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Research Article

Potentiometric Biosensor for Highly Toxic Pb (lead) and Cd (cadmium) Detection in Milk Using Layer-by-Layer Immobilization

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Abstract: Milk contamination with heavy metals such as lead (Pb) and Cadmium (Cd) poses a serious threat to public health, particularly in industrial regions. To address the need for rapid and on-site detection of these contaminants, a potentiometric biosensor was developed for the quantification of Pb^{2+} and Cd^{2+} ions in milk and dairy products. The biosensor functions by measuring the change in electrical potential resulting from the inhibition of catalase, an enzyme that catalyzes the decomposition of hydrogen peroxide, by toxic metal ions. The enzyme was immobilized on a graphite substrate using a five-layer Layer-By-Layer (LbL) coating of chitosan and sodium alginate to improve sensor sensitivity and stability. Milk samples were artificially contaminated with lead at concentrations of 0.001 mg/L, 0.1 mg/L, and 1.0 mg/L, and with cadmium at 0.003 mg/L, 0.03 mg/L, and 0.3 mg/L, representing levels below, at, and above the maximum permissible concentrations (MPCs). Catalase activity decreased by 30 and 54% at MPC levels of Pb (0.1 mg/L) and Cd (0.03 mg/L), respectively, and by 60 and 50% at 10× MPC levels. These findings suggest that the developed biosensor is a reliable, low-cost, and field-deployable tool for real-time monitoring of milk contamination, offering practical benefits over conventional laboratory-based methods.

Keywords: Potentiometric Biosensor, Pb (lead), Cd (Cadmium), Milk, Dairy Products, Catalase, Enzyme Immobilization, Layer-by-layer Technology, Chitosan, Sodium Alginate

Introduction

Contamination of food raw materials with toxic metals, pathogens, and other harmful substances presents a significant global concern (Lebelo *et al.*, 2021). Heavy metals such as lead (Pb), cadmium (Cd), zinc (Zn), and copper (Cu), often originating from industrial activities and environmental pollution, can accumulate in edible products, posing serious risks to human health. In addition, microbial pathogens and their toxins further exacerbate the hazards in the food supply chain (Sarsembayeva *et al.*, 2020; Zhang *et al.*, 2025). Effective detection of these contaminants is essential for ensuring consumer safety and maintaining public confidence in the food industry.

Rapid detection methods are essential to mitigate these risks and ensure that contaminated products do not reach consumers. Traditional analytical methods, such as high-performance liquid chromatography, liquid chromatography-mass spectrometry, and spectroscopic techniques-offer high sensitivity and specificity but usually require complex sample preparation, expensive equipment, and specialized personnel. They typically involve laborious sample preparation, complex detection protocols, and expensive instrumentation operated by

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highly skilled personnel. Such limitations restrict their applicability in real-time monitoring and on-site analysis (Dong *et al.*, 2024; Vanegas *et al.*, 2017).

Biosensors have emerged as highly promising analytical devices for rapid, on-site determination of toxic and pathogenic substances in food. Biosensors integrate biological recognition elements, including enzymes, antibodies, nucleic acids, and cells, with physicochemical transducers that convert biological interactions into measurable signals (Pisoschi *et al.*, 2024; Mishra *et al.*, 2018). This integration enables the detection of a broad range of toxins with high sensitivity and specificity while significantly reducing analysis time. Furthermore, the compact design and portability of many biosensor systems allow for their use directly in the field or at production sites, bypassing the need for centralized laboratories (Malitesta and Guascito, 2005; Shen *et al.*, 2021).

A variety of biosensor platforms have been developed to suit different analytical needs. For example, electrochemical biosensors detect changes in electrical properties upon binding of the target analyte (Fernández *et al.*, 2017; Wasiewska *et al.*, 2023), whereas optical biosensors monitor variations in light absorption or fluorescence (Sforza *et al.*, 2024; Aihaiti *et al.*, 2024). Other types, such as thermal, piezoelectric, and nanobiosensors, also contribute to the versatile detection capabilities available today (Thakur *et al.*, 2022; Kakimova *et al.*, 2023; Gupta *et al.*, 2021). The choice of biosensor type and its biological recognition component can be tailored to the specific contaminant, ensuring rapid and selective detection even at trace concentrations.

Among the wide range of food sectors, the dairy industry stands out as both a major economic contributor and a sector highly susceptible to contamination challenges. Milk and dairy products are particularly vulnerable to contamination from environmental toxins and microbial agents, which can compromise product quality and endanger consumer health (Kakimov *et al.*, 2023). Monitoring milk quality in real-time is therefore imperative to prevent unsafe products from entering the consumer market. Recent advancements have demonstrated the efficacy of biosensors in this sector. For instance, amperometric biosensors have been successfully utilized for the rapid detection of pathogens such as Salmonella in milk (Alexandre *et al.*, 2016; Alexandre *et al.*, 2018), while colorimetric biosensors have been developed to identify bacterial toxins without the need for extensive sample processing (Feng *et al.*, 2024). Moreover, enzyme-based sensors exploiting the inhibition of horseradish peroxidase by heavy metals like lead and cadmium have proven effective in quantifying these contaminants with high accuracy (Nomngongo *et al.*, 2011; Silwana *et al.*, 2014).

In regions experiencing intensive industrial activities, such as East Kazakhstan, concerns about heavy metal contamination in milk and other raw materials are particularly acute. Studies from several districts (Borodulikha, Ulan, Beskaragai, Shemonaikha, Katon-Karagai) have reported that lead and cadmium often exceed Maximum Permissible Concentrations (MPC) (Zharykbasov *et al.*, 2023).

Despite significant advancements, existing biosensor technologies still present several limitations, particularly regarding sensitivity, cost-efficiency, portability, and stability under diverse environmental conditions. For instance, the biosensor developed by Malitesta and Guascito (2005), although effective in detecting Hg^{2+} and Cu^{2+} through enzyme inhibition, relies on electropolymerization for enzyme immobilization, which might lead to challenges in achieving uniform enzyme layers and controlling the thickness precisely. In contrast, the layer-by-layer (LbL) immobilization technique allows precise control over the enzyme deposition process, ensuring reproducible biofilm thickness, enhanced sensitivity, and improved operational stability. Furthermore, the simplicity, portability, and robustness of potentiometric biosensor tailored specifically for rapid on-site detection of Pb and Cd in milk directly address the practical needs of dairy industries operating in regions with significant heavy metal contamination.

In response to these challenges, research and development efforts are increasingly focused on optimizing biosensor technologies for food safety applications. In the dairy industry, the deployment of biosensors for continuous, real-time monitoring can enhance quality control, improve traceability, and facilitate immediate corrective actions in case of contamination. This research aims to develop a potentiometric biosensor for the rapid and reliable detection of toxic heavy metals (lead - Pb and cadmium - Cd) in milk and dairy products.

Hypothesis: Increasing concentrations of lead (Pb^{2+}) and cadmium (Cd^{2+}) in milk samples will significantly inhibit the catalytic activity of immobilized catalase, resulting in measurable changes in electrode potential, thereby enabling their quantitative detection using a potentiometric biosensor.

Materials and Methods

Cow milk samples were obtained between September and October 2024 from five different districts in the East Kazakhstan region: Borodulikha, Ulan, Beskaragai, Shemonaikha, and Katon-Karagai. For each district, milk (approximately 2–3 liters per farm) was collected from five to seven private farms. Subsequently, samples from each district were combined and stored in refrigerated tanks. These particular districts were strategically chosen based on their geographical orientation relative to Ust-Kamenogorsk, a significant industrial center. The proximity of these districts to industrial activities increases the likelihood of heavy metal transfer into the environment, potentially contaminating water, soil, plants, and ultimately milk. Additionally, these regions possess extensive pasture areas and support significant dairy farming activities, making them primary contributors of raw milk to local dairy processing facilities.

Hydrogen peroxide solution (3%, LLP "Dosfarm"), chitosan from crab shells (high viscosity, Sigma-Aldrich), sodium alginate (Sigma-Aldrich), carboxymethyl cellulose (Sigma-Aldrich), crystallized catalase (Sigma-Aldrich), and phosphate buffer solution, pH 7.0 (Reagecon) were purchased from different companies. Instrumentation included a two-beam UV spectrophotometer SPECORD-210 Plus, a gasometric setup consisting of a burette, flask, gas outlet tube, and water-filled vessel, and an Arduino Nano (HK ShanHai, China) with a 1602A display and a receptacle for the biosensor were used in this study.

Sample Preparation

For this research, a total of 15 ml of milk at a temperature of 25°C, 5 ml of sterile 3% hydrogen peroxide solution, and 0.001 g of crystallized catalase were measured and prepared. The milk sample was placed in a flask, and the hydrogen peroxide solution and catalase were quickly added. The flask was immediately sealed with a rubber stopper, and a gas outlet tube connected to a 50 ml burette was attached to the stopper. Prior to the experiment, the water level in the burette was carefully adjusted to the zero mark. The contents of the flask were continuously stirred at a controlled temperature of 25 ± 1°C. The pressure was monitored by measuring the volume of oxygen accumulated in the burette over a period of 1 minute. The evolved oxygen volume was recorded as the result of the experiment.

For the analysis, a plate with immobilized enzyme was immersed in a cuvette containing 2 ml of distilled water for 30 minutes. After that, the cuvette was analyzed using a spectrophotometer. The results of the measurements were used to assess the degree of enzyme transfer from the plate surface to water and the degree of decomposition of multilayers formed during immobilization.

We employed a layer-by-layer immobilization method due to its multiple advantages. Firstly, this method allows us to form a thin film on the sensor's surface, thereby enhancing the efficiency of enzyme immobilization. Secondly, by alternating the layers of positively and negatively charged polyelectrolytes, we enhance the stability of the immobilization, reducing enzyme leakage. Most importantly, this approach also enables us to perform a breakdown analysis of the multilayer films to assess the immobilization quality, which is crucial for the success of our research. The film is formed by applying alternating layers of oppositely charged polymers. To do this, the substrate with the enzyme applied to its surface was sequentially immersed in solutions of chitosan (0.161 g chitosan per 100 ml of 3% acetic acid) and sodium alginate (0.198 g sodium alginate per 100 ml of water). The sensor with the adhered biomaterial was immersed into the solution of positively charged polyelectrolyte and then into the solution of negatively charged polyelectrolyte to a depth of 5 mm with a 2-second dwell time in each solution. The number of immersions varied from 1 to 9 bilayers. The samples were dried at a temperature of 35°C for 2 hours.

To conduct the measurement, we used a basic experimental setup, which included a burette, flask, gas outlet tube, and a vessel filled with water. The flask was placed on a magnetic stirrer with heating.

Biosensor Assembly

A biosensor consists of a biological sensing element, a transducer, and an electronic interface. The biological element (e.g., enzyme or antibody) specifically interacts with the target analyte. The transducer converts this interaction into a measurable signal, and the electronic interface processes and displays the result. As shown in Fig. 1, the biosensor assembly includes a display (1), a sensor (2), an external power cord (3), fine (4) and coarse (5) tuning controls, and a power button (6) for start/stop operation (Fig. 1).

The detection part of the device is made of a plastic substrate with conductive graphite tracks deposited on it. On top, a layer of adhesive film with a cell measuring 1.5x3 mm is applied for precise dosing of the tested material that comes into contact with the sensor.

Fig. 1: Biosensor assembly: 1 - display; 2 - sensor; 3 - external power cord; 4 - biosensor fine tuning; 5 - biosensor coarse tuning; biosensor power button

Biosensor working principle: when the biosensor sensor with immobilized enzyme comes into contact with the tested milk containing heavy metal ions, an electrochemical reaction occurs. In the process of the electrochemical reaction, an electric current is generated depending on the content of heavy metal ions in the product. The instrument measures the current strength and displays the measurement results on the screen. Determination of heavy metal ions in the tested product should be carried out at a temperature of 10-35 °C. Biosensor detectors are intended for one-time use only. The technical characterization of the biosensor is presented in Table 1.

Biosensor Testing

To verify the quality of the graphite track deposition on the substrate surface, a multimeter was used. It measures the resistance of the deposited tracks, which should not exceed 10 Ohms.

Selection of Biological Material and Determination of its Activity

In the proposed biosensor for the detection of heavy metal salts, the enzyme catalase was used, which decomposes hydrogen peroxide into water and oxygen. The enzyme solution was prepared by dissolving it in a phosphate buffer solution with a pH of 7.0. The activity of catalase was determined by measuring the volume of oxygen evolved over a specific time period. The sensitivity of catalase to heavy metal salts, such as Pb and Cd, was established.

Enzymatic Optimization

The concentration of catalase enzyme and reaction time were investigated. Concentrations of the enzyme ranging from 0.0005 g to 0.05 g per 0.001 L of buffer solution were studied. The duration of the enzymatic reaction was measured by monitoring the complete decomposition cycle of hydrogen peroxide and the volume of oxygen evolved within 1 minute.

Table 1: Biosensor technical characteristics

Name	Characteristics and technical data
Display type 1601	Two-line LCD with 16 characters
Working fluid mass, µl	0.5-2
Timing (measuring time), sec	60
Transducer type	Amperometric
Range of results, mg/l	0.001-0.5
Substrate storage temperature	25 °C
Relative humidity operating range, %	85-90
Operation duration of power supply elements, number	≈ 1000 measurements
Type of power supply elements	AA battery - 4 pieces
Geometric parameters of the unit	
- length, mm	- 190
- width, mm	- 100
- height, mm	- 50

Determination of Heavy Metal Salt Concentrations

According to the Technical Regulation of the Customs Union "On the Safety of Milk and Dairy Products" (TR CU 033/2013), the maximum permissible level of Pb in milk and dairy products is 0.1 mg/kg, and for Cd, it is 0.03 mg/kg. Based on this, the concentrations of heavy metal salts in this study were varied as follows: 10 times below the maximum permissible level, equal to the maximum permissible level, and 10 times higher than the maximum permissible level for each metal.

Biosensor Investigation (Enzyme Deposition, Immobilization, Determination of Layer Composition and Quantity)

In the proposed biosensor, catalase enzyme was used as the biorecognition element for heavy metal salts, which inhibit its activity. The enzyme solution was prepared by dissolving it in a buffer solution with a pH of 7.0. A 0.01 mL of the enzyme solution was applied to the sensor cell and dried in a convection drying cabinet for 30 minutes at a temperature of 35 °C. The enzyme is completely adsorbed onto the sensor. Subsequently, protection against external influences needs to be created.

The combination of "Chitosan-Sodium Alginate" and "Chitosan-Sodium Carboxymethyl Cellulose" was used as a carrier for immobilization. A chitosan solution of 0.161 g was prepared using 3% acetic acid. The sodium alginate solution was prepared by dissolving 0.198 g/100 mL in water. The sodium carboxymethyl cellulose solution was prepared by dissolving 0.223 g/100 mL in water. The solutions were left on a magnetic stirrer at a temperature of 20 °C for 24 hours with a rotation speed of 120 rpm.

The enzyme immobilization was performed using the layer-by-layer method as follows: the sensor with the enzyme was immersed in a solution of positively charged polyelectrolyte and a solution of negatively charged polyelectrolyte to a depth of 5 mm with a 2-second interval in each solution. The number of immersions varied from 1 to 9 bilayers. The sensor was then dried at a temperature of 35 °C for 2 hours. Subsequently, a series of test samples were prepared with the fixation of the electrode potential. By applying the Nernst equation, the electrode potential value for the oxidation-reduction reaction of hydrogen peroxide decomposition was calculated to be -667.2 mV.

System Stability Under the Influence of External Factors

The assembled biosensor was tested for stability against factors such as ionic strength, pH, and temperature.

Deposition of Metal Ions

To avoid errors in the identification of Pb and Cd salts, the substrate needs to be prepared in a way that only one of the metals is present in the environment during measurement. This is achieved by precipitating one of them in the tested sample.

Linear Range, Detection Limit, and Quantification Limit

Solutions of heavy metal salts with different concentrations were applied to the prepared biosensor. The magnitude of the electrode potential was recorded. To obtain a calibration curve, a graph was plotted showing the dependence of the electrode potential indicator on the concentration of heavy metal salts in the substrate.

Interference Effects on the Detection of Heavy Metal Salts

The main indicator of the fabricated biosensor is the potential difference resulting from the enzyme inhibition reaction by heavy metal salts. To prevent false identification during the determination of Pb content in the substrate, the precipitation of Cd ions was performed prior to the measurement. Similarly, for the determination of Cd content, Pb ions were precipitated. This eliminates interference in the identification of metal ions that exhibit inhibitory properties towards catalase.

Statistics

The study employed one-way ANOVA to assess differences in catalase activity under varying concentrations of Pb and Cd, ensuring statistical significance. Each measurement was performed in triplicate ($n = 3$) to enhance data reliability. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant, confirming that the observed inhibition of catalase activity was not due to random variation but rather the presence of heavy metal ions. For lead (Pb), one-way ANOVA yielded an F-statistic of $F(3,8) = 26{,}979.29$, indicating an extremely strong effect of Pb concentration on catalase inhibition. The corresponding effect size, calculated using eta-squared (η^2), was 0.9999, reflecting a nearly complete association between Pb levels and enzymatic activity reduction. Similarly, for cadmium (Cd), the analysis produced an F-statistic of $F(3,8) = 51{,}727.52$, with an effect size (η^2) of 0.9999, also indicating a very strong inhibitory effect. These results confirm the high sensitivity of the biosensor to increasing concentrations of both heavy metals. All statistical analyses were performed using STATISTICA 14 (StatSoft, USA) and Microsoft Excel 2016 (Microsoft, Redmond, WA, USA).

Results and Discussion

Biosensor Assembly

Selection of Plastic Material

Two commonly used plastics, Polyethylene Terephthalate (PET) and Polypropylene (PP), were chosen as the substrate materials.

PET is a thermoplastic polymer widely used in various applications. PET is known for its transparency, high strength, good flexibility (both at high and low temperatures), and chemical resistance. It exhibits excellent resistance to substances like gasoline, oils, fats, alcohols, diluted acids, and alkalis. PET is insoluble in water and many organic solvents but can be dissolved in certain conditions. However, PET tends to undergo spontaneous crystallization over time, which can result in changes in properties and dimensions of Pb (lead). PET is also somewhat hygroscopic (Panowich et al., 2021; Nistico, 2020).

PP, on the other hand, is a chemically resistant material widely used in industries such as chemical manufacturing and ventilation systems. It does not react with most acids, making it suitable for various applications, including medical devices. PP is known for its high environmental and human safety ratings. It does not release harmful substances into the environment and can be used for containers of drinking water and food without concerns of contamination (Li *et al.*, 2022).

Application of Conductive Layers

The application of tracks was carried out using the method of sputtering in a vacuum sputter coater JEE-420 (Japan). This apparatus allows for the deposition of conductive layers using materials such as graphite and various metals like copper, silver, gold, platinum, and others. The thickness of the deposited layer can range from 3 nm and above, depending on the sputtering time.

Silver exhibits a silver-white color, often with yellow, brown, or blackish hues. It readily oxidizes on the surface when exposed to air, and the extent of oxidation increases with the presence of impurities, resulting in a change of surface color to various shades of black. It has a hardness of 2.5-3 and a density of 9.6-12. Silver is highly ductile, flexible, and malleable, and it boasts one of the highest thermal and electrical conductivities among metals. It reacts with HCl, forming a white AgCl. One drawback of using silver as a conductive layer in biosensor construction is its tendency to oxidize, Pb ion to potential distortions in the obtained results (Gahlaut *et al.*, 2022; Sotiriou and Pratsinis, 2011).

Platinum ranges in color from silver-white to steel-black. It has a hardness of 4-4.5 and can reach up to 6-7 in alloys with iridium. Platinum is malleable and exhibits a hooked fracture. It is an excellent electrical conductor and is one of the most inert metals, remaining insoluble in acids and alkalis. Upon stronger heating, platinum reacts with carbon and silicon, forming solid solutions, similar to metals in the iron group. The only disadvantage of platinum is its relatively high cost (Soto and MacLachlan, 2023).

Graphite, resistant to chemical and natural influences, is quite strong and a good conductor of electricity. It has a low hardness, relative softness, and solidifies after exposure to high temperatures. Its density is 2.23 g/cm³. Graphite possesses a metallic luster and dark gray color. Due to its relatively high thermal conductivity, graphite is commonly used for manufacturing components in electrical equipment. Graphite products exhibit enhanced operational characteristics. Compared to silver and platinum, this material is relatively inexpensive (Zhang *et al.*, 2021; Sengupta *et al.*, 2011).

In this study, the deposition of graphite was adjusted to achieve track resistance not exceeding 10 Ohms. Sputtering was carried out at a pressure of approximately 4 x 10^{-4} Pa. To obtain clear track boundaries, a mask with openings shaped like tracks was used. Consequently, the sputtered material was applied only to the exposed areas of the substrate.

Biosensor Block Diagram

The biosensor block diagram is shown in **Figure 2** which consists of sensor B containing biocatalyst A, which interacts with heavy metal salts S to produce a product. As a result of a chemical reaction at the interfaces of the transducer B, an electrical potential P is generated. The electrical potential exits the transducer as an electrical signal and is amplified by an operational amplifier C. The amplified signal is processed by an Arduino Nano D microcontroller using a loaded program and displayed on the display E as a digital image.

Selection of Biological Material and Determination of its Activity

Based on an analysis of literature sources, it has been determined that enzymes of the oxidoreductase class, specifically the enzyme catalase, are most sensitive to heavy metal salts (Elsebai *et al.*, 2017; Takio *et al.*, 2021; Kurbanoglu *et al.*, 2020, Gholivand and Khodadadian, 2014). Therefore, catalase was chosen as the biological element in the proposed biosensor. The activity of catalase was determined using the gasometric method based on the Warburg method. For this purpose, 0.001 g of crystalline catalase was taken and added to 15 ml of substrate (distilled water, milk) in a round-bottom flask at a temperature of 37°C with constant stirring. Then, 5 ml of 3% hydrogen peroxide was added, and the flask was tightly closed with a stopper connected to a volumeter through a silicone tube to ensure airtightness. The volume of the evolved gas was measured and its value was normalized to standard conditions using the combined gas law of Gay-Lussac:

$$\frac{P_1V_1}{T_1} = \frac{P_2V_2}{T_2}, \text{ or } \frac{PV}{T} = const, \quad (1)$$

- P_1 is the initial pressure in (kPa)
- P_2 is the final pressure in (kPa)
- V_1 is the initial volume in (mL)
- V_2 is the final volume in (mL)
- T_1 is the temperature of the first gas in (K)
- T_2 is the final temperature in (K)

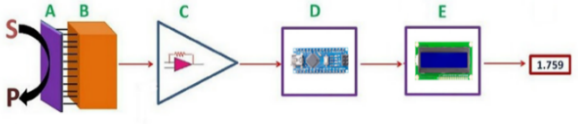

Fig. 2: Block diagram of the biosensor

The study of the influence of heavy metal ions was conducted using a similar procedure, with the addition of 0.02 mg/kg of heavy metal salts (Pb and Cd). Cow's milk was used as the medium for the investigation. The enzyme activity was measured in three variations of the medium:

- In a medium without heavy metal salts (control)
- In a medium containing Cd salts
- In a medium containing Pb salts

Based on the obtained data, the degree of substrate conversion was calculated. The results are presented in **Figure 3**. As seen from Fig. 3, in the presence of heavy metal salts, the activity of the enzyme decreases. While the activity decreases slightly in the presence of Cd ions, it decreases by approximately 13% in the presence of Pb ions. These findings indicate that catalase can be used as an indicator for detecting heavy metal salts.

Enzymatic Optimization

The concentration of catalase was optimized based on the rate of hydrogen peroxide decomposition into water and oxygen per unit time. A low enzyme concentration renders the biosensor system inefficient, resulting in a weak response. On the other hand, a high enzyme concentration Pb to an explosive reaction that often extinguishes before the desired time. In the study, the enzyme concentration was examined ranging from 0.0005 to 0.005 g. It is known that the Michaelis-Menten constant for the reaction of hydrogen peroxide decomposition by catalase is 25 mM (Jones & Suggett, 1968). Applying the *Km* value to our conditions, it was determined that 1 unit of catalase decomposes 0.025 moles of hydrogen peroxide in 1 minute. The maximum possible volume of evolved oxygen under the given conditions was calculated to be 2200 μmol.

From the results shown in Figure 4, it can be observed that at enzyme concentrations below 0.001 g, the reaction proceeds slowly, and the decomposition of hydrogen peroxide exceeds the predetermined time. At concentrations above 0.001 g, oxygen is evolved actively, and the reaction terminates prematurely. At a catalase concentration of 0.001 g, a complete cycle of hydrogen peroxide decomposition occurs within 1 minute, resulting in the evolution of 1560 μmol of oxygen. Therefore, this enzyme concentration is considered optimal.

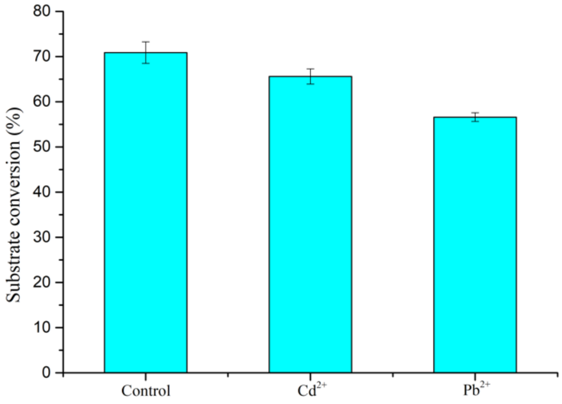

Fig. 3: Substrate conversion (%), In the presence of heavy metal salts in the milk

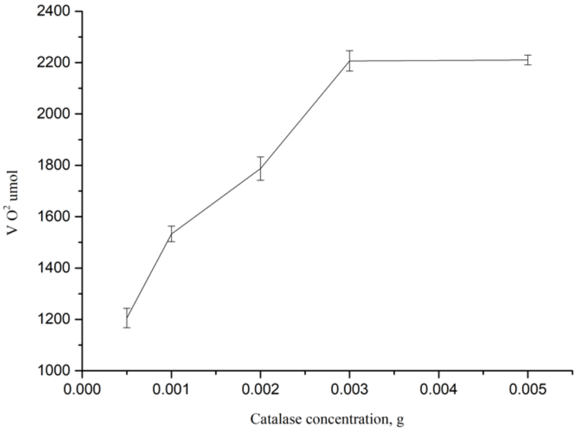

Fig. 4: The dependence of the volume of released oxygen on the concentration of the enzyme

Determination of Heavy Metal Salt Concentrations

The intake of toxic elements within the limits regulated by legislative acts and established as maximum permissible concentrations (MPC) does not have a detrimental effect on the human body as a whole. However, enzymes are highly sensitive to external factors, especially toxicants. Under their influence, the enzyme molecule loses its native structure, Pb ions to a decrease in enzyme activity.

The MPC for Pb in milk and dairy products is 0.1 mg/L, and for Cd, it is 0.03 mg/L. To assess the degree of enzyme activity inhibition by heavy metal salts, the concentrations of Pb and Cd were varied as follows: 10 times below the MPC level, equal to the MPC, and 10 times higher than the MPC for each metal. The quantitative values are presented in Table 2.

Table 2: Quantitative values

Toxic Element	MPC*, mg/l	10 times > MPC, mg/l	10 times < MPC, mg/l
Pb	0.1	1	0.01
Cd	0.03	0.3	0.003

To assess the reaction intensity and enzyme activity in the presence of heavy metal salts, we introduced 0.001 grams of crystalline catalase into a 15-milliliter round-bottom flask containing milk as a substrate, maintaining the temperature at 37°C with continuous stirring. Subsequently, 5 milliliters of 3% hydrogen peroxide (H_2O_2) were added, and the flask was hermetically sealed with a silicone tube connected to a volumeter. We conducted a series of sample tests by introducing the corresponding amounts of heavy metal salts. The volume of evolved gas was measured in milliliters.

The graph in Figure 5 presents the average oxygen volume generated for each metal. When compared to the control group, we observed that at heavy metal salt concentrations lower than ten times the permissible exposure limits (PEL), Cd resulted in a 6% reduction in enzyme activity, while Pb showed an 8% reduction. When the concentrations of Cd and Pb were increased to levels equal to the PEL, enzyme activity decreased by 30 and 54%, respectively. Elevating the concentrations of Cd and Pb to ten times above the PEL led to a reduction in enzyme activity by 50 and 60%, respectively.

Biosensor Probe Investigation

In the development of enzyme biosensors, special attention is given to the process of immobilizing the biological material on the substrate surface, as the method of attachment will affect the biosensor's sensitivity and measurement quality. In this study, the layer-by-layer technology was employed, where oppositely charged polyelectrolytes form multilayers on the substrate surface. Two types of multilayers were used for enzyme immobilization: the first one consisted of a combination of chitosan and sodium alginate, and the second one utilized chitosan and sodium carboxymethyl cellulose (CMC).

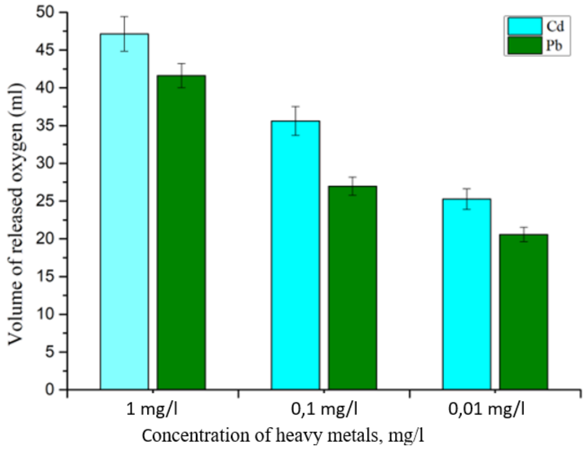

Fig. 5: Dependence of the volume of released oxygen (ml) on the concentration of salts of heavy metals

Using the Nernst equation, the electrode potential for the redox reaction of hydrogen peroxide decomposition was calculated:

$$E = E^0 - \frac{RT}{nF}\ln\frac{a(Red)}{a(Ox)} \qquad (2)$$

R - The universal gas constant, equal to 8.31 J/(mol·K)
T - Absolute temperature
F - Faraday's constant, equal to 36,500 C/mol
N - The number of moles of electrons involved in the process

The calculated electrode potential under these conditions was -667.2 mV. The electrode potential of the experimental samples was measured using a digital dual-channel storage oscilloscope, model ASK-2034 (Russia, 2008), in the constant current mode. The results of multiple measurements on the substrate with the multilayer combination of "chitosan-CMC" exhibited a large scatter of data, indicating the impracticality of using this combination for enzyme immobilization. The measurement results on the substrate with the multilayer combination of "chitosan-sodium alginate" are shown in Figure 6.

As can be seen from Figure 6, the actual measured value of the electrode potential for the reaction conducted on the polymer substrate with immobilized enzyme is slightly lower than the theoretically possible value. However, the difference is insignificant and falls within an acceptable range. This is because, during the experiment, a systematic dependence of enzyme activity on the influence of environmental components was established.

To achieve an active response of the enzyme to the effect of inhibitors, it is recommended to apply it at a concentration of 0.1 g/mL in a phosphate buffer solution. Using a dosimeter, 0.01 mL of the enzyme solution is applied to the open cell of the biosensor and dried. The enzyme adheres to the substrate cell of the sensor.

Subsequently, enzyme immobilization was performed. The sensor with the dried enzyme was sequentially immersed in positively charged and negatively charged polyelectrolytes. Variations were prepared by applying 1, 3, 5, 7, and 9 bilayers to form a dense multilayer membrane.

As shown in Figure 7, when increasing the number of bilayers to 7 and 9, low values of electrode potential were observed (-152 mV and -264 mV, respectively), likely due to the limited diffusion of substrate and product molecules to the surface of the support with increasing membrane thickness. In samples with 5 or fewer bilayers, a sufficiently high electrode potential of -432 mV or higher was observed. However, in samples with fewer than 5 bilayers, a small amount of protein precipitation was observed. Experimental findings indicate that samples with 5 bilayers demonstrate reliable enzyme immobilization and a high electrode potential.

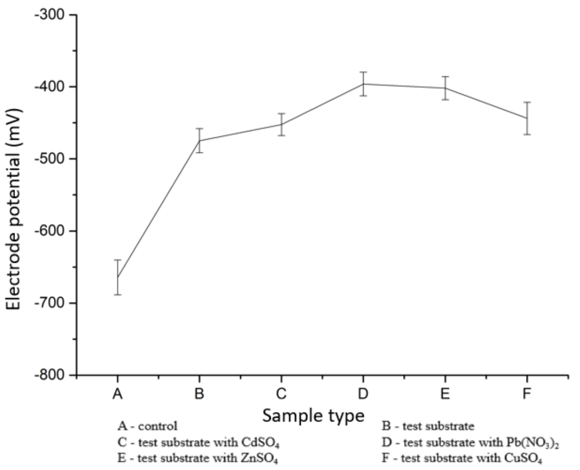

Fig. 6: Index of electrode potential in samples

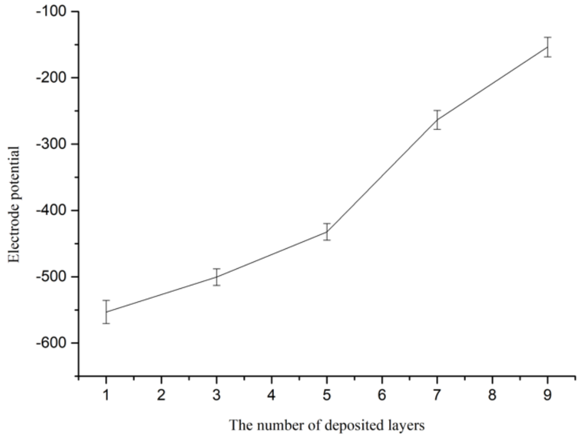

Fig. 7: Dependence of the value of the electrode potential on the number of deposited layers during immobilization with a combination of "chitosan-sodium alginate"

Stability of the System Under the Influence Of External Factors.

When selecting the temperature optimum for the carrier with immobilized enzyme, the characteristics of the system components were taken into account. The storage temperature range for catalase enzyme in crystalline form is from -2 to -12 ºC. The research results showed that the upper temperature limit for the polymer system of chitosan-sodium alginate was 25 ºC. The upper temperature limit for the polymer system of chitosan-(CMC) was 20 ºC.

Considering that hydrogels can recover from a frozen state without losing their native properties and taking into account the lower temperature limit of catalase, the experiment was conducted at a maximum low temperature of -12 ºC and a maximum high temperature of 36 ºC. However, a decrease in the aggregate stability of the investigated systems (chitosan-sodium alginate and chitosan-CMC) was observed at temperatures above 25 ºC.

The temperature ranges from -12 ºC to 25 ºC is considered acceptable for the storage of the investigated systems, as the activity of the redox reaction was preserved, the electrode potential remained within the theoretically acceptable value (-667.2 mV), and no visual changes were observed on the substrate surface.

At this stage, the stability of the carriers to variations in ionic strength and the efficiency of enzyme immobilization in the tested systems were determined. The immobilization of catalase was performed by layer-by-layer deposition of oppositely charged polyelectrolytes. The system was tested using a glycine buffer, and the efficiency of enzyme immobilization was determined by measuring the transmitted protein in the liquid medium using a dual-beam UV spectrophotometer, SPECORD-210 Plus.

To assess the system's stability, glycine buffer solutions with concentrations of 0.01, 0.05, 0.1, and 0.5 M were used. The substrate with the immobilized system was kept in the buffers with the specified concentrations for 2 hours. At glycine buffer concentrations of 0.01 M and 0.05 M, no visual changes were observed in the chitosan-sodium alginate and chitosan-CMC systems. Spectrophotometric measurements also indicated the integrity of the multilayers.

When increasing the buffer concentration to 0.1 M and 0.5 M, the substrate surfaces of both carrier systems became mucous, indicating the degradation of the multilayer film. As shown in the graph, spectrophotometric measurements revealed that the protein loss from the substrate at a buffer concentration of 0.1 M was approximately 18-20% for the chitosan-sodium alginate system and about 25% for the chitosan-CMC system. At a concentration of 0.5 M, the protein loss was approximately 46-48% for the chitosan-sodium alginate system and 52-55% for the chitosan-CMC system. It was determined that variations in ionic strength up to 0.05 M were acceptable for the tested carriers.

Precipitation of Heavy Metal Ions

Pb and Cd ions have an inhibitory effect on the catalase activity. To determine Cd concentration, the precipitation process of Pb ions needed to be carried out. And to determine Pb concentration, the precipitation of Cd ions was required. To determine the concentration of Cd ions in the substrate, it was necessary to remove Pb ions by precipitating them with sodium chloride. Pb cations form a white precipitate of $PbCl_2$ with chloride ions (at pH <7): $Pb^{2+} + 2Cl^{-} \rightarrow PbCl_2$.

Practical Considerations, Limitations, and Future Perspectives

Addressing potential limitations is critical for ensuring the practical applicability of the developed biosensor. In this study, specific strategies were employed to minimize interference from co-existing metal ions, including the selective precipitation of either Pb^{2+} or Cd^{2+} prior to measurement. This approach effectively enhanced the biosensor's selectivity for the target ion. Additionally, the operational stability of the biosensor was evaluated under varying environmental conditions, including a temperature range of −12 to 25 °C and ionic strength levels up to 0.05 M glycine buffer. The biosensor demonstrated consistent performance within these parameters. However, long-term stability under continuous use in real-world field environments remains to be fully validated. Further studies are recommended to assess durability and sensor integrity over extended operational periods and repeated measurements.

Future research should focus on further validating and expanding the applicability of the biosensor under real-world conditions. Planned next steps include conducting extensive field testing on dairy farms located in regions with known heavy metal contamination, particularly in East Kazakhstan. Moreover, the integration of the biosensor with Internet-of-Things (IoT) platforms is proposed to enable continuous, real-time monitoring and automated data transmission. Such integration would significantly enhance traceability, facilitate early detection of contamination, and support rapid decision-making in dairy production quality control systems.

The developed potentiometric biosensor achieved detection limits ranging from 0.01 to 1 mg/L for Pb and 0.003 to 0.3 mg/L for Cd, offering rapid, real-time results within 1 minute. Although traditional methods such as Inductively Coupled Plasma Mass Spectrometry (ICP-MS) typically offer lower detection limits (sub-µg/L levels), they require expensive instrumentation, extensive sample preparation, and trained operators. In contrast, our biosensor provides a practical alternative characterized by portability, simplicity of operation, minimal sample preparation, and cost-efficiency, making it suitable for routine on-site monitoring, especially in resource-limited or field conditions.

Conclusion

A potentiometric biosensor has been developed for the determination of highly toxic Pb and Cd ions in milk and dairy products. Catalase enzyme was chosen as the test object for detecting heavy metal salts. An optimal concentration of 0.001 g of catalase enzyme was determined based on the rate of hydrogen peroxide decomposition into water and oxygen per unit time. In this work, the layer-by-layer technology was employed for creating enzymatic biosensors, where oppositely charged polyelectrolytes form multilayers on the substrate surface. A multilayer combination of "chitosan-sodium alginate" was used for enzyme immobilization. Experimental results showed that samples with five layers exhibited reliable enzyme immobilization and high electrode potential. The stability of the carriers to variations in ionic strength and the effectiveness of enzyme immobilization in the tested systems were established.

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Author’s Contributions

All authors equally contributed in this work.

Ethics

The authors should address any ethical issues that may arise after the publication of this manuscript.

Patents

Patent for invention No. 37011 of the Republic of Kazakhstan “Biosensor test system based on immobilized enzyme for determination of heavy metal salts in milk” was obtained. Number and date of the bulletin - No. 6 dated 07 February 2025.

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