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Original research

An amperometric hand-held Nitrate biosensor based on Nitrate reductase immobilized on a thin film electrode: a tool for in-filed water and food analysis

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ABSTRACT

This study presents the design and evaluation of a portable amperometric biosensor for detecting nitrates, utilizing a gold three-electrode system with immobilized nitrate reductase. With a sensitivity of 12.01 μA mM^{-1} cm^{-2} over a 0.16–0.96 mM range and a regression coefficient of 0.98, the biosensor outperforms traditional UV spectrophotometric methods (0.01–0.19 mM) by offering a broader range (0.16–0.96 mM) and greater accuracy, as evidenced by a 6.69% relative standard deviation for reproducibility and a detection limit of 0.14 mM. Maintaining 63% of its initial response after four days and showing minimal interference from nitrite (1 mM), this biosensor is highly suitable for water testing, although pre-treatment is required for analyzing food samples due to phenolic compounds.

Keywords: Nitrate; Biosensor; Amperometric; Enzyme; Immobilization

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1. Introduction

Nitrate (NO_3^-) is an inorganic compound of nitrogen. Its residues in food, water, and the environment have attracted attention due to its high concentrations and potential health concerns. Besides its importance in food and water analysis, there is a lack of a simple, convenient, and accurate method to determine this substance. Biosensors are gaining increasing importance for the rapid and reliable detection of analytes for both lab-based analysis and outdoor applications.

Inorganic nitrate NO_3^- anion is present under a variety of both natural and artificial environmental conditions. Nitrate is usually used in meat products for the improvement of their color and flavor and also to inhibit the growth of food poisoning microorganisms such as Clostridium botulinum (Wang et al. 2017). Nitrate levels may also increase in drinking water due to contamination from different sources. Nitrate is not toxic to humans, but when it is converted to nitrite in the body by bacteria, it causes Methemoglobinemia (blue baby syndrome) and, consequently, oxygen deficiency in the body (Sohail and Adeloju 2016). In addition, it is known that nitrite can also react with secondary amines and amides in the stomach or during cooking to form carcinogenic N-nitrosamines (Wang et al. 2017). As per the recommendations of the World Health Organization (WHO) and the US Environmental Protection Agency (EPA), the tolerance limit of nitrate levels in drinking water is 44 ppm (10 ppm as nitrogen per liter) (Sohail and Adeloju 2016). Furthermore, nitrate has unfavorable effects on fish and aquatic life (Alahi and Mukhopadhyay 2018). Therefore, it is necessary to determine nitrate levels in drinking water, food, and in aqueous ecological systems conveniently and regularly for the sake of the environment and human health.

Considering the importance of determining nitrate concentrations, several methods have been reported for determining nitrate, including spectrophotometric, chromatographic, electrochemical analysis, capillary electrophoresis, flow injection, fluorescence, electromagnetic sensors, fiber-optic sensor and others (Alahi and Mukhopadhyay 2018; Sohail and Adeloju 2016; Wang et al. 2017). While these methods can provide good sensitivity and accurate information about the levels of nitrate ion, the mentioned methods suffer from many drawbacks, and they are mainly laboratory-based methods that require chemicals and skillful operators, which is not suitable for portable applications. Therefore, the development of fast, inexpensive, simple, reliable and even

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portable tools and methods for the detection of nitrate is of particular importance.

Biosensors have been studied as alternative methods for the detection and quantification of target compounds providing appropriate sensitivity, selectivity, and accuracy. Biosensors are one of the potential technologies which can minimize the shortcomings of the current detection technologies mentioned above, providing simple, sensitive, and useful analytical tools for fast, reliable, and convenient detection of analytes. Biosensors are usually composed of a biological element (bioreceptor) and a physicochemical detector (transducer). An enzyme-based electrochemical biosensor is a biosensor with an electrochemical transducer and the enzyme as a biological element that is immobilized on it and selectively reacts with the substrate (Sohail and Adeloju 2016). Enzyme-based biosensors can determine nitrate with high sensitivity and acceptable performance. Most of the biosensors for nitrate detection have been based on nitrate reductase for biocatalysis of nitrate reduction to nitrite (Bollella and Gorton 2018; Jadán et al. 2017; Strehlitz et al. 1994; Umar and Nasar 2018). Several studies have recently been conducted on nitrate biosensors; however, to the best of our knowledge, determination of nitrate by portable and cheap biosensors have received less attention. Furthermore, the determination of nitrate using gold microchip platform has not yet been investigated.

Nitrate biosensors have been developed on sophisticated lab-based instruments and no portable devices have been introduced. In the present paper, a chip with a three-sectioned thin-film gold electrode was fabricated by sputtering technique followed by laser ablation technique. In addition, nitrate reductase was immobilized on the gold electrode after modification with self-assembled monolayers (SAM) of L-cysteine, and the electrode was coupled to a fabricated portable amperometric device. It was aimed to design a simple, cost-effective, reproducible and accurate method for fast quantification of nitrate.

2. Material and Methods

2.1. Reagents and instruments

Nitrate reductase (NR, EC 1.7.1.1, from *Arabidopsis thaliana*) was purchased from Sigma Aldrich (St. Louis, Missouri, US), L-cysteine, glutaraldehyde, potassium ferricyanide, potassium ferrocyanide, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, potassium nitrate, potassium nitrite, gallic acid were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical grade and were prepared in deionized water.

The scanning electron microscope (SEM, VEGA\TESCAN-LMU) used to characterize the surface of the prepared biosensors. The images were taken using an acceleration voltage of 15 kV. Surface topology images of the electrode were explored via an AFM instrument (NT-MDT, Moscow, Russia). Imaging was carried out at ambient temperature in non-contact mode. Experiments of cyclic voltammetry (CV) were performed with a PalmSens potentiostat (Palm Instruments BV, Netherlands) at room temperature (25 °C). Chronoamperometric measurements were performed using a portable handmade potentiostat that is explained in section 2.2. Nitrate analysis was done by preparation of calibration graph by the American Public Health Association method (American Public Health Association, 1995) and spectrophotometric measurements in absorption experiments were carried out using a UV-Vis spectrophotometer (Spectrum SP-UV500DB). Ion chromatography (IC, Dionex 500, Sunnyvale, CA) was used for the nitrate real sample analysis.

Cucumber juice was obtained by a kitchen juicer. Clarified juice was produced by treating with 1000 mg/l of activated charcoal (Merck, Germany) at 50 °C for 1 h and then the juice was filtered by a filter paper and then through a syringe membrane.

2.2. Portable potentiostat

The potentiostat is a device that can run the most commonly used electrochemical techniques, such as cyclic voltammetry (CV) and chronoamperometry (CA) and it is necessary for performing the electroanalysis and read out the signal of an electrochemical biosensor. Because of its expensiveness and bulkiness, it is not suitable for portable biosensors. Thus (Lopin and Lopin 2018) and (Muñoz-Martínez et al. 2018) reported recently a portable low-cost potentiostat based on PSOC technology which was capable to send data to a system with USB and wirelessly. In this work, an improved version of this potentiostat is presented. The previous designs were modified with an optional smoothing function, which can improve the results processing and display. For this purpose, we used the Savitzky-Golay (SG) filter, which is reported to be used to smooth and differentiate time series, especially biomedical data (Dai et al. 2017).

2.3. Sensor Fabrication

The three-electrode system on a glass substrate was manufactured by sputtering and laser ablation techniques. The working electrode diameter was 6 mm. The process sequence for the fabrication of the sensors is schematically illustrated in Fig. 1. Briefly, a layer of Ti is applied onto the glass substrate as an intermediary adhesive layer. Then, a gold layer (100 nm) was deposited by sputtering technique on a glass substrate. Afterward, the resulting layer was directly patterned by laser ablation in order to create micro-scale electrodes. A general view of the sensor is presented in Fig. 1c.

2.4. Preparation of nitrate biosensor

Before immobilization procedure, electrodes were pretreated in 0.5 M H_2SO_4 solution by cyclic voltammetry (CV) from 0 to 1.2 V until a reproducible voltammetric response was obtained. Then the immobilization procedure was performed. Initially, the gold electrode was immersed into 30 mM cysteine aqueous solution for 5 h at room temperature. Then the electrode was rinsed using phosphate buffer (0.2 M pH 7.5) to remove cysteine loosely attached molecules. Afterward, 2.5% of glutaraldehyde solution prepared in phosphate buffer (0.2 M, pH 8.0) was spread over the surface of the Au-Cys modified electrode and dried for 1 h at room temperature. It was then washed with the same phosphate buffer solution. Finally, 10 uL nitrate reductase solution (100 µg/mL) in phosphate buffer (0.2 M pH 7.5) was dropped on the Au-Cys-GA modified electrode (4 °C, 3 h). The obtained enzyme electrode Au-Cys-GA-Nar was washed with the same buffer (pH 7.5) and used in further experiments. The nitrate biosensor and solutions were stored at 4 °C until used.

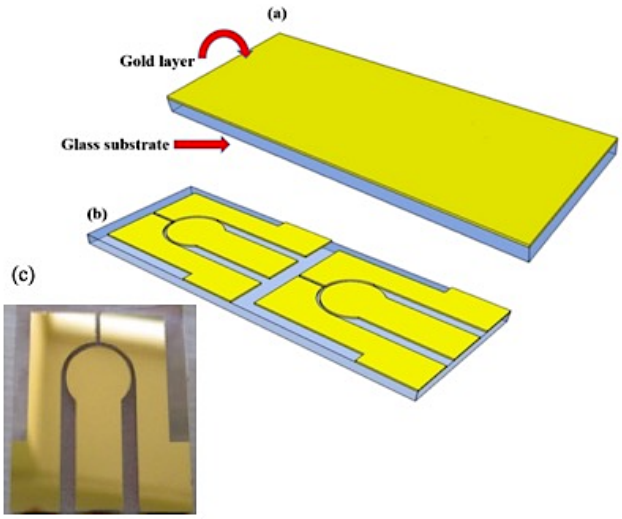

Fig. 1. Process sequence of fabrication of the sensor platform: (a) Deposition of the Au layer on glass substrate; (b) Laser ablation of Au layer (c) Optical image of the fabricated sensor.

3. Results and Discussion

3.1. Electrochemical characterization of the fabricated sensor

Before using the fabricated sensor to measure nitrate, the electrochemical response of the sensor was evaluated. The electrochemical activity of this system was evaluated by cyclic voltammetry (CV) as a commonly used and standard electrochemical method. Cyclic voltammetry is a versatile method for studying the mechanism of the electrode reactions, their kinetic parameters, and it can be used for quantitative analysis (Chooto, 2019). Potassium ferricyanide was used to obtain information about the electron transfer process and also as a model redox-active compound to characterize the electrode system. The characteristic voltammograms of 5 mM $K_3Fe(CN)_6$ in phosphate buffer as a function of scan rate are shown in Fig. 2a. The peak-to-peak current ratio was close to 1.0, and the separation between the cathodic and anodic peak potentials increased from 76 mV to 121 mV, which are larger than the theoretical value of 59 mV. Based on these observations, the electrodes fulfilled the requirements for a quasi-reversible system with a fast electron transfer (Bard and Faulkner 2001; Reich et al. 2017). Moreover, as shown in Fig. 2b, the cathodic and anodic peak currents were linearly proportional to the square root of the scan rate (25–100 mV/s) (R^2 is 0.99 and 0.98, respectively). These results showed that the behavior of the electrodes was similar to the traditional electrochemical cells, and the diffusion of potassium ferricyanide governed the electrochemical reaction rate into the surface of the electrode (Bard and Faulkner 2001). Our results are in good agreement with other studies performed on various thin-film gold electrodes (Ben-Yoav et al. 2015; Branch et al. 2017; Lee et al. 2017; Uludag, Olcer, and Sagiroglu 2014; Wang et al. 2019; Zhang et al. 2017). Also, for the evaluating the reproducibility of the sensor, the cyclic voltammograms of the three sensors were carried out in 0.5 M H_2SO_4 (Fig. 2), and the RSDs of peak current and peak potential for the reduction of gold oxide were 1.15% and 0.23%, respectively (Table 1). Therefore, the constructed three-electrode configuration can be favorably adopted for the analytical application.

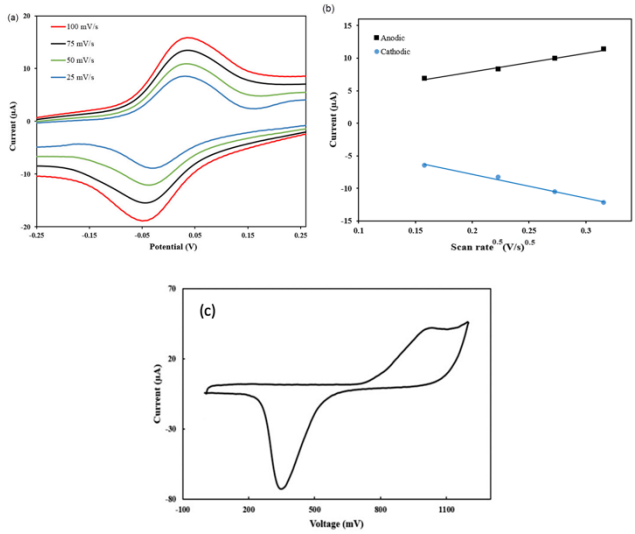

Fig. 2. Electrochemical characterization of the three-electrode system: (a) cyclic voltammograms of the sensor at various scan rates (25-100 mV/s) in 0.2 M potassium phosphate buffer (pH 7.5); (b) Plot of the peak current versus the square root of the scan rate; (c) Cyclic voltammogram of the fabricated sensor in 0.5 M H_2SO_4. Potential range: 0 V to 1.2 V, scan rate: 50 mV/s.

Table 1. Response of the fabricated sensor with or without nitrate reductase for the reduction of gold oxide and nitrate.

	Sample	Reduction of gold oxide	
		Potential (mV)	Current (µA)
Sensor without enzyme	gold oxide	348±1.00 *	-70.62±0.99**
	Background	-	0.99±0.05
Sensor with enzyme	Nitrate 1 mM	-	4.10±0.20
	Nitrate 1 mM/Nitrite 1mM	-	3.99±0.20

*(RSD = 0.23%)

**(RSD = 1.15%)

3.2. Immobilization of nitrate reductase on the electrode surface

Self-assembling monolayers (SAMs) is an interesting and powerful method to immobilize enzymes on electrode surfaces. The Au surface can be modified by self-assembled monolayers (SAMs). The thiol-group compounds (-SH) can be chemisorbed onto the gold surface forming the gold-thiol bond. Cystamine, cysteine, and mercaptan compounds are often recognized as SAMs reagent. Following this strategy, nitrate reductase from *Arabidopsis thaliana* was immobilized on the surface of self-assembled monolayer gold electrode with the assistance of cysteine and glutaraldehyde to obtain a biosensor for nitrate determination. In the first step, covalent bonds between the sulfur and gold atoms of the gold electrode were formed by cysteine solution which is an alkanethiols compound. Furthermore, glutaraldehyde solution which is a well-known powerful crosslinker with two aldehyde groups, was used and

formed stable and strong bonds between amino group on gold electrodes and amino group of enzymes.

The surface morphology of the Au electrode, as well as Au-Cys-GA-Nar, were examined using AFM technique. The 3D morphologies of the fabricated electrodes are shown in Fig. 3. As shown in Fig. 3b, the surface morphology of the Au-Cys-GA-Nar electrode after the enzyme molecules were immobilized on the Au electrode is obviously different from that of bare Au electrode (Fig. 3a), and some small dots appeared and uniformly dispersed on the surface. It could be observed that enzyme molecules were covalently attached onto the Au electrode. Moreover, the average surface roughness was raised from 0.99 nm to 2.38 nm. Results reveal the immobilization of the enzyme on the surface of the electrode.

The immobilization of the enzyme on the electrode surface was also confirmed by SEM analysis. The obtained images could be seen in Fig. 3. Data were obtained from SEM image supported AFM results. It was clear that the surface of the electrode was changed after enzyme immobilization. Compared to the bare electrode (Fig 3c), there was a uniform coverage of the enzyme on the surface of the modified electrode (Fig. 3d). These observations were in accordance with the obtained images from immobilization of ChOx on Au surface by (Matharu et al. 2012). Thus, the SEM images show the successful immobilization of nitrate reductase on the surface of the electrode.

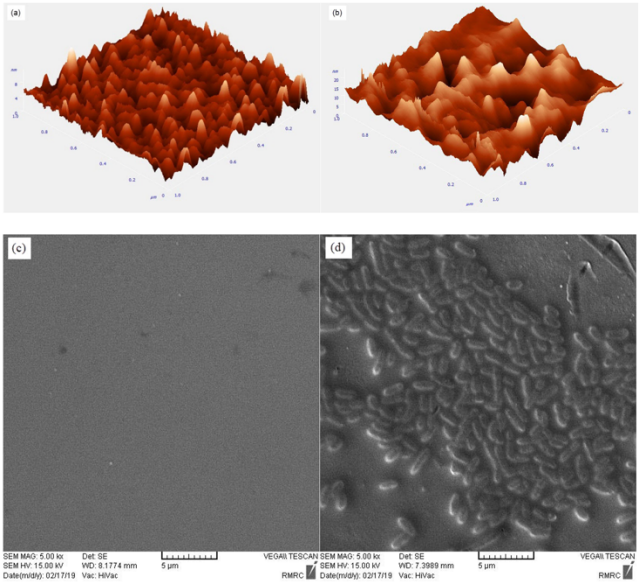

Fig. 3. AFM and SEM images of: (a,c) bare Au electrode; (b,d) immobilized surface.

3.3. Performance of the biosensor

The analytical performance of the biosensor towards the detection of nitrate was performed using chronoamperometric measurements. The applied working potential was set to -75 mV for the reduction of enzymatically produced $K_3[Fe(CN)_6]$. Sensor calibration and sample analysis were performed at room temperature in 100 µL of measurement solution (0.2 M potassium phosphate buffer, pH 7.5, with 5 mM $K_4[Fe(CN)_6]$). These conditions represented the optimal working environment for the amperometric biosensor with $K_4[Fe(CN)_6]$ mediator (Sohail and Adeloju 2016; Monošík et al. 2012; Amor-Gutiérrez et al. 2017).

Fig. 4, presents the amperometric responses to nitrate concentrations ranging from 0.16 to 0.96 mM NO_3^- (10-60 ppm). A well-defined amperometric response of the biosensor was observed when the applied potential was stepped at -75 mV. According to the reaction mechanism, the enzyme catalytic reaction between the immobilized NR and nitrate on the electrode surface caused the current response to increasing with the increase in nitrate concentration. This rise was a result of the oxidized NR reduction to its native form by electron capture from the potassium ferrocyanide. The reduction of the enzyme converted potassium ferrocyanide to potassium ferricyanide and the current of this reaction was proportional to nitrate concentration. For the quantification purposes, the current at 50 s for different concentrations was collected from the chronoamperometric graph (Fig. 4a) and the data was employed for the calibration curve calculation. The derived calibration curve is shown in Fig 4b. As can be seen from Fig. 4b, the amperometric currents of the biosensor demonstrated good linear relationship with the concentration of nitrate in the range of 0.16 to 0.96 mM NO_3^- (10-60 ppm), with a correlation coefficient of 0.98. Based on the slope of the linear fit, the sensitivity of the biosensor was calculated as 12.01 µA mM^{-1} cm^{-2}, and the detection limit of 0.14 mM (8.68 ppm, S/N = 3). The sensitivity and linear range of our biosensor could be used for nitrate measurements. The present biosensor's analytical capabilities were assessed alongside previously reported enzyme-based nitrate biosensors (Table 2). This analysis revealed that the biosensor possesses superior analytical properties, notably a wide linear range and an adequate limit of detection, making it highly effective for in-field applications.

The analytical parameters of the fabricated biosensor for the determination of nitrate were compared with the UV spectrophotometric assay, and the results are showed our biosensor had a wider detection range (0.16-0.96 mM) with a regression coefficient of 0.98 for nitrate determination than the UV spectrophotometric assay (0.01–0.19 mM).

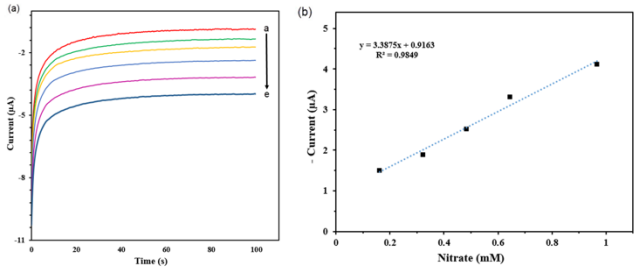

Fig. 4. Chronoamperograms (a) and calibration plot (b) of the nitrate biosensor with nitrate concentration of 0.16 to 0.96 mM (plot "a" to plot "e").

3.4. Stability, reproducibility and interference study

One of the disadvantages of biosensors is that their biological components usually have a short life span. The stability of enzyme-based biosensors mainly depends on the activity of the employed proteins (Pilas et al. 2018). For this purpose, several nitrate biosensors were prepared and kept in a refrigerator at 4 °C and the stability of the prepared biosensor has been investigated. The results show that the response current of the biosensor is reduced by less than 37% of its initial response after four days' storage. This reduction in electrocatalytic activity could be due to degradation of the enzyme (Kalimuthu et al. 2015). Calibration of the data may be

used to overcome this issue, but this problem remains a major issue, as reported repeatedly for other biosensors as well (Can et al. 2012; Kalimuthu et al. 2015; Ferreyra and Solís 2004).

Table 2. Comparison of the analytical performances of various nitrate biosensors incorporating NR.

Electrode	Linear range/µM	Detection Limit/µM	Ref.
GC/PVA/NR	15–300	4.1	(Quan et al. 2005)
Pt/PPY/CNT/SOD/NR	0.5–10000	0.2	(Madasamy et al. 2014)
GC/PPY-Viologen/NR	5–160	0.5	(Da Silva et al. 2004)
GC/chitosan-NR	0–20	0.011	(Kalimuthu et al. 2021)
CNT/PPy/NR	440-1450	170	(Can et al. 2012)
Au/NR	160 –970	140	This work

Abbreviations: PVA – polyvinyl alcohol, CNT – carbon nanotubes, PPY - polypyrrole

In addition, we investigated the reproducibility which is considered as one of the key factors of a biosensor performance. Responses of three biosensors toward 0.48 mM nitrate (30 ppm) were used to calculate the relative standard deviation (RSD), and the RSD of those three biosensors was 6.69%, which confirmed the satisfactory performance of the method used for the determination of nitrate concentration. The good reproducibility may be due to the high sensitivity and efficiency of the immobilization of the enzyme on the SAM–Au electrode (Moccelini et al. 2008).

Anti-interference capability is an important factor for the practical use of biosensors because many compounds can adversely affect the accuracy of measurements. Since samples for nitrate analysis often also contain nitrite and some of the reported nitrate biosensors exhibit a large interfering response in the presence of nitrite (Ferreyra et al. 2000; Glazier et al. 1998), it was important to check the selectivity of the biosensor to nitrite. The effect of the interference was examined by measuring the amperometric response with 0.96 mM nitrate ions (60 ppm) and 1 mM of nitrite ions. The results obtained are reported in Table 1. Results showed that there was no significant difference in the current after the addition of 1 mM nitrite during the analysis of nitrate ions this observation suggests the selective behavior of biosensor to nitrite for nitrate detection.

3.5. Analysis of real samples

The proposed biosensor was evaluated for the determination of nitrate concentrations in real samples. The determination of the biosensor was compared to the results obtained from ion chromatography and spectrophotometry methods. Initially, the Biosensor was tested for the analysis of two types of samples in several runs. One of the samples was tap water and the other sample was a model food solution sample containing 1 ppm nitrate and 1 ppm gallic acid. The values for nitrate concentration obtained with the nitrate biosensor and ion chromatography methods are shown in Table 3. For water samples, as the results of the Table 3 show, there is no significant difference between the results of the methods and fabricated biosensor can be successfully used for the determination of nitrate in water. However, for the model food sample, the biosensor response decreased significantly. This was possibly due to the reaction of gallic acid with the mediator compounds because they are highly reactive compounds and strong oxidizers of phenolic substances (Can et al. 2012). It should be noted that the choice of gallic acid was based on several investigations and literature review (Amor-Gutiérrez et al. 2017; Can et al. 2012). Gallic acid, as an antioxidant, chemically active substance, a reducing agent and, a member of widely spread chemicals in food (phenolics), was found to be a proper candidate to represent food samples. Considering the interfering signal of gallic acid, this biosensor was not suitable to detect the nitrate concentration of samples that have high concentrations of antioxidants or phenolic compounds. Testing the biosensor with cucumber juice, as a real food sample, confirmed these results and the sensor response was not satisfactory. However, clarification of the juice with a simple process to remove interfering compounds resulted in acceptable results with high recovery values.

Table 3. Determination of nitrate concentration (mM) in real samples. mM=1000*(g/L) /62.

Sample	Spiked	Ion chromatography	Measured	Recovery (%)
Water	-	1.35	1.45±0.07*	107
Model Food Sample**	0.97	-	0.41±0.02	42
Cucumber juice	0.97	-	0.62±0.11	64
Clarified cucumber juice	0.97	0.45	1.31±0.15	92

*Data are given as average ± standard deviation (n =3).
**The model food sample was chosen based on comprehensive literature review (Can et al. 2012). Gallic acid was found to be a proper candidate and was used to prepare the sample.

4. Conclusion

In this study, a portable three-electrode biosensor was successfully constructed for nitrate determination. The biosensor accurately measures nitrates in the 0.16-0.96 mM range in water samples and demonstrates potential after sample pretreatment for food analysis, particularly in clarified cucumber juice. Despite challenges with direct food sample analysis due to phenolic compounds, the biosensor's portability and rapid response make it a promising tool for in-field analysis of water, food, and agricultural products. Future research should explore the use of additional biological materials for biosensor fabrication, such as other enzyme varieties or bio-recognition elements, which could further enhance sensitivity and specificity, particularly in complex matrices such as food samples. The integration of advanced data analytics and machine learning algorithms could refine the device's analytical capabilities, enabling a more nuanced interpretation of results and even real-time monitoring of nitrate levels.

Acknowledgments

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Conflict of interest

The authors declare that there is no conflict of interest.

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