Biosensor based on immobilised Nitrate Reductase for the quantification of nitrate ions in dry cured ham

Felipe Jadán, María-Concepción Aristoy, and Fidel Toldrá*

Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Avd. Agustín Escardino 7, 46980, Paterna, Valencia, Spain

* Corresponding author: Tel: +34963900022 ext.2112; fax: +34963636301.

E-mail address: ftoldra@iata.csic.es

Abstract

A nitrate reductase immobilized system with an oxygen electrode has been developed and optimised to determine the nitrate content in dry-cured ham. The obtained amperometric signal was recorded at 5 s in the immobilised nitrate reductase sensor and the reaction rates (slope) were related to the nitrate content. A linear relationship between the reduction nitrate rate by action of nitrate reductase and the nitrate concentration was found within the range 10-70µM (R^2=0.9761). The immobilized enzyme showed a high specificity and sensibility and was stable enough to allow the reutilization of the membranes up to 8 times without loss of activity. This reduces the cost of the analysis as well as the necessary equipment that is cheap and the short average measurement time for each sample. The analysis of nitrate in dry-cured ham samples with the sensor and by HPLC revealed very good agreement (R^2= 0.971). The use of this sensor may constitute an interesting and valid alternative for the quantification of nitrate in dry cured ham.

Keywords: Biosensor, Nitrate, Nitrate Reductase, HPLC, dry-cured ham.

1. Introduction

Nitrates and nitrites are widely distributed in nature and can reach our organism through drinking water and vegetables. Indeed, plant takes nitrate from ground and water and uses it as a source to synthesize their proteins. In a study on the exposition to nitrate through diet, it was concluded that a dietary inclusion of 400 g per day of mixed vegetables meant a consumption of 157 mg nitrate/day (EFSA, 2008). Other sources of nitrate through the diet are cured meat products. Nitrates and nitrites are used for preserving meat products against *Clostridium botulinum*. Although nitrite is responsible for the desired action, nitrate is also added as a slow source of nitrite as preservative. Nitrite also improves the color of cured meat because of its antioxidant action. However, the use of nitrates and nitrites is controversial since they can contribute to the generation of carcinogenic nitrosamines (Sindelar et al., 2012). It is also responsible for the oxidation of hemoglobin to methemoglobin, which is unable to carry oxygen, and thus leads to tissue hypoxia (cyanosis) (Bruning-Fann et al., 1993).

An Acceptable Daily Intake (ADI) for nitrate of 3.7 mg/Kg b.w./day, equivalent to 222 mg nitrate per day for a 60 Kg adult was established by the former Scientific Committee on Food (SCF) and was reconfirmed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2002 (EFSA Journal, 2008). In this context it is necessary to control the levels of these compounds in the meat products industry to verify the permitted maximum levels in the finished product using feasible and reliable analytical instruments.

There are many methods to quantify nitrate in water, food or biological fluids. The most used are chromatographic, spectrophotometric, or electrochemical techniques (Alonso et al., 1992; Bilal et al., 2001; Kitamaki et al., 2003; Hsu et al., 2009; Wang et al., 2013). In addition to these, the analysis of nitrate using enzymatic sensors based on

the nitrate reductase as recognition element has been of great interest in recent times, especially in clinical analysis (Kaneene, 1993; Dykhuizen et al 1996).

The basis for the measurement using this method is the reversible reduction of nitrate in the presence of the nitrate reductase enzyme according to the following scheme (Gross et al., 2015). (Eq. 1).

$$NO_3^- + 2H^+ + 2e^- \rightleftharpoons NO_2^- + H_2O \quad (1)$$

In this enzyme system, a cathodic reduction of the oxygen present in the medium to hydrogen peroxide and to water can occur simultaneously (Plumeré et al 2013) (Eqs 2, 3).

$$O_2\,(g) + 2H^+ + 2e^- \rightleftharpoons H_2O_2 \quad (2)$$

$$O_2\,(g) + 4H^+ + 4e^- \rightleftharpoons 2H_2O \quad (3)$$

Since this could constitute a possible interference in the analysis of nitrate some strategies that were summarized by Plumeré (2013) have been proposed and include, i) the removal of oxygen by using oxygen scavenging systems, ii) the use of more positive potentials for analyte detection or iii) the increase in the overpotential for the oxygen reduction reaction (Eq 2 and 3) which means to shift the overpotential for the cathodic oxygen reduction to values as negative as possible. All these strategies complicate the analysis or require modifications in the standard oxygen sensors that makes them more expensive and not easily applicable for on-site monitoring.

However, some little adjustments can reduce or even avoid the oxygen interferences without the need of changing the structure of the sensor. By taking into account that kinetics of the catalytic process involving nitrate reduction (Eq 1) is much faster than that of the oxygen reduction, the latter can be diminished a lot if the analysis is performed quickly with short reaction times. There have been reported sensors that work in the presence of oxygen (De Quan et al., 2005; Gross et al 2015). Other possibility is using

pH reactions in which Eq 1 predominates over Eq 2 and 3 that can be performed if allowing the enzyme activity.

The nitrate and nitrite biosensors should remove the oxygen from the sample by working at enough negative potentials for analyte detection (Plumeré, 2013). It has been described that the effect of oxygen is small when using eukaryotic nitrate reductase in small volume in an electrochemical system for fast reading (Plumeré et al., 2012).

The majority of reported biosensors for the determination of aqueous nitrate concentrations are based on immobilized or co-immobilized nitrate reductase (Cosnier et al., 1994; Quan et al., 2005; Madasamy et al 2014; Talekar et al., 2014; Ankush et al., 2015). The main objective of the immobilization is to reduce the cost and, for this reason, it is important to optimize the immobilization process especially with regard to the minimum required amount of enzyme. The previous activation of the membrane facilitates the linkage of the enzyme on the membrane which will determine the activity of the enzyme.

The main objective of this study is to evaluate the use of an optimized nitrate reductase-based sensor combined with an electrode of Pt/Ag to quantify nitrate in meat products..

Materials and methods

Chemical and reagents

Nitrate Reductase (NiR, NAD(P)H, EC 1.6.6.1-3 from *Aspergillus niger* (each vial contains 7.8 mg of protein with $\geq$ 300 U /g protein).

Methanol, acetonitrile, and tetrahydrofurane, grade HPLC gradient were from Sigma (Sigma-Aldrich (St. Louis, MO, USA). MOPS 3-(N-morpholino) propanesulfonic

acid and sodium nitrate were from Sigma-Aldrich. Hydrochloric acid (37%), sodium phosphate, potassium di-hydrogen phosphate, sodium acetate anhydrous, sodium hydroxide, glacial acetic acid and acetone were from Panreac (Panreac Química, Barcelona, Spain). Doubly distilled milli-Q water was used throughout.

The preactivated immunodyne ABC membrane (Nylon 66, pore size 0.45 μm) was supplied by Pall Europe (Porsmounth, UK).

Preparation of the Nitrate Reductase Solution and Measure of the Enzyme Activity

Commercial enzyme reagent (7.8 mg) was dissolved in 15 ml of 0.1M potasium phosphate buffer, pH 7.1 aliquoted in 0.5 ml eppendorf tubes and frozen stored at -80 °C until use. The activity of each new vial of the commercial enzyme was analysed by using a spectrophotometric Enzymatic assay protocol (EC 1.9.6.1 Sigma-Aldrich).

Materials

Muscle (*Biceps femoris*) from 3 traditional Serrano dry-cured hams at 2 months of processing was obtained from a local ham industry.

Equipment

An enzymatic sensor composed by an oxygen electrode with an immobilized enzyme as described in Hernández-Cázares et al, (2010) for the hypoxanthine analysis was used here in the analysis of nitrate. The oxygen electrode (Rank Brothers, Bottisham, Cambridge, England) consists of a central 2 mm diameter platinum disk (working electrode), with a surrounding silver ring (Ag/AgCl, reference electrode), a thermostated (by water recirculation) reaction cell and an oxygen permeable Teflon membrane (12.7 μm). The current output was recorded by a datalogger ADC-16 (Tip Technology Limited, St, Net.

Cambridgeshire, United Kingdom) and displayed like a variation of the current (mV) during the nitrate reduction reaction.

The chromatographic analysis was performed in an 1100 Agilent liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an autosampler and a diode array detector.

Sample preparation

Ground muscle pork ham (5 g) was homogenized with 0.6 M perchloric acid (15 ml) for 10 min at 4 ºC in a stomacher (IUL Instrument, Barcelona Spain). The homogenate was centrifuged at 10,000xg at 4 ºC and the supernatant filtered through glass wool. Supernatant was neutralized up to pH in the range 6.5 - 7 by the addition of solid potassium carbonate and leaving to stand until no more precipitate is formed. The neutralized extract was centrifuged (20 min at 3000 rpm), filtered and used for the analysis of nitrate by HPLC and by the enzyme sensor.

Biosensor preparation

A good electrical communication between the electrodes is necessary. So, a damp tissue with 3 M KCl and oxygen permeable Teflon membrane over it was placed covering the sensor electrodes as described in Hernández-Cázares et al (2010). The potential of polarization was fixed at -600 mV with respect to the Ag/AgCl reference electrode and the temperature of the reaction cell was maintained at 22 ºC. In these conditions, with water filling the reaction cell, the system was allowed to equilibrium.

Immobilised Enzyme Sensor

The immobilization procedure was based on that described for lysine oxidase in Jadán et al, (2016). The optimal percentage of glutaraldehyde, assayed in the range 2 to 10%, was fixed in 8%. Thus, a mixture (total 50 µl) containing 15 µl nitrate reductase solution (0.038 U), 27 µl of 100 mM MOPS pH 7.1 and 8 µl of a solution of 50 % glutaraldehyde, was placed drop by drop on a immunodyne ABC membrane (1cm^2). The membrane was dried at air for 1h to complete the cross-linking. The nitrate reductase membrane was attached onto the Teflon membrane in the oxygen electrode by an o-ring and clampled to the thermostated reaction cell.

The nitrate reductase membrane can be used until 8 times before changing for a new one, and 3 min were necessary to restore the equilibrium in the system before each new injection. The enzymatic rate ($mV \bullet s^{-1}$) value at 10 s was used and extrapolated in the calibration curve to calculate the nitrate content in meat samples. To build the calibration curve, nitrate concentrations 0.01, 0.03, 0.05, and 0.07 mM were used.

Actuating Mechanism of Nitrate Reductase onto Nitrate

In order to study the kinetics of nitrate reduction in the presence of nitrate reductase, 1.5 mL of the enzyme solution (3.85 U) plus 1.5 mL of a nitrate standard solution (0.01 mM) or meat extract (dilution 1/5) were brought to 15 mL of 100mM MOPS, pH 7.1, and allowed to react at 20 ºC under gently stirring. During the enzymatic reaction 900 µl aliquot was removed each 30s and immediately mixed with 100 µl of hydrochloric acid (37%) to stop the reaction. Aliquots were centrifuged (12,000 rpm for 5 min at 4ºC) and analysed by HPLC. This assay was also conducted to study the stability of the enzymatic solution at different storage temperatures.

Chromatographic method

The chromatographic analysis of nitrate content in ham extracts or standards was performed by HPLC using a Spherisorb S5 SAX, (5µm, 250 x 4.6 mm) column (Waters, Milford, MA, USA) thermostated at 35 ºC and 7.5 mM orthophosphoric acid adjusted to pH 3.5 with NaOH at 0.8 mL/min as mobile phase. Twenty microlitres of sample or standard were injected and detection was by ultraviolet at 215 nm (Alonso, 1992). The mobile phase was filtered through a 0.45 µm nylon membrane filter and degassed prior to use.

Validation

The linearity of the immobilized enzyme system was obtained from a triplicate analysis of each respective standard concentration solutions: 0.01-0.07 mM applying the least- squares method.

The measurement of repeatability was assayed by 25 consecutive injections of 0.05 mM nitrate standard or meat samples which were analysed within the same day with the same equipment under the same conditions in the immobilized enzyme sensor, but using a new membrane each time. The overall mean concentration and the coefficient of variation (%) were calculated.

The operational stability of the nitrate reductase membrane was evaluated. Thus, successive injections of 0.05 mM nitrate were made in a same membrane and the response recorded until a 50 % loss in the activity was observed.

The stability of nitrate reductase stored at 4 ºC; in solution and immobilized on the membrane was evaluated. In the first study, fifteen enzymatic solutions (3.75 ml) were prepared and stored at 4 ºC. Each enzymatic solution was tested each week in triplicate,

by injecting 0.01 mM Nitrate standard solution. In the second approach several freshly prepared nitrate reductase membranes were maintained at 4, 10, 15, 22, and 30 ºC and then the enzyme activity was assayed by injecting 0.05 mM nitrate as usually, in triplicate each membrane for each time. The results were compared by plotting their response against time for each temperature and calculating the average half-life time.

Statistical method

Linear correlation analysis between the nitrate content measured by the enzyme sensor and HPLC was carried out. The statistical analysis was done using Statgraphics Plus (v5.1) software.

Results and discussion

During the catalytic reduction of nitrate by nitrate reductase, a sharp decrease in the electrode response takes place, which is proportional to the concentration of substrate and can be amperometrically monitored in an electrochemical cell.

The effect of pH and the temperature on the biosensor response was studied. The pH studied was in the range of 6.6-7.6 using MOPS 100 mM, pH 7.1. Different temperatures were studied (within the range 15-35ºC) and the optimal temperature was found at 20º C (Data not shown). The enzyme may start denaturation at temperatures above 25ºC and this explains the sharp decrease in activity above such temperature (Ntoko and Senwo, 2012).

Actuating Mechanism of the Nitrate Reductase onto Nitrate

The disappearance of nitrate in the presence of the enzyme was analysed by HPLC in a model system using initial nitrate concentration of 0.145 mM in the reaction vessel, and submitting it to reaction with the soluble enzyme. The results shown in the Fig. 1 indicate that the time required for the complete reduction of nitrate was 70 seconds, indicating a good affinity of nitrate reductase over the nitrate and a negligible level of interference from nitrite. In this type of analysis, the main possible interference that could occur is the oxidation of nitrite to nitrate due to the reversible reaction (Eq. 1) (Madasamy et al 2014). It has been described that nitrate reductase acts in a complete and specific way on nitrate (Gross et al 2015). In this study, the use of nitrate reductase from *Aspergillus niger*, the analytical conditions in the sensor, the voltage of – 600 mV, low volumes of reaction and the very short time for monitoring (10 s), reduces any possible interference resulting from oxygen reduction or nitrite oxidation (Plumeré et al., 2012).

Effect of Immobilization on Enzyme

The enzyme immobilization is a necessary process to achieve economic viability in food analysis. Immobilization would allow the reutilization of membranes and spend less enzyme. Nevertheless, the process of immobilization may diminish enzyme activity because of changes in conformation resulting in a loss of activity. This makes necessary the optimization of the parameters involved in the immobilization process like the glutaraldehyde concentration (Can et al 2012, Talekar et al 2014). Different concentrations of glutaraldehyde from 2 to 10 % were tested and the highest enzymatic activity was observed when using 8 % for a fixed nitrate reductase concentration (0.038U) (data not shown). The effect of the immobilization in the enzyme activity was evaluated by measuring the apparent Michaelis-Menten constant, $K_{M(app)}$ after immobilization, at

the optimized conditions. Thus, $K_{M(app)}$ was calculated using a Hanes-Woolf method and was 0.23 mM what indicate a very good affinity of the enzyme for the substrate. Similar results $K_{M(app)}$= 0.21 mM were reported by Da Silva (2004).

Biosensor Characteristics

The relationship between the nitrate concentration and the enzyme sensor response ($mV \cdot s^{-1}$) is shown in Fig. 2. A linear response was observed in the range 10 to 70 µM of nitrate (R^2=0.9761); the lower limit in this sensor is at a low concentration of nitrate, showing a good sensitivity. The sensor could not read at concentrations of nitrate above 70 µM because high amounts of nitrate would inhibit the enzyme by excess of substrate. Thus, it was necessary to dilute the ham sample to fit inside the linear detection range (Fig. 2).

The obtained repeatability (CV) in standards and dry cured ham samples were 5.14% and 6.49 %, respectively, that shows quite good performance of the sensor. The variability, somehow higher in the sample extract than in standard solutions may be attributed to possible fouling problems (Hernández-Cázares et al. (2010).

The stability of the nitrate reductase in solution at storage temperature (4 ºC) was evaluated by analysing 35 µM of nitrate in solution. Thus, the activity of the enzymatic solution maintained at 4ºC and used during five consecutive weeks, were analyzed and compared. Ninety percent of its initial activity remained after six days, while its half-life was of 21.2 days.

The stability of the nitrate reductase-membrane was strongly dependent on temperature. It must be taken into account that membranes placed in the sensor and ready for the analysis, are submitted to 20ºC for stabilization and analysis what will influence

the results of stability of those membranes stored at lower temperatures (4, 10 and 15 ºC). In these conditions, membranes stored at 4ºC were analyzed daily and showed to be stable, with 90 % of the initial activity retained, within 6.6 days and a half-life of 21.2 days. It allows having membranes stored at 4 ºC and ready for use during a working week. Membranes stored at higher temperatures had to be tested more frequently because they lost activity quicker. Thus, the half-life was reduced to 70 min when stored at 22 ºC. Based on these results and the time required for equilibration and analysis, it is estimated that one membrane located in the sensor can be re-used up to 8 consecutive times if these analyses are carried out straight without stopping.

Analysis of Nitrate in Ham Samples.

The effectiveness of the nitrate reductase sensor to determine nitrate in the processing of dry cured ham was analyzed.

In general the higher concentration of nitrate in hams is found at 2 months of the curing process, after the post salting stage (Armenteros et al., 2012). At this time, nitrate has penetrated to the inner part of the ham where muscle *Biceps femoris* is located with an amount of 4.48 mg /100g of ham (wet weight) analyzed by the HPLC method. Simultaneously, a desirable reduction to nitrite takes place, progressively up to the almost total disappearance of the nitrate at the end of the process (by 10 months).

Nitrate concentrations obtained with the nitrate reductase sensor analysis of 6 dilutions of extracts made from dry-cured ham sampled at two months of processing were validated by comparison to those obtained when analyzing the same samples with the HPLC method. The correlation is shown in Fig. 3. In this model there was a good

agreement between the results obtained by both techniques, being the R^2= 0.971 for the enzymatic sensor *vs* HPLC.

Conclusion

Based on these results, this enzyme sensor constitutes a valid alternative method for measuring the amount of nitrate in cured meat products. The sensitivity and precision of the immobilized enzyme system was good enough for the analysis of nitrate in meat products in comparison to those values obtained using the HPLC method. The average measurement time for each sample is 3 min. The immobilization process allowed the reutilization of the membranes up to 8 times without loss of activity and this reduces the cost of the analysis. Also the necessary equipment is cheap and available for meat industry.

Oxygen interference was not observed because of the use of small volumes, a negative potential and a reductase enzyme.

Acknowledgements

The research leading to these results has received funding from the Spanish Ministry of Economy, Industry and Competitiveness, AGL2014-57367-R and FEDER funds.
Scholarship from Secretaria de Educación Superior, Ciencia, Tecnología e Innovación. (SENESCYT) Ecuador to F. A. Jadán Piedra is acknowledged for their support in this research.

Compliance with Ethical Standards

Funding: This study was funded by the Spanish Ministry of Economy, Industry and Competitiveness, AGL2014-57367-R and FEDER funds. Felipe Jadán enjoyed a scholarship from Secretaria de Educación Superior, Ciencia, Tecnología e Innovación. (SENESCYT), Ecuador.

Conflict of Interest: Author Felipe Jadán declares that he has no conflict of interest. Author M-Concepción Aristoy declares that she has no conflict of interest. Author Fidel Toldrá declares that he has no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: Not applicable.

References

Alonso, A., Etxaniz, B., Martinez, M.D. 1992. The determination of nitrate in cured meat products. A comparison of the HPLC UV/VIS and Cd/ spectrophotometric methods, Food Additives and Contaminants, vol.9 No. 2, 111-117.

Ankush, A. G., Jue, L., Rankothge R. W., Jing, Y., Ilsoon, L. 2015. Amperometric detection and quantification of nitrate ions using a highly sensitive nanostructured membrane electrocodeposited biosensor array. Electroanalysis 27, 1127-1137.

Armenteros, M., Aristoy, M.C., Toldra, F. 2012. Evolution of nitrate and nitrite during the processing of dry-cured ham with partial replacement of NaCl by other chloride salts. Meat Science 91, 378-381.

Bilal, Butt, S., Riaz, M., Zafar Iqbal, M. 2001. Simultaneous determination of nitrite and nitrate by normal phase ion –pair liquid chromatography. Talanta, 55, 789-797.

Bruning-Fann, CS., Kaneene, JB. 1993. The effects of nitrate, nitrite and n-nitroso compounds on human health: A Review. Veterinary and Human Toxicology 35, 521-538.

Can, F., Korkut Ozoner, S., Ergenekon, P., Erhan, E. 2012. Amperometric nitrate biosensor based on Carbon nanotube/Polypyrrole/Nitrate reductase biofilm electrode, Materials Science and Engineering C 32, 18-23.

Cosnier, S., Innocent, C., Jouanneau, Y. 1994. Amperometric detection of nitrate via a Nitrate Reductase immobilized and electrically wired at the electrode surface, Analytical Chemistry, 66, 3198-3201.

Da Silva, S., Shan, D., Cosnier, S. 2004. Improvement of biosensor performances for nitrate determination using a new hydrophilic poly (pyrrole- viologen) film. Sensors and Actuators B, 103, 397-402.

De Quan, Jun Ho Shim, Jong Dae Kim, Hyung Soo Park, Geun Sig Cha, Hakhyun Nam. 2005. Electrochemical determination of nitrate with nitrate reductase-immobilized electrodes under ambient air. Analytical Chemistry, 77, 4467-4473.

Dykhuizen, RS., Masson, J McKnight, G., Mowat, ANG., Smith, CC., Smith LM., Benjamin, N. 1996. Plasma nitrate concentration in infective gastroenteritis and inflammatory bowel disease. Gut 39, 393-395.

EFSA Journal. 2008. Nitrate in vegetables. 689, 1-79.

Gross, A., Holmes, S., Dale, S., Smallwood, M., Green, S., Winlove, C., Benjamin, N., Winyard, P., Marken, F. 2015. Nitrite/nitrate detection in serum based on dual-plate generator-collector currents in a microtrench. Talanta 131 228-235.

Hernandez-Cázares, A.S., Aristoy, MC., Toldrá, F. (2010). Hypoxanthine-based enzymatic sensor for determination of pork meat freshness. Food Chemistry 123, 949-954.

Hsu, J., Arcot, J., Alice Lee, N. 2009. Nitrate and nitrite quantification from cured meat and vegetables and their estimated dietary intake in Australians. Food Chemistry 115, 334- 339.

Kitamaki, Y., Jin, Ji-Ye., Takeuchi, T. (2003). Determination of inorganic anions via postcolumn reaction with iodide in ion chromatography. Journal of Pharmaceutical and Biomedical Analysis 30, 1751-1757.

Madasamy, T., Pandiaraj, M., Balamurugan, M., Bhargava, K., Sethy, N., Karunakaran, Ch. 2014. Cooper, zinc superoxide dismutase and nitrate reductase coimmobilized bienzymatic biosensor for the simultaneous determination of nitrite and nitrate, Biosensors and Bioelectronics 52, 209-215.

Ntoko, F., Senwo, Z. 2012. Nitrate reduction by commercially available nitrate reductases: Bio-catalytic potentials and enzymatic activities in the presence of metal ions. Journal of Environmental Science and Health, Part A, 47, 2028-2034.

Plumeré, N., Hening, J., Campbell, W. 2012. Enzyme-Catalyzed O_2 Removal System for Electrochemical Analysis under Ambient Air: Application in an Amperometric Nitrate Biosensor. Analytical Chemistry. 84, 2141-2146

Plumeré, N. 2013. Interferences from oxygen reduction reactions in bioelectroanalytical measurements: the case study of nitrate and nitrite biosensors, Analytical & Bioanalytical Chemistry, 405, 3731- 3738.

Quan, D., Shim, J.H., Kim, H.S., Park, G.S., Cha, H. 2005. Electrochemical determinación of nitrate with nitrate reductase immobilized electrodes under ambient air, Analytical Chemistry, 77, 4467-4473.

Sindelar, J.J., Milkowski, A.L. 2012. Human safety controversies surrounding nitrate and nitrite in the diet. Nitric oxide, 26, 259-266.

Talekar, S., Joshi, G., Chougle, R., Nainegali, B., Desai, S., Joshi, A., Kambale, S., Kamat, P., Haripurkar, R., Jadhav, S., Nadar, S. 2014. Preparation of stable cross-linked enzyme aggregates (CLEAs) of NADH-dependent nitrate reductase and its use for silver nanoparticle synthesis from silver nitrate, Catalysis Communications 53, 62-66.

Wang, T., Schlueter, K., Riehl, B., Jhonson, J., Heineman, W. 2013. Simplified Nitrate-Reductase-Based Nitrate Detection by a Hybrid Thin- Layer Cotrolled Potential Coulometry/Spectroscopy Technique. Analytical Chemistry, 85, 9486-9492.

Figure Captions

Fig 1. Nitrate disappearance with time of reaction in the presence of nitrate reductase.

Fig 2. Calibration curve obtained with the nitrate reductase biosensor at 2 0 ºC in MOPS 100mM pH 7.1.

Fig. 3. Correlation between the nitrate content analyzed by the immobilized nitrate reductase biosensor at 20 ºC in MOPS 100mM pH 7.1, and HPLC. The study was done with three replicates per assay.

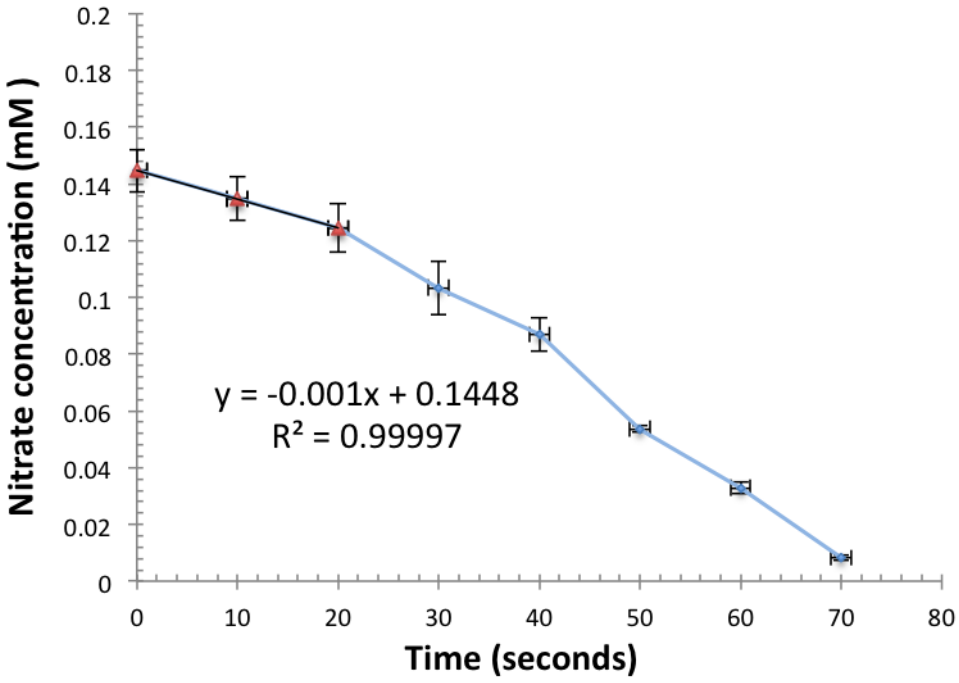

Fig. 1 . Nitrate disappearance with time of reaction in the presence of nitrate reductase.

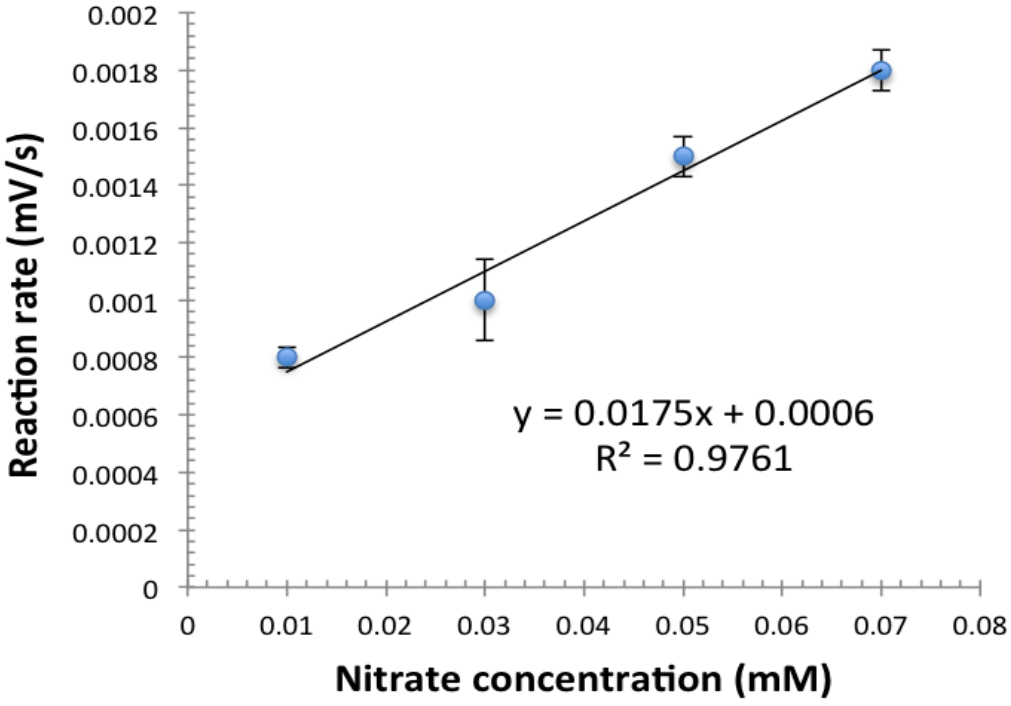

Fig. 2. Calibration curve obtained by the enzyme biosensor in MOPS 100mM pH 7.1.

Figure 3 Click here to download Figure Fig 3.docx

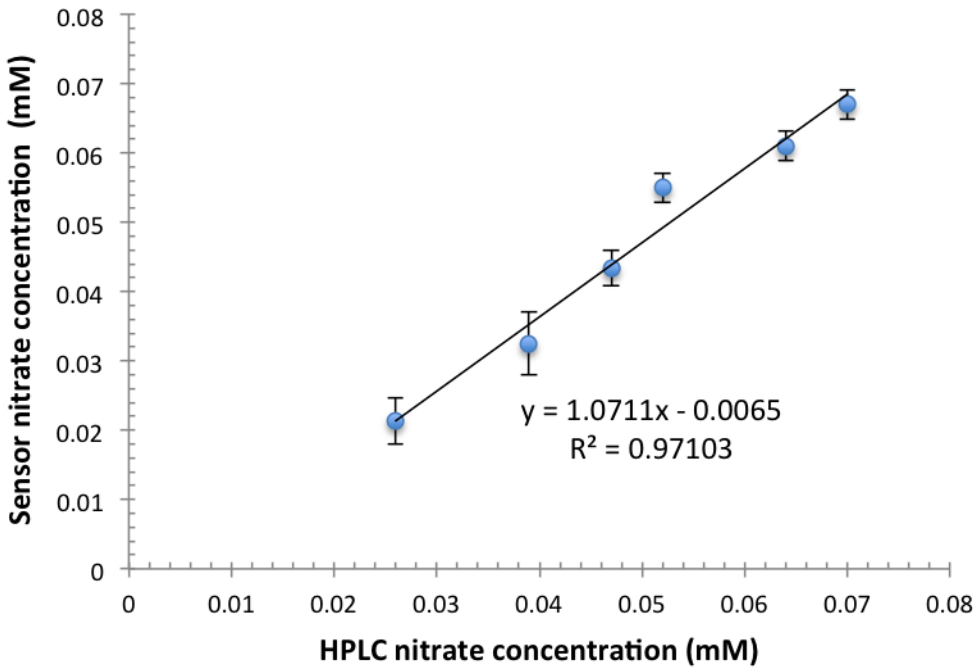

Fig. 3. Correlation between nitrate content measured by the enzyme sensor and HPLC. The study was done with three replicate per assay.