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DEVELOPMENT AND COMPARISON OF TWO ALANINE AMINOTRANSFERASE-SENSITIVE BIOSENSORS BASED ON PYRUVATE OXIDASE AND GLUTAMATE OXIDASE

Alanine aminotransferase (ALT) is a clinically important biomarker widely used in the diagnosis of liver diseases. The currently known traditional methods of measuring ALT activity (e.g., spectrophotometry, colorimetry, etc.) have a number of limitations, mainly the inability to monitor in real time, relatively high cost of analysis, the need for well-trained personnel, and bulky equipment. In contrast to traditional methods, biosensor methods of analysis are becoming more widespread, as they are mostly free from the disadvantages of the traditional methods. ***Objective.*** *To compare two developed biosensors based on pyruvate oxidase (POx) and glutamate oxidase (GlOx) for measuring alanine aminotransferase activity and to determine their main advantages and disadvantages.* ***Methods.*** *A three-electrode amperometric measurement scheme was used in the study. Platinum disk electrodes were used as working electrodes, and the signal was recorded with a PalmSens potentiostat. Immobilized POx and GlOx were used as recognition biomaterials.* ***Results.*** *The method and the optimal conditions for immobilization of each of the enzymes were selected. The procedure for creating the ALT-sensitive biosensors based on POx and GlOx was optimized. The optimal operating parameters for each sensor were chosen and analytical characteristics of both proposed biosensors were compared. POx biosensor has 1.5 times higher sensitivity, wider linear range and 2.5 lower detection limit, and unlike GlOx biosensor, can be used in a multibiosensor system for simultaneous measurement of ALT and AST.* ***Conclusions.*** *Thus, the biosensor based on POx has more advantages to be used for ALT activity determination in the biological fluids.*

Keywords: *alanine aminotransferase, biosensor, amperometry, clinical significance, in vitro testing, kinetics.*

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Introduction

Alanine aminotransferase (ALT) is a highly specific multisubstrate enzyme that belongs to the class of transferases and is localized mainly in the cytoplasm of liver cells. ALT catalyzes the reversible transamination reaction between L-alanine (amino group donor) and α-ketoglutarate (amino group acceptor) to form pyruvate and L-glutamate [1].

$$\text{alanine + ALT + ketoglutarate} \leftrightarrow \text{pyruvate + ALT-NH}_3\text{ + ketoglutarate} \leftrightarrow \text{pyruvate + ALT + glutamate} \quad (1)$$

This enzyme is an important link in the synthesis of energy molecules, in particular in the tricarboxylic acid cycle [2], glucose-alanine cycle [3], as well as in the metabolism of amino acids [4] and nitrogen metabolism, including the urea cycle [5].

In healthy people, the serum ALT level is usually <30 U/l [6]. In the case of liver damage (heart, muscle, *etc.*), the enzyme is released into the intercellular space and its concentration increases significantly, making it a useful biomarker. For example, in cirrhosis, ALT can reach an average of 162 ± 354 U/l, which is 8—35 times higher than norm [7].

The traditional methods for determination of the alanine aminotransferase activity include colorimetry, spectrophotometry, chemiluminescence, chromatography, and fluorometry [8]. However, such methods are always accompanied by certain disadvantages, such as the need for valuable equipment, expensive reagents, trained personnel, and careful sample preparation. Therefore, the biosensor technologies become of high relevance [9, 10], since they offer rapid and low-cost diagnostics that could be used in the clinical practices. Moreover, development of the ALT-specific biosensors represents a novel approach to improve early detection of liver dysfunction and to enable continuous monitoring of disease progression, which is not achievable with the conventional assays [11—14].

Among biosensors, amperometric biosensors are the most common due to their simple design, cost-effectiveness, relatively high speed, and the ability to provide continuous real-time monitoring.

Many biosensors that determine enzymatic activity are currently known [15—17]. Among the biosensors that measure ALT activity, the most common bio-recognition elements are based on oxidase class enzymes. Glutamate oxidase (GlOx)-based biosensors are widely used for detecting transaminase activity due to their simplicity, stability, and high selectivity after immobilization. GlOx catalyzes the oxidation of glutamate — the common product of the ALT reaction (1) — into α-ketoglutarate and electroactive hydrogen peroxide (2), which is then electrochemically detected to indirectly quantify enzyme activity. GlOx is especially popular because it uses only one coenzyme (FAD), which simplifies biosensor design and reduces the need to optimize complex reaction conditions [18—20]. Pyruvate oxidase (POx) is another enzyme commonly used in the ALT-specific biosensors. It catalyzes the oxidative decarboxylation of pyruvate — another product of ALT activity (1) — into acetyl phosphate and hydrogen peroxide (3), which is then electrochemically detected. Unlike GlOx, POx requires multiple substrates and coenzymes (such as FAD, Mg^{2+}, thiamine pyrophosphate, and inorganic phosphate ions), making biosensor development more complex and requiring careful optimization of assay conditions [21—23].

$$\text{glutamate + } O_2 + H_2O \xrightarrow[\text{GlOx}]{} \text{α-ketoglutarate + } NH_3 + H_2O_2 \quad (2)$$

$$\text{pyruvate + Pi + } O_2 + H_2O \xrightarrow[\text{POx}]{\text{FAD, TPP, } Mg^{2+}} \text{acetylphoshate + } CO_2 + H_2O_2 \quad (3)$$

The electroactive hydrogen peroxide formed during reaction (2, 3) decomposes on the sensitive surface of the platinum disk electrode at an applied potential of +0.6 V. The electrons formed during reaction (4) are drawn into the electric circuit, changing the current recorded by the potentiostat.

$$H_2O_2 \xrightarrow{+0,6\text{ V}} 2H^+ + 2e^- + O_2 \quad (4)$$

Thus, the goal of our work was to develop biosensors based on both enzymes, compare their manufacturing procedures, operating parameters, operational and analytical characteristics, and find out a more promising biomaterial for further usage.

Materials and Methods

Materials

Pyruvate oxidase from *Aerococcus viridans* (35 units/mg protein) and alanine aminotransferase from porcine heart (84 units/mg protein) were purchased from Sigma-Aldrich, recombinant glutamate oxidase was obtained from *Streptomyces sp.* with activity of 7 units/mg protein was purchased from Yamasa Corporation, Japan. HEPES, glutaraldehyde (GA), sodium pyruvate, magnesium nitrate, thiamine pyrophosphate, pyridoxal phosphate, α-ketoglutarate were from Sigma-Aldrich. Polyvinyl alcohol with stearyl pyridinium groups (PVA-SbQ) was from Toyo Gosei Kogyo Co.Ltd, Japan. Other inorganic compounds used in the experiment (in particular, hydrogen peroxide, ethyl alcohol, potassium dihydrogen phosphate, etc.) were of domestic production and had reagent purity grade.

Amperometric equipment

For the experiments, a standard three-electrode scheme for amperometric analysis was used. Experimental setup consists of a PalmSens potentiostat (Palm Instruments BV, the Netherlands), an 8-channel PalmSens multiplexer, and 3 types of electrodes: several working electrodes (platinum disc electrodes), a counter electrode (platinum), and a reference electrode (Ag/AgCl). More details can be found in our previous work [24].

Methods of bioselective membrane manufacturing

For immobilization of POx, a gel was prepared containing 10% glycerol, 5% BSA, and 4.86 units/μl of POx in 25mM HEPES buffer (pH 7.4). Glycerol ensured membrane flexibility and prevented its premature drying, while BSA enhanced membrane density, thereby reducing enzyme leaching. In the case of covalent crosslinking, BSA molecules provide additional cross-linking sites, thereby preventing the enzyme from excessive binding and consequently reducing its activity. The prepared enzyme gel and an aqueous solution of PVA-SbQ photopolymer with a concentration of 19.8% were mixed in a 1 : 2 ratio, after which 0.15 μl of the mixture was immediately applied onto the electrode's sensitive surface and polymerized in a UV lamp at a light wavelength of 365 nm until total irradiation of 2.4 J was reached (approximately 8 min). Before use, the biosensor was washed 2—3 times for 3 minutes in working buffer solution.

Covalent crosslinking with glutaric aldehyde (GA) was chosen as the method of the GlOx immobilization. Accordingly, a gel containing 10% glycerol, 4% BSA, 8% GlOx dissolved in 100 mM phosphate buffer (pH 6.5) was prepared. The enzyme gel was mixed with GA (0.5% concentration) in a 1 : 2 ratio. Immediately, 0.05 μl of the resulting mixture was applied to the sensitive electrode surface and air-dried for 35 minutes. Before use, the biosensor was washed 2—3 times for 3 minutes in the working buffer solution.

To evaluate the performance of the formed bioselective membranes, series of measurements were carried out with the corresponding analytes (pyruvate or glutamate), to analyze the shape, speed, and magnitude of the sensor responses.

Methodology of the amperometric ALT measurement

Measurements were performed in a fixed-volume cell (2 ml) with constant stirring at room temperature. A potential of +0.6 V (optimal decomposition potential of H_2O_2 on a platinum surface) was applied to the working electrodes relative to the Ag/AgCl reference electrode. Aliquots of the necessary reaction components were sequentially added to the cell in the same order. To ensure accuracy, the measurements were taken only after baseline stabilization, following reagent addition but prior

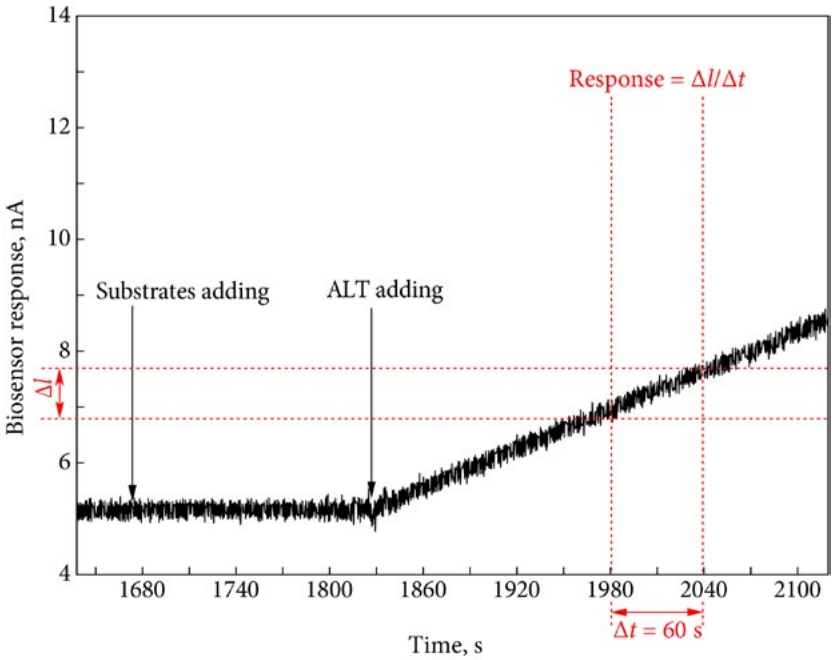

Fig. 1. Principle of operation of the biosensor for ALT determination.

to ALT addition. The results were calculated as follows: a linear section of the biosensor response was selected, response noise was linearized, and the change in current over 60 s was calculated using the formula: *response* = $\Delta l/\Delta t$ (where Δl is the difference in current strength and Δt is the time interval during which the measurement was performed) (Fig. 1).

Calibration curves were built to determine the main analytical characteristics of the developed biosensors (LOD, linear range, sensitivity). A linear equation $y = k \times x + b$, where y — current value and x — analyte concentration (ALT), was used to fit the biosensor signal.

Results and Discussion

Optimization of GlOx and POx immobilization methods

During the development of ALT-sensitive biosensors, several immobilization strategies of the bioselective element were tested, taking into account the physicochemical properties of GlOx and POx.

The choice of immobilization approach depended on the characteristics of the enzymes. For GlOx, GA proved to be the optimal immobilizing agent. During covalent cross-linking, GA minimally blocks the active sites of GlOx, practically not changing its activity in the immobilized state and providing high stability. However, applying this method to POx did not yield satisfactory results (the sensitivity of the biosensor did not allow measurements within the required limits (40—100 μM pyruvate), and the manufacturing reproducibility was critically low). Therefore, a different approach was chosen for the POx enzyme, namely, encapsulation of the enzyme in a photopolymer, which maximized the sensitivity of the biosensor to the analyte.

The POx-based sensor was characterized by a fourfold faster immobilization process and the absence of GA toxicity, respectively, making this method both safer and more efficient in manufacturing.

Analysis of the response shapes obtained on POx and GlOx sensors showed that the POx sensor responses are slower and have a flatter shape,

which may affect the slope (response speed) of the ALT response. In contrast, the response of the GlOx sensor is twice as fast and has a sharp shape, so the response to ALT will be almost instantaneous and more accurate.

The analytical characteristics of biosensors for intermediate analytes (pyruvate, glutamate) obtained during the measurements (Table) clearly describe the suitability of the created bioselective membranes for further work. The key parameters we considered were response noise, LOD, and the linear range of the sensor.

Comparison of working solution parameters

To ensure high sensitivity of the biosensor, optimization of the working medium — especially the buffer type, its concentration, and pH — is essential. It was necessary to select the correct proportions and concentrations of substrates and coenzymes required for the enzymatic reactions in each biosensing material. Additionally the ratios and concentrations of substrates and coenzymes required for the ALT-catalyzed reaction itself had to be determined.

Comparison of basic characteristics of the bioselective elements

Parameters	Values for POx	Values for GlOx
Sensitivity for intermediate analyte, nA/mM	307 ± 11	174
Linear range for intermediate analyte, μM	10—500	5—600
LOD for intermediate analyte, μM	0,88	3
Dynamic range for intermediate analyte, μM	10—4000	1—5000
Response reproducibility for intermediate analyte, %	5,46	8,8
Baseline current for intermediate analyte, nA	4,1 ± 1,8	2.3 ± 0.9
Baseline noise for intermediate analyte, nA	0,201 ± 0,12	0,7
Response noise for intermediate analyte, nA	0,609 ± 0,28	1,1
Response time for intermediate analyte, s	55 ± 11	20 ± 8

The first step was to select a buffer system — HEPES (pH 7.4) was chosen for both biosensors. This pH value is of physiological value, so further work with real samples will not change the pH of the system and the conformation of the reagents.

In addition to the buffer system, for the correct functioning of the biosensor, it is necessary to ensure the presence of substrates and coenzymes in the bioselective material. Both enzymes (POx and GlOx) are FAD-dependent. FAD is already associated with enzymes, so there was no need to study its effect. POx, unlike GlOx, additionally requires inorganic phosphates as an additional substrate, free divalent metal ions (e.g., magnesium ions) as a cofactor, and thiamine pyrophosphate as a coenzyme. Therefore, the optimal concentrations of these substances were determined: 5 mM, 0.5 mM, and 2 mM, respectively (Fig. 2).

Finally, the concentrations of substrates and coenzymes required for ALT (alanine, α-ketoglutarate, and pyridoxal phosphate) were optimized, since ALT possesses a single active site where substrates with different affinities compete. The following concentrations of substances proved to be optimal for the POx-based biosensor: 16 mM alanine, 2 mM α-ketoglutarate, 10 μM pyridoxal phosphate. In contrast, the GlOx sensor requires 4 mM alanine, 50 μM α-ketoglutarate, and 50 μM pyridoxal phosphate (Fig. 3). *The higher concentrations used in the POx system likely resulted from substrates competition at the ALT active site, reducing the efficiency of enzyme-substrate interactions.* Accordingly, to maintain the proper rate of ALT reaction in POx-based biosensors, it's substrates (alanine and ketoglutarate) were added in higher concentrations compared to GlOx-based biosensors.

Thus, establishing stable operating conditions for the POx-based sensor was more difficult due to the need to optimize more parameters, whereas

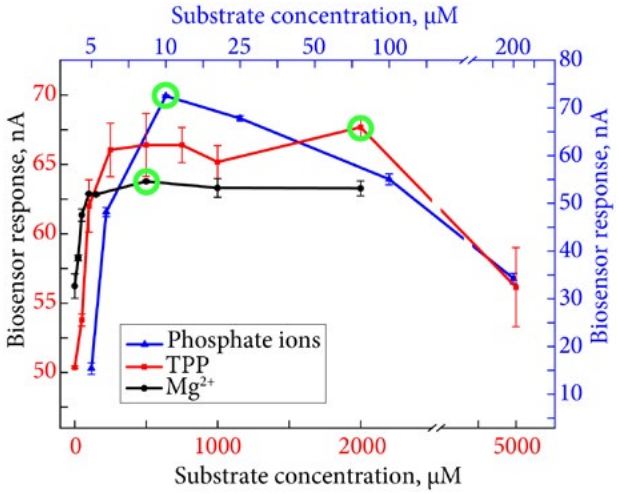

Fig. 2. Comparison of the optimal concentrations of the POx substrates

the GlOx-based biosensor required fewer components, that simplifies its optimization.

Comparison of the analytical characteristics of the biosensors

The final stage of the work was the analysis and comparison of the analytical characteristics of two developed biosensors for the determination of ALT activity. The biosensors were compared by the following main parameters: ALT detection limit, linear range of the biosensor, and sensitivity to the analyte. For the GlOx-based biosensor, the detection limit was 2.5 U/l of ALT, the linear range was 10—500 U/l of ALT, and the sensitivity was 0.5 nA/min per 100 U/l of ALT. For the sensor based on POx, the detection limit is scaled at 1 U/l ALT, linear range 1—500 U/l, sensitivity 0.75 nA/min per 100 U/l ALT. All of the above characteristics were calculated from the obtained calibration graphs (Fig. 4). We established that the biosensor based on POx had better analytical characteristics, which is more promising for the analysis of ALT content in real samples.

It is known that, along with the ALT determination, the aspartate aminotransferase (AST) activity is often used in medicine as a diagnostic parameter

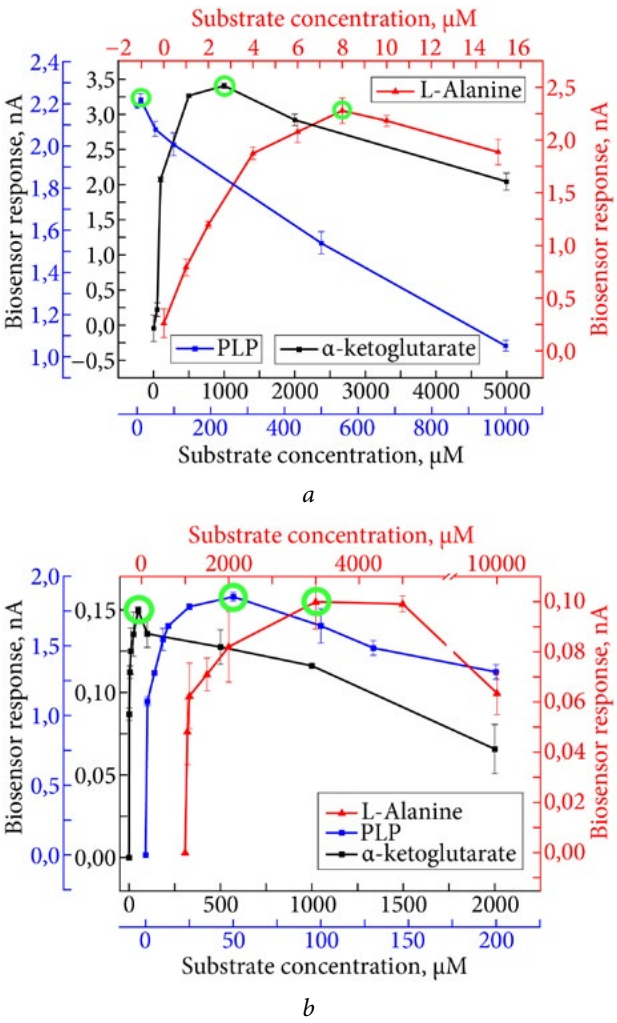

Fig. 3. Comparison of optimal ALT substrates for *a* — POx and *b* — GlOx. Green circle is optimal substrate concentration

[25]. It has the same biochemical reaction step with ALT and, accordingly, the same substrate and product of this step (α-ketoglutarate and glutamate, respectively) (4).

$$\text{aspartate + AST + ketoglutarate} \leftrightarrow \leftrightarrow \text{oxaloacetate + AST-NH}_3\text{ + ketoglutarate} \leftrightarrow \leftrightarrow \text{oxaloacetate + AST + glutamate} \quad (5)$$

Since the GlOx-based biosensor detects ALT through glutamate, it is not able to distinguish

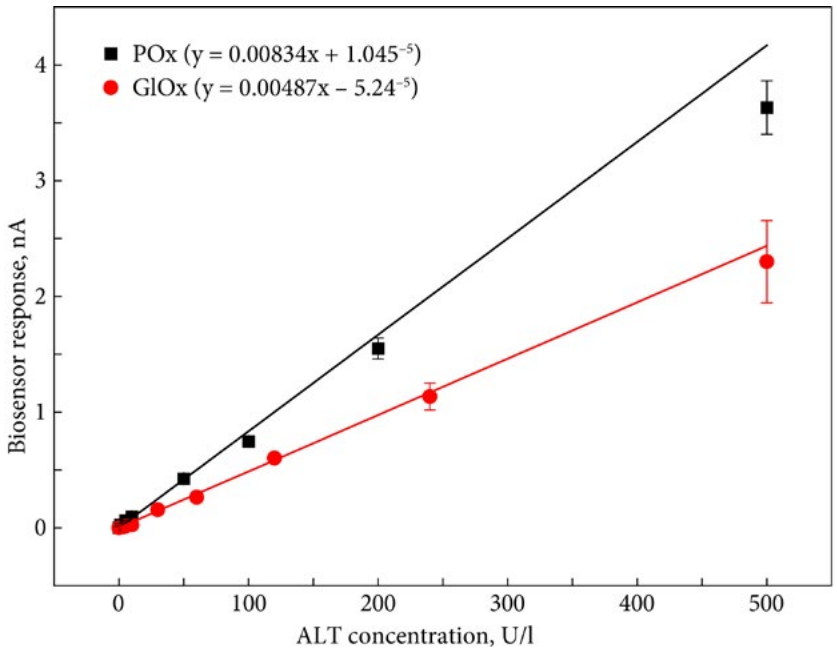

Fig. 4. Calibration curves of the ALT-sensitive biosensors based on POx and GlOx (The electronic version of the article contains a color figure)

between signals from ALT and AST, unlike the POx-based biosensor. Accordingly, if a system is developed for the simultaneous detection of ALT and AST, the POx-based sensor will be required.

Conclusions

In this work, we developed two biosensor variants for the detection of ALT activity (based on pyruvate oxidase and glutamate oxidase), and evaluated the advantages, disadvantages, and limitations of each system.

The GlOx-based biosensor proved to be simpler and more cost-effective to manufacture, as the enzyme immobilization process does not require additional equipment, and the enzyme itself does not depend on extra coenzymes and substrates for functioning. The analytical characteristics of the developed GlOx-based biosensor are potentially sufficient for detecting clinically significant changes in ALT activity in the blood serum samples.

In contrast, the POx-based biosensor is more demanding to prepare because it requires a UV lamp for the photopolymerization. However, the use of a photopolymer eliminates the need of toxic glutaraldehyde. The operation of this biosensor is complicated by the requirement of multiple additional substances for proper enzyme functioning in the bioselective element, which in turn increases the assay cost. Nevertheless, despite the more complex preparation process, the POx-based biosensor demonstrated superior analytical performance: it exhibited 1.5 times higher sensitivity (0.75 nA/min vs. 0.5 nA/min per 100 U/l ALT), a wider linear range (1—500 vs. 10—500 units per liter) and a 2.5 times lower detection limit (1 vs. 2.5 units per liter). Furthermore, the POx-based biosensor offers the unique ability to differentiate signals between ALT and AST, which makes it promising candidate for use in multibiosensor systems for the simultaneous determination of ALT/AST. Therefore, despite the relatively high cost and complexity of manufacturing the POx-based biosensor, its superior analytical performance and potential integration into a multibiosensor system for simultaneous ALT/AST determination as an ALT selective part

of the biosensor make it the more promising candidate for further development and optimization.

Conflict of Interests. The authors declare that there is no conflict of interests regarding the publication of this paper.

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РОЗРОБКА ТА ПОРІВНЯННЯ ДВОХ АЛАНІНАМІНОТРАНСФАРАЗО-ЧУТЛИВИХ БІОСЕНСОРІВ НА ОСНОВІ ПІРУВАТОКСИДАЗИ ТА ГЛУТАМАТОКСИДАЗИ

Аланінамінотрансфераза (АЛТ) — клінічно важливий біомаркер, що широко застосовується при діагностиці проблем з печінкою. Відомі наразі традиційні методи вимірювання активності АЛТ (наприклад спектрофотометрія, колориметрія тощо) мають ряд обмежень, головним чином неможливість проводити моніторинг в реальному часі, відносно велика ціна аналізу, необхідність добре навченого персоналу та громіздкого устаткування. На противагу традиційним методам, розповсюдження набувають біосенсорні методи аналізу, котрі здебільшого позбавлені недоліків, властивих традиційним методам. ***Мета.*** Порівняти два розроблених біосенсори на основі піруватоксидази (ПОкс) та глутаматоксидази (ГлОкс) для вимірювання активності аланінамінотрансферази та визначити їхні основні переваги та недоліки. ***Методи.*** В роботі використовувалась триелектродна амперометрична схема вимірювання. В якості робочих електродів використано платинові дискові електроди, реєстрація сигналу проводилась за допомогою потенціостата PalmSens. В якості розпізнавального біоматеріалу використано іммобілізовані ПОкс та ГлОкс. ***Результати.*** В роботі було проведено вибір методу та підібрано оптимальні умови іммобілізації кожного із ферментів. Було оптимізовано процедуру створення розроблених АЛТ-чутливих біосенсорів на основі ПОкс та ГлОкс. Підбрано оптимальні робочі параметри для кожного з сенсорів. Порівняно аналітичні характеристики обох запропонованих біосенсорів. ***Висновки.*** Аналіз аналітичних характеристик розроблених біосенсорів показав, що біосенсор на основі ПОкс мав більше перспектив для визначення активності АЛТ у біологічних рідинах.

***Ключові слова*:** аланінамінотрансфераза, біосенсор, амперометрія, клінічна значущість, *in vitro* тестування, кінетика.