Romanian Biotechnological Letters *Vol. 18, No.3, 2013*

ORIGINAL PAPER

AMPEROMETRIC TYROSINASE BASED BIOSENSORS FOR SEROTONIN DETECTION

Received for publication, October 10, 2012
Accepted, January 25, 2013

IRINA MIRELA APETREI[1], CONSTANTIN APETREI[2,*]
[1]*Department of Pharmaceutical Sciences, Faculty of Medicine and Pharmacy, "Dunarea de Jos" University of Galati, Romania*
[2]*Department of Chemistry, Physics and Environment, Faculty of Sciences and Environment, "Dunarea de Jos" University of Galati, 47 Domneasca Street, 800008 Galati, Romania;*
E-mail: apetreic@ugal.ro

Abstract

An amperometric biosensor for serotonin detection and quantification, using a carbon paste electrode modified with cobalt(II) phthalocyanine and tyrosinase is described. The tyrosinase immobilization on carbon nanopowder was made using drop-and-dry method followed by cross-linking with glutaraldehyde. The novel biosensor response was optimized using serotonin and it presented the best performance in 0.01 mol L^{-1} phosphate buffer (pH=7.0). Under optimized working conditions at -0.1 V versus Ag/AgCl, a linear response range from 4 to 140 □M was obtained. The detection limit was 0.84 □M and the response time was lower than 5 s. The novel biosensor presented a stable response during 100 cycles under continuous monitoring by cyclic voltammetry. The variation of the response between four biosensor preparations was lower than 2%. The biosensor was applied in the determination of serotonin in walnut samples obtaining a good recovery average of 102%.

Key words: biosensor, carbon paste electrodes, tyrosinase, phthalocyanine, serotonin

Introduction

Among several different species of clinical interest, neurotransmitters are ones of the most investigated compounds [1]. Neurotransmitters have a crucial role in the nervous system, because they are the key to communication between neurons [2]. Biogenic amines, including catecholamines such as dopamine, norepinephrine, epinephrine and indoleamines such as serotonin, are neurotransmitters that have special roles in neuroscience [3]. Dopamine has been related to Parkinson's disease [4]. Epinephrine and norepinephrine have been a special interest in the origin of neurological tumors [5]. Serotonin (5-hydroxytryptamine) plays an important role in a variety of physiological functions (i.e. sleep regulation) and pathological states (psychiatric disorders, mental retardation, autism, etc.) [6]. Low levels of serotonin are associated with several disorders, including depression, anxiety, and migraines [7]. High levels of serotonin can manifest toxicity and potentially critical effects known as serotonin syndrome [8].
It has been reported that some foods contain significant quantities of serotonin. Foods reported to contain high amounts include banana, tomatoes, avocado, pineapple and walnuts [9].
Numerous techniques have been employed for the determination of serotonin such as high performance liquid chromatography [10], spectrofluorimetric method [11] and, capillary electrophoresis [12]. However, these techniques necessitate expensive instruments, time consuming pretreatment and/or derivatization processes which results in long and expensive procedures. Electrochemical techniques based on sensors and biosensors have been developed to solve these difficulties [13-15]. The determination of serotonin has been reported at carbon paste and diversity of other electrodes [17,18]. However, determination of serotonin by

electrochemical methods remained a challenge due to the interference of other biomolecules such as ascorbic acid, uric acid and dopamine present in biological sample.
Therefore, in this work the research interest has focused on developing a novel biosensor for electrochemical determination of serotonin. The biosensor has been fabricated from carbon nanopowder, tyrosinase as biocatalyst and cobalt(II) phthalocyanine as electron mediator. The capability of biosensor to detect serotonin has been evaluated. The biosensor characteristics including kinetics, calibration curve and limit of detection in the detection of serotonin has been investigated. The capability of biosensor to quantify the serotonin in walnut samples has been studied.

Materials and Methods

Chemical and solutions

The sources of materials and reagents used were as follows: serotonin and cobalt(II) phthalocyanine (CoPc) from Sigma-Aldrich. The enzyme, tyrosinase (EC 1.14.18.1, from mushroom) was purchased from Sigma. A 60μg·μL^{-1} solution of tyrosinase in buffer phosphate solution (0.01 M, pH=7) was used for the enzyme immobilization. The phosphate buffer solutions were prepared from potassium monobasic and dibasic phosphate salts from Aldrich. Ultrapure water (18 MΩ·cm, Millipore Milli-Q) was used for preparation of all aqueous solutions.

Carbon paste based biosensor

Carbon paste electrodes (CPE) were prepared using procedure previously reported [14,19], by mixing carbon nanopowder (<50 nm particle size (TEM), ≥99% trace metals basis, Sigma-Aldrich) and the cobalt(II) phthalocyanine (15%, w/w). Nujol was used as the binder of the multi-component composite mixture. Paste was packed into the body of a 1mL PVC (polyvinylchloride) syringe and compacted. A metallic copper wire was used as an electrical contact.
The tyrosinase (Ty) was immobilized on the above CoPc-CPE (CPE modified with CoPc) by drop-and-dry technique followed by cross-linking with glutaraldehyde. 10μL of 0.01 M phosphate buffer (pH 7.0) containing 50μg·μL^{-1} of enzyme, was added onto CoPc-CPE surface. After drying, the biosensor was exposed to a 5% (v/v) glutaraldehyde solution (in phosphate buffer 0.01M of pH 7) for 10 minutes at room temperature. The enzyme-immobilized electrode was dried at 10ºC and rinsed with phosphate buffer solution thrice to remove any unbound enzyme from the biosensor surface and was further dried at 10ºC and stored at 4ºC [19].

Apparatus

Voltammetric measurements were performed on an Biologic Science Instruments SP 150 potentiostat/galvanostat using the EC-Lab Express software. An Elmasonic S10H ultrasonic bath was used for dissolving and homogenization of solutions. For pH measurements an Inolab pH 7310 was used.
A three-electrode configuration was used in all cases, a Princeton Applied Research Ag|AgCl/KCl 3 mol L^{-1} and a Pt plate being used as reference electrode and counter electrode, respectively. Biosensor (Ty/CoPc-CPE) was used as working electrode for serotonin detection. All the electrochemical experiments were carried out in 0.01 M phosphate buffer solution (PBS) of pH=7 as supporting electrolyte.
Cyclic voltammograms were registered from -0.5 to +0.5V (the scan started at 0V) at a sweep rate of 0.05Vs^{-1} (except otherwise indicated).
The amperometric measurements were carried out in 50 mL of buffer solution and applying an adequate potential. Initially the current was continuously monitored until it reaches the

steady state. After that, additions of standard solution of serotonin were made into the buffer solution, which was stirred for a few seconds, in order to homogenize the solution before current monitoring.

Samples

Walnut (*Juglans regia*) samples of were obtained from local supermarkets. Samples 1 and 2 were entire walnuts, samples 3 and 4 were walnut seeds and sample 5 was roasted walnut seeds. 5 g of grounded sample was added in 25 mL of phosphate buffer solution. The mixture was ultrasonicated during 5 minutes. The liquid phase was separated by filtration. All the samples were prepared in triplicate and each measurable sample was analyzed with biosensor in triplicate.

Results and Discussions

Tyrosinase is a copper-containing enzyme that catalyze conversion of phenolic derivatives to the corresponding quinones in the presence of oxygen [19]. In order to explore the performance of proposed biosensors, they were immersed in 10^{-4} M serotonin in phosphate buffer solutions (0.01 M, pH 7).

Cyclic voltammetry studies

The response towards serotonin solution of the biosensor was registered in the range from -0.5 V to +0.5V at a scan rate of 0.05 $V \cdot s^{-1}$ (Figure 1).

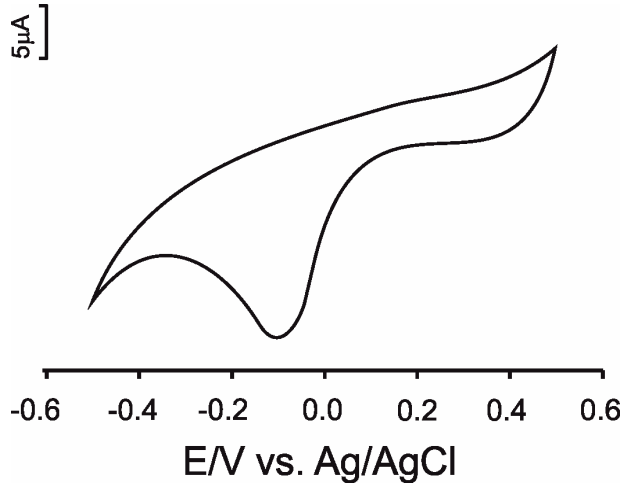
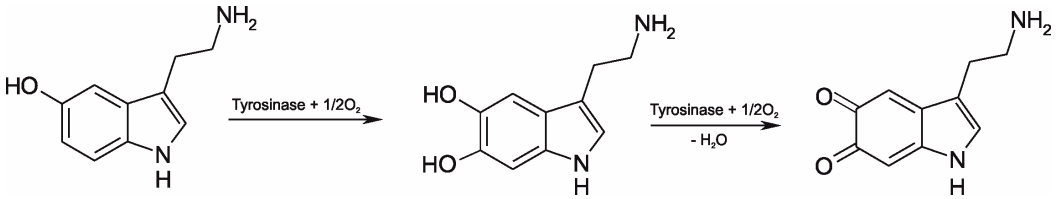


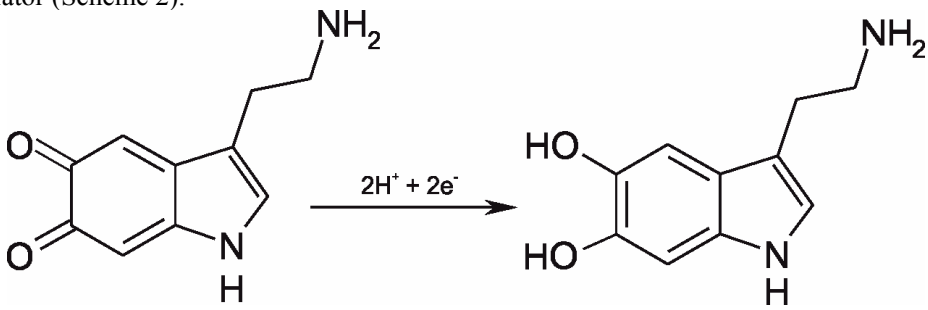
Figure 1: Cyclic voltammogram of biosensor in 10^{-4}M serotonin solution (0.01M PBS pH 7.0)

The cyclic voltammogram of biosensor in serotonin solution do not show the peaks related with phthalocyanine. As is show in the Figure 1, only the peak corresponding to reduction of the o-quinone derivative enzymatically formed at electrode surface appearing at -0.1V is observed. The presence of reduction peak indicates that the immobilization process retains the biocatalytic activity of tyrosinase immobilized onto solid substrate.

Tyrosinase catalyzes the oxidation of serotonin to o-serotonin-quinone derivative in two steps, as shown in Scheme 1.



The generated o-quinones can be reduced electrochemically at low potential without any mediator (Scheme 2).



Therefore, serotonin can be detected by electrochemical reduction of the o-serotonin-quinone [19].

The cobalt(II) phthalocyanine immobilized in carbonaceous matrix increase the peak current, therefore, the sensibility of the biosensor.

Optimization of the experimental parameters

Influence of scan rate in biosensors response were performed by registering the cyclic voltammograms of the Ty/CoPc-CPE biosensor in 10^{-4}M serotonin solution (in PBS 0.01 M, pH of 7) at different scan rates, from 0.05 to 0.30 V s^{-1} (Figure 2).

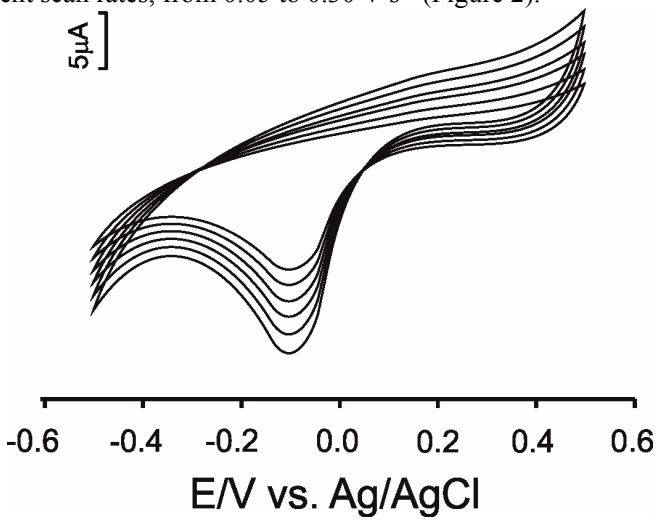


Figure 2. Cyclic voltammograms of Ty/CoPc-CPE biosensor registered at different scan rates in 10^{-4}M serotonin solution (0.01M PBS pH 7.0)

As observed in Figure 2 a linear regression is obtained representing the cathodic current (i / A) in function of scan rate (/ V s^{-1}). The equation of this regression is i = -3×10^{-5} − 6×10^{-6}

with $R^2 = 0.9962$. It was observed that peak height varies directly with the scan rate, showing that there is no diffusion limitation [20].
From the relationship of cathodic peak potential and logarithm of scan rate for an irreversible process is given by Eq. (1) [20].

$$E_c = \frac{2.3RT}{\alpha n_\alpha F} \log \nu + K \quad (1)$$

where E_c is the potential of cathodic peak (V), □ is the electron transfer coefficient, $n_□$ is the number of electrons involved in the redox process, F is the Faraday constant (F = 96,485 C mol^{-1}), ν is the potential scan rate (V s^{-1}), R is the ideal gas constant (8.314 J K^{-1} mol^{-1}), K is a constant, and T is the temperature (K).
The Tafel slope was found to be 124.4 mV/decade highlighting the issue of biosensor passivation since they are higher than the 60 mV/decade estimated for a two-electron rate determining step [20]. Considering $n_□$=2, the transfer coefficient determined from the slope of the representation E_c=f(log (ν)) is 0.284.

Optimization of experimental parameters
The modification of biosensor response on the applied potential was investigated over a potential range of −0.5 to +0.5 V, using 10^{-4}M serotonin in 0.01 M phosphate buffer (pH 7.0). The effect of applied potential for the Ty/CoPc-CPE biosensor on the amperometric signal and background current is shown in Figure 3.

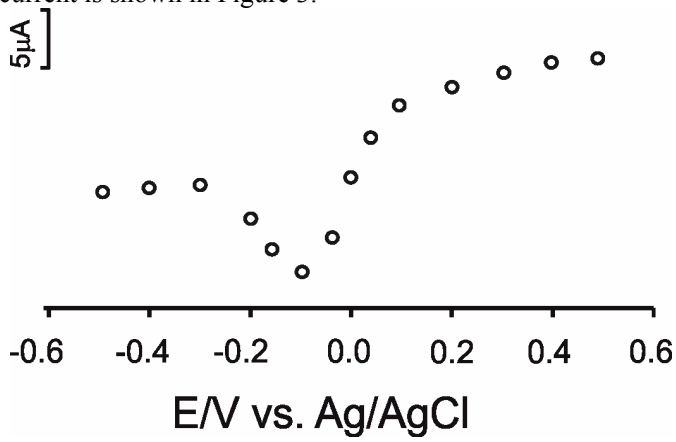


Figure 3. Cathodic current-applied potential dependence in 0.01 M PBS (pH 7.0) and 10^{-4} M serotonin under constant stirring.

The maximum of the signal vs. background current is obtained at -0.1 V. When applied potential more negative than -0.1 V, a higher current is achieved, but background current increases more rapidly because of possible reduction of dissolved oxygen. Therefore, −0.1 V was used as the applied potential. This potential is favorable, since only some chemical species expected to be present in samples are reduced at such a low potential [21].
The performance of the Ty/CoPc-CPE biosensor is affected by the pH value of solution due to the participation of protons in the enzymatic reaction. The effect of pH for the Ty/CoPc-CPE biosensor in 0.01 M PBS containing 10^{-4}M serotonin is shown in Figure 4.

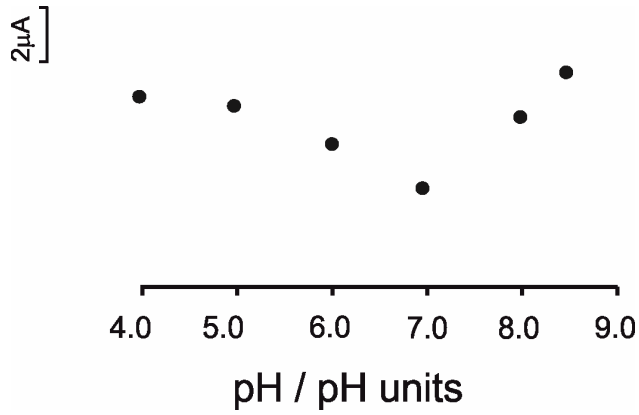


Figure 4. The effect of pH value in biosensor response. Conditions: 0.01 M PBS containing 10^{-4} M serotonin. Applied potential: -0.1 V.

The reduction current increases slightly as the pH changing from 4.0 to 7.0, and then decreases gradually from 7.0 to 9.0. The current achieves the maximum value at a pH of 7. This pH value is in accordance with the pH at which the enzymatic activity is maximum in solution [22]. To obtain the maximum response, a pH of 7.0 for the PBS was selected for the following studies.

Amperometric response of the biosensor

Figure 5 illustrates a characteristic amperometric response for the Ty/CoPc-CPE biosensor at -0.1V after the addition of successive aliquots of serotonin to the 0.01 M PBS (pH 7.0) under constant stirring.

A well-defined reduction current proportional to the concentration of serotonin is observed, which results from the electrochemical reduction of o-quinone derivatives enzymatically formed.

The Ty/CoPc-CPE biosensor achieves 95% of steady-state current in almost 5 s. Such rapid response is attributed to a fast electron transfer between the enzymatically-produced quinone derivative and the biosensor surface.

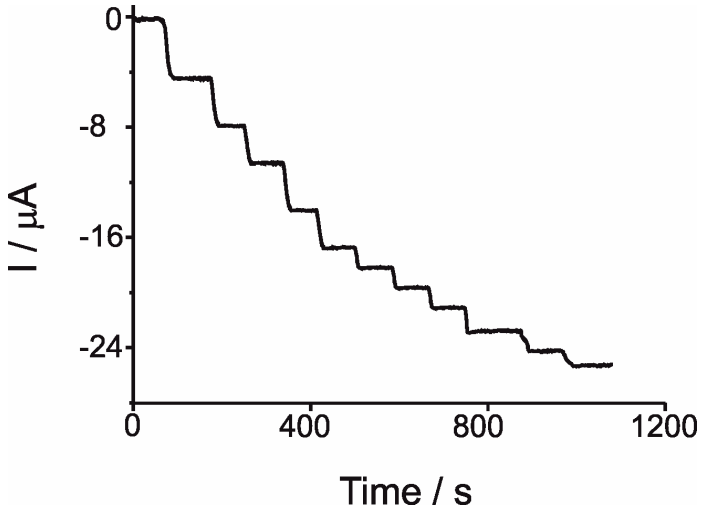


Figure 5. Amperometric response of biosensor to serotonin in 0.01 M PBS solution (pH 7.0). Applied potential -0.1 V.

Figure 6 showed the relationship between the response current of the biosensor and the serotonin concentration in PBS (pH 7.0) at -0.1V (calibration curve).

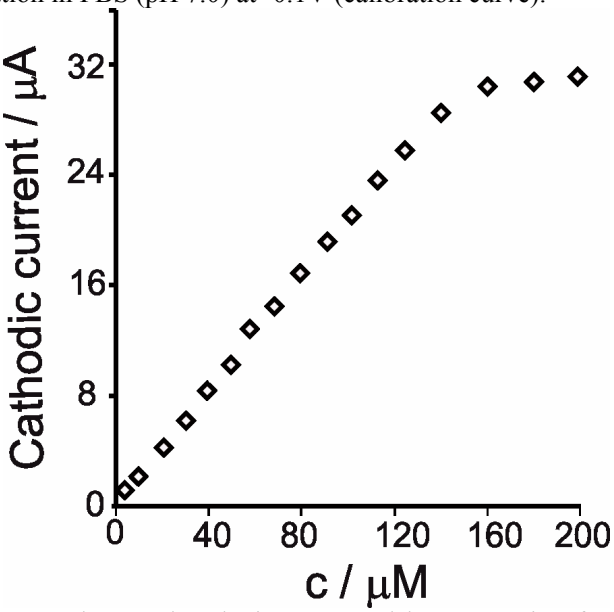


Figure 6. The calibration curve between the reduction current and the concentration of serotonin in PBS (pH 7.0) at -0.1 V.

It can be seen from Figure 6 that the response current is linear with serotonin concentration in the range from 4 to 140 □M. The sensitivity of the biosensor is 0.2034 A M^{-1}. The corresponding detection limits were calculated according to the $3s_b/m$ criterion. In this equation m was the slope of the calibration graph. The s_b was estimated as the standard deviation (n = 5) of the amperometric signals from different solutions of the substrate at the concentration level corresponding with the lowest concentration of the calibration plot. The detection limits calculated were 0.84 □M.

From the calibration data, the Hill coefficient (h) can be calculated by representing the $\log[I/(I_{max}-I)]$ vs. log [serotonin]. A Hill coefficient of 1.06 ± 0.02 was calculated for the reduction process of o-quinone derivative formed from the enzymatic reaction on the electrode surface (R^2 = 0.9976). The value obtained for the h parameter, obtained from the corresponding Hill's plot was close to unity demonstrating that the kinetics of the enzymatic reaction fitted into a Michaelis-Menten type kinetics. The value slightly higher than 1 obtained for biosensor immersed in serotonin solution demonstrates a positive cooperative effect between the occupied active sites [17].

The parameters for Michaelis-Menten kinetics were calculated from the steady-state currents and the electrochemical adaptation of the Lineweaver-Burk equation (Eq. 2):

$$\frac{1}{I} = \frac{1}{I_{\max}} + \frac{K_M^{app}}{I_{\max}[S]} \tag{2}$$

where: I is the steady-state current after the addition of analyte, [S] is the concentration of serotonin, I_{max} is the maximum rate of the enzymatic reaction, and K_M^{app} is the apparent Michaelis-Menten constant.

The I_{max} and K_M^{app} can be calculated from the intercept and slope. The results obtained for the Ty/CoPc-CPE biosensor towards serotonin are: I_{max} is 32.52 □A, and K_M^{app} *is* 78.54 □M.

From the above results could be concluded that Ty/CoPc-CPE biosensor has good quality performances. Moreover, they are similar or better to those reported for other biosensors used for serotonin detection [16-18].

Biosensor repeatability and stability

To investigate the repeatability of the biosensor, the cyclic voltammetry measurements were performed in a 100 □M serotonin solution using the same biosensor. The relative standard deviation (RSD) of the measurements corresponding to 100 cycles was 4.8%. Repeatability of the biosensor was better than the result from previous literature [16-18].

In order to study the reproducibility of the biosensor fabrication four different biosensors were prepared. The amperometric signal towards 100□M serotonin solution were recorded. The differences between the amperometric signals in the term of relative standard deviation are lower than 2%. Therefore, the biosensor construction is highly reproducible.

Application of the biosensor to determination of serotonin in walnut samples

The proposed biosensor was tested in the determination of serotonin in the walnut samples. Thus, Table 1 shows the results obtained in the biosensor application for serotonin determination, obtained through the standard addition method in the walnut samples.

Table 1. Values of serotonin concentration, obtained with the proposed biosensor, through standard addition method

Sample number	Serotonin concentration (□g g^{-1})
1	70 ± 6
2	74 ± 6
3	52 ± 5
4	51 ± 4
5	24 ± 2

In order to evaluate the matrix effect Table 2 shows the results obtained in the recovery experiments of five different samples. The results obtained suggest that this proposed biosensor can be applied enough well in biological samples with no significant influence of the matrix. The recoveries obtained were very good giving an average recovery of 102±2%.

Table 2. Recovery % obtained with the biosensor after serotonin addition (50□M) in each walnut samples

Sample number	Recovery value (%)
1	104 ± 2[a]
2	97 ± 3[a]
3	104 ± 3[a]
4	103 ± 2[a]
5	102 ± 2[a]

[a] Relative standard deviation for three replicates

The biosensor presented a linear response range for serotonin determination in the range between 4 to 140 □M of serotonin. Considering that in walnut the serotonin level is around 87±20 □g g^{-1} [9], therefore it could be employed for serotonin determination in food samples.

Conclusions

This work reports an easy biosensor construction for serotonin determination, modifying carbon paste with CoPc, tyrosinase and glutaraldehyde. The biosensor construction is highly reproducible, allowing to obtain biosensors with quite similar sensitivities (RSD < 2%). The biosensor presented a linear response range between 4 and 140 □M of serotonin, with a good sensitivity, making possible the determination of serotonin in walnut samples, and opening the possibilities for quantification of serotonin in food samples. In this sense, the proposed biosensor offer a good alternative to the existing methods, allowing carried out fast, simples and low-priced analysis with minimal pre-treatment of the sample.

Acknowledgments

This work was supported by a grant of the Romanian National Authority for Scientific Research, CNCS - UEFISCDI, project number PN-II-ID-PCE-2011-3-0255.

References

1. [1] D.T. MARC, J.W. AILTS, D.C.A. CAMPEAU, M.J. BULL, K.L. OLSON, Neurotransmitters excreted in the urine as biomarkers of nervous system activity: validity and clinical applicability. *Neurosci. Biobehav. Rev.* **35**, 635-644 (2011).
2. [2] R.R. GONZALEZ, R.F. FERNANDEZ, J.L.M. VIDAL, A.G. FRENICH, M.L.G. PEREZ, Development and validation of an ultra-high performance liquid chromatography-tandem mass-spectrometry (UHPLC–MS/MS) method for the simultaneous determination of neurotransmitters in rat brain samples. *J. Neurosci. Methods*, **198**, 187-194 (2011).
3. H.E. HIMWICH, W.A. HIMWICH (Eds.), *Biogenic Amines*, Elsevier Publishing Company, Amsterdam, 1964.
4. K.L. ESKOW JAUNARAJS, J.A. GEORGE, C. BISHOP, L-DOPA-induced dysregulation of extrastriatal dopamine and serotonin and affective symptoms in a bilateral rat model of Parkinson's disease. *Neuroscience*, **218**, 243-56 (2012).
5. S.G. PALOP, A.M. ROMERO, J. M. CALATAYUD, Oxidation of adrenaline and noradrenaline by solved molecular oxygen in a FIA assembly. *J. Pharmaceut. Biomed.*, **27**, 1017-1025 (2002).
6. E. HAMMOCK, J. VEENSTRA-VANDERWEELE, Z. YAN, T.M. KERR, M. MORRIS, G.M. ANDERSON, C.S. CARTER, E.H. COOK, S. JACOB, Examining Autism Spectrum Disorders by Biomarkers: Example From the Oxytocin and Serotonin Systems. *J. Am. Acad. Child Psy.*, **51**, 712-721 (2012).
7. L. IMERI, M. MANCIA, S. BIANCHI, M.R. OPP, 5-Hydroxytryptophan, but not l-tryptophan, alters sleep and brain temperature in rats. *Neuroscience*, **95**, 445-452 (2000).
8. L.N. SQUIRES, K.N. TALBOT, S.S. RUBAKHIN, J.V. SWEEDLER, Serotonin catabolism in the central and enteric nervous systems of rats upon induction of serotonin syndrome. *J. Neurochem.*, **103**, 174-180 (2007).
9. J.M. FELDMAN, E.M. LEE, Serotonin content of foods: effect on urinary excretion of 5-hydroxyindoleacetic acid. *Am. J. Clin. Nutr.*, **42**, 639-643 (1985).
10. S. UMEDA, G.W. STAGLIANO, M.R. BORENSTEIN, R.B. RAFFA, A reverse-phase HPLC and fluorescence detection method for measurement of 5-hydroxytryptamine (serotonin) in Planaria. *J. Pharmacol. Toxicol. Methods*, **51**, 73-76 (2005).
11. A.G. BRACAMONTE, A.V. VEGLIA, Spectrofluorimetric determination of serotonin and 5-hydroxyindoleacetic acid in urine with different cyclodextrin media. *Talanta*, **83**, 1006-1013 (2011).
12. A. ZINELLU, S. SOTGIA, L. DEIANA, C. CARRU, Reverse injection capillary electrophoresis UV detection for serotonin quantification in human whole blood. *J. Chromatogr. B*, **895–896**, 182-185 (2012).
13. I.M. APETREI, C. APETREI, Amperometric biosensor based on polypyrrole and tyrosinase for the detection of tyramine in food samples. *Sens. Actuators B*, **178**, 40-46 (2013).
14. M.L. RODRIGUEZ-MENDEZ, M. GAY, C. APETREI, J.A. DE SAJA, Biogenic amines and fish freshness assessment using a multisensor system based on voltammetric electrodes. Comparison between CPE and screen-printed electrodes. *Electrochim. Acta*, **54**, 7033–7041 (2009).

15. R.N. GOYAL, B. Agrawal, Ag ion irradiated based sensor for the electrochemical determination of epinephrine and 5-hydroxytryptamine in human biological fluids, *Anal. Chim. Acta*, **743**, 33-40 (2012).
16. T. JACOMETO CASTILHO, M.P. TABOADA SOTOMAYOR, L. TATSUO KUBOTA, Amperometric biosensor based on horseradish peroxidase for biogenic amine determinations in biological samples. *J. Pharm. Biomed. Anal.*, **37**, 785-791 (2005).
17. C. APETREI, M.L. RODRIGUEZ-MENDEZ, J.A. DE SAJA, Amperometric tyrosinase based biosensor using an electropolymerized phosphate-doped polypyrrole film as an immobilization support. Application for detection of phenolic compounds. *Electrochim. Acta*, **56**, 8919-8925 (2011).
18. J. LI, X. LIN, Simultaneous determination of dopamine and serotonin on gold nanocluster/overoxidized-polypyrrole composite modified glassy carbon electrode. *Sens. Actuators B*, **124**, 486-493 (2007).
19. I.M. APETREI, M.L. RODRIGUEZ-MENDEZ, C. APETREI, J.A. DE SAJA, Enzyme sensor based on carbon nanotubes/cobalt(II) phthalocyanine and tyrosinase used in pharmaceutical analysis. *Sens. Actuators B*, **177**, 138-144 (2013).
20. A.J. BARD, L.R. FAULKNER, *Electrochemical Methods*, John Wiley and Sons, New York, 2001.
21. R.S. FREIRE, N. DURAN, L.T. KUBOTA, Electrochemical biosensor-based devices for continuous phenols monitoring in environmental matrices. *J. Brazil. Chem. Soc.*, **13**, 456-462 (2002).
22. M. FRIEDMAN, H.S. JURGENS, Effect of pH on the stability of plant phenolic compounds. *J. Agr. Food Chem.*, **48**, 2101-2110 (2000).