensor is based on the inhibition effect of benzoic acid on the biocatalytic activity of tyrosinase, polyphenol oxidase. In the biosensor, tyrosinase is entrapped in titania gel modified with multiwalled carbon nanotubes (MWCNT) and Nafion. The procedure of optimization of experimental conditions affecting the biosensor response, as well as its analytical characteristics and results of the approach verification using synthetic samples and a reference material are presented. The developed biosensor exhibits linear range up to 2.46 μM, good sensitivity toward benzoic acid (1.06 μA μM^{-1}) and low detection limit (0.03 μM). The approach has been applied to benzoic acid determination in a real beverage sample giving the result consistent with that obtained using the HPLC method.

1. Introduction

Benzoic acid is widely used as a preservative of food, beverages, cosmetics and pharmaceuticals to prevent their decomposition by microbiological, enzymatic or chemical changes [1–3]. However, at higher than permitted safety levels it can be harmful for human health. Therefore, monitoring of benzoic acid concentration is important for both quality assurance and consumer protection.

A variety of analytical methods were developed for benzoic acid determination. In recent years, applications of chromatography [4–8] and capillary electrophoresis [9,10] techniques have been frequently reported. Regarding flow methods, approaches based on sequential injection (SI) technique coupled with monolithic column [11], flow injection (FI) on-line dialysis coupled to FID-HPLC system [12] or second-order spectrophotometric data generated by a pH gradient formed in FI technique [13] were developed to determination of benzoic acid in pharmaceutical preparations [11], soft drinks [12] or juices [13]. The determination in juices was also proposed to be performed using PLS-2 multivariate calibration [14] or a net analyte signal based methodology named HLA/GO [15], both applied to UV spectrometric data.

Electrochemical methods for benzoic acid determination, especially those employing amperometric biosensors, have been regarded as promising, because of their effectiveness, simplicity and selectivity. They are based on tyrosinase, a copper-containing polyphenol oxidase, which possesses ability to oxidize mono- and diphenols to corresponding *o*-quinones. Quinones can be reduced at electrode surface to enable low potential detection of phenolic compounds. The addition of benzoic acid causes the enzyme inhibition, hence a decrease of steady-state current signal, proportional to the inhibitor concentration, can be observed. Several biosensors based on tyrosinase inhibition have been developed for detection of benzoic acid [16–20]. A polyaniline–poliphenol oxidase biosensor was proposed for determination of benzoic acid in beverages and food samples [16]. Morales et al. employed a graphite-teflon-tyrosinase composite biosensor for analysis of foodstuffs [17]. Biosensor based on inhibition of tyrosinase entrapped in polyaniline–polyacrylonitrile composite matrix was reported by Shan et al. [18]. Other authors reported a highly reversible biosensor based on immobilization of tyrosinase by calcium carbonate nano-materials for monitoring of benzoic acid in beverages [19]. The evaluation of benzoic acid in aqueous and organic media was also realized with the use of inhibition biosensor based on calcium phosphate [20].

The monosegmented flow approach generally consists in locating a sample zone between two gas bubbles in order to form a monosegment [21]. This way, the influence of the liquid carrier on the analyte concentration in the sample can be limited to such an extent that the dilution effect can be omitted during results calculation. Combining the approach with a sequential injection system, makes it possible to control the volume of a solution introduced into the monosegment [22–24].

In the present work, an amperometric tyrosinase based biosensor for inhibitive determination of benzoic acid with the use

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* Corresponding author. Tel.: +48 126632014; fax: +48 126632232.
E-mail address: kochana@chemia.uj.edu.pl (J. Kochana).

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of a flow-batch monosegmented sequential injection system is proposed. In the biosensor, tyrosinase is entrapped in titania gel modified with multiwalled carbon nanotubes (MWCNT) and Nafion.

Titania gel was chosen for entrapment of enzyme in construction of biosensor because it is known to be hydrophilic and biocompatible, and it may be easily prepared by the sol–gel procedure [25,26]. Moreover, sol–gel glasses are promising matrixes for the immobilization of biomolecules as they are thermal and chemical stable, are able to entrap large amount of enzyme and can retain their catalytic activity [26]. Carbon nanotubes (CNTs) are attractive components of biosensor matrices due to their excellent electronic properties, high surface area, and chemical and mechanical stability [27]. Nafion, perfluorinated sulfonate ionomer, was reported as a component that decreased enzymes leakage and simultaneously enhanced the sensor stability [28].

The procedure of optimization of experimental conditions affecting the biosensor response (potential of working electrode, temperature of measurements, concentration of an enzyme substrate) and the biosensor analytical characteristics are described. The results of the approach verification using synthetic samples and a reference material, and its application to determination of benzoic acid in a real sample are presented.

2. Experimental

2.1. Chemicals and solutions

Titanium(IV) isopropoxide, tyrosinase from mushroom (EC 1.14.18.1; 1181 U/mg) and multi-walled carbon nanotubes MWCNT (O.D. 10–15 nm, I.D. 2–6 nm, length 0.1–10 μm, >90% purity) were purchased from Sigma (USA); catechol was from Merck (Germany), ethanol (96%), 2-propanol, HCl (35%) and L-(+)-ascorbic acid were from POCh (Poland); HNO_3 (65%), NH_{3aq} (25%) and acetone were obtained from LACHNER (Czech Republic); 0.3 μm alumina powder, used for polishing working electrode surfaces, was from Buehler Micropolish (USA); Nafion, perfluorinated sulfonate ionomer, 5% (w/v) solution in mixture of low aliphatic alcohol and water, was purchased from Fluka. Buffer solutions of pH 6.0 and 7.0, in concentration of 0.1 and 0.5 M, respectively, were prepared by mixing appropriate volumes of KH_2PO_4 and Na_2HPO_4 solutions. Tyrosinase solution (0.144 mg/10 μL) was prepared in 0.5 M phosphate buffer solution, pH 7. Catechol solutions were prepared daily in deoxygenated 0.1 M phosphate buffer solution, pH 6.

Benzoic acid solutions used as standards or samples were prepared from Benzoic acid, Argon Łódź (Poland). Cola drink reference material, T0395, FAPAS® TEST MATERIAL was obtained from The Food and Environmental Research Agency (USA). A non-alcoholic beverage, commercially available on the Polish market, was used as a sample.

All chemicals were analytical-grade reagents. Double distilled water was used throughout.

2.2. Apparatus

Electrochemical analyzer M161 (mtm-anko, Poland) was employed for the electrochemical detection. All experiments were performed with a conventional three-electrode system: the graphite working electrode (spectral carbon rod, $d = 6$ mm) covered with nanocomposite layer, the saturated silver/silver chloride reference electrode (mtm-anko, Poland) and the platinum auxiliary electrode. Amperometric experiments were carried out in thermostatic cabinet Pol-Eko-Aparatura (Poland), in an electrochemical cell under constant stirring with a magnetic bar, and under free access of air. In the flow-batch SI mode, glass flow electrochemical cells, made in our laboratory, were used. The supporting electrolyte was 0.1 M phosphate buffer solution, pH 6.

A Sonic 3 Ultrasonic bath (POLSONIC, Poland) was employed for matrix composite homogenization and for beverage degassing. Nitrogen was passed through buffer solution in order to deoxygenate it. A combined glass electrode ERH-11 (HYDROMED, Poland) and a CP-501 pH-meter (Elmetron, Poland) were employed for pH measurements. A vortex (IKA, Germany) was used to obtain homogenous nanocomposite mixtures.

SEM images were taken using Hitachi S-4700 SEM at Laboratory of Field Emission Scanning Electron Microscopy and Microanalysis at the Institute of Geological Sciences of the Jagiellonian University.

2.3. Reference method

As the reference, the HPLC-UV method was applied. The separation was performed using UltiMate 3000 Rapid Separation LC System by Dionex (Germany) and the Brownlee Spheri-5 C18 chromatographic column (4.6 mm × 100 mm, 5 μm) by PerkinElmer (USA), coupled to appropriate column guard (C18, 4.6 mm × 10 mm). As the mobile phase a mixture of 125 mM ammonium acetate buffer (pH 4.0) with acetonitrile (75:25, v/v) was applied at a flow rate of 1 mL min^{-1}. The acquisition was performed at 230 nm.

2.4. Measurement procedure

Tyrosinase biosensor is proposed for benzoic acid determination. The method is based on the inhibition effect of benzoic acid on the biocatalytic activity of the tyrosinase. The biosensor functioning was optimized in batch mode. Method verification was performed by chronoamperometry in batch mode and with the use of flow-batch monosegmented sequential injection system for introducing solutions into electrochemical cell.

In batch mode solutions were introduced into the cell manually (using pipettes). Determination of benzoic acid in a real beverage sample was performed using flow-batch sequential injection system. In order to compensate possible matrix effects, the standard addition method was used.

In both, batch and sequential injection modes, determinations were performed in electrochemical cell of capacity ca. 5 mL. The first stage included introduction of phosphate buffer (pH 6) and catechol solutions into electrochemical cell. After establishing the baseline, an initial current signal was recorded. Subsequently, a sample was added and the current response in the presence of benzoic acid was registered. The sensor displayed a response time about 30 s. Afterwards, the standard was added and the corresponding current response was measured. The procedure of standard addition was repeated fourfold. In order to keep the catechol concentration at a constant level during the analysis, appropriate portions of diluted catechol solution were added additionally along with sample and standard additions.

2.5. Preparation of TiO_2/MWCNT/Nafion nanocomposites

Titania sol was prepared by acid hydrolysis and further polycondensation of titanium(IV) isopropoxide as described in our earlier reports [25,26,29]. To prepare the enzyme-containing matrix, the enzyme solution was mixed with sol, added to appropriate amount of MWCNT and shaken. Next, portion of Nafion was incorporated and shaken again. Finally, the composite of Tyr/TiO_2/MWCNT/Nafion was sonicated to obtain a homogenate.

To prepare a biosensor, 20 μL (in portion of 10 μL) of nanocomposite was deposited on the surface of a pretreated graphite electrode [25,26,29]. After adding each of composite portion, the

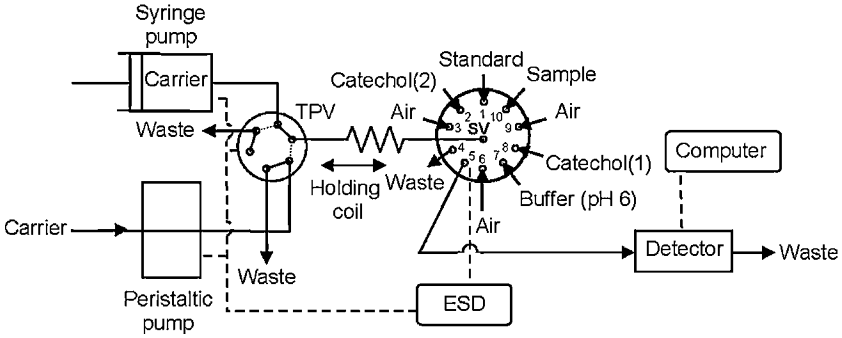

Fig. 1. Sequential injection system developed for the method implementation (in position 1 of TPV); TPV—two-positional valve, SV—selection valve, ESD—electronic steering devise; catechol (1) and (2)—catechol solutions of different concentration (details in the text).

surface of electrode was dried in air for ca. 10 min. Finally, the electrode was allowed to dry over saturated disodium phosphate solution for 20 h at 4 °C. When not in use, sensors were stored at 4 °C in phosphate buffer solution (pH 6.0, 0.1 M), with their active surface touching the surface of solution. Between measurements the biosensor was rinsed with water and stored in buffer solution (pH 6).

2.6. Flow-batch monosegmented sequential injection system

The sequential injection system presented in Fig. 1 was applied to the method mechanization. Using the system, established volumes of appropriate solutions, necessary for analytical curve construction according to the procedure described in Section 2.4, were successively introduced in the form of monosegments into an electrochemical cell. Time of their introduction and a flow rate of carrier were optimized to enable the whole volume of solution contained in a monosegment to reach the cell. The flow rate of aspiration and pumping was 3 mL min^{-1}.

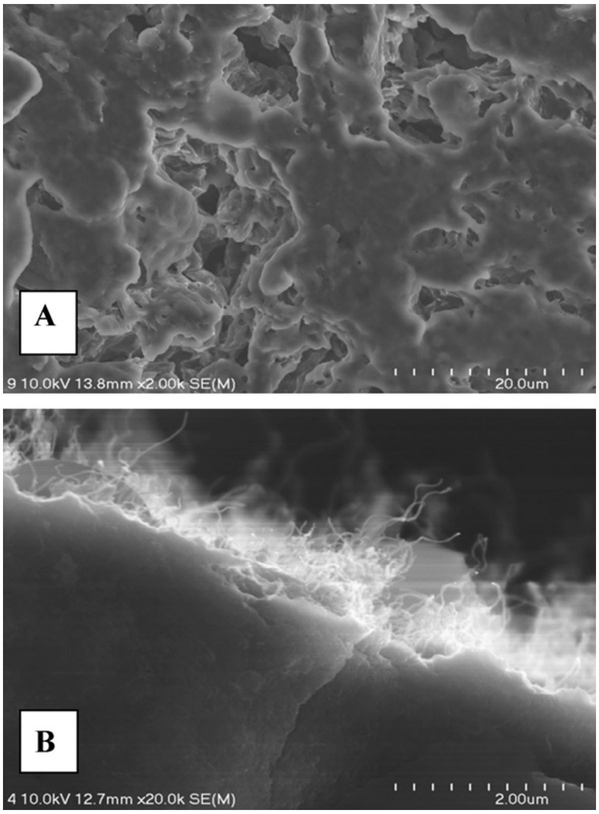

Fig. 2. SEM view of titania gel/MWCNT/Nafion nanocomposite on the surface of graphite electrode at magnification: 2000 (A); 20,000 (B).

3. Results and discussion

3.1. Morphology of matrix composite

The morphology of matrix nanocomposite (Ti$_2$O/MWCNT/Nafion) was characterized by SEM images. Our earlier studies showed that addition of CNT and ionomer into titania sol increased porosity of titania gel [30]. In the SEM picture (Fig. 2A), it is seen that titania gel modified with carbon nanotubes and Nafion exhibits three-dimensional micro-porous structure. As a result, substrates can easily diffuse onto and out of matrix layer. Moreover, the presence of carbon nanotubes (noticeable in Fig. 2B), due to their unique electric properties, promotes electron transfer between substrate and electrode surface and improves analytical performance of the sensor.

3.2. Optimization of experimental conditions

The biosensor response can be influenced by enzyme loading, pH of background electrolyte, potential of working electrode and temperature of measurement.

The optimum tyrosinase loading (193 U/electrode) and background solution (0.1 M phosphate buffer solution, pH 6) were chosen on the basis of earlier studies [26]. Working electrode potentials were tested between −200 mV and 200 mV vs. Ag/AgCl. The increase of reduction current for potential more negative than 200 mV (up to −50 mV), observed in Fig. 3, is caused by an enhancement of driving force for *o*-quinones reduction at low potential [31].

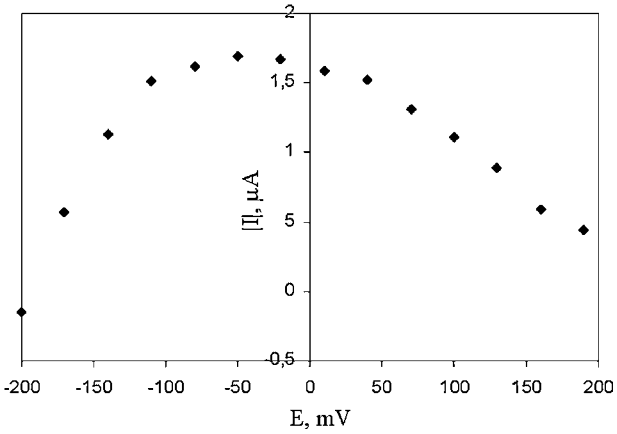

Fig. 3. Effect of working electrode potential on biosensor response in 8 µM catechol solution; 0.1 M phosphate buffer solution, pH 6; 35 °C.

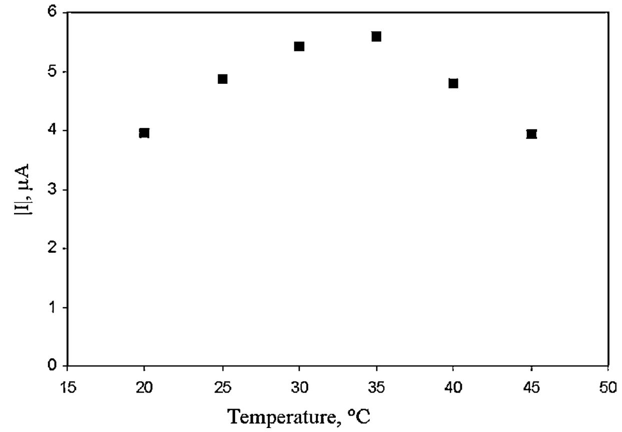

Fig. 4. Effect of temperature on the amperometric response of biosensor in 8 μM catechol solution, E_{app} = −50 mV; 0.1 M phosphate buffer solution, pH 6.

For potentials more negative than −50 mV, the decrease in cathodic current was noticed. The reason of the effect can be polymerization of *o*-quinones accompanying the enzymatic reaction at such negative potentials [32]. According to the results, the optimal potential of working electrode was −50 mV.

The influence of measurement temperature on the activity of tyrosinase entrapped in TiO_2/MWCNT/Nafion layer is shown in Fig. 4. The optimal temperature was found to be 35 °C.

The response current of biosensor employed to inhibition-based determination is regulated by enzyme kinetics because the amperometric signal is usually proportional to the activity of the entrapped enzyme [18]. It was reported that benzoic acid is a competitive inhibitor of tyrosinase [16–20]. For competitive inhibition, the substrate competes with the inhibitor, thus at too high concentration of substrate the inhibition effect is not noticed [33]. Therefore, the influence of catechol concentration on the biosensor response toward benzoic acid was examined. The reason of choosing the catechol as the tyrosinase substrate was the observation that biosensor based on tyrosinase immobilized in titania gel exhibited the highest sensitivity toward that phenolic compound [26]. In order to find the optimum catechol concentration chronoamperometric measurements toward benzoic acid were carried out in catechol solutions of concentrations: 1, 4, 10, 20 and 40 μM. As expected, substrate concentration noticeable affected the biosensor behavior. The lowest value of sensitivity toward analyte occurred in 1 μM catechol solution, while the highest in 40 μM solution, 0.001 and 6.217 μA μM^{-1}, respectively. Simultaneously, with an increase of biosensor sensitivity, narrowing of linear range was observed. For determinations of benzoic acid, the catechol concentration of approximately 20 μM was chosen, because of relatively high sensitivity of biosensor and acceptable linear range.

3.3. Analytical characteristics of biosensor

Analytical characteristics of proposed biosensor toward benzoic acid were performed chronoamperometrically under optimized conditions in batch mode. Biosensor response displayed linear dependence on the inhibitor concentration within the range 0.10–2.46 μM with mean sensitivity of 1.06 μA μM^{-1}. The obtained limit of detection (LOD) was equal 0.03 μM. It was estimated according to the equation LOD = $3S_x/a$, where a is a mean sensitivity of biosensor and S_x constitutes the standard deviation (n = 10) of signals recorded for 1 μM benzoic acid [17]. $I_{0.5}$, the inhibition concentration corresponding to 50% of the inhibition signal was calculated to be 2.5 μM. Biosensor repeatability was estimated in 0.475 μM benzoic acid solution giving RSD value of 4.7% (n = 5).

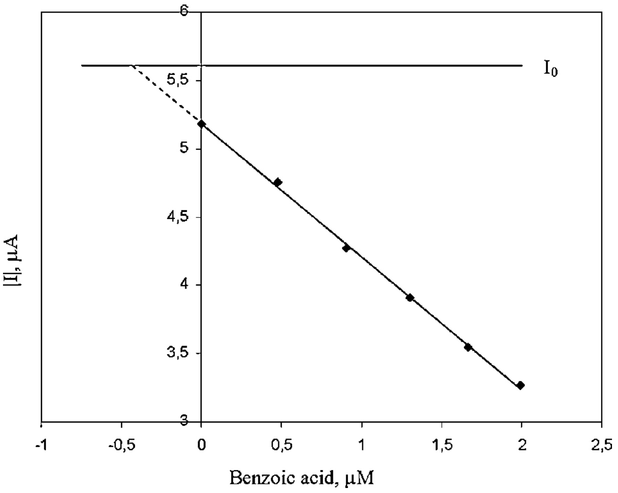

Fig. 5. Example of calibration curve for inhibition-based benzoic acid determination in 20 μM catechol solution; I_o—initial signal for 20 μM catechol solution; $y = -0.9743x + 5.1852$, $R^2 = 0.9981$, x—benzoic acid concentration (μM), y—current, I (μA); E_{app} = −50 mV; 0.1 M phosphate buffer solution, pH 6; 35 °C.

For a series of five electrodes prepared at different time, RSD equal 6.2% for 0.243 μM benzoic acid solution was obtained. The received values of RSD indicate the satisfying performance of the proposed biosensor in terms of repeatability and reproducibility. The operational stability of biosensor was examined by 30 consecutive measurements of its response in 1.62 μM analyte solution. A noticeable decrease of response current after 15 measurements was observed. The limitation of the developed biosensor is its long term stability. After 5 days of storage in buffer solution (pH 6) at 4 °C, the biosensor retained 89% of its initial current response and after an additional week – only 34%. This response decrease may be attributed to enzyme leaching from the matrix and the inherent instability of tyrosinase.

According to regulations, the content of benzoic acid in non-alcoholic beverages should be lower than 150 mg L^{-1} (1.23 mM) [34]. Taking into account the linear range of the proposed method, for some samples, an appropriate dilution before the measurements should be necessary. On the other hand, good sensitivity of the developed biosensor and consequently the low limit of detection achieved, makes it possible to apply the biosensor to detect the traces of the analyte.

3.4. Monosegmented sequential injection procedure

Monosegmented sequential injection procedure was employed during the proposed approach verification and application. The course of the procedure is schematically presented in Table 1. It can be seen (Table 1) that, in order to keep the catechol concentration at a constant level, appropriate portions of diluted catechol solution were added additionally along with sample and standard additions.

3.5. Method verification

In order to verify the developed method, determinations of benzoic acid in synthetic solutions and in cola reference material were performed in both, batch and flow-batch SI systems. The reference material was diluted 50-fold with 0.1 M phosphate buffer solution (pH 6.0). In synthetic solutions three concentration levels of benzoic acid were tested. Each synthetic sample was analyzed twice, whereas cola reference material–trice. Example of a calibration curve obtained for inhibition-based determination of benzoic acid is shown in Fig. 5. Results of determinations

Table 1
Schematic presentation of a single analytical procedure based on employing monosegmented sequential injection system; TPV—two-positional valve, SV—selection valve; catechol (1) and (2)—catechol solutions of different concentrations, 654.00 and 56.244 μM, respectively (details in the text).

Stage	Pump		Valve position		Volume (μL)	Action
	Syringe	Peristaltic	TPV	SV/substance		
1	ON	OFF	I	6/air	500	Aspiration
2	ON	OFF	I	7/buffer	3500	Aspiration
3	ON	OFF	I	8/catechol(1)	100	Aspiration
4	ON	OFF	I	9/air	500	Aspiration
5	OFF	ON	II	5/air-buffer, catechol(1)—air	4500[a]	Pumping: introducing catechol(1) into the electrochemical cell
6	OFF	OFF	II	5/–	–	Signal I_0 (Fig. 5) measuring (for buffer + catechol(1))
7	ON	OFF	I	9/air	500	Aspiration
8	ON	OFF	I	10/sample	50	Aspiration
9	ON	OFF	I	2/catechol(2)	50	Aspiration
10	ON	OFF	I	3/air	500	Aspiration
11	OFF	ON	II	5/air-sample, catechol(2)—air	1000[a]	Pumping: introducing sample and catechol(2) into the cell
12	OFF	OFF	II	5/–	–	Signal measuring (for buffer + catechol + sample)
13	ON	OFF	I	9/air	500	Aspiration
14	ON	OFF	I	1/standard	100	Aspiration
15	ON	OFF	I	2/catechol(2)	100	Aspiration
16	ON	OFF	I	3/air	500	Aspiration
17	OFF	ON	II	5/air-standard, catechol(2)—air	1100[a]	Pumping: introducing standard and catechol(2) into the cell
18	OFF	OFF	II	5/–	–	Signal measuring (for buffer + catechol + sample + standard)
19	Three repetitions of stages 13–18 in order to introduce the subsequent standard additions into the electrochemical cell					

[a] 100 μL of air remained in the tube connecting the selection valve with the cell.

are presented in Table 2. It is seen that the developed approach exhibits acceptable accuracy, as the obtained values of |RE| are generally lower than 4.5%. In two cases, values of |RE| exceed 7%, which can be caused by insufficient concentration of standard addition [35].

Repeatability of results obtained with the use of the monosegmented sequential system was estimated on basis of five determinations of benzoic acid in solution of concentration 0.475 μM. The precision equal 2.1% (RSD) was obtained. Comparing both, batch and flow-batch monosegmented SI approaches, it should be noted, that the latter mode lasts about 15 min longer. The reason is the time necessary for realization of aspiration and pumping stages.

Among substances which are usually encountered in beverages, electroactive are sugars, caffeine, citric and ascorbic acids. The reference material we used contained caffeine and sugars at levels usually met in drinks. Relatively good accuracy obtained for analysis of the reference material shows that these substances probably did not influence the response of biosensor toward benzoic acid. The possible positive interferences of citric and ascorbic acid could be eliminated by employing a polycarbonate membrane coated with the negatively charged hydrogel layer [36].

Table 2
Method verification; results of determinations of benzoic acid in synthetic solutions and cola reference material.

Benzoic acid concentration (μM) Synthetic solution		
Expected	Found[a] (RE, %)	
	Batch system	Flow-batch system
0.243	0.238 (−2.1)	0.250 (2.9)
0.475	0.458 (−3.6)	0.487 (2.5)
0.696	0.715 (2.7)	0.754 (8.3)
Reference material		
Assigned	Found[b] (RE, %)	
	Batch system	Flow-batch system
0.555	0.597 (7.6)	0.580 (4.5)

[a] Mean value of two determinations.
[b] Mean value of three determinations.

The performance of the developed biosensor was compared with other tyrosinase based biosensors developed for determination of benzoic acid (Table 3). To collate sensitivity values, sensitivity of the proposed sensor was expressed additionally (in Table 3) in alternative units. The inhibition progress can be described as a degree of inhibition Inh [%] = $((I_0 - I)/I_0) \times 100$, where I_0 is the initial current signal in catechol solution, and I constitutes the current signal after addition of inhibitor [16]. In the approach, sensitivity of biosensor can be expressed in %μM^{-1} or in %μM^{-1} cm^{-2} units. Taking into account values presented in Table 3, it is apparent that sensitivity of the proposed biosensor is higher (in several cases significantly) than sensitivity of other reported biosensors [16–19]. Linear range of the proposed sensor is comparable to that obtained for sensor based on tyrosinase adsorbed on calcium phosphate materials [20], and noticeable narrower than ranges demonstrated for other biosensors [16–19]. The achieved limit of detection is the same as that reported in [20], lower than limit calculated for enzyme entrapped by calcium carbonate nanomaterial [19], and one order of magnitude better than limits of other biosensors described [16–18]. Taking into account $I_{0.5}$ parameter, the proposed biosensor is characterized by the lowest value among the other reported.

Regarding the comparison of the developed approach with methods employing different analytical techniques, it allows results to be obtained with acceptable accuracy and comparable precision [4–12]. However, it should be noted, that most of the of cited methods, enable simultaneous determination of at least two analytes [4,5,7,9–12]. Comparing analytical ranges, the approach is characterized by a narrow linear range. Taking into account the limit of detection achieved, it is considerable better than limits of other reported approaches [4–6,8,10–12,15] and slightly better than those reported in [7,9].

3.6. *Analysis of a real sample*

The proposed procedure was employed for determination of benzoic acid in a sample of beverage. As no benzoic acid in the sample was detected, it was spiked with the analyte of concentration close to the concentration found in the reference material (ca. 120 mg L^{-1}). No special sample pretreatment was performed; it was only diluted 50-fold with 0.1 M phosphate buffer solution (pH 6.0). As a reference, HPLC method was applied. The sample

Table 3
Comparison of analytical characteristics toward benzoic acid for reported inhibitive biosensors based on tyrosinase.

Method	Sensitivity	Linear range (μM)	LOD (μM)	$I_{0.5}$[a] (μM)	Ref.
Tyrosinase immobilized in polyaniline	Not reported	Up to 40	0.3	35	[16]
Tyrosinase entrapped in graphite—teflon composite	0.017 μA μM^{-1}	1–40	0.9	Not reported	[17]
Tyrosinase immobilized in polyaniline—polyacrylonitrile layer	1.67% μM^{-1}	Up to 20	0.2	40	[18]
Tyrosinase entrapped by calcium carbonate nano-material	1.061 μA μM^{-1} cm^{-2}	0.56–92	0.08	12.9	[19]
Tyrosinase adsorbed on calcium phosphate materials	7.1×10^{-6}% μM^{-1} cm^{-2}	0.03–3	0.03	7.5	[20]
Tyrosinase encapsulated in titania gel/MWCNT/Nafion	1.06 μA μM^{-1}[b] 3.79 μA μM^{-1} cm^{-2} 19.95% μM^{-1} 71.25% μM^{-1} cm^{-2}	0.01–2.46	0.03	2.5	Present work

[a] The inhibition concentration corresponding to 50% of the inhibition signal.

[b] Sensitivity of the proposed biosensor was recalculated and expressed in various units to compare with other reported biosensors; current density was estimated using the assumption that the matrix biolayer covered the whole surface of graphite electrode.

was analyzed three times. Content of benzoic acid determined in the tested beverage with the use of the developed procedure was 105.6 ± 2.0 mg L^{-1}. Chromatographic analysis gave result of 109.9 ± 0.1 mg L^{-1}. It is seen, that both received values are in good agreement.

4. Conclusions

A developed tyrosinase biosensor for determination of benzoic acid is presented. Determination is based on the inhibition effect of benzoic acid on the biocatalytic activity of the tyrosinase. The results show that analytical features of the proposed biosensor are competitive in relation to features of other biosensors, especially on the account of its good sensitivity and limit of detection. Acceptable values of the proposed biosensor repeatability and reproducibility were obtained. Nevertheless, the lifetime of the developed biosensor appeared to be not satisfactory, suggesting further research. A monosegmented sequential injection system was successfully employed for rationalization of sample and standard introduction into an electrochemical cell. This way the risk of random errors was constrained and analytical results were allowed to be obtained in less laborious way. The approach has the potential to be adapted to other electrochemical systems. Research in this direction is being conducted in our laboratory.

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