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Construction of a Potentiometric Glutamate Biosensor for Determination of Glutamate in Some Real Samples

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Abstract: The potentiometric glutamate biosensor based on ammonium-selective poly(vinylchloride) (PVC) membrane electrode was constructed by chemically immobilizing glutamate oxidase. Ammonium ions produced after an enzymatic reaction were determined potentiometrically. We determined the optimum working conditions of the biosensor such as buffer concentration, buffer pH, lifetime, response time, linear working range, kinetic constants (K_m and V_{max}) of glutamate oxidase enzyme used for biosensor construction values, and other response characteristics. Additionally, glutamate assay in some real samples such as chicken bullion, healthy human serum, and commercial multipower amino acid mixture were also successfully carried out. The results showed good agreement with previously reported values.

Keywords: Glutamate oxidase; Glutamate biosensor; Enzyme electrode; Potentiometric; Glutamate determination

INTRODUCTION

L-glutamate is an important amino acid playing a significant role in areas such as protein metabolism, manufacturing of pharmaceutical products, medicine and food industry. In the mammalian central nervous system, it is an excitatory amino acid neurotransmitter. Since it plays a key role in a wide variety of brain functions, glutamate in an exess amount may cause neuronal death and some neurological disorders such as epilepsy, schizophrenia, stroke, ischemia, and Alzheimer's and Parkinson's diseases [1–4]. Although glutamate is not essential for human nutrition, it is widely used as a flavor enhancer in foodstuffs, especially fast foods, and it has been associated with the Chinese restaurant syndrome [5]. Thus, there is a need for rapid and sensitive methods to detect it in a variety of biological and food samples [1].

For glutamate determination, several methods have been developed. These methods have different detection techniques. There are many methods such as spectrophotometric [6], fluorescence [7], chromatographic techniques [8–10], and capillary electrophoresis [11,12].

The electrochemical approach to glutamate determination is advantageous because it has a potential to provide a fast and sensitive quantification of this amino acid. The approach is based on the enzymatic reaction between the glutamate and one of the redox enzymes such as glutamate oxidase or glutamate dehydrogenase. Alternatively, an enzyme electrode can be combined with an electrochemical sensor to produce an electrode that will measure the spesific substrate to that enzyme. Such electrodes, known as enzyme electrodes, have enabled the assay of many compounds of biochemical interest. However, they remain largely unexploited in medical and pharmaceutical measurements [13]. Biosensors containing an immobilized enzyme or enzymes as biologically active material basically translates biological material(s) to electrochemical signals on this electrode surface. Ion-selective electrodes immobilized on one or more enzyme, the determination of which various substrate gained importance in recent years. The glutamate determination methods based on biesensors have also been pointed out. Most of these have been constructed as amperometrically [14–20].

In this study, ammonium-selective potentiometric glutamate biosensor was constructed by immobilizing glutamate oxidase enzyme onto a PVC membrane electrode containing palmitic acid and nonactine as an ammonium ionophore. Glutamate oxidase enzyme catalyses the hydrolysis of glutamate:

$$\text{Glutamate} + O_2 + H_2O \rightarrow \alpha\text{-Keto Glutarate} + H_2O + NH_4^+$$

Ammonium ions produced were determined potentiometrically. The buffer concentration, buffer pH, lifetime,

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response time, optimum working range, Km and Vmax values, interference effects of ascorbic acid, some ions and amino acids were also determined. Furthermore, we investigated whether these biosensors can be used to determine glutamate level in several real samples, such as chicken bullion, multipower amino acid mixture, and human serum samples.

MATERIALS AND METHODS

Reagents and Apparatus

Nonactin, bis-(2-ethyl)hexylsebacate (DOS), poly(vinylchloride) (PVC), palmitic acid were obtained from Fluka. Glutamate oxidase (E.C.1.4.3.11) (5 U/mg), 1-ethyl-3-(3-dimetylaminopropyl) carbodiimide (EDC), trisma base, trisma-HCl, ammonium chloride, monosodium glutamate, L-glutamine, L-alanine, L-arginine, L-valine, L-ascorbic acid, L-lysine monohydrate, L-histidine, L-methionine, L-serine, L-glycine and L- cysteine were obtained from Sigma Chem. Co. (St. Louis, MO, USA). Tetrahydrafurane (THF) was purchased from Merck. All other chemicals used were of analytical reagent grade. Standard solutions and buffer solutions were prepared with deionized water.

The human serum samples and multipower amino acid mixture for glutamate assay were provided from Yildiz Technical University Health Center and GNC Live Well Firm, respectively. Chicken bullion was purchased in a local market. Potential and pH measurements were carried out with an ORION 4 Star Benchtop pH-ion meter. The potential values were given against the Ag/AgCl double junction reference electrode (ORION 90-02).

Construction of Ammonium Electrode

To prepare ammonium-ion selective electrode, 118 mg PVC, 13.6 mg palmitic acid, 12.6 mg nonactin and 255.8 mg bis-(2-etil) hekzil sebakat (DOS) were prepared by dissolving in 5 mL of tetrahydrofurane (THF). The solution was poured on a glass plate inside a glass ring (42 mm diameter). After 24 hours of drying at room temperature (20–25°C), the membrane was formed (approximately 0.5 mm thickness). Disks of 5 mm diameter were cut out and attached to the glass electrode body. The inner electrode solution was 1.0×10^{-2}M ammonium chloride (pH 7.0).

Construction of Glutamate Biosensor

For chemical immobilization of glutamate oxidase enzyme onto ammonium-selective membrane electrode surfaces, first the first stock glutamate oxidase enzyme solution was prepared by dissolving 5 U glutamate oxidase enzyme in 1 mL bidistilled water. 200 μL of this solution was mixed with 1 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. 50 L of this solution was dropped on the membranes of ammonium-selective electrodes and left overnight. Then, 25 L of 2.5% glutaraldehyde solution was deposited on the surface of this biosensor, the biosensor was left for 25 minutes, and the electrode surface was washed with bidistilled water to remove the excess glutaraldehyde. Then, 50 L of second stock glutamate oxidase enzyme solution (5U glutamate oxidase/1 mL bidistilled water) was deposited on the glutamate biosensor surface. The biosensor was left overnight. To remove the excess of unbounded enzymes all types of biosensors were left for 1 hour in a vigorously stirred phosphate buffer (10 mM, pH 7.0). When not in use, biosensors were stored in a refrigerator at 4°C.

Potentiometric Measurement

For glutamate biosensor, potential measurements were made by varying glutamate concentration in a steady-state condition. 10 mM of TRIS buffer was used as a working buffer solution (pH 8.2). Measurements were made with the proposed glutamate biosensor and Ag/AgCl reference electrode. The biosensor was immersed to a depth of 1.5 cm in glutamate solution and stirred by a magnetic stirrer. The pH values were determined using an Orion combinated glass-pH electrode. All the experimental works were carried out at room temperature (20–25°C).

The calibration curves were obtained by plotting the potential values of a series of standard glutamate solution against the logarithm of glutamate concentration.

Procedure for Determination of Glutamate in Real Samples

The glutamate amount in chicken bullion, human serum, and commercial multipower amino acid mixture were determined separately by using the standard addition method. The glutamate biosensor was immersed in the 10 mM TRIS buffer containing a certain amount of these biological samples. Then, stock glutamate solution (10^{-3}M pH 8.2) was added to this solution step by step. After each step, potential values against added amount (mL) of glutamate solution were plotted and ammonium amount was determined by using our prepared ammonium-selective glutamate biosensor. To check our results obtained from proposed glutamate biosensors, these values were compared with reported values.

RESULTS AND DISCUSSION

Sensitivity of Glutamate Biosensors Towards Ammonium and Glutamate

The calibration curves of glutamate biosensor for ammonium ions and glutamate are shown in Figures 1 and 2. It can be seen from Figures 1 and 2 that the responses of glutamate biosensor with PVC containing palmitic acid towards ammonium ions and glutamate were about Nernstian after immobilizing the enzymes onto a PVC membrane containing palmitic acid. The sensitivities of glutamate biosensor towards ammonium ions and glutamate are very good in the concentration ranges of 1.0×10^{-1}–1.0×10^{-5}M and $1.0\times10^{-1}-1.0\times10^{-3}$M, respectively. The slope of the biosensor was 53.18 ± 1.2 mV/p[NH_{4+}] and 45.23 ± 0.58 mV/p[glutamate] closed to Nernstian value of 59 mV/substrate concentration. Therefore we have decided that the glutamate biosensor can be used for glutamate determination in biological samples.

The linear working range of amperometric glutamate biosensor obtained $5{,}0\times10^{-6}-7{,}8\times10^{-5}$M [18]. The linear response was at the range of $1{,}0\times10^{-1}$–$1{,}0\times10^{-3}$M with our prepared potentiometric glutamate biosensor. Because our prepared glutamate biosensor based on PVC membrane has an operating range of $1.0\times10^{-1}-1.0\times10^{-3}$M, it can be said that the glutamate biosensor can be used to determine glutamate in biological samples with many more advantages.

Effect of Buffer Concentration

For examination of buffer concentration on the response of the glutamate biosensor, the slopes of the biosensor were measured at six different buffer concentrations varying from 5 mM to 30 mM. Nernstian responses for all glutamate biosensor were obtained at 10 mM TRIS buffer (Figure 3). First reference to Figure 3.

As shown in Figure 3, the highest slope obtained for glutamate biosensor optimum buffer concentration was found to be 10mM. Some researcher pointed out that the rise in buffer concentration caused sensor sensitivity in the phosphate buffer [18,21]. This is compatible with our results. We also showed the decrease in biosensor response when the buffer concentration of our biosensor increased. Although we tried to study with the phosphate buffer, we could not obtain good results.

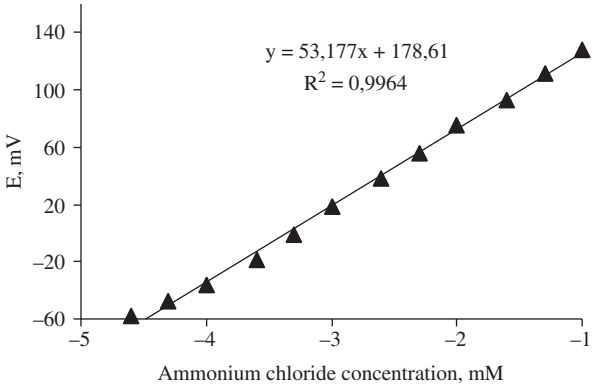

Figure 1. The response of glutamate biosensor against ammonium ions. The study was carried out with 10^{-1}–10^{-6}M ammonium chloride calibration solutions in 10 mM TRIS buffer (pH 8.2).

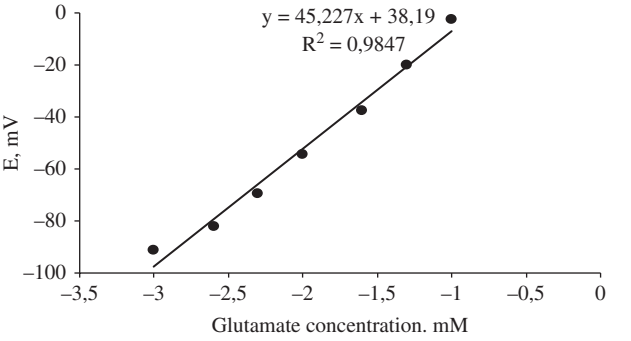

Figure 2. The calibration graphs of glutamate biosensor. The study was carried out with 10^{-1}–10^{-3}M glutamate calibration solutions in 10 mM TRIS buffer (pH 8.2).

Effect of pH

Effect of pH on the analytical signal of the glutamate biosensor was investigated by measurements in 10 mM TRIS buffer at six different pHs and in the range of 6.0–9.0.

As shown in Figure 4, the slope of the glutamate biosensor gradually increases until pH 8.5 and then falls. Glutamate oxidase immobilized on the biosensor can be denaturized after pH 8.5 and the enzymes activity is

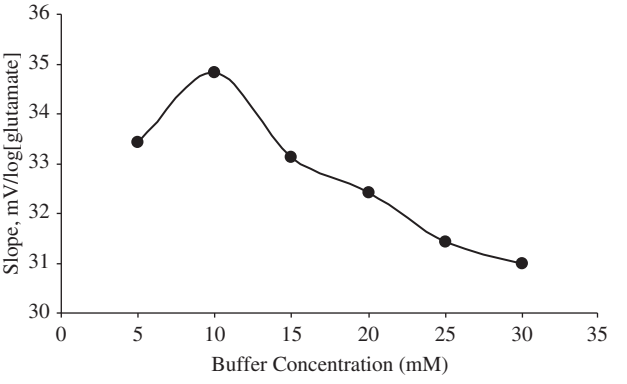

Figure 3. Effect of buffer concentration on glutamate biosensor. The study was carried out with 10^{-1}–10^{-5}M glutamate calibration solutions in TRIS buffer (pH 8.2).

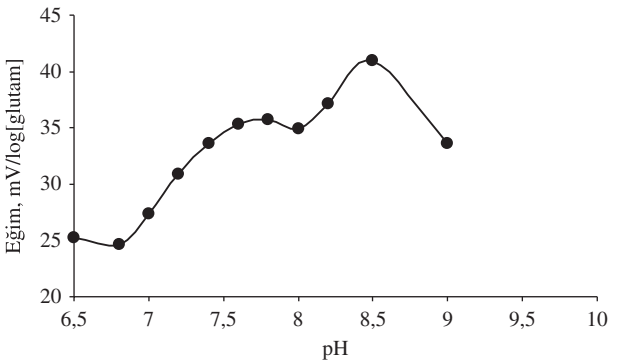

Figure 4. Effect of pH on glutamate biosensor. The study was carried out with $10^{-1}-10^{-5}$M glutamate calibration solutions in 10mM TRIS buffer at changing pH values.

reduced [22]. The response was also diminished with increase in buffer pH, which are common properties of the potentiometric systems [23]. Because the maximum activity of the biosensor was shown at pH 8.4, this pH value was chosen as optimum pH and used for all other measurements.

Because glutamate oxidase has low pI value and it is negatively charged at lower pHs, it was studied at basic pHs [18,24]. The optimum pH of glutamate oxidase was determined amperometrically as near-neutral pHs in phosphate buffer [1,16,25]. The pH ranges we studied were much broader than that of other related studies in literature and we determined as the optimum pH value of 8.2.

Effect of Temperature

To examine the effect of temperature on our glutamate biosensor response, potential measurements were separately made in prepared glutamate calibration solutions at six different temperatures varying from 20°C to 45°C and determined at optimum buffer concentration and pH, then the slope values of the biosensor were determined

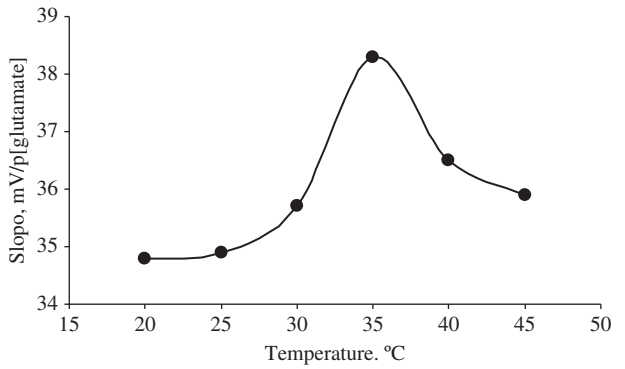

Figure 5. Effect of temperature on glutamate biosensor. The study was carried out with $10^{-1}-10^{-5}$M glutamate calibration solutions in 10 mM TRIS buffer (pH 8.2).

Table 1. The selectivity coefficients of glutamate biosensor.

İon	Na^+	K^+	Ca^{2+}
Selectivity coefficient	$3{,}8\times10^{-2}$	$2{,}4\times10^{-1}$	$1{,}3\times10^{-3}$

by plotting calibration graphs for each temperature (Figure 5). First reference to Figure 5.

As seen from Figure 5, the slope of biosensor increased until 35°C. The decrease in the slope of the biosensor after 35°C shows that glutamate oxidase activity immobilized on biosensor fallen. The optimum operating temperature of biosensor was determined as 35°C. The studies related to glutamine biosensor can also be carried out at high temperatures because glutamate oxidase is a thermally stable enzyme [26]. Pan et al. determined 48°C of optimum temperature in their study and reported thermal decomposition of the enzyme immobilized on the biosensor [26]. This value was determined as 37°C in a different study [25]. Although we determined optimum temperature as 35°C, we studied all experiments at room temperature because of cost and operation simplicity.

Selectivity on Ammonium-selective Glutamate Biosensor

For determining the interference effects of Na+, K+ and Ca^{+2} ions (as chlorides, pH 7.0) on the response of the glutamate biosensor, we tested in view of its sensitivity towards sodium, potassium and calcium ions (as chlorides, pH 7.0) with our proposed glutamate biosensor, and their selectivity coefficients were evaluated by the separated solution method according to the IUPAC recommendation [27]. As seen in Table 1, interference effects of these ions is low, but K+ ions showed the most interference among these cations.

The interference effects of some amino acids, -glycine, alanine, valine, serine, cysteine, histidine, methioinine, glutamate, arginine and lysine-, the stock solutions of glutamate and 11 different amino acids of 10^{-2}M were prepared. The glutamate and amino acid solution at the same concentration was prepared separately for each amino acid at optimum conditions (10^{-3}M, pH 7.4) and we carried out mV measurements for eachamino acid. In addition, measurements were made in the glutamate solution (10^{-3}M, pH: 8.2) without the addition of any other amino acid. In this way, the interference effect of amino acids was determined as a percentage. The effect of interference of each amino acid is given in Table 2. Although glutamine showed a more important interference effect than other amino acids, the others did not show any negative effect on biosensor response. In addition, the interference effect of ascorbic acid that can present in human serum medium was studied and this substance did not show any interference effect on the biosensor.

Table 2. Selectivity of glutamate biosensor towards ascorbic acid and some amino acids.

Amino acid	Relative Response %
Lysine	0,19
Serine	0,29
Methionine	1,28
Glycine	0,59
Valine	0,99
Arginine	1,68
Alanine	1,97
Histidine	2,47
Cysteine	4,5
Glutamine	41
L-Ascorbic Acid	1,38

Effect of Stirring Rate

To study the effect of stirring rate on the responses of glutamate biosensors, the measurements were done in 10 mM TRIS buffer (pH 8.2) at three different stirring rates (100, 250, and 750 rpm) after reaching stable potential. When stirring was made at 100 and 750 rpm levels, the time to reach stable potential was longer than those of the 250 rpm rate level. A decrease of stirring rate (100 rpm) has not resulted in any influence on the magnitude of the analytical signal, but has caused an increase in the response time of sensors. This situation can be explained in that enzymatic reaction is slow at low stirring rate. At a high stirring rate (750 rpm), the rate of enzymatic reactions also decreases because of possible changes in the enzyme conformation; therefore, the magnitude of analytical signal was found to be lower. For this reason, 250 rpm is determined as the optimal stirring rate.

Response Time

Response time is very important for biosensors. An ideal biosensor should respond in a short time. The response time of the glutamate biosensors was determined by recording the time elapsed to reach a stable potential value after the biosensor and the reference electrode were immersed in calibration solutions. This response time changed between 1-2 minutes; however, the response time of the IC biosensor was less than 1 minute.

Storage Stability of the Glutamate Bioensors

To determine the lifetime of the glutamate biosensor for a period of 45 days, potential measurements were made 2 or 3 times per week and the linear working range and slopes were determined by drawing calibration graphs. It was shown that there was 12% of activity loss response after 15 days and there was no additional activity decrease until the forty-fifth day for a glutamate oxidase-based biosensor (Figure 6).

Because the lifetime of our glutamate biosensor based on glutamate oxidase and PVC membrane containing palmitic acid shelf life is very long, it can be easily used in bioanalytical studies. The lifetime of glutamate biosensor was determined as 2 or 3 weeks for the amperometric glutamate biosensor [1,25]. The lifetime of our potentiometric glutamate biosensor is much better than these amperometric biosensors.

Reproducibility of the Glutamate Biosensors

To determine the reproducibility of the biosensor prepared, mV measurements were made in 10 serial glutamate calibration solutions (pH 8.2) on the same day and the slope of calibration graphs was determined after each measurement (Figure 7). Numerically the largest slope value was assumed to be 100% and the other slope values were calculated according to it. It was determined that the lowest slope percent was 85%. The results obtained

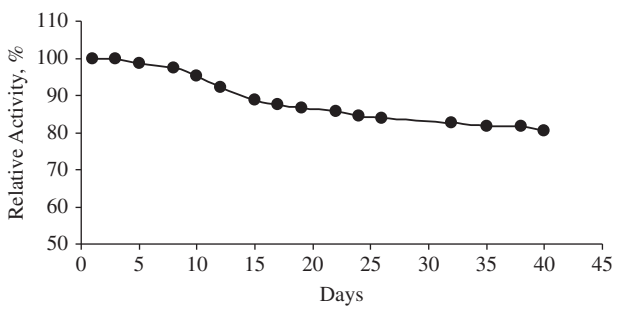

Figure 6. The lifetime of glutamate biosensor for a period of 45 days. The study was carried out with 10^{-1}–10^{-5}M glutamate calibration solutions in 10 mM TRIS buffer (pH 8.2).

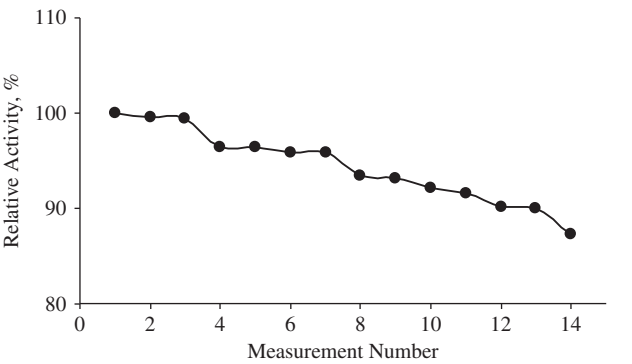

Figure 7. Reproducibility of the glutamate biosensors within a day. The study was carried out with $10^{-1}-10^{-5}$M glutamate calibration solutions in 10 mM TRIS buffer (pH 8.2).

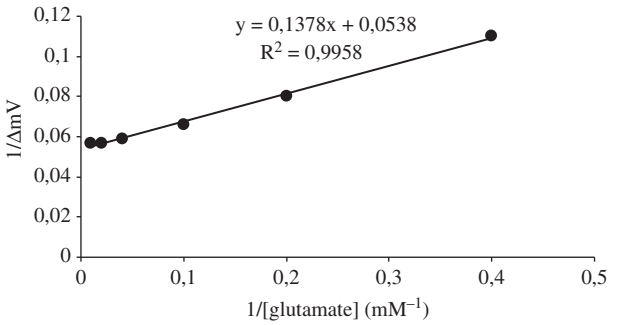

Figure 8. The Lineweaver-Burk plot of glutamate oxidase immobilized in the glutamate biosensor's membrane. The measurements were made using our proposed biosensor.

show that the reproducibility of our glutamate biosensor is very good.

Determination of Kinetic Constants with Glutamate Biosensor

In order to determine the maximum velocity (V_{max}) and Michaelis-Menten constant (K_m) of the glutamate oxidase enzyme presented in the glutamate biosensor, initial reaction rates of glutamate oxidase at optimal pH and temperature values were measured using glutamate calibration solutions at different concentrations (1–10 mM). Km and Vmax values were calculated by means of the Lineweaver-Burk plot [28]. K_m and V_{max} values of glutamate oxidase containing glutamate biosensor were found to be 162 mM and 21.1 mV/p[glutamate], respectively.

Glutamate Determination in Chicken Bullion, Human Serum Samples, and Multipower Amino Acid Mixture

We investigated if our prepared glutamate biosensor based on a PVC membrane containing palmitic acid can be used in real samples, chicken bullion, human serum samples, and multipower amino acid mixture for glutamate determination. For this purpose, glutamate assay in these samples was carried out using a standard addition method. The results obtained with the biosensor and reported values are shown in Table 3.

Monosodium glutamate used as a food additive was determined with the glutamate biosensor we prepared in chicken bullion. The maximum amount of monosodium glutamate was coded as E621 in the official newspaper of the Agriculture and Rural Affairs Ministry published in 2008 at Annex-1. According to this newspaper, it should be added as 10 g/kg of monosodium glutamate or between 0.1% and 0.8% in foods. The value obtained from our prepared biosensor is compatible with these values.

As seen in Table 3, references values show good correlation with that of our glutamate biosensor for each sample. Therefore, glutamate assays were successfully made with the proposed glutamate biosensor without any interfering effect presented in the sample medium.

Table 3. The results of glutamate content obtained from the proposed glutamate biosensor in several samples

	Reported value	Glutamate biosensor
Chicken bullion (mg/10g tablet)	10g/kg	63,4
Multipower amino acid mixture (mg /100(mue)L)	1,48	1,38
Human serum (mg / 100μL)	$6{,}6 \times 10^{-4}$	$6{,}5 \times 10^{-4}$

CONCLUSION

An ammonium-selective glutamate biosensor was constructed by immobilizing glutamate oxidase onto a PVC membrane containing prepared palmitic acid of ammonium electrode. The analytical characteristics, such as sensitivity towards glutamate, dynamic stability, response time, repeatibility, and lifetime of the biosensor, are very good. The response time is 1-2 minutes at the linear concentration range of 10^{-1}–10^{-4}M and pH:8.2. The rapid determination of glutamate amounts in chicken bullion, human serum samples, and multipower amino acid mixture was also made possible. The interference effects of Na+, K+ and Ca++ ions, ascorbic acid, and other several amino acids reported in the text that can present in the biological medium were not shown on the response of the proposed potentiometric glutamine biosensor. This is an advantage for glutamate determination in serum and aminoacid mixture when it is compared with other amperometric biosensors.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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