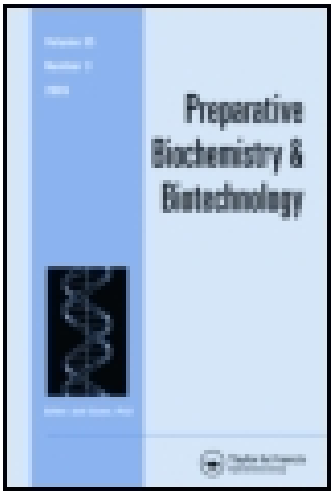

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SENSITIVE NITRATE DETERMINATION IN WATER AND MEAT SAMPLES BY AMPEROMETRIC BIOSENSOR

Erhan Dinçkaya [a], Erol Akyilmaz [a], M. Kemal Sezgintürk [a] & F. Nil Ertaş [a]
[a] Department of Biochemistry, Faculty of Science, Ege University, Bornova-Izmir, Turkey
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Erhan Dinçkaya, Erol Akyilmaz, M. Kemal Sezgintürk, and F. Nil Ertaş
Department of Biochemistry, Faculty of Science, Ege University, Bornova-Izmir, Turkey

□ *This study describes a novel biosensor method for specific determination of nitrate in food and water samples by using nitrate reductase (NR) (EC 1.9.6.1) biosensor based on the detection of oxidation peak current of redox mediator, methyl viologen, related to nitrate concentration. The method was shown to be selective and sensitive to determine the nitrate levels of water samples and processed meat samples. Immobilization procedure and also working conditions of the biosensor were optimized. Dynamic range attained with this method was established as (5.0–90.0* $\times 10^{-9}$ *M) for nitrate concentration with a 10 s response time. Limit of detection (LOD) and quantification (LOQ) of the biosensor were calculated as* 2.2×10^{-9} *M and* 5.79×10^{-9} *M, respectively. Reproducibility experiments was established on repetitive measurements by using a freshly prepared biosensor for avoiding the memory effect. The RSD was calculated as 1.22% at a nitrate concentration of* 4.7×10^{-8} *M (*$n = 7$*).*

Keywords amperometric, biosensor, mediator, nitrate, nitrate reductase

INTRODUCTION

Nitrate is a well-known environmental contaminant largely encountered in ground water and waste water of food industry. Nitrate and nitrite have been added to certain meat products at the curing process. To determine the contents of nitrates and nitrites in foodstuffs is very important because of their harmful effects. Nitrates and nitrites, in slight amounts, are natural components of higher plants, and are involved in biochemical and physiological processes.[1] Human nitrate intake is mainly from vegetables, water supplies and from additives/preservatives used in meat. About 87% of the total nitrate concentration in a normal diet is believed to be a direct result of vegetable intake.[2]

Address correspondence to Dr. Erol Akyilmaz, Ege University, Faculty of Science, Biochemistry Department, 35100 Bornova-Izmir, Turkey. E-mail: erol.akyilmaz@ege.edu.tr

The main concern for the public health is the link between nitrates and stomach cancer. It is due to the fact that nitrates help in the formation of carcinogenic nitrosamines. The elevation of gastric pH > 5.5 leads to bacterial growth followed by rapid conversion of nitrate to nitrite. Nitrite is a precursor in the formation of nitrosamines. The nitrates in foods can be reduced to nitrite. It is known that nitrite causes methaemoglobinaemia and, with secondary and tertiary amines, yields the cancerogen nitrosoamines.[3] Due to these toxic effects, it is important to develop new analysis methods for the simultaneous determination of nitrate in real samples.

Various methods have been developed to determine nitrates in food, water and other matrices. Over the years, analytical techniques, such as spectrophotometry,[4–8] fluorescence,[9] potentiometry,[10,11] ion chromatography,[12] polarography,[13] conductometric,[14,15] capillary electrophoresis,[16,17] as well as high-performance liquid chromatography[18] have been used for the determination of nitrate.

The acceptable daily intake for nitrate was set as 0–3.65 mg/kg body weight by the Joint FAO/WHO Expert Committee on Food Additives (JECFA).[19] These regulations have increased the demand for accurate, sufficiently sensitive and selective analytical procedures for nitrate.

Nitrate reductase (NR) has a multiredox center containing a molybdoproteine cofactor which is the active site of the enzyme, an iron–sulfur center which is involved in the enzymatic electron transport and a heme-b carrying subunit. However, these redox centers are deeply embedded in the protein structure and thus prevent the direct electron transfer with the electrode.[20–22]

Biosensors are attractive alternatives due to their simple use in complex sample and the possibility of fabricating fast portable biosensors. NR from algae, higher plants and different microorganisms has been used by different authors for the construction of amperometric biosensors including whole cells, membranes and isolated enzymes.[23–29]

Besides, depending on the immobilization method, the inadequate orientation of donor to acceptor sites of the enzyme results in less signal formation. The redox mediators with low molar mass such as methyl viologen were therefore used as electron shuttle between the immobilized enzyme and the electrode surface.

In view of the increase in interest in the quality of drinking water, sensitive, selective and simple methods for the determination of nitrates are desirable. Present study describes a voltammetric method for specific determination of nitrate in meat and water samples by using NR biosensor based on redox mediator of methyl viologen.

EXPERIMENTAL

Chemicals

Nitrate reductase (EC 1.9.6.1) from *E.coli*, methyl viologen dichloride hydrate, azure-A, thionine, nile blue, toluidine blue, gelatin, glutaraldehyde (25%) and all other chemicals used in the experiments were purchased from Sigma-Aldrich Chemie GmbH (Germany).

Apparatus and Reagents

The chemicals used were of analytical reagent grade. Voltammetric analysis was carried out with a Metrohm 694 VA Processor and Stand (Switzerland). A three electrode system including Ag/AgCl reference electrode and platinum auxiliary electrode was used throughout the work. The working electrode was a glassy carbon (GCE) support with a 3 mm diameter supplied from Metrohm. Sonication was made with Ultrasonic LC30 (Germany).

Procedure

The GCE surface was polished on a piece of velvet with alumina slurry, rinsed with distilled water and then sonicated for 10 min. Nitrate reductase (usually 1.9 μU/ml) and 225 bloom gelatin (10 mg/ml) were mixed in 0.050 M phosphate buffer (pH 7.5) and dissolved at 38°C. A 100 μl of this solution was injected onto the GCE surface and allowed to dry at 4°C for 20 min. After this period the electrode was immersed in 2.5% glutaraldehyde solution in phosphate buffer for 3 min. Then, the electrode was rinsed with distilled water and kept in buffer solution at room temperature before use. This biosensor was utilized in constructing a calibration curve for nitrate in the presence of methyl viologen (100 mg/l) in phosphate buffer by monitoring the change in the peak current while the potential was scanning from −250 mV to −750 mV. The buffer in the reaction cell was thoroughly purged with nitrogen before potential was applied and stirred by nitrogen bubbling during the measurements.

Sample Analysis

The nitrate content of drinking water and mineral water samples was determined by method based on UV photometric measurements after necessary dilution.[30] Nitrate determination in the meat samples was made by using the biosensor and polarographic method[31] employing hanging mercury drop electrode (HMDE).

For the preparation of meat samples the procedure is as follows;

10 g sample was cut into small pieces, 100 ml distilled water was added and mixed for 5 min. in a high frequency mixer. After that, allow to stand for 1 h in a water bath at 90°C. 2 ml [$Zn(CH_3COO)_2$] 30% (w/v) and 2 ml [$K_4Fe(CN)_6$] 15% (w/v) were added into the mixture and mixed (Carrez precipitation). Finally, the mixture was filtered through a paper filter and through a membranous filter (0.45 μm). The obtained clear solution was used for the analysis.

RESULTS AND DISCUSSION

Optimization of Immobilization Parameters

Initial studies comprised developement of the biosensor and detailed investigation of constituents of bioactive layer namely the NR enzyme activity, gelatin amount and glutaraldehyde percentage. The effect of enzyme activity on the biosensor response was studied first and the bioactive layer was prepared by enclosing three different levels (0.038, 0.19 and 0.95 μU/100 μL) of NR by the aid of 3.3 mg/100 μL gelatin and crosslinking with 2,5% glutaraldehyde. These electrodes were then utilized to construct calibration graphs for nitrate in the presence of 100 mg/l methyl viologen at 35°C. On comparing the slope of the graphs it can be concluded that biosensor response slightly differs for the combinations studied. Therefore, 0.19 μU/100 μl enzyme activity was chosen for further studies since a larger linear range including more sensitive region (5×10^{-9}–9.0×10^{-8} M) was obtained with this electrode. Figure 1 shows

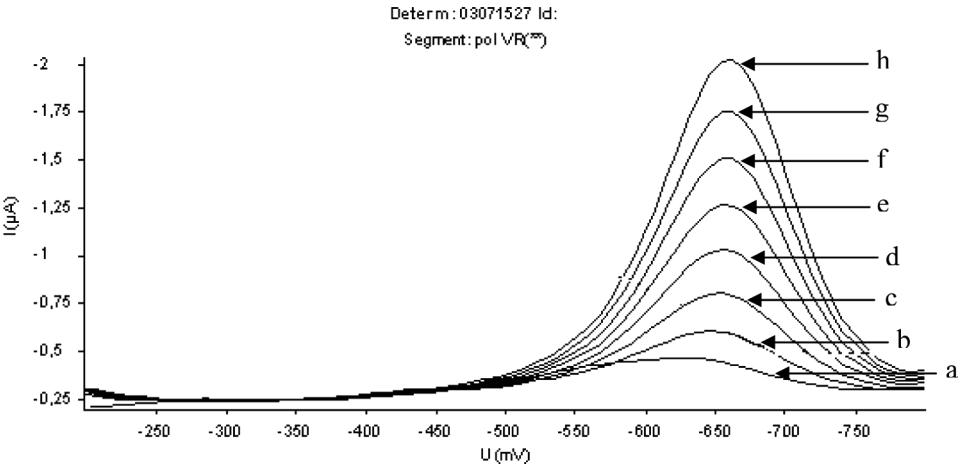

FIGURE 1 Differential pulse voltammograms of nitrate standards in phosphate buffer (pH 7.5; 50 mM, $T = 35°C$) containing methyl viologen (100 mg/L). a: baseline, b: 5×10^{-9} M, c: 10×10^{-9} M, d: 25×10^{-9} M, e: 50×10^{-9} M, f: 75×10^{-9} M, g: 90×10^{-9} M, h: 125×10^{-9} M.

the reduction peak currents of the mediator were plotted against the nitrate concentration.

The amount of gelatin was optimized in a similar way by adding three different amounts (5, 10 and 20 mg) per 300 µl injection volume and by comparing the biosensor response. The procedure given above was applied in the presence of methyl viologen as the mediator and resulting peak currents were plotted against the nitrate concentration As can be followed from Figure 2, more dense gelatin results in a lower response indicating a diffusion barrier for the substrate. On the other hand, less gelatin amounts can not provide mechanical stability and the linear response sub limit was found higher (9.8×10^{-9} M) than that of prepared with denser gelatin. Consequently, the optimum gelatin amount was chosen as 10 mg/300 µl. By this means a linear response was obtained for nitrate concentration in a range of $5.0–90 \times 10^{-9}$ M.

Similarly, the cross-linking agent namely glutaraldehyde percentage was optimized by using 1.25, 2.5 and 5% in the same procedure. Biosensor response was lower for highest glutaraldehyde percentage probably due to the diffusion blockage with increased cross-linking. Therefore 2.5% glutaraldehyde was used for further studies. The linear response range was established as $5.0–90 \times 10^{-9}$ M nitrate concentration.

It is well known that use of mediators improves the performance in amperometric biosensors. Proper selection of mediator is important since it markedly effect the peak potential and the sensitivity as well. Performances of the biosensors were compared by using buffer solutions containing 100 mg/L methyl viologen, Azure-A, Thionine, Nile Blue and Toluidine

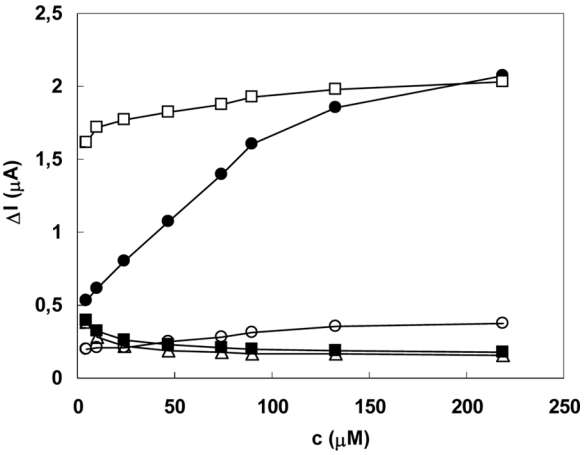

FIGURE 2 Determination of the most suitable mediator for nitrate reductase biosensor. All the mediators were prepared in phosphate buffer (pH 7.5; 50 mM, $T = 35°C$) to be 100 mg/L. (-●- : Methyl viologen, -△-: Azure A, -■-: Nil Blue, -□-: Thionine, -▲- : Toluidine Blue).

Blue. From the experiments, it was determined that methyl viologen was an excellent choice for the nitrate reductase based electrode. From the results it is obvious that except methyl viologen we didn't obtain a calibration curve for nitrate in the experiments. Figure 2 shows the results obtained from the experiments.

Medium Characteristics

The medium pH has a major effect on the results as it has an influence on the enzymatic reaction as well as on the reduction of the mediator. The biosensor response was monitored for 4.7×10^{-8} M nitrate concentration in a pH range of 6.0 to 8.5. All the phosphate buffer solutions contain 100 mg/l methyl viologen. As can be seen from Figure 3, best results were obtained with pH 7.5 phosphate buffer at 35°C. If we consider the optimum pH value for free nitrate reductase (pH 7.0) it can be said that the immobilization method used in the biosensor preparation is suitable and didn't affect the activity of the enzyme. Then, the effect of buffer concentration on the response was searched and among the concentrations studied (25, 50, 100 ve 200 mM) best results were obtained with 50 mM buffer solutions. Higher concentrations resulted in lower responses and this may be attributed to the increased ionic strength that effects the activity of the enzyme.

The effect of temperature on the biosensor response was also investigated. The activity of the biosensor was plotted against the tepmperature in range of 20–40°C and a bell shaped graph was obtained. Best results were obtained at 35°C and further studies were performed at this temperature. It was noted that a deformation of the enzyme layer was observed at 40°C.

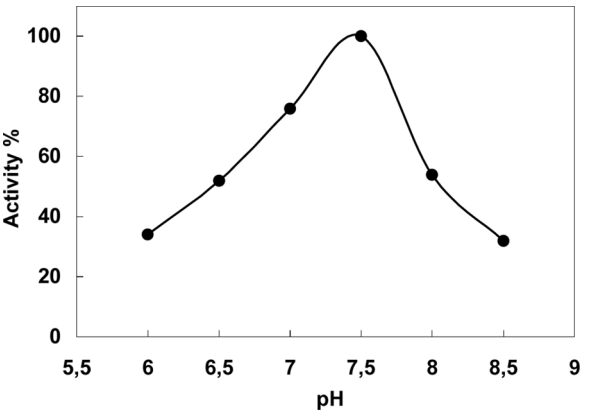

FIGURE 3 Effect of pH on the biosensor. Phosphate buffer (50 mM) containing 100 mg/L methyl viologen. $T = 35$°C.

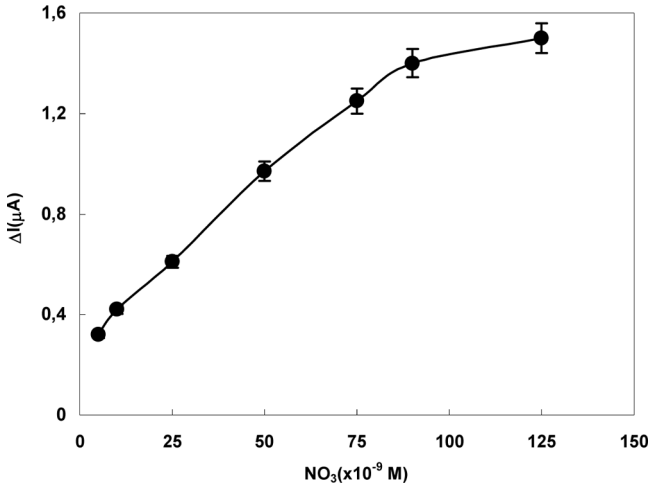

FIGURE 4 Calibration graph for nitrate obtained at pH 7.5 phosphate buffer (50 mM) containing 100 mg/L methyl viologen. $T = 35°C$.

Analytical Characteristics

Analytical performance of the biosensor developed was tested upon addition of nitrate in buffer solutions containing 100 mg/l methyl viologen as described above. As can be followed from Fig. 4, dynamic range attained with this method was established as $5.0–90.0 \times 10^{-9}$ M. Here it should be emphasized that each dot on the calibration graph was obtained by using a freshly prepared biosensor for avoiding the memory effect that may be resulted from the substrate accumulation inner site of gel layer in particular at high concentrations of nitrate. Limit of detection (LOD) of the biosensor is 2.2×10^{-9} M and the limit of quantification (LOQ) of the biosensor is 5.79×10^{-9} M.

TABLE 1 Nitrate Levels of Drinking and Sparkling Water Samples Obtained by Both Proposed and Standard Enzymatic Method

Sample	Nitrate Stated (mg/L)	[a]Nitrate Found (mg/L)*	[a]Recovery (%)	[b]Nitrate Found (mg/L)*	[b]Recovery (%)
Spring water 1	8.360	8.270	98.9	8.150	97.5
Spring water 2	1.236	1.196	96.7	1.157	93.6
Spring water 3	3.520	3.182	90.4	3.205	91.0
Spring water 4	1.000	1.049	104.9	1.013	101.3
Spring water 5	1.000	1.074	107.4	1.070	107.0
Mineral water	0.200	0.212	106.0	0.210	105.0

[a]With the biosensor.
[b]With reference method.
*Average of four measurements.

TABLE 2 Nitrate Levels in Processed Meat Samples Obtained by Both the Proposed Method and the Reference Method

	Biosensor Method			Reference Method		
Sample	*Nitrate Found (mg/kg)	SD (mg/kg)	CV (%)	*Nitrate Found (mg/kg)	SD (mg/kg)	CV (%)
Pastrami	377	±14.68	3.89	403	±35.65	8.85
Sausage	1467	±30.50	2.08	1474	±40.70	2.76
Salami	280	±6.92	2.47	267	±26.38	9.87

*Average of four measurements.

The stability of the biosensor was tested upon monitoring the response of two biosensors one kept at +4°C 24 h and the other 48 hours to compare the performances. No significant change was observed for nitrate solutions at different concentration levels with both biosensor responses.

Reproducibility of the results was established on repetitive measurements by using a freshly prepared biosensor for aviding the memory effect. The RSD was calculated as 1.22% at a nitrate concentration of 4.7×10^{-8} M ($n = 7$).

Application of the Biosensor

The method developed was applied for determination nitrate levels in selected foodstuff including drinking water and processed meat samples such as salami, pastrami and sausage obatined from local markets. The accuracy of the results was tested upon comparing with those obtained by using reference methods. The results obtained for the spring and mineral water samples are summarized in Table 1.

Table 2 shows the results obtained from the meat sample analysis. As can be followed from Table 2, the results of both methods are in well agreement. The reproducibility figures were improved with the proposed method. In addition, the detection limit of the proposed method lies in nanomolar levels and this constitutes another advantage of the method.

CONCLUSION

This paper describes a very sensitive and selective method based on a nitrate reductase based electrode related to an electron mediator, methyl viologen, for nitrate determination. The biosensor exhibited a low detection limit for nitrate and rapid response to changing nitrate concentration. It demonstrated the ability to assay real samples such as spring and mineral water, and meat products. The biosensor does not require any expensive or

complex associated instrumentation, unlike techniques such as ion chromatography, polarography, fluorometry or chemiluminescence. Because enzymatic reactions are specific, the biosensor developed can be used in a wide variety of difficult samples. High nitrate level of the water and food products is a growing problem because of cancer risk. So, nitrate monitoring capabilities may help solve this problem. An accurate, sensitive and safe nitrate measurement method can be a useful tool for food security to control the level of nitrate in the drinking water and food products.

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