БІОСЕНСОРИ
BIOSENSORS

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APPLICATION OF AMMONIUM-SELECTIVE ZEOLITE FOR ENHANCEMENT OF CONDUCTOMETRIC BI-ENZYME BIOSENSOR FOR L-ARGININE DETECTION

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Abstract. L-arginine conductometric biosensors were developed on the basis of arginase and urease cross-linked by glutaraldehyde in a single bioselective membrane and modified with clinoptilolite. The clinoptilolite intrinsic properties helpful for conductometric detection of L-arginine were investigated using three approaches to the creation of zeolite-containing enzymatic membranes. The developed biosensors were compared with the L-arginine biosensor, not modified with clinoptilolite, for the sensitivity, linear and dynamic range, detection limit, response time, operational and storage stability in L-arginine analysis. It was shown that the incorporation of ammonium-selective zeolite to the bioselective membrane of L-arginine biosensor allows increasing the biosensor sensitivity when applying all proposed approaches to the formation of zeolite-containing biomembranes. In addition, excellent values of both detection limit and linear range (1.0×10^{-5} M and 0.01–6 mM, respectively) were achieved for the biosensor based on arginase, urease and zeolite distributed in a single bioselective layer. The clinoptilolite-based biosensors for L-arginine demonstrated short response time, high operational stability (a coefficient of variations reached 0.74%), their lifetime exceeded four months.

Keywords: L-arginine; Clinoptilolite; Conductometric biosensor; Arginase; Urease

ЗАСТОСУВАННЯ АМОНІЙ-СЕЛЕКТИВНОГО ЦЕОЛІТУ ДЛЯ ПОКРАЩЕННЯ КОНДУКТОМЕТРИЧНОГО ДВОФЕРМЕНТНОГО БІОСЕНСОРА ДЛЯ ВИЗНАЧЕННЯ L-АРГІНІНУ

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Анотація. Розроблено кондуктометричні біосенсори для визначення L-аргініну на основі аргінази та уреази, іммобілізованих в одній біоселективній мембрані методом поперечного зшивання за допомогою глутарового альдегіду та модифікованій клиноптилолітом. Характерні властивості клиноптилоліту, корисні для кондуктометричного визначення L-аргініну, були досліджені за допомогою трьох підходів для створення цеоліт-вмісних ферментних мембран. Розроблені біосенсори порівнювали з біосенсором для визначення L-аргініну, який не був модифікований клиноптилолітом, за такими показниками, як чутливість, лінійний і динамічний діапазон, нижня межа визначення, час відгуку, операційна стабільність і стабільність при зберіганні. Показано, що включення амоній-селективного цеоліту до складу біоселективної мембрани L-аргінінового біосенсора дозволяє підвищити чутливість біосенсора, застосовуючи всі запропоновані підходи для формування цеоліт-вмісних біомембран. Крім того було отримано чудові значення нижньої межі визначення та лінійного діапазону ($1{,}0\times10^{-5}$ М та 0,01–6 мМ відповідно) у біосенсора, у якому аргіназа, уреаза та цеоліт були розподілені в єдиному біоселективному шарі. Біосенсори для визначення L-аргініну на основі клиноптилоліту мали короткий час відгуку, високу операційну стабільність (коефіцієнт варіації досягав 0,74%), а їх стабільність при зберіганні була вища за чотири місяці.

Ключові слова: L-аргінін; Клиноптилоліт; Кондуктометричний біосенсор; Аргіназа; Уреаза

ИСПОЛЬЗОВАНИЕ АММОНИЙ-СЕЛЕКТИВНОГО ЦЕОЛИТА ДЛЯ УЛУЧШЕНИЯ КОНДУКТОМЕТРИЧЕСКОГО ДВУХ-ФЕРМЕНТНОГО БИОСЕНСОРА ДЛЯ ОПРЕДЕЛЕНИЯ L-АРГИНИНА

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Аннотация. Разработаны кондуктометрические биосенсоры для определения L-аргинина на основе аргиназы и уреазы, иммобилизованных в одной биоселективной мембране методом поперечной сшивки с помощью глутарового альдегида и модифицированной клиноптилолитом. Характерные свойства клиноптилолита, полезные в кондуктометрическом измерении L-аргинина, были исследованы с помощью трех подходов к созданию цеолит-содержащих ферментных мембран. Разработанные биосенсоры сравнивали с биосенсором для определения L-аргинина, который не был модифицирован клиноптилолитом, по таким показателям, как чувствительность, линейный и динамический диапазоны, нижняя граница определения, время отклика, операционная стабильность и стабильность при хранении. Показано, что включение аммоний-селективного цеолита в состав биоселективной мембраны L-аргининового биосенсора позволяет увеличить чувствительность биосенсора, используя все предложенные подходы к формированию цеолит-содержащих биомембран. Кроме того, были получены замечательные значения по нижней границе определения и линейному диапазону ($1{,}0\times10^{-5}$ М и 0,01–6 мМ соответственно) у биосенсора, в котором аргиназа, уреаза и цеолит были распределены в од-

ном биоселективном слое. Биосенсоры для определения L-аргинина на основе клиноптилолита имели короткое время отклика, высокую операционную стабильность (коэффициент вариации достигал 0,74%), а их стабильность при хранении превышала четыре месяца.

Ключевые слова: L-аргинин; Клиноптилолит; Кондуктометрический биосенсор; Аргиназа; Уреаза

1. Introduction

An estimation of plasma or urinary amino acids is useful for disease diagnostics, treatment monitoring, prevention and prognosis in errors of amino acid metabolism. It is known that such a disorder as argininemia which results from arginase deficiency, usually manifests itself in elevated arginine in blood (544 to 1,074 μmol/L versus normal 61 to 173), cerebrospinal fluid (88 μmol/L versus normal 6 to 29) and urine [1]. This is usually caused by the deficiency in arginase activity in liver and stratum corneum suggesting its total deficiency. This autosomal recessive clinical disorder caused by the deficient arginase gene, located on a chromosome 6, is one among a variety of progressive mental and motor deteriorations [2–4]. The affected patients have skeletal muscle spasticity and hypperreflexia as well as seizures.

Being a conditionally essential amino acid for humans, L-arginine is required for children in the growth phase (since it stimulates a production of the somatotropic hormone) and under some pathological conditions in adults [5]. For that reason, the monitoring of arginine-based therapeutic medications within body fluids as well as the control of their quality are of the vital importance. The L-arginine ability to enhance the T-cell-dependent immunity is a cause of its immune-modulating effect on the organism [6]. Intravenous arginine significantly decreases the losses of nitrogen and body weight in post-traumatic and postoperative period, slows down cancer growth, while arginine deficiency increases the risk of the endothelium dysfunction in type 2 diabetes [5].

Classical analytical practice of amino acid measurement consists of complex multi-stage laboratory procedures requiring costly chemicals and/or complex instrumentation. Commonly L-arginine is determined using its direct reaction with colorimetric agents such as ninhydrin [7,8]. However, colorimetric methods have low specificity to L-arginine; besides, a large amount of probe is needed. The arginine determination based on arginase coupled with urease with the following spectrophotometric detection is also used [9,10]. However, it is possible only if protein hydrolizates without any urea are available. Highly accurate quantification of L-arginine in a complex mixture can be obtained by liquid or ion-exchange chromatography [11,12]. In this case, the pre- or post-column analyte derivatisation is usual since L-arginine is not amenable to direct optical or electrochemical detection. The electrochemical biosensors have already demonstrated their potential to be a nice alternative for the conventional analytical methods for the variety of compounds. This is due to the analytical characteristics of the biosensors, simplicity of their use, portability, ease of mass manufacture.

To date, a great number of publications have reported about L-arginine biosensors based on amperometric [13–15] and potentiometric [16–21] transduction modes. For example, in [15] a biosensor system, consisting of amperometric biosensors for determination of L-arginine, L-histidine, L-lysine and L-glutamic acid, is presented. Each of them, being a part of the integral system, included an oxygen electrode and a gas-permeable membrane with immobilized enzymes. In particular, L-arginine was detected by means of arginine decarboxylase (E.C. 4.1.1.19). Another amperometric biosensor for L-arginine has been reported by Sarkar et al. [13]. The biosensor was elaborated based on amino acid oxidase from *Crotalus adamateus* (E.C. 1.4.3.2) immobilized on the surface of a screen-printed three-electrode amperometric transducer. The developed biosensor was used to monitor milk ageing effects. There is also reported a bi-enzyme biosensor with arginase (E.C. 3.5.3.1) and urease (E.C. 3.5.1.5) immobilized on the surface of the carboxylated poly(vynil chloride) ion-selective membrane using carbodiimide

and glutaraldehyde [19]. Another arginase-urease biosensor was developed by Karacaoğlu et al. [17]. The authors immobilized enzymes on the pH electrode surface using the gelatine membrane.

Another electrochemical technique, conductometry, has rarely been applied for the determination of L-arginine [22]. A major advantage of the conductometric method of detection results from the fact that almost all enzymes catalyse the reactions involving either consumption or production of charged species, which cause changes in the ionic composition of the reaction medium [23]. Thus, conductometry offers a generic sensing technique requiring immobilization of the chosen enzyme onto the transducer. In addition, conductometric biosensors have some benefits regarding their potentially wide application, which is determined by the development of microsystem technologies. Thin-film electrodes are suitable for miniaturization and large scale production using inexpensive technology; there is no need in the reference electrodes (costly and of macroscopic size); differential mode of measurements allows avoiding numerous interferences; transducers are light insensitive; the driving voltage can be significantly lowered that decreases the power consumption.

Our previous work on the L-arginine biosensor [24] has demonstrated for the first time the feasibility of the fabrication of a conductometric biosensor, operating on the basis of the enzymatic transformation of L-arginine with arginase and urease (Eqn. 1 and Eqn. 2).

$$\text{L-arginine} \xrightarrow{\textit{Arginase}} \text{L-ornithine} + \text{urea} \quad (1)$$

$$(NH_2)_2CO + 2H_2O + H^+ \xrightarrow{\textit{Urease}} 2NH_4^+ + HCO_3^-. \quad (2)$$

Noteworthy, despite the prospective features of such biosensor, it is evident that two-step enzymatic system tends to random substrate losses within the interim period, and therefore, to a decrease in the biosensor sensitivity to L-arginine. In this work, we present an approach to overcome this drawback, applying a natural zeolite clinoptilolite, possessing high adsorption and cation-exchange capacities.

Zeolites are crystalline, hydrated aluminosilicates of alkali and alkaline earth cations that have a three-dimensional structure (i.e., tektosilicates). The negative charge occurring when Al^{3+} replaces Si^{4+} in the tetrahedral structure, is counterbalanced by cations (e.g., Na^+, K^+ and Ca^{2+}, Mg^{2+}). These sites are located in large channels and cavities throughout the structure and are called zeolitic exchange sites [25]. Clinoptilolite is distinguished from other zeolites of the heulandite group by lower void volume and higher silica content (Si/Al, >4). This structural feature addresses to clinoptilolite a pronounced ability to adsorb proteins on its surface [26]. Here, the type of protein adsorption is strongly dependent on the acidic properties of each participant of the process. In particular, the adsorption of urease and BSA on clinoptilolite is determined by the repulsive and attractive forces (hydrophobic interaction). This is caused by the following relations between the medium conditions and specific properties of the zeolite lattice and proteins:

$$PZC_{clinoptilolite} < pH_{buffer} > pI_{urease} \quad (3)$$

(pH 3.0<pH 7.4>pH (5.0–5.2),

$$PZC_{clinoptilolite} < pH_{buffer} > pI_{BSA} \quad (4)$$

(pH 3.0<pH 7.4>pH below 5.0),

where PZC – the pH value, at which the net crystalline charge of the zeolite is equal to zero (for clinoptilolite, this value is 3); pI – isoelectric point of the protein; buffer – a phosphate buffer solution used for the immobilization (40 mM phosphate buffer, pH 7.4).

Arginase is adsorbed on the clinoptilolite in the electro-attractive conditions, as it goes from the relationship:

$$PZC_{clinoptilolite} < pH_{buffer} < pI_{arginase} \quad (5)$$

(pH 3<pH 7.4<pH 9.3).

In a current work, we proceed with our previous investigations of clinoptilolite applicability to enzyme-based conductometric biosensors [27]. As it was shown, a combination of ammonium-sieving and ion exchange properties of clinoptilolite with unique biorecognition capacity of urease allowed significant increasing of sensitivity of

the conductometric detection of urea. Here we continue studying the effects of the enzyme immobilization in the presence of the clinoptilolite on the analytical features of the L-arginine biosensor based on arginase and urease.

2. Materials and methods

2.1. Materials

Arginase from bovine liver (E.C. 3.5.3.1) with initial activity of 136 U/mg solid, urease from jack beans (E.C. 3.5.1.5) with initial activity of 100 U/mg solid, bovine serum albumin (BSA), glutaraldehyde (GA, 25 % aq. solution), urea (60.06 g/mol), L-arginine monohydrochloride (210.7 g/mol) were purchased from Sigma-Aldrich (France). The working solution used was prepared from KH_2PO_4 and Na_2HPO_4, in the concentration of 5 mM and pH 6.0–6.1. Glycerol was purchased from Macrokhim (Ukraine). The chemicals were at least of analytical grade purity. All solutions used were prepared using Milli Q water (the water was obtained from a Millipore purification system; its resistivity was no less than 18.2 MΩ·cm).

2.2. Characteristics of clinoptilolite

A powdered sample of clinoptilolite of Romanian origin (natural zeolite ZN-C1BF-R was generously provided by Mediterranean Society of Zeolites, France) and its unit cell formula was $(Na_{0.10}K_{0.57})(Ca_{0.47}Mg_{0.15})(Al_{1.97}Fe_{0.12})(Si_{9.96}Ti_{0.02})O_{24}$, $7H_2O$ (chemical composition determined by elemental analysis using fluorescence). The average size of clinoptilolite particles was 0.4 μm (90% between 0.2 and 1.0 μm). The sample was microporous, and its specific surface area was 101 $m^2\ g^{-1}$, with total pore volume of 0.036 $cm^3\ g^{-1}$, as determined by BET analysis, performed from N_2 adsorption isotherms. The cation exchange capacity determined was 2.6 meq g^{-1}. Purity was checked by X-ray diffraction, thermogravimetric analysis, ^{29}Si and ^{27}Al nuclear magnetic resonance. The structure was typical for the Heulandite family of tectosilicates (sheet-like) and its morphology revealed monoclinic crystal form with platelets 10–20 nm thick (Fig. 1). SEM images were obtained using FEI Quanta™ 50.

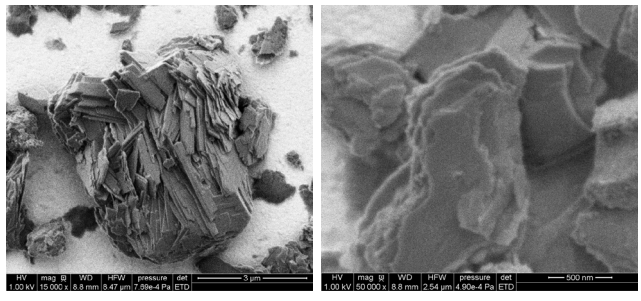


Fig. 1. SEM images of the clinoptilolite sample used.

As it was shown in [28], the clinoptilolite selectivity to ammonium is a result of replacement of all sodium ions from the zeolitic exchange sites with ammonium. The ion exchange between Na^+ and NH_4^+ in the clinoptilolite matrix was also reported by Ming and Dixon [25] and Jha and Hayashi [29].

2.3. Transducers

Each conductometric transducer consisted of two identical interdigitated electrodes; the sensitive surface area of each electrode was about 2.9 mm^2. The electrodes were fabricated by vapor deposition of gold onto a non-conducting pyroceramic substrate (5×30 mm). A 50 nm thick intermediate chromium layer was used to improve the gold adhesion to the substrate. The digit width and interdigital distance were 10 μm, and their length was ~ 1.5 mm. Such geometry was taken to provide complete and stable transduction of the biochemical reaction effect from the selective membrane to sensitive electrodes of the sensor. Conductive buses were covered with a protective insulation material, except for the active region and the contact zone. The first pair of electrodes together with an adjacent non-reactive BSA layer, served as a reference sensor; the second pair, covered with a bioselective layer, was a working sensor.

2.4. Preparation of selective elements of conductometric biosensors for L-arginine determination

2.4.1. Bioselective membrane of L-arginine biosensor (control)

The enzyme and BSA membranes were prepared using a cross-linking technique with glutaraldehyde as a bi-functional agent. For the prepa-

ration of the enzyme-containing solution, arginase (1.2 mg), urease (2.9 mg) and BSA (3.8 mg) were thoroughly dissolved in 40 μL of 40 mM phosphate buffer (pH 7.4), containing glycerol (15%). Afterwards, 0.15 μL of the prepared solution and 0.15 μL of the GA aqueous solution (2 %, v/v) were vigorously homogenized and deposited onto the sensitive surface of one pair of electrodes (such biomembrane configuration was further considered as a working membrane). The reference membrane was prepared by the same procedure except that arginase and urease were replaced by BSA (in respect to their total percentage content). Time of the biomaterials immobilization was 22–25 min. Before measurements, the biosensor (hereafter considered as a biosensor control) was carefully washed for 15–20 min in 5 mM phosphate solution, pH 6.1.

2.4.2. Bioselective elements of L-arginine biosensors based on clinoptilolite

Clinoptilolite-modified biosensors were prepared applying three approaches for the incorporation of zeolite particles into the bioselective membrane of the L-arginine biosensor. For all variants, the clinoptilolite suspension (50 mg/mL) was prepared by the sample incubation in Milli Q water, firstly under mechanical stirring and then under sonication in series of three times for 15 min each.

The first variant of L-arginine biosensor based on clinoptilolite was prepared by depositing a thin layer of homogenized mixture of zeolite and protein-based gel onto the sensitive surface of conductometric transducer. The enzyme-based solution, GA and clinoptilolite suspension (50 mg/mL) were spread onto one pair of pre-treated electrodes. The enzyme-based solution contained arginase (3.0%), urease (7.3%), BSA (8.0%) and glycerol (15%) dissolved in 40 mM phosphate buffer (pH 7.15). The arginine-selective membrane of each biosensor contained 0.15 μL of clinoptilolite suspension (50 mg/mL), 0.15 μL of enzyme-based solution, and 0.15 μL of GA (3% v/v). The reference membrane was prepared using the same protocol as a reference membrane described in *2.4.1*. The obtained L-arginine biosensor (the biosensor Variant 1) was stored dry at 4°C until analysis.

Another possibility of protein immobilization on zeolite was the following. One pair of interdigitated electrodes was covered with 1.2 μL of clinoptilolite (50 mg/mL) and left in a dark and dry place for 1 h. After that the sensor was thoroughly washed in 5 mM phosphate solution, pH 6.1. The following immobilization of bioselective layer on it was performed according to the procedure given in *2.4.1*. The reference membrane was prepared likewise the reference membrane in *2.4.1*. The L-arginine biosensor incorporating clinoptilolite (the biosensor Variant 2) was stored dry at 4°C until analysis.

The third variant of zeolite-modified L-arginine biosensor was prepared by depositing 1.2 μL of clinoptilolite (50 mg/mL) onto one pair of interdigitated electrodes with the following immobilization of zeolite-containing enzymatic membranes. Specifically, after zeolite adhesion on one pair of electrodes (1 h), the sensor was thoroughly washed in the phosphate buffer and dried. The zeolite-containing enzymatic membrane consisted of 0.15 μL of enzyme-based solution, 0.15 μL of GA (3% v/v) and 0.15 μL of clinoptilolite suspension (50 mg/mL). The enzyme-based solution was prepared from arginase (3.0%), urease (7.3%), BSA (8.0%) and glycerol (15%) dissolved in 40 mM phosphate buffer, pH 7.15. Subsequently, the solutions containing clinoptilolite and enzymes were thoroughly homogenized and immobilized onto a pair of electrodes, previously covered with a zeolitic layer. The reference membrane was prepared likewise that described in *2.4.1*. After the immobilization of biomaterials (22–25 min), the biosensor (the biosensor Variant 3) was thoroughly washed for 15–20 min in 5 mM phosphate solution, pH 6.1, and stored dry at 4°C until further use.

Before the immobilization procedure, all conductometric transducers were treated with piranha solution and thoroughly rinsed in Milli Q water. Afterwards, electrodes were carefully degreased with ethanol.

2.5. Electrochemical measuring system

The conductometric measurements of L-arginine, using the prepared biosensors, were realized in a differential measuring mode. This allowed achieving the satisfactory detection accuracy and suppression of non-informative effects of the environment (variations of temperature, pH and background conductivity of the working solution). Here, the difference between the transducers active resistances and their deviation from the nominal did not exceed 10%, their capacitances did not differ by more than 30%, and the value of tan φ did not outreach 0.5.

The bridge circuit (Fig. 2) allowed us to counterbalance the components of output signals of transducers with active and passive membranes and, therefore, to receive the bridge output signal, which depended only on the change in electrical conductivity of the active membrane. The scheme (Fig. 2) consists of the sinusoidal test signal generator (G) containing an additional outlet of the in-phase reference signal for the synchronous rectifier SR, difference amplifier DA with a transfer coefficient k=3, and a display device DD of the output signal. The bridge consisted of impedances of conductometric transducers Z_1, Z_2 and resistors R_3, R_4.

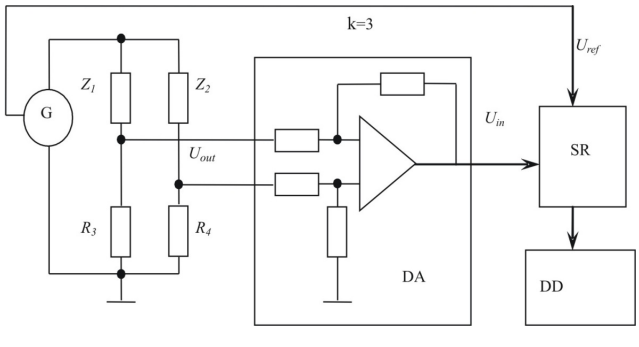


Fig 2. A compensation-bridge circuit of a differential conductometric transducer.

The applied sinusoidal potential with frequency of 100 kHz and amplitude of 10 mV allowed avoiding such effects as faradaic processes, double-layer charging and polarization of the microelectrodes. Illumination and temperature variations had practically no influence on the biosensor characteristics. The measurements were carried out in a glass cell filled with phosphate buffer (volume 3.5 mL), under vigorous magnetic stirring.

Electrochemical impedance spectroscopy (EIS) measurements were performed in a frequency range of 100 mHz to 100 kHz using VoltaLab 80, Model PGZ 301 (*Radiometer Analytical*, France), in a two-electrode configuration. Experiments were carried out in a glass cell filled with electrolyte solution, under vigorous magnetic stirring. EIS measurements were performed 1–2 min after the analyte injection into the cell to attain a steady-state situation at the electrode/(working solution) interface.

3. Results and discussion

3.1. Electrical properties and analytical characteristics of conductometric clinoptilolite-based L-arginine biosensors

As it is shown in [24], the optimal medium for maintaining the arginase and urease activity, immobilized in a single bioselective membrane of the L-arginine conductometric biosensor, was 5 millimolar phosphate solution (pH 6.0). On the other hand, optimization of the working solution parameters for clinoptilolite-based conductometric microsensor showed that 5 mM phosphate solution with pH 6.0 provided also the highest sensitivity of the microsensor toward ammonium (Fig. 3). The details on the microsensor development were reported in [30].

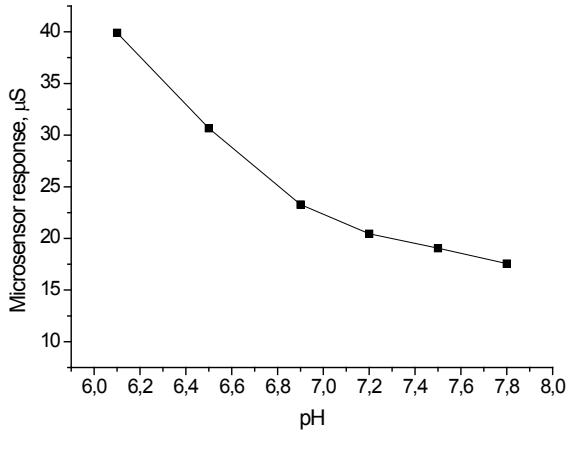


Fig. 3. Effect of pH on the sensitivity of the clinoptilolite-based microsensor to ammonium. Measurements in 5 mM phosphate buffer.

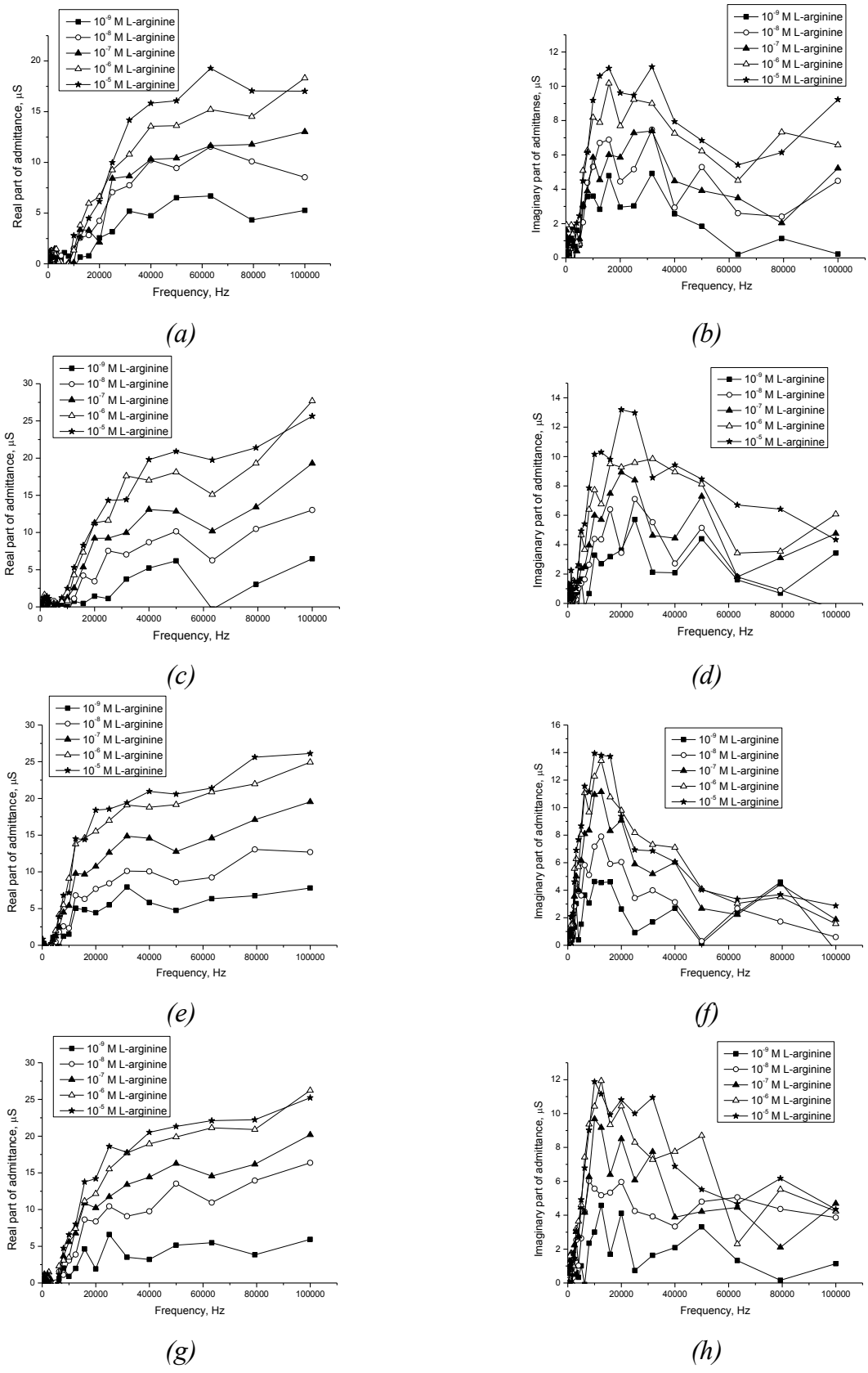
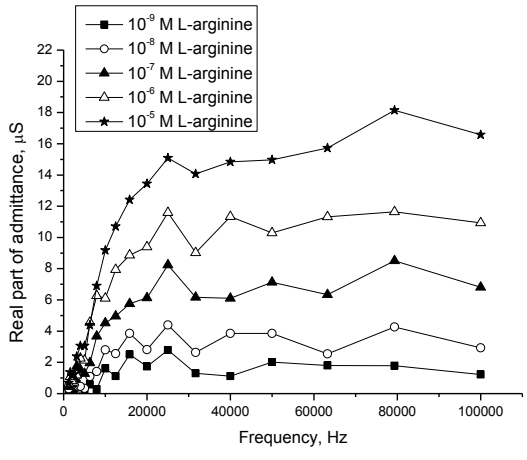
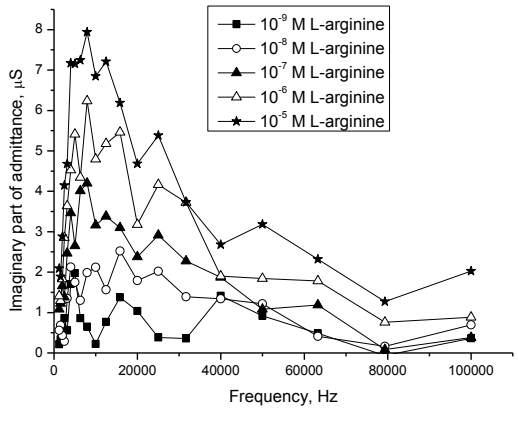


Fig. 4. Frequency dependence of real and imaginary parts of admittance at low concentrations of L-arginine, studied at the working membrane of the L-arginine biosensors: *a* and *b* – the biosensor control; *c* and *d* – the biosensor Variant 1; *e* and *f* – the biosensor Variant 2; *g* and *h* – the biosensor Variant 3. Measurements in 5 mM phosphate solution, pH 6.0.

The optimal pH value, found here, corresponds well with the data reported by Karadag et al. [31] and Vassileva and Voikova [32] where the authors show that pH value near to 6 provides high adsorption capacity of clinoptilolite toward ammonium. Thus, 5 mM phosphate buffer (KH_2PO_4-Na_2HPO_4) with pH 6.0 was used further as a working solution for the clinoptilolite-based L-arginine biosensors.



(a)



(b)

Fig. 5. Frequency dependence of real and imaginary parts of admittance at low concentrations of L-arginine studied at the reference membrane of the L-arginine biosensor. Measurements in 5 mM phosphate solution, pH 6.0.

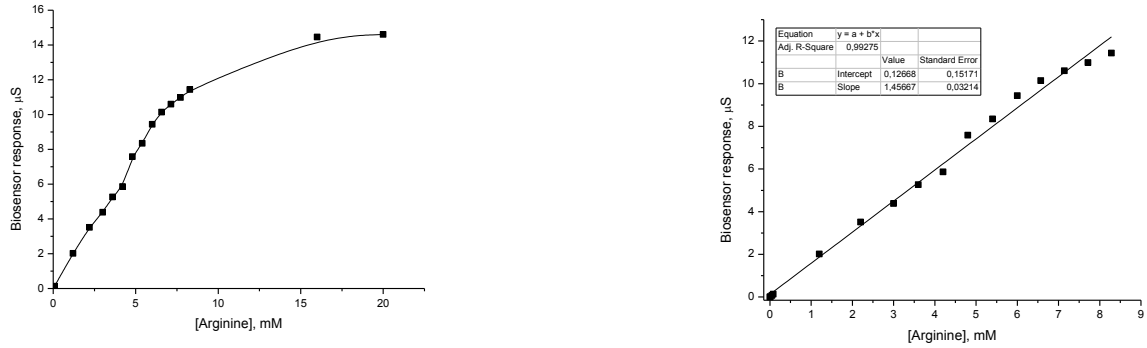
Study on the frequency dependence of the admittance real and imaginary parts of the developed biosensors (excluding the biosensors' admittance obtained in the fresh buffer) revealed that at low concentrations of L-arginine all biosensor variants had a considerable increase in the real part of admittance ΔY_{Re} (at the working electrode), which corresponded to the conductivity growth there (Fig. 4(*a,c,e,g*)).

At the same time, no visible increase was demonstrated in the imaginary part of admittance ΔY_{Im} (Fig. 4(*b,d,f,h*)). This finding is a reason for us to believe that shifts in the active resistance at the electrode/(working solution) interface was caused by one of the processes: (a) pre-concentration of ammonium, generated in the enzymatic reactions, on clinoptilolite; (b) pre-concentration of L-arginine, which is usually highly charged in the medium close to neutral, on clinoptilolite. The studies, described below, were useful then to reveal that the relevant admittance shifts were caused by the L-arginine pre-concentration.

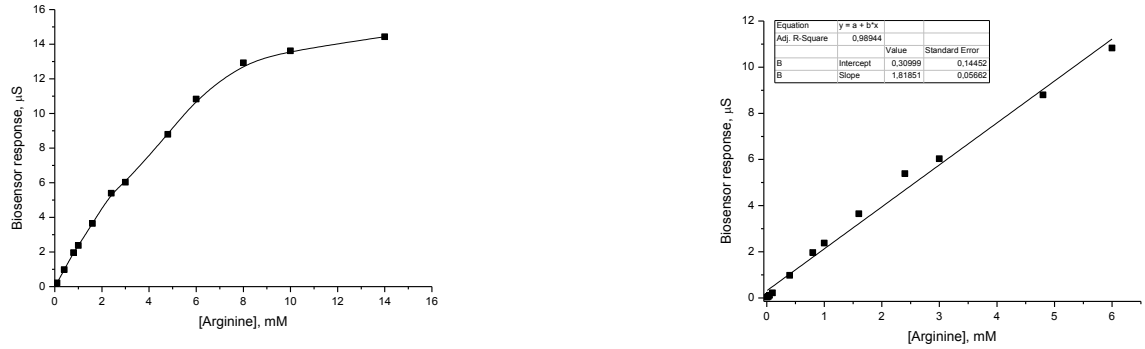
As it is seen in Fig. 4(*a,c,e,g*), high curve resolution from one concentration to another was obtained only at high frequencies: 40–100 kHz (the biosensor control); 30–100 kHz (the biosensor Variant 1) and 20–100 kHz (the biosensors Variant 2 and Variant 3).

The corresponding frequency dependence of the admittance real and imaginary parts of the BSA-membrane (Fig. 5(*a,b*)) showed that the reference membrane was almost insensitive to L-arginine.

The linear range of the developed biosensors, calculated in accordance with the equation $Y_{Re} = Increment + Slope \times C$ and being consistent with the adjusted squared correlation coefficient Adj. $R^2 \geq 0.99$, was the following: 0.01–8.29 mM (the biosensor control); 0.01–6 mM (the biosensor Variant 1); 0.5–6 mM (the biosensor Variant 2) and 0.01–0.1 mM (the biosensor Variant 3), Fig. 6(*a–d*). If compared the linear ranges of the developed bi-enzyme biosensors toward L-arginine (Fig. 6(*a–d*)) and urea (Fig. 7(*a–d*)), remarkably, that for all the configurations, the biosensors' linear range to urea was similar: 0.001–2 mM.



(a)



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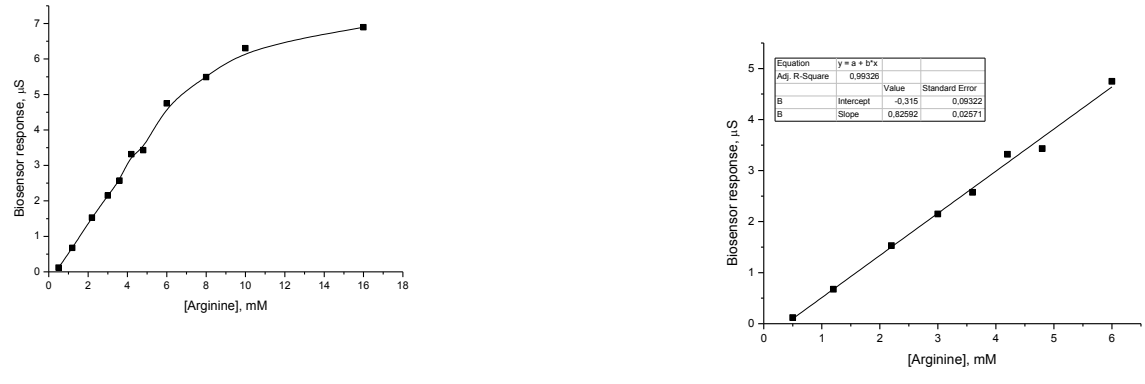
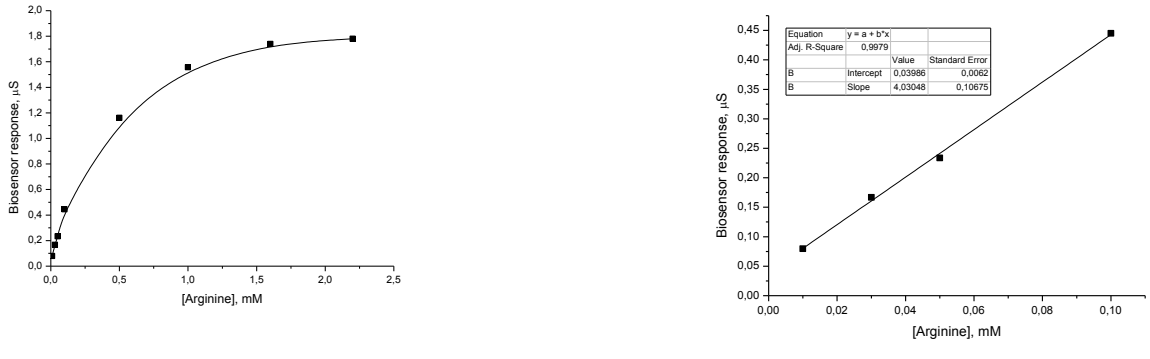
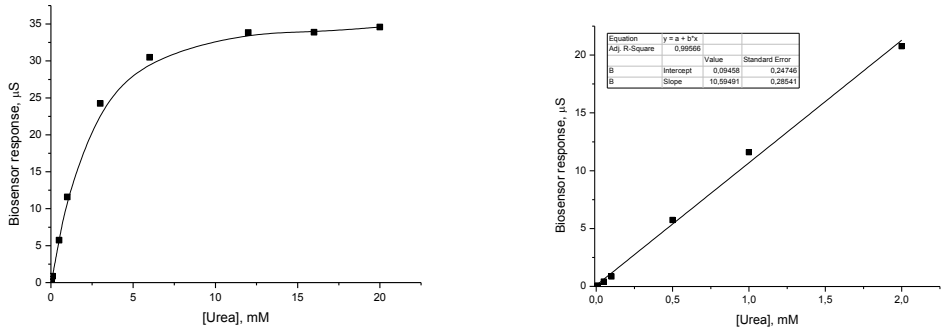


(c)

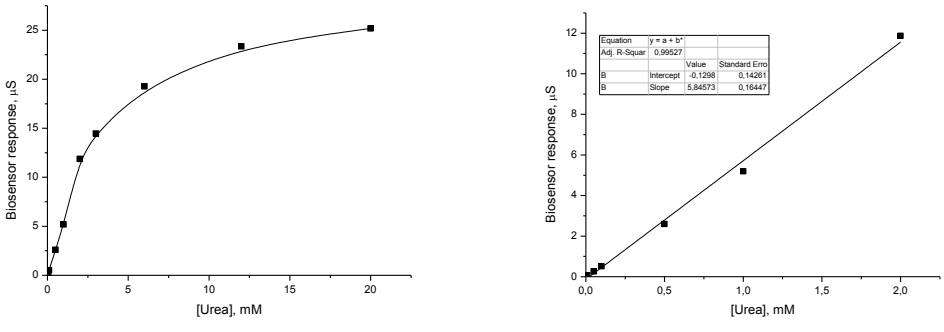


(d)

Fig. 6. Dynamic range and linear range approximation for the L-arginine biosensors based on clinoptilolite: *a* – the biosensor control; *b* – the biosensor Variant 1; *c* – the biosensor Variant 2; *d* – the biosensor Variant 3. Measurements in 5 mM phosphate solution, pH 6.1.



(a)



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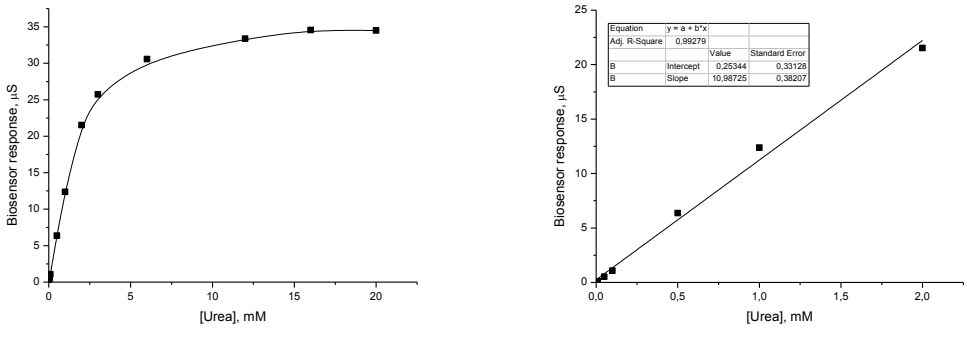
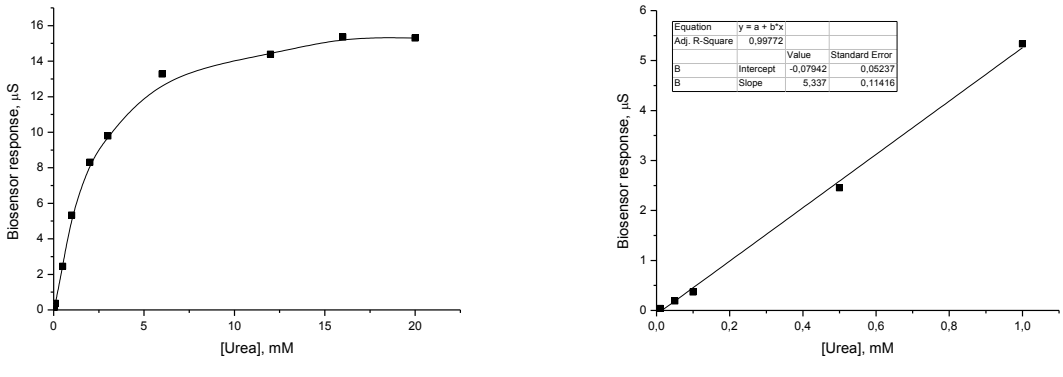


(c)



(d)

Fig. 7. Dynamic range and linear range approximation for the L-arginine biosensors based on clinoptilolite: *a* – the biosensor control; *b* – the biosensor Variant 1; *c* – the biosensor Variant 2; *d* – the biosensor Variant 3. Measurements in 5 mM phosphate solution, pH 6.1.

Interestingly also, that the sensitivity of the biosensor Variant 3 toward L-arginine was significantly lower in comparison with other types of the bioselective membranes, containing zeolite (the L-arginine loading of the biosensor here reaches only 2 mM, Fig. 6*d*). We assume that this observation may be a result of the enhanced adsorption capacity of the zeolite toward L-arginine, which was provoked by the increase in the zeolite percentage content in the biomembrane (the clinoptilolite adsorption of L-arginine can be deduced from the following reaction conditions:

$$PZC_{clinoptilolite} < pH_{buffer} < pI_{L\text{-}arginine} \quad (6)$$

(pH 3<pH 7.4< pH 10.76).

Summarizing the analytical characteristics of the developed biosensors (Table 1), it is well observed that clinoptilolite incorporation into bioselective elements of the conductometric biosensor for L-arginine determination results in the improvements in the biosensor sensitivity. We suppose that such achievement may be a result of several processes: (1) formation of the high concentration field of ammonium ions in close proximity to the zeolite surface (at the zeolite surface, high concentration of NH_4^+, produced in the enzymatic reactions, is created by the migration of these ions in the direction of zeolite due to the high adsorptive properties of the latter); (2) intensive ion migration within the biomembrane due to replacement of extra-framework sodium with ammonium. The sensitivity of the clinoptilolite-based biosensors increases in the following order: the biosensor Variant 1 < the biosensor Variant 3 < the biosensor Variant 2.

Estimation of the detection limit of the L-arginine biosensors (the value was obtained according to a signal-to-noise ratio=2–3) unfortunately did not reveal some improvements when enzymes were co-immobilized in the presence of zeolite. However, in comparison with the previously reported biosensors for L-arginine [16–21], the detection limit and range of the linearity of the developed here biosensors (the biosensor control and the biosensor Variant 1) are very promising and comparable with that obtained for the potentiometric biosensor developed by Koncki et al. [19]. At the same time, all the clinoptilolite-based biosensors had significantly shorter response time than that of the biosensor reported in [19]: (46±0.6)–(58±0.6) s versus 90–240 s, respectively.

Here we would like to pay attention to the result inaccuracy, which we got in our previous works on the L-arginine biosensor [24]. There we stated that the detection limit of the biosensor to L-arginine was 5.0×10^{-7} M (for the biomembranes prepared by cross-linking with GA) and 2.5×10^{-5} M (for the biomembranes prepared by enzyme entrapment in PVA-SbQ). Using additional experimental data, we concluded that values of 5.0×10^{-7} M and 2.5×10^{-5} M of L-arginine, taken for the detection limit, corresponded only to the nonspecific conductivity changes within biomembranes. This assertion was based on the fact that L-arginine is capable to interact with histidine residues, present in a large quantity within BSA, urease and arginase molecules. Therefore, the interaction was seen as a response which had nonlinear nature. The revised values of the linear range and detection limit of the biosensor prepared by cross-linking (from [24]) were 0.75–5 mM and 1.0×10^{-4} M, respectively; the revised values of the linear range and detection limit of the biosensor prepared by enzyme entrapment in polymeric membrane (from [24]) were 1–3 mM and 5.0×10^{-4} M, respectively. If compared these data with current results on the detection limit of the biosensor, the difference in the values is observed: the diminution of the detection limit from 1.0×10^{-4} M to 1.0×10^{-5} M. We explain this with the difference in the substrate solubility: L-arginine in the form of salt (arginine monohydrochloride, applied here) is more soluble than L-arginine. That's why the difference in the sensitivities is also observed when using L-arginine and L-arginine monohydrochloride. For the further application of the developed biosensor in real sample analysis it is essential to pay attention for this difference when preparing model solutions for the calibration.

As it is also seen in Table 1, the deposition of clinoptilolite as a preparatory layer for the further immobilization of arginase and urease, gives the improvements in the biosensor response time in comparison with the biosensor variant when clinoptilolite-containing enzymatic layer was immobilized simply on the bare electrode.

Table 1. Analytical characteristics of the clinoptilolite-based bi-enzyme conductometric biosensors for L-arginine determination. Measurements in 5 mM phosphate buffer, pH 6.1.

Biosensor configuration	*Sensitivity*, μS/mM*	*Linear range, mM*	*Dynamic range, mM*	*LOD**, M*	*Response time, s*	*Biosensor regeneration time, min*
Control	11.205±1.203	0.01–8.29	0.01–20	1.0×10^{-5}	55±0.7	10
Biosensor Variant 1	17.5±2.166	0.01–6	0.01–15	1.0×10^{-5}	58±0.6	–″–
Biosensor Variant 2	19.025±2.649	0.5–6	0.5–16	5.0×10^{-4}	46±0.3	–″–
Biosensor Variant 3	18.3±2.516	0.01–0.1	0.01–2	1.0×10^{-5}	47±0.6	–″–

** – the result is presented with standard error of the mean (accounting the sensitivity values obtained from 3–4 different biosensors);*
***LOD – limit of detection.*

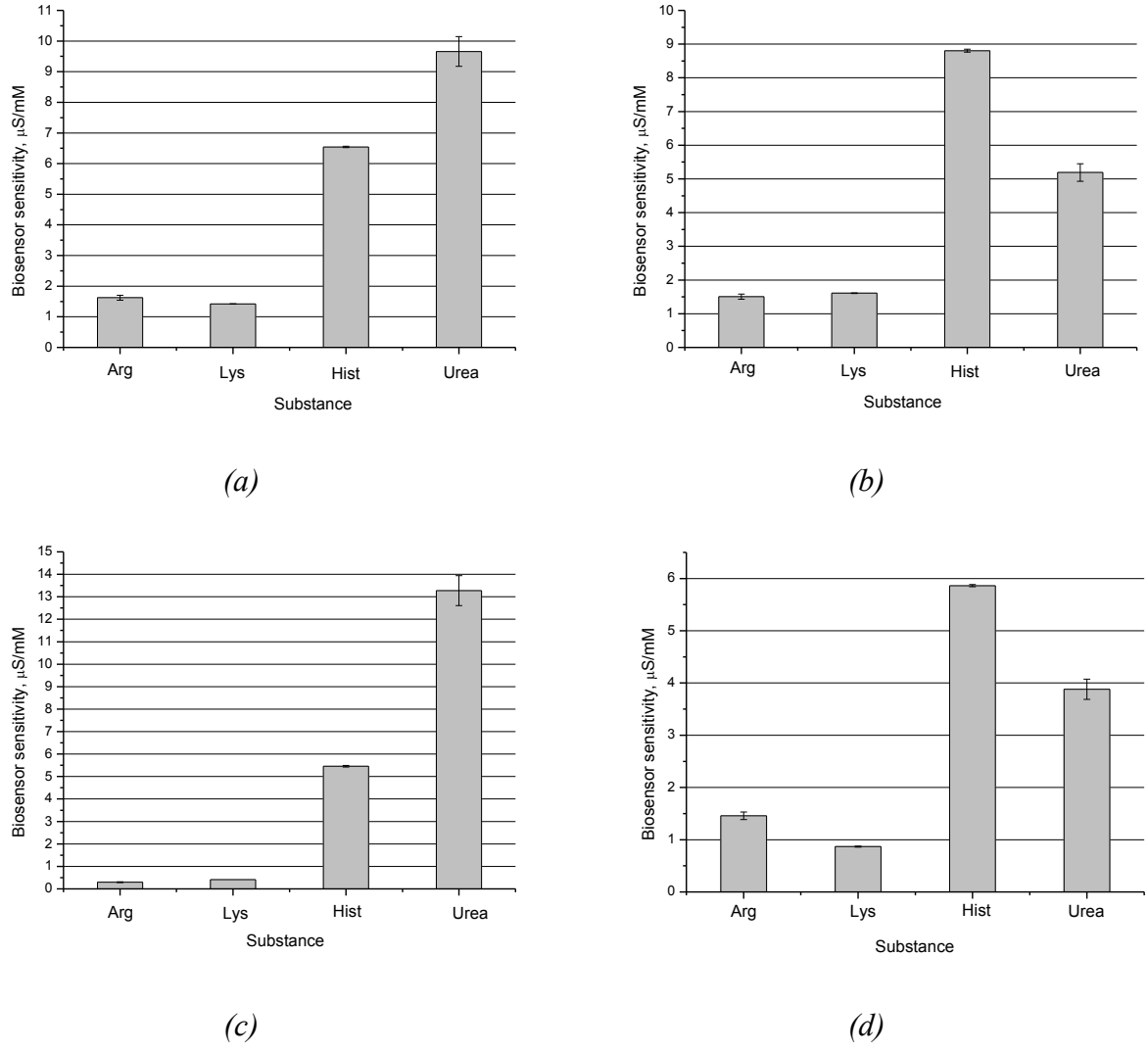


Fig. 8. Selectivity studies of the L-arginine biosensors based on clinoptilolite. Type of the bioselective membrane: *a* – the biosensor control; *b* – the biosensor Variant 1; *c* – the biosensor Variant 2; *d* – the biosensor Variant 3. Measurements in 5 mM phosphate buffer, pH 6.1.

3.2. Selectivity of the L-arginine biosensors based on clinoptilolite

As it was previously found for the L-arginine biosensor [24], the biosensor demonstrated high sensitivity to L-lysine and L-histidine. That's why we aimed to study the responsiveness of the L-arginine biosensors based on clinoptilolite to these amino acids, in order to investigate the zeolite behavior toward these compounds and probably to achieve the better selectivity of the L-arginine biosensor.

As it is seen in Fig. 8, the biosensors, comprising clinoptilolite within their bioselective elements, do respond to L-lysine and L-histidine. The ratios of the response amplitudes were similar to that of the biosensor control. Therefore, the specific properties of the zeolite toward L-lysine and L-histidine were not observed and, thus, we can't propose the procedure of the biosensor modification with clinoptilolite as mean which enhances the biosensor selectivity. For this particular purpose, it would be more desirable to overview the composition of the bioselective membrane of the L-arginine biosensor since it has already become evident that only this factor is largely responsible here for the selectivity of the biosensor detection.

3.3. Operational stability of clinoptilolite-based conductometric L-arginine biosensors

Monitoring of the biosensors' signal amplitudes measured at injections of 0.2 mM of L-arginine showed high reproducibility of the signal of all the clinoptilolite-containing biosensors (Fig. 9).

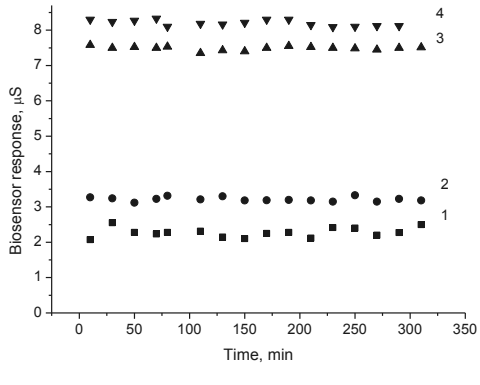


Fig. 9. Operational stability of clinoptilolite-based conductometric L-arginine biosensors: 1 – the biosensor control; 2 – the biosensor Variant 1; 3 – the biosensor Variant 2; 4 – the biosensor Variant 3. Biosensors responses to 0.2 mM of L-arginine in 5 mM phosphate solution, pH 6.0.

The coefficients of variation calculated for the biosensor Variant 1, the biosensor Variant 2 and the biosensor Variant 3 were 1.91%, 0.76% and 1.04%, respectively while for the L-arginine biosensor not modified with zeolite it was about 6%. Apparently, high operational stability of the modified biosensors could be a result of the good adhesive properties of zeolites towards gold and ceramic surfaces. Operational stability of the developed biosensors gives also evidences to the efficiency of the comparably easy immobilization techniques that were applied. At the same time, enzyme immobilization in the presence of zeolite was proven to be mild in respect to the influence on the enzyme activity.

Therefore, clinoptilolite incorporation into the L-arginine biosensor may be a reasonable technological approach for the improvement of the biosensor reproducibility.

3.4. Storage stability of zeolite-based conductometric biosensors for L-arginine determination

A storage stability of all L-arginine biosensors was studied for both L-arginine and urea. The biosensor responses as a function of storage time are shown in Fig.10; here each point corresponds to an arithmetic average of 2–3 measurements. It was taken 3–4 biosensors for each variant of L-arginine biosensor. After each measurement, the biosensors were stored dry at 4°C.

As it is seen in Fig. 10, in the end of the first month, the biosensor Variant 1 had around 86% of its initial sensitivity to L-arginine and the biosensor Variant 3 lost less than 10% of its initial activity, i.e. they demonstrated high stability. Noteworthy, that in all cases, except for the biosensor Variant 2, the biosensors residual activity toward L-arginine was higher than that to urea. In particular, in the end of the first month, the developed biosensors had the following residual activities toward L-arginine: the biosensor control – 91%, the biosensor Variant 1 – 86%, the biosensor Variant 2 – 55%, and the biosensor Variant 3 – 90%, while the biosensors' activity toward urea was 66%, 65%, 75%, and 70%, correspondingly. Here we suppose that high catalytic activity and stability of the zeolite-modified biosensors were caused by the arrangement of arginase and urease in close

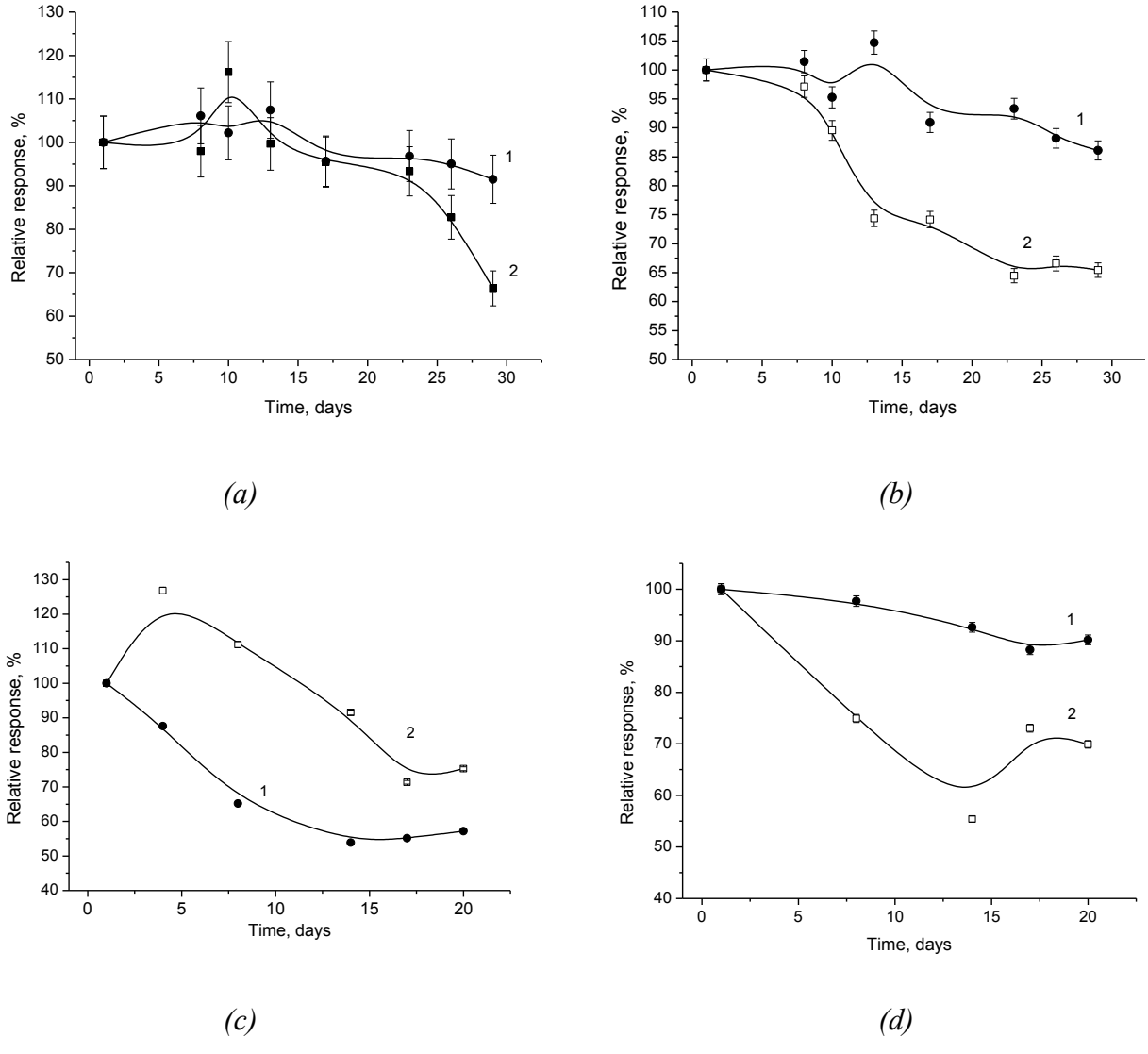


Fig. 10. Storage stability of L-arginine biosensors: a – the biosensor control; b – the biosensor Variant 1; c – the biosensor Variant 2; d – the biosensor Variant 3. Biosensor responses to 1 mM of L-arginine (curve 1) and 1 mM of urea (curve 2) in 5 mM phosphate solution, pH 6.0.

proximity to each other in one bioselective membrane due to their attachment to zeolite particles.

Evidently, that the biosensor Variant 2 has insufficient stability: its initial response to L-arginine drops to about 55% during the first 20 days. Interestingly, that just this variant is characterized by the shortest response time (46±0.3 s). Generally, the lifetime of all studied biosensors was no less than four months. Thus, it is an actual challenge to recommend the most appropriate immobilization technique for the development of L-arginine biosensor depending on the analysis purpose.

4. Conclusions

Application of ammonium-selective zeolite, clinoptilolite, in bi-enzyme conductometric biosensors allowed considerable increasing of the biosensor sensitivity toward L-arginine. Three variants of L-arginine biosensor were tested: 1) comprising zeolite phase in the arginase-urease cross-linked membrane, 2) possessing a zeolite layer adsorbed on the electrode together with an enzymatic layer, and 3) based on the primary zeolite layer and the secondary arginase-urease-clinoptilolite layer. They had the following sensitivities:

(17.5±2.166) μS/mM, (19.025±2.649) μS/mM, and (18.3±2.516) μS/mM, correspondingly. The sensitivity of L-arginine biosensor not modified with clinoptilolite was (11.205±1.203) μS/mM. The values of linear and dynamic range, detection limit and response time of the biosensor control were found to be quite comparable with the biosensor comprising zeolite within arginase-urease cross-linked membrane. Operational stability of the developed biosensors was notably higher in comparison with L-arginine biosensor that was not modified with zeolite. The mild immobilization techniques had also a favorable effect on the biosensors' storage stability. Analytical characteristics of the developed biosensors make them promising devices for routine L-arginine determination in control of pharmaceuticals quality. The adaptation of highly sensitive L-arginine biosensors based on clinoptilolite for the real sample analysis is ahead.

Acknowledgements

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